Optimizing Pancreatic Islet Transplantation: A Translational Research Approach

by

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ABSTRACT

Type 1 diabetes (T1D) is caused by autoimmune-mediated destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans. People with T1D need exogenous insulin to survive, but also to avoid complications from uncontrolled glycemia. Unfortunately, insulin treatment commonly causes hypoglycemia, which severely limits optimal glycemic control. While technological advances can ameliorate hypoglycemia, some people remain recalcitrant to the most advanced interventions and progress to severe hypoglycemia, hypoglycemia unawareness, and potentially, death. Pancreatic islet transplantation (ITx) corrects this dire scenario. In ITx, islets obtained from deceased-donor pancreata are infused into the recipient's intraportal circulation using a percutaneous approach. While ITx has progressed substantially, long-term outcomes to optimize patient care are scarce. Additionally, although ITx enables abrogation of hypoglycemia, improvements in glycemic control and, in many cases, insulin independence, it is not a cure in its current form, predominantly because patients require lifelong immunosuppression to avoid immune rejection. Hence, strategies to attain immunosuppression-free ITx are necessary.

This thesis presents studies contributing to enhance informed shared decision-making in clinical ITx, but also introduces experimental strategies to circumvent lifelong immunosuppression post-transplant. Each chapter provides context for the problem being addressed and detailed methodology to evaluate the strength of our conclusions.

In the first chapter, long-term outcomes with ITx are evaluated by analyzing the 20-year experience at our center. Patient survival post-transplant is >90%, which is reassuring in the context of chronic immunosuppression. Indeed, while immunosuppression-related complications are common, these have no impact on patient survival. Graft survival rates are

~50% at 20-years post-transplant, and insulin independence is achieved in 80% of patients, although 20-year insulin independence rates are only 8%. Improvements in glycemic control and measures of hypoglycemia are observed throughout follow-up. Finally, an exploratory analysis shows that the use of anakinra plus etanercept for ≥1 islet infusion and a BETA-2 score ≥15 within 1-year post-first transplant increases the odds of maintaining graft survival.

The second chapter evaluates whether ITx and whole pancreas transplantation (PTx) have similar mortality, morbidity, and metabolic benefits. Over a 20-year period, we show that ITx and PTx have similar patient survival, however, ITx has shorter hospital lengths-of-stay, and fewer procedural complications and hospital readmissions. Conversely, insulin independence rates and glycemic control were better with PTx.

Chapter three explores extrahepatic ITx. While safe, intraportal ITx can cause bleeding and thrombosis sporadically. Thus, alternative "extrahepatic" implantation sites are desirable. Herein, a comparative analysis shows that extrahepatic ITx does not support islet engraftment compared to intraportal ITx. This occurs across all evaluated extrahepatic sites (omentum, subcutaneous space or gastric submucosa). Importantly, patients having intraportal ITx after a failed extrahepatic transplant show similar outcomes to those undergoing upfront intraportal ITx.

These studies address relevant issues to inform clinical practice and identify opportunities to optimize ITx. The next chapters propose two experimental strategies to enable immunosuppression-free ITx.

Chapter four studies the potential of regulatory T cells (Treg) in preventing immune rejection after ITx. Herein, we use antibodies directed at the tumor necrosis factor receptor superfamily member 25 (TNFR25), which is intrinsically expressed by Tregs. We demonstrate that antibody-mediated agonistic stimulation substantially expands endogenous

Tregs, and that a single injection of TNFR25-antibodies prior to transplantation significantly prolonged graft survival in a mouse model of allogeneic ITx. This approach circumvents the need for *ex vivo* Treg expansion, a complex and costly procedure that limits clinical translation.

Chapter five presents a sequential approach harnessing controlled inflammation-induced neovascularization to create a subcutaneous cavity that supports optimal function of customized islet encapsulation devices. This study builds on two successful strategies previously introduced by the Shapiro and Ma laboratories. This combinatorial approach enabled diabetes reversal in syngeneic, allogeneic, and xenogeneic murine models without the use of immunosuppression. Human islet survival was also observed in an immunocompetent xenogeneic islet transplant model. Notably, we demonstrate that impaired devices can be readily replaced *in situ* into the existing vascularized cavity, with prompt return to normoglycemia. Finally, we present protocols to guide scalability of our approach using a minipig model.

These experimental studies introduce two promising platforms to support immunosuppression-free clinical ITx.

Beyond current limitations, β-cell replacement therapies represent a hope for a true cure for T1D. The clinical and experimental perspectives presented in this thesis contribute to achieving this goal.

PREFACE

Dear Reader,

This thesis entitled "Optimizing Pancreatic Islet Transplantation: A Translational Research Approach" is submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Surgery in the Department of Surgery at the University of Alberta. The work presented herein evaluates strategies to optimize pancreatic islet transplantation and advance this therapy forward as a potential cure for type 1 diabetes (T1D).

This thesis is divided in chapters containing clinical and pre-clinical research, in which the author held a leading role within a collaborative and interdisciplinary team. These chapters are presented as a paper-based format, from manuscripts that are either published or submitted for publication.

Chapter 1 presents the current state of pancreatic islet transplantation and is composed of two parts. ***Part 1*** consists of an introductory component discussing contemporary clinical evidence, as well as challenges and potential solutions to move pancreatic islet transplantation forward as a true cure for T1D. This section is presented from a first-author manuscript published in the ***Journal of Hepato-Biliary-Pancreatic Sciences*** *(*Marfil-Garza BA, Shapiro AMJ, Kin T.), entitled *Clinical islet transplantation: Current progress and new frontiers.* J Hepatobiliary Pancreat Sci. March 2021; 28:3, 243-254. For this publication, I performed the bibliographical review, prepared the figures and legends, and wrote the manuscript. AMJS and KT provided revisions to the final manuscript. ***Part 2*** builds on the previous section and the recognition of the lack of robust evidence as one of the major limitations for a more widespread application of pancreatic islet transplantation. This section presents long-term outcomes following allogeneic pancreatic islet transplantation at the University of Alberta. Herein, we present the largest report on

allogeneic pancreatic islet transplantation and describe relevant outcomes, such as patient and graft survival, measures of glycemic control, insulin independence, as well as adverse effects post-transplant. This section is presented from a first-author manuscript published in ***The Lancet – Diabetes & Endocrinology*** (Marfil-Garza BA, Imes S, Verhoeff K, Hefler J, Lam A, Dajani K, Anderson B, O'Gorman D, Kin T, Kneteman N, Bigam D, Ryan E, Senior P and Shapiro AMJ) with the title *Pancreatic Islet Transplantation in Type 1 Diabetes: 20-year Experience from a Single-Centre Cohort in Canada.* My role for this manuscript was writing the research ethics proposal, designing the study, retrieving, curating and analyzing the data, preparing the figures and tables, and writing of the manuscript. SI, KV, HJ and AL provided support with data retrieval. AL, KD, BA, NK, DB, ER, PS, AMJS were involved in patient care over the last 20 years. DO'G and TK performed the islet isolations. All co-authors provided revisions. AMJS provided final edits and revisions as the senior corresponding author.

Chapter 2 continues exploring current limitations with pancreatic islet transplantation. This chapter is also divided in two parts. ***Part 1*** introduces an in-depth review of pancreatic islet transplantation and whole pancreas transplantation. As both therapies continue to be refined, this chapter serves as a comprehensive and much-needed framework to contextualize outcomes. This section is presented from a first-author manuscript currently included in the ***Textbook of Diabetes 6th edition*** (Marfil-Garza BA, Senior P, Shapiro AMJ), entitled *Whole Pancreas and Islet Cell Transplantation.* This book has established itself as the foremost international guide to diabetes. My role for this manuscript was designing and performing the bibliographical review, writing manuscript, and preparing the figures and tables. PS provided critical review of the manuscript and revisions. AMJS provided final edits and revisions as the senior author. ***Part 2*** extends on the areas of opportunity identified

in the previous section and presents a comparative analysis of whole pancreas and pancreatic islet transplantation over 20 years at the University of Alberta. Herein, we present the largest comparative analysis of these two important treatment modalities. We include outcomes, such as patient and graft survival, measures of glycemic control, insulin independence, as well as adverse effects post-transplant. This section is presented from a first-author manuscript currently under review in ***Annals of Surgery*** (Marfil-Garza BA, Hefler J, Verhoeff K, Lam A, Dajani K, Anderson B, O'Gorman D, Kin T, Senior PA, Bigam D, and Shapiro AMJ), entitled *"Whole Pancreas and Pancreatic Islet Transplantation: Comparative Outcomes of a Single-centre Cohort over 20-years"*. My contribution to this work was writing the research ethics proposal, designing the study, retrieving, curating and analyzing the data, preparing the figures and tables, and writing manuscript. JH, KV and AL provided support with data retrieval. AL, KD, BA, PS, DB, and AMJS were involved in patient care over the last 20 years. DO'G and TK performed the islet isolations. All co-author provided revisions. AMJS provided final edits and revisions as the senior corresponding author.

Chapter 3 deals with strategies to improve the safety and accessibility of pancreatic islet transplantation, focusing on alternative implantation sites for pancreatic islet transplantation. In this chapter, we present a comparative analysis of extrahepatic vs intraportal pancreatic islet transplantation. We report outcomes such as stimulated and fasting C-peptide levels, fasting plasma glucose levels, measures of graft function, such as the BETA-2 score, as well as 5-year data on the clinical course of intraportal after extrahepatic pancreatic islet transplantation. We identified that extrahepatic pancreatic islet transplantation, in its current form, does not enable optimal engraftment and clinically-relevant C-peptide production. However, extrahepatic pancreatic islet transplants do not have

a negative impact on subsequent intraportal transplants. This work is presented from a co-first-author manuscript published in ***Transplantation*** (Verhoeff K, Marfil-Garza BA, Sandha Gurpal, Cooper D, Dajani K, Bigam D, Anderson B, Kin T, Lam A, O'Gorman D, Senior P, Ricordi C, Shapiro AMJ), entitled "*Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study"*. My contribution to this work was writing the research ethics proposal, designing the study, retrieving, curating and analyzing the data, preparing the figures and tables, and writing 50% of the manuscript. KV provided support with data retrieval and wrote 50% of the manuscript. GS, DC, KD, DB, BA, AL, PS, AMJS were involved in patient care. DO'G and TK performed the islet isolations. All co-authors provided revisions. AMJS provided final edits and revisions as the senior corresponding author.

In the next chapters, the thesis transitions into the basic research component of the translational approaches evaluated to optimize pancreatic islet transplantation. In previous chapters, in-depth analysis of the clinical evidence identified a major limitation preventing the potential of pancreatic islet transplantation to offer a true cure for T1D: the need for life-long immunosuppression. In the following chapters, two experimental strategies were evaluated to address this issue.

In **Chapter 4,** we evaluate the use of regulatory T cells (Tregs) as co-adjuvant immunomodulatory therapies to diminish or completely abrogate the use of chronic immunosuppression following pancreatic islet transplantation. This chapter is presented in two distinct parts. ***Part 1*** provides a review on the role of Tregs in T1D and pancreatic islet transplantation, dissecting the immunological relevance of these cells, current therapeutic applications, and future directions. This section is presented from a first-author manuscript published in ***Endocrine Reviews*** (Marfil-Garza BA, Hefler J, Bermudez-De-Leon M,

Pawlick R, Dadheech N, Shapiro AMJ), entitled *"Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation"*, Endocrine Reviews, 2021, Vol.42(2);198-218. For this publication, I performed 90% of the bibliographical review, prepared the figures and legends, and wrote 90% of the manuscript. JH contributed with 10% of the bibliographical review and a section of the manuscript. All co-authors provided revisions. AMJS final edits and revisions as the senior corresponding author. ***Part 2*** evaluates a novel antibody directed at the tumor necrosis factor receptor superfamily member 25 (TNFRSF25) as a means to induce endogenous *in vivo* Treg expansion, and its potential to delay or prevent graft rejection in an allogeneic murine islet transplant model. The work included in this section is presented from a first-author manuscript published in the ***American Journal of Transplantation*** (Marfil-Garza BA, Pawlick R, Szeto J, Kroger C, Tahiliani V, Hefler J, Dadheech N, Seavey M, Wolf J, Jasuja R, Shapiro AMJ), entitled *"Tumor Necrosis Factor Receptor Superfamily Member 25 (TNFRSF25) Agonists in Islet Transplantation: Endogenous In vivo Regulatory T Cell Expansion Promotes Prolonged Allograft Survival",* Am J Transplant, 2021, Vol 22:1101-1114. My role in this publication was designing the experiments, performing 50% of islet isolations and transplants, 100% of animal care and monitoring, conducting 80% of flow cytometry experiments, analyzing data, preparing figures and legends, and writing 100% of the manuscript. RP assisted with 50% of islet isolations and transplants. JS, CK, VT also assisted with 20% of flow cytometry experiments. MS, JW, RJ provided reagents for the experiments (mPTX-35). All co-authors provided revisions to the manuscript. AMJS provided final edits and revisions as a senior corresponding author.

In **Chapter 5,** we shift our efforts on preventing graft rejection and avoiding lifelong immunosuppression using cellular encapsulation. This chapter consists of two parts. ***Part 1***

includes an in-depth review of cellular encapsulation in T1D. This section is presented from a first-author thorough review published in ***Comprehensive Physiology*** (Marfil-Garza BA, Polishevska K, Pepper AR, Korbutt GS), entitled "*Current State and Evidence of Cellular Encapsulation Strategies in Type 1 Diabetes"*, Comprehensive Physiology, Vol 10(July 2020):839-878. For this publication, I performed 70% of the bibliographical review, prepared the figures and legends, and wrote 60% of the manuscript. KP also contributed to the 30% of the bibliographical review and 40% of the manuscript. All co-authors provided revisions. ARP and GK provided final edits and revisions as senior corresponding authors (GK [Prof. Gregory Korbutt] serves as a co-supervisor in my committee). ***Part 2*** evaluates a sequential prevascularization and encapsulation approach to enable subcutaneous pancreatic islet transplantation. In this collaborative project with Dr. Minglin Ma's group at Cornell University, we evaluate islet macroencapsulation using a thread-reinforced alginate-based macroencapsulation device coupled with our device-less approach for prevascularization of the subcutaneous site in syngeneic, allogeneic, concordant rat-to-mouse xenogeneic, and discordant human-to-mouse mouse models of pancreatic islet transplantation. This work is presented as a co-first author manuscript undergoing revisions for acceptance in ***Nature Biomedical Engineering*** (Wang LH, Marfil-Garza BA, Ernst AU, Pawlick RL, Okada K, Epel B, Viswakarma N, Kotecha M, Flanders JA, Datta AK, Ma M, and Shapiro AMJ) entitled "*Immunosuppression-free islet transplantation with a replaceable and scalable cell encapsulation device into a vascularized subcutaneous site".* My role in this publication was to conceive and design the experiments, conduct 50% islet isolations and transplants, 50% of animal care and follow-up, perform 30% of experiments, perform 50% of data analysis, prepare 40% of figures and legends, and write 50% of the manuscript. LHW, AMJS and MM also conceived and designed the experiments. LHW conducted 50% of islet isolations and

transplants, performed 70% of experiments, prepared 60% of figures and legends, and wrote 50% of the manuscript. AUE performed experiments and 100% of the computational models. RLP helped with islet isolations and transplants. JAF and LHW performed 100% of experiments with minipigs. BE, NV and MK contributed by providing technology for local O_2 measurements. AMJS and MM provided supervision and expert feedback for the final version of the manuscript.

Finally, **Chapter 6** provides an overview of the topics encompassed in this thesis and their implications for future research. Herein, I discuss the current challenges and unanswered questions in the field of pancreatic islet transplantation. In this section, I also provide some personal views on the priorities of clinical and basic research in the field of pancreatic islet transplantation, and the potential avenues to move forward in the future.

This thesis is an original work by Braulio Alejandro Marfil Garza. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Names: 1) "Comparison of Pancreas vs islet Transplantation at the University of Alberta", No. PRO00087040; 2) "Review of islet and progenitor cell transplant patients", No. PRO00001120. Animal studies also received ethics approval from the University of Alberta Animal Care and Use Committee (AUP00000331).

Chapter 5, Part 2 of this thesis forms part of an international research collaboration, led by Prof. A.M.J. Shapiro from the University of Alberta and Dr. Minglin Ma, from Cornell University. The encapsulation device used in this section of the thesis was designed by Dr. Minglin Ma's group at Cornell University, while the subcutaneous "device-less" implantation approach was developed in Prof. A.M.J. Shapiro's laboratory.

I hope that you find this thesis valuable and useful, and I sincerely wish that the work hereby included has a palpable repercussion in optimizing islet transplantation.

DEDICATION

I want to dedicate this thesis and all the work I have done throughout my PhD to my wonderful, unique and brave wife, Martha. Mi Boshita: your unwavering and unconditional support throughout the good and the bad times always carried me forward. Without you I would not have enjoyed these past few years as much as I have. You are my best friend and I will forever love you.

I also present this thesis in loving memory of all the people I lost throughout the years of my PhD and to whom I could not say goodbye in person. My cousin, but also my brother, Bernardo. Life will not be the same without you. I will miss you and carry you in my heart and in my thoughts always. You changed my perspective on life and taught me how to be brave in the most difficult moments. I will cherish that and honor your memory by always being the best I can be. Also, I want to mention my aunt Magda. You were like a second mother to me, and I will always remember you as one of the most loving persons I have met. Throughout your life, you gave so much to make us happy and I will thank you for that forever. To my grandmothers, abuelita Lilia and abuelita Yola. I miss you a lot and I will always remember you very fondly. To all of you, and many others that I have lost along the way, it profoundly hurts me that I could not say goodbye to you in person, but I know that you are happy and in peace. Please keep an eye on us, we need it. Big hugs to all of you wherever you are! I love you Berni, tía Magda, abuelita Lilia and abuelita Yola.

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I would like to use this opportunity to acknowledge and thank all the individuals and institutions that have supported my work and personal growth during my PhD.

First and foremost, I would like to extend my most profound gratitude to Prof. James Shapiro for his support and patience during my time as a PhD student. Dr. Shapiro, you are an inspiration for all of us working in the field of islet transplantation. The success you have achieved was the main reason I decided to come to Edmonton to train and learn from you. I will forever be grateful for your advice and guidance, but also for pushing me to achieve my fullest potential. I will also always remember and cherish our interactions during these last four years and I hope that this is only the beginning of a long-lasting and fruitful partnership.

I want to also thank the members of my supervisory committee, Dr. Gregory Korbutt and Dr. Peter Senior. It has been a pleasure and a privilege to have you as a part of my journey and I hope that we can continue to collaborate in the future.

I would like to acknowledge Dr. Fred Berry, Director of Graduate Education within the Department of Surgery. Thank you for always making yourself available to address my concerns. You are a great leader and I recognize your efforts in improving the quality of graduate research within the Department of Surgery.

I would like to appreciate Dr. Colin Anderson for serving as an arms-length examiner during my candidacy. Your profound knowledge in immunology is admirable and you motivated me to do my best during that important part of my journey. Additionally, I want to thank Dr. Patrick MacDonald for serving as an arms-length examiner during my candidacy and an external examiner during my final examination. I sincerely appreciate your openness to collaborate and, as we have discussed, I hope that we can work together in the future.

I want to extend my sincerest regards to Dr. Jose Oberholzer for agreeing to serve as an external examiner for my defence. Dr. Oberholzer, I admire the way you are leading the path forward for β-cell replacement therapies to become an accessible therapy for patients with T1D. You are a giant in the field and I feel honored to have met you.

A multitude of people in the laboratory have supported and carried me carry forward to conclude my projects. I want to specially than Rena Pawlick. Rena: none of the work I did would have been possible without your help. It has been a privilege to learn from you and to have you as one of my most important mentors. Beyond my appreciation of the time we spent working together, I will always cherish our honest conversations and sincere moments of friendship. You are an incredible person and I really appreciate your patience and understanding along the way. Next, I would like to thank Dr. Andrew Pepper. Andrew, you are a brilliant scientist, but most importantly, you are an extraordinary human being. I cannot thank you enough for all the talks we had over the years, both the academic, but also the personal. I want to continue with Anissa Gamble. Although our time together was short, we built a very good friendship and we enjoyed some great talks. In particularly, you gave me invaluable insight into what it is to live with type 1 diabetes. Your perspectives helped me guide the way I want to approach my research in the clinic. I want to continue with Joshua Hefler. Josh, you are a brave soul. Your resilience is an example for all of us. I will always remember our wings Wednesdays and your incursions into the office to talk and vent. To Nidheesh Dadheech, you are a brilliant researcher and what you've accomplished during your time in the lab is monumental. I will always be grateful for inviting us to your home and trying to create bonds beyond work. Kevin, we've only known each other for less than a year, but I want to thank you for motivating me to work hard and aim high. You are a tour de force and I really admire your energy and enthusiasm. Nerea, you are a brilliant scientist and a

great friend. Thank you for the good times outside the lab and the conversations we've shared over the last few months. I will also like to mention Ila Jasra. Ila, you are a kind soul and I appreciate our time together in the office. I will also like to thank the students that participated directly or indirectly in my projects and that helped me carry out my work in the laboratory. These include Patrick Albers, Sirtaj Jhutty, Mark Farinas, Zhe Fan, Sofia Varelas, Sandhya Sapkota, Omar Mouhammed, and many others. Specially, I want to thank Jake Szeto and Steffane McLennan, both of you taught me how to be a supervisor and I can only wish you learned something valuable from me and that you forgive my many shortcomings.

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I want to allocate a few words of gratitude to the researchers, and now friends, that I met during my time at Cornell University. The week I spent in Ithaca was one of the highlights of my PhD. Long-Hai, my brother, and probably the hardest working researcher that I have met. I wish you the best in this new stage of your life as a principal investigator in China. You will always have a friend and a colleague in me, wherever my life takes me. Alec, an exemplary PhD student and a brilliant individual. Finally, Dr. Minglin Ma, a great leader that I profoundly admire for the work he's doing at Cornell.

I want to also extend a word of recognition to the tremendous work done by all the members of the Clinical Islet Transplant Program. In particular, I want to thank Dr. Tatsuya Kin for welcoming me in such a friendly way, and for including me in his research projects. I consider myself lucky to have your support for my future endeavors and I know we will do great things together in the future. Doug, I am really excited for the projects that we have

started together, I am sure this is only the beginning of a great partnership. Finally, I want to thank Feiyue, Indri, Rayna, Dayne, Parastoo and Rosemary. Additionally, I want to honor Andrew Malcolm, a kind soul and a foundational pillar within the Clinical Islet Transplant Program which will be missed greatly by all of us. Together, all of you have helped me in innumerable ways and I will always be in debt to you. Beyond the work aspects, you have always treated me kindly, which I have always appreciated.

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Thank you to HSLAS staff that so diligently took care of our mice. Mirjana and Erin, I want to thank you for having so much patience with me during my training and for helping me along the way.

I want to recognize the tremendous support that has been given to me by the different funding institutions, the Ray Rajotte Graduate Award, the Alberta Graduate Excellence Scholarship, but most importantly, the Patronato del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and the Fundación para la Salud y la Educación Dr. Salvador Zubirán (FunSaEd). I want to thank Luz María Aguilar Valenzuela and Rosa María Hernández for your unwavering support and patience throughout my studies. Without your help, I would not be here.

Finally, I want to thank my family, for making me the person I am today, for carrying me forward through the difficult times and for always being there for me when I needed you. I truly missed you having here throughout my journey, and I hope to share more moments together in the future. You are my rock and I love you all.

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95% CI – 95% confidence interval

99mTc DTPA – Technetium 99 diethylene-triamine-pentaacetate

ACT – adoptive cell transfer

APC – antigen-presenting cell

ATG – anti-thymocyte globulin

ATT – adoptive Treg transfer therapies

BAP – bioartificial pancreas

BMI – body mass index

CAD – coronary artery disease

CAR – chimeric antigen receptor

CCR2 – C-C chemokine receptor type 2

CD – cluster of differentiation

CITR – Collaborative Islet Transplant Registry

CKD – chronic kidney disease

CKD-EPI – chronic kidney disease epidemiology collaboration

CMV – cytomegalovirus

CNI – calcineurin inhibitor

cPRA – calculated panel reactive antibodies

CRISPR – clustered regularly interspaced short palindromic repeats

CsA – cyclosporine A

CTLA4 – cytotoxic T-lymphocyte antigen 4

DCCT – diabetes control and complications trial

DPP-4 – dipeptidyl peptidase-4

DR3 – death receptor 3

DSA – donor-specific antibodies

EDIC – epidemiology of diabetes interventions and complications

EFA – efalizumab

eGFR – estimated glomerular filtration rate

EPITA – European Pancreas and Islet Transplant Association

EPR – electron paramagnetic resonance

ESRD – end-stage renal disease

ETA – etanercept

FACS – fluorescence activated cell sorting

FBR – foreign-body response

FDA – Food and Drug Administration

FOXP3 – forkhead box protein 3

FPG – fasting plasma glucose

GCSF – granulocyte-colony stimulating factor

GFR – glomerular filtration rate

GLP-1 – glucagon-like peptide-1

GSIS – glucose-stimulated insulin secretion

GWAS – genome-wide association studies

HbA1c – hemoglobin A1c

HBSS – Hank's Balanced Salt solution

hESC – human embryonic stem cells

hiPSC – human induced pluripotent stem cells

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HR – hazard ratio

HSCT – hematopoietic stem cell transplant

IAK – islet-after-kidney transplant

IBMIR – instant blood-mediated inflammatory reaction

IEQ – islet equivalent

IL – interleukin

IM – intramuscular

INSERM – institut national de la santé et de la recherche médicale

IPEX – immunodysregulation polyendocrynopathy enteropathy X-linked syndrome

IPGTT – intraperitoneal glucose tolerance test

IPITA – International Pancreas and Islet Transplant Association

IPTR – international pancreas transplant registry

IQR – interquartile range

IS – immunosuppression

ITA – islet transplant alone

ITx – pancreatic islet transplantation

IV – intravenous

IVGTT – intravenous glucose tolerance test

KC – kidney capsule

LBL – layer-by-layer coating

LDL – low-density lipoproteins

LOI – lumee oxygen index

MHC – major histocompatibility complex

MMF – mycophenolate mofetil

MMTT – mixed-meal tolerance test

MPDN – methylprednisolone

MSC – mesenchymal stem cells

mTOR – mammalian target of rapamycin

NHP – non-human primates

NK – natural killer

NOD – non-obese diabetic

nPOD – network for pancreatic organ donor with diabetes

NSGS – non-sustained graft survival

OGTT – oral glucose tolerance test

OR – odds ratio

OT – operational tolerance

P-PASS – pancreas allocation suitability score

PAD – peripheral artery disease

PAK – pancreas-after-kidney transplant

PAN – polyacrylonitrile

PCL – polycaprolactone

PDRI – pancreas donor risk index

PEC – pancreatic endocrine cells

PEG – poly(ethylene glycol)

PERV – porcine-endogenous retrovirus

PGA – poly-glycolide

PLA – poly-lactide

PLGA – poly(lactic-co-glycolic acid)

PLL – polycation poly-L-lysine

PLN – pancreatic lympho nodes

PLO – poly-L-ornithine

polyHEMA – poly(2-hydroxyethyl methacrylate

PTA – pancreas transplant alone

PTFE – polytetrafluoroethylene

PTx – whole pancreas transplantion

PU – polyurethane

PVC – polyvinyl chloride

SC – subcutaneous

SGLT2 – sodium-glucose transporter-2

SGS – sustained graft survival

SHE – severe hypoglycemic episode

SHEATH – subcutaneous host-enabled alginate thread

SIK – simultaneous islet-and-kidney transplant

SOT – solid organ transplantation

SPK – simultaneous pancreas-and-kidney transplant

SRL – sirolimus

STZ – streptozotocin

SUITO – secretory unit of islet transplant objects

T1D – type 1 diabetes

T2D – type 2 diabetes

TAC – tacrolimus

TCR – T cell receptor

TGF-beta – transforming growth factor beta

TLA1 – tumor necrosis factor-like cytokine 1A

TNF – tumor necrosis factor

TNFR1 – tumor necrosis factor receptor 1

TNFRSF25 – tumor necrosis factor receptor superfamily member 25

Treg – regulatory T cell

UKPDS – UK prospective diabetes study

UNOS – united network for organ sharing

VEGF – vascular endothelial growth factor

VIF – variance inflation factors

Xeno-ITx – islet xenotransplantation

CHAPTER 1

PART 1 - CLINICAL ISLET TRANSPLANTATION: CURRENT PROGRESS AND NEW FRONTIERS

CHAPTER 1, PART 1 - CLINICAL ISLET TRANSPLANTATION: CURRENT PROGRESS AND NEW FRONTIERS

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TOPIC

Clinical islet transplantation: Current progress and new frontiers

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Abstract

Islet transplantation (IT) is now a robust treatment for selected patients with type 1 diabetes suffering from recurrent hypoglycemia and impaired awareness of hypoglycemia. A global soar of clinical islet transplant programs attests to the commitment of many institutions and researchers to advance IT as a potential cure for this devastating disease. However, many challenges limiting the widespread applicability of clinical IT remain. In this review, we will touch on the milestones in the history of IT and its path to clinical success, discuss the current challenges around IT, propose some possible solutions, and elaborate on the frontiers envisioned in the future of clinical IT.

KEYWORDS

beta-cell, diabetes, insulin, islet, transplantation

1 | INTRODUCTION

Islet transplantation (IT) has progressed substantially in the last two decades, and is now an effective therapy for many patients with type 1 diabetes (T1D), particularly those suffering from recurrent hypoglycemia and impaired awareness of hypoglycemia.[1] While IT is becoming accessible through funding and implementation of clinical islet transplant programs all over the world,[2] its widespread applicability is hampered by several challenges. Innovative efforts to surmount these obstacles are being carried out by researchers all over the world. Herein, we will touch on the milestones in the path to clinical success, discuss the current challenges and solutions, and elaborate on the frontiers envisioned for the future of clinical IT.

2 | THE PATH TO CLINICAL SUCCESS

It is nearly 100 years since the discovery of insulin. Frederick Banting, Charles Best, John MacLeod, and James Collip changed medical history by isolating and purifying insulin, the pivotal hormone in glucose homeostasis and the central (absent) player in the pathophysiology of diabetes. Before discovering insulin, the link between the pancreas and diabetes, first proposed by von Mering and Minkowski in 1890, had prompted attempts to reverse diabetes through pancreatic tissue transplantation. For example, Watson-Williams and Harsant performed xenotransplantation of fragmented sheep pancreatic tissue for the first time in 1894; Pybus followed with the first allotransplants of sliced pancreatic tissue into two patients with diabetes in 1924.[3] The modern era of IT came with Lacy's work, who spearheaded a paradigm shift from transplantation of pancreatic tissue to transplantation of purified islets. Building on Moskalewski's methods for pancreatic tissue digestion, Lacy's group introduced intraductal injection and, subsequently, density gradient separation to improve islet yield and quality, which ultimately allowed diabetes reversal in animal models.[4] Lacy's group also introduced the liver's intraportal circulation as an implantation site for islets,[5] which is now the clinical gold standard. These achievements strongly supported migration to the clinical realm.

Clinical outcomes have been improved in parallel with refinements in islet isolation techniques (Figure 1). Najarian et al.

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Title: Clinical Islet Transplantation: Current Progress and New Frontiers

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1.1.1 - Abstract

Islet transplantation (ITx) is now a robust treatment for selected patients with type 1 diabetes suffering from recurrent hypoglycemia and impaired awareness of hypoglycemia. A global soar of clinical islet transplant programs attests to the commitment of many institutions and researchers to advance ITx as a potential cure for this devastating disease. However, many challenges limiting the widespread applicability of clinical ITx remain. In this review, we will touch on the milestones in the history of ITx and its path to clinical success, discuss the current challenges around ITx, propose some possible solutions, and elaborate on the frontiers envisioned in the future of clinical ITx.

1.1.2 - Introduction

Islet transplantation (ITx) has progressed substantially in the last two decades, and is now an effective therapy for many patients with type 1 diabetes (T1D), particularly those suffering from recurrent hypoglycemia and impaired awareness of hypoglycemia.[1] While ITx is becoming accessible through funding and implementation of clinical islet transplant programs all over the world,[2] its widespread applicability is hampered by several challenges. Innovative efforts to surmount these obstacles are being carried forward by researchers all over the world. Herein, we will touch on the milestones in the path to clinical success, discuss the current challenges and solutions, and elaborate on the frontiers envisioned for the future of clinical ITx.

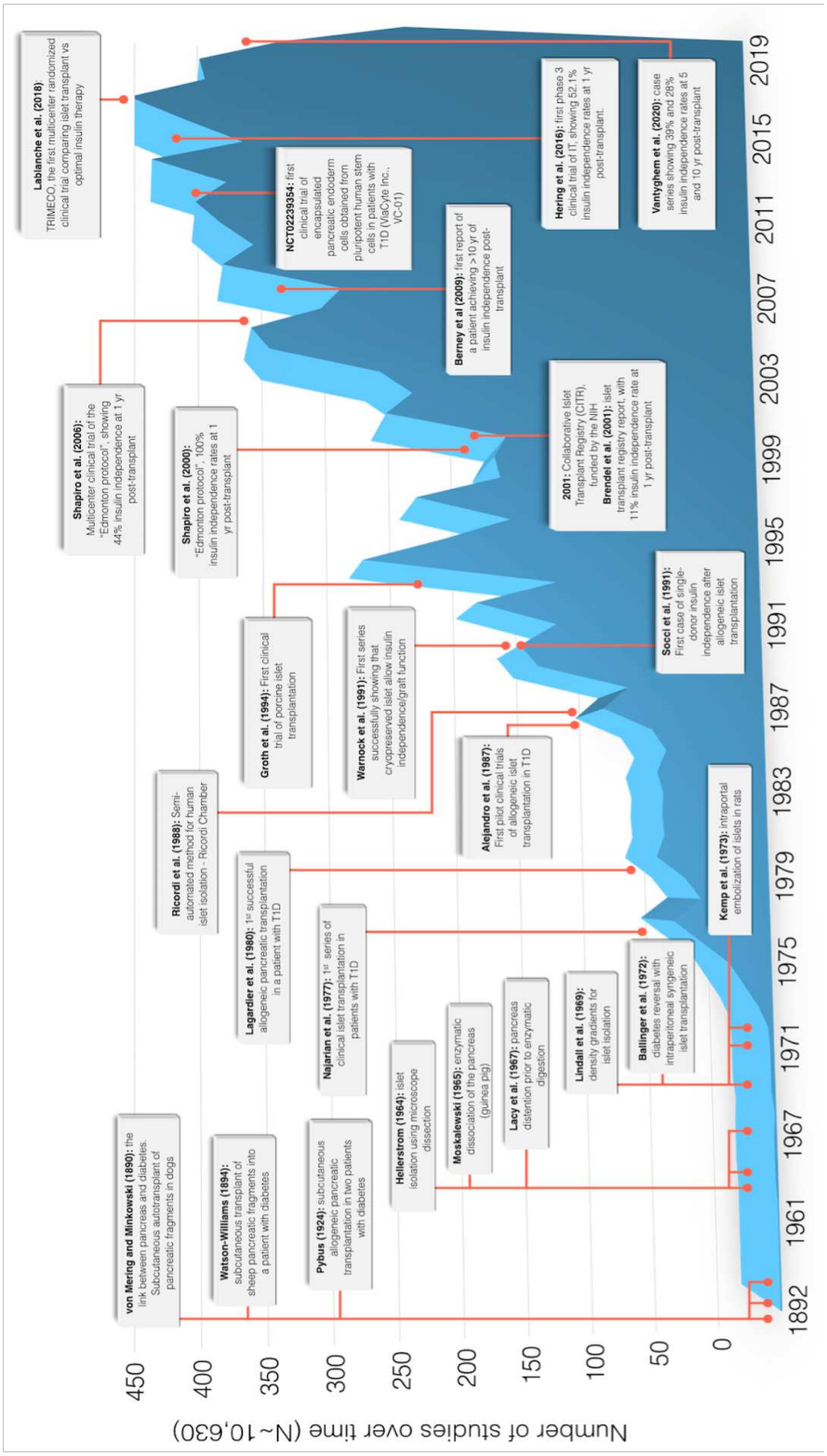
1.1.3 - The Path to Clinical Success

It is nearly 100 years since the discovery of insulin Frederick Banting, Charles Best, John MacLeod and James Collip changed medical history by isolating and purifying insulin, the pivotal hormone in glucose homeostasis and the central (absent) player in the pathophysiology of diabetes. Before discovering insulin, the link between the pancreas and diabetes, first proposed by von Mering and Minkowski in 1890, had prompted attempts to reverse diabetes through pancreatic tissue transplantation. For example, Watson-Williams and Harsant performed xenotransplantation of fragmented sheep pancreatic tissue for the first time in 1894; Pybus followed with the first allotransplants of sliced pancreatic tissue into two patients with diabetes in 1924.[3] The modern era of ITx came with Lacy's work, who spearheaded a paradigm shift from transplantation of pancreatic tissue to transplantation of purified islets. Building on Moskalewski's methods for pancreatic tissue digestion, Lacy's group introduced intraductal injection and, subsequently, density gradient separation to

improve islet yield and quality, which ultimately allowed diabetes reversal in animal models.[4] Lacy's group also introduced the liver's intraportal circulation as an implantation site for islets,[5] which is now the clinical gold standard. These achievements strongly supported migration to the clinical realm.

Clinical outcomes have been improved in parallel with refinements in islet isolation techniques (**Figure 1.1.1**). Najarian et al. first showed the feasibility to achieve insulin independence following ITx, this was demonstrated in patients undergoing autotransplantation (i.e., requiring no immunosuppression).[6] Shortly thereafter, Largiadèr et al. reported the first case of insulin independence following allotransplantation of pancreatic microfragments into the spleen.[7] Subsequent clinical success, however, was hampered by impure islet preparations leading to complications such as portal vein thrombosis.[8] In this regard, a major leap forward was achieved with the semi-automated method for islet isolation developed by Ricordi et al..[9] This method substantially improved islet yield and quality, while increasing replicability of islet isolation protocols. Incorporation of an automated continuous gradient cell separation by Lake et al., and the addition of cold preservation solutions to these gradients by Olack et al., ultimately set the stage for standardized clinical islet isolation protocols.[10] Clinical reports achieving variable rates of success,[11-13] even using cryopreserved islets,[14] followed (**Figure 1.1.1**). However, up to the year 2000, only ~11% of patients achieved insulin independence beyond 1-year post-transplant. This changed with the groundbreaking "Edmonton protocol", which was the first to show 100% 1-year insulin independence rates post-transplant in non-uremic patients with T1D.[15] Beyond the scientific milestone, the "Edmonton protocol" reinvigorated ITx research and marked a new era in the field.

Figure 1.1.1. The path to clinical success, a timeline of the major landmarks in the field of islet transplantation.



Note: A non-systematic search in PubMed for publications using the terms islet transplant* AND diabetes up to September 2, 2020. Relevant studies before 1942 were selectively added to the figure due to their importance in the field. Additionally, we included Moskalewski, 1965 in the figure, despite not appearing in the original search, due to its relevance.

The most recent report of the Collaborative Islet Transplant Registry shows that five-year abrogation of severe hypoglycemic episodes is achieved in >90%, optimal glycemic control (HbA1c <7%) in ~60%, and insulin independence in ~30% of patients following ITx.[2] A recent multicenter report of a phase 3 study supports these numbers, with rates of a composite outcome of optimal glycemic control (HbA1c < 7.0%) and complete absence of severe hypoglycemic episodes (SHEs) of 87.5% and 71% at 1- and 2-years post-transplant.[16] Islet-after-kidney transplantation seems to be also effective, as recently described in a recent Clinical Islet Transplant consortium trial including 24 patients. In this trial, similar outcomes (e.g., HbA1c <6.5% + absence of SHEs) were achieved in 62.5%, 58.3% and 45.8% at 1, 2, and 3 years post-ITx. Notably, awareness of hypoglycemia was almost completely restored at 1 year post-ITx.[17] Recent reports from the integrated UK programme,[18] from San Raffaele Hospital in Milan[19] presented at the International Pancreas and Islet Transplant Association's congress in 2019 and from the Edmonton group presented at the 80th Scientific Session of the American Diabetes Association[20] consistently show substantial improvement in glycemic control, reductions in insulin doses and a nearly complete abrogation of SHEs. This level of glycemic control and its effect on abrogation of SHE and awareness of hypoglycemia has not been achieved with state-of-the-art insulin therapies; results from the only published randomized clinical trial also support this notion.[21] Of note, some centers have reported metabolic outcomes comparable to whole pancreas transplantation,[19, 20, 22-24] however, significant heterogeneity between centers remains an issue. Ten-year outcomes have begun to appear more frequently, many of these showing optimal glycemic control and insulin independence rates of 18-28%.[20, 25, 26] Lastly, ITx has shown to ameliorate progression of diabetic complications as compared to intensive insulin therapy.[27] This accumulated experience has been recently published by Freige and cols. from the Canadian Agency for Drugs and Technologies in health in a systematic review[28] and the

interested reader is referred to this report. Overall, these studies highlight the potential for ITx to positively impact the lives of an extremely complex subset of patients living with T1D.

1.1.4 - Indications and Procedural Considerations

ITx is a safe procedure. The most recent Collaborative Islet Transplant Registry report shows a mortality rate at 5 years of 1.6% in patients undergoing an islet transplant alone.[2] Longer follow-up shows a mortality of 0.3-3.28% per 100 patient-years.[20, 25, 29] However, adverse events related to infusion and immunosuppression are frequent, with rates of life-threatening events as high as 24.0% in the early eras of ITx (1999-2002);[30] these have substantially decreased in most recent eras (~ 3-8%).[2] Thus, a favorable risk-benefit ratio must be present to proceed with ITx. Currently, this is the case for patients with T1D suffering from problematic hypoglycemia and/or impaired awareness of hypoglycemia, in which the perceived benefits outweigh the risks related to the procedure and the commitment to lifelong immunosuppression. Evidence-informed clinical practice recommendations by the American Diabetes Association[31] state that islet or pancreas transplantation should be considered for patients whose problematic hypoglycemia persists (stage 4) despite structured education programs (stage 1) and the use of continuous subcutaneous insulin infusion or continuous glucose monitoring (stage 2) with sensor-augmented insulin pumps (stage 3).[31] Although this step-wise approach follows an appropriate risk gradient, these recommendations may be limited by local resources, insurance reimbursement, and patient choice. Guidelines by the TREPID working group and the IPITA/EPITA opinion leader workshop provide more context-specific recommendations accounting for physiological age, weight, cardiovascular risk, the presence of end-organ damage, previous organ transplantation (and sensitization state), current use of immunosuppression, and importantly, the patient's capacity to deal with hypoglycemia.[32, 33]

Instruments to assess severity of hypoglycemia, glycemic lability and impaired awareness of hypoglycemia, such as the HYPO score, the glucose coefficient of variation and the Clarke and Gold scores, respectively, are suggested as part of clinical assessment to decide on eligibility for β-cell replacement therapies, but also to define appropriate patient-centered outcomes and realistic expectations.[33] Proposed indications for ITx are summarized in **Table 1.1.1**.

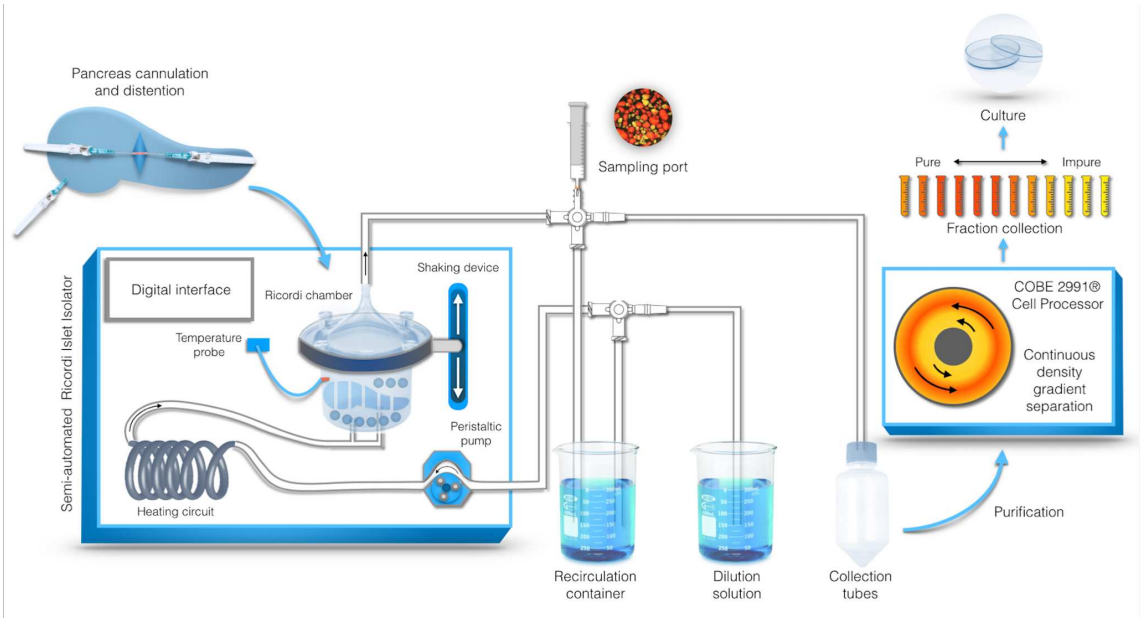
Table 1.1.1. Indications for Islet Transplantation

Indications for Islet Transplantation*
Confirmed T1D (absent C-peptide), with a duration >5 years, and a HbA1c > 7.5-8.0%
Age > 18 years old
Insulin requirements <1.0 U/kg/day
Absence of malignancy or untreated infection
Ability to comply with immunosuppression and proper follow-up
Problematic hypoglycemia (≥ 2 episodes/year of severe hypoglycemia) despite optimal glycemic management with insulin pump and adequate monitoring by a diabetologist or endocrinologist - Recurrent episodes of diabetic ketoacidosis and/or severe, rapidly progressing complications of diabetes may also be considered
One episode of severe hypoglycemia/year *plus* evidence of impaired awareness of hypoglycemia and/or extreme glycemic lability using objective scores, such as the Clark score (≥ 4), HYPO score (≥ 1000) or lability index (≥400). - Major fear or maladaptive behavior related to hypoglycemia may also be considered

*Adapted from Dajani KZ and Shapiro AM, 2019,[4] Wojtusciszyn A et al., 2018[32] and Rickels MR et al., 2018.[33]

ITx is a unique type of transplant because it demands processing of the donor pancreas to conduct islet isolation, which is done by tissue specialists at dedicated facilities, following good-manufacturing practices. This process is described in **Figure 1.1.2**.

Figure 1.1.2. Islet isolation process.



Note: After "decontaminating" the pancreas (removing spleen, duodenum and peripancreatic fat), a small incision in the mid-body of the pancreas is done to expose the main pancreatic duct, which is then cannulated using two catheters; the outflow tract at the major papilla is sealed using a third catheter. Pancreas distention with cold collagenase infused through both catheters using a perfusion machine (not shown) follows. The pancreas is then sliced and placed in the Ricordi chamber for digestion. Enzymatic digestion is sustained by activating collagenase through controlled warming (~ 36ºC) using a heating circuit and a temperature probe. Mechanical digestion is facilitated using orbital shaking of the chamber and silicon nitride/metal marbles inside the chamber. The solution is recirculated through the circuit until the pancreas is appropriately digested and islets are visibly dislodged from the exocrine tissue; this is evaluated by taking samples at predefined time points through a sampling port and staining them with dithizone. Once digestion is appropriate, the circuit is "opened" to dilute the solution and stop enzymatic activity. The islets are then collected. The collected tissue is introduced into a cell processor for purification using continuous density gradient centrifugation. Tissue fractions are collected from the cell processor and the highly-pure fractions are selected to be cultured and, subsequently, transplanted

After islet isolation, several quality measures should be ensured to "release" the tissue for transplantation. These may include sterility, potency (insulin stimulation index >1), cell volume (<5 cc of packed cell volume), as well as sufficient purity (>30%) and viability (>70%). Suitable islet preparations are then infused into a patient through percutaneous cannulation of the portal vein by interventional radiologists; portal pressures are measured at baseline and during infusion, being careful not to surpass a 5-mmHg pressure change in portal pressure due to the risk of portal vein thrombosis.[34] Finally, the cannulation tract is obliterated using a

thrombostatic paste to minimize the risk of bleeding. Peritransplant infusion of heparin and insulin is then started, which has now shown to be correlated with better engraftment and higher rates of insulin independence post-transplant.[35]

A relevant aspect for the global applicability of ITx is its cost-effectiveness. Unfortunately, there is scarce evidence on this topic. Early studies from the GRAGIL consortium showed that the average cost of an ITx was €77,745, with 30% attributed to islet isolation and preparation.[36] The authors reported that costs were significantly higher than those associated with pancreas transplantation, which was later supported by comparative analysis of patients undergoing simultaneous islet-kidney and pancreas-kidney transplants.[37] In 2016, Moassesfar at al. found no significant differences in cost between ITx alone and pancreas transplantation alone; these authors, however, reported costs of $138,872 USD and $134,748 USD for ITx alone and pancreas transplantation alone, respectively.[24] Cost-utility analyses of ITx have shown that ITx is cost-effective, albeit at high willingness-to-pay thresholds (e.g., $100,000 CAD) and over the long-term.[38-40] These studies have compared ITx to insulin therapy, which are not mutually exclusive or equivalent, and will have to be updated as procedural and immunosuppression costs decrease, as reports on long-term outcomes become more prevalent, and as stem cell therapies enter the clinic.[40] Of note, ITx is reserved for patients failing multiple interventions to prevent problematic hypoglycemia. In these highly complex patients, ITx would be justified, even if costly.

1.1.5 - Challenges and Potential Solutions

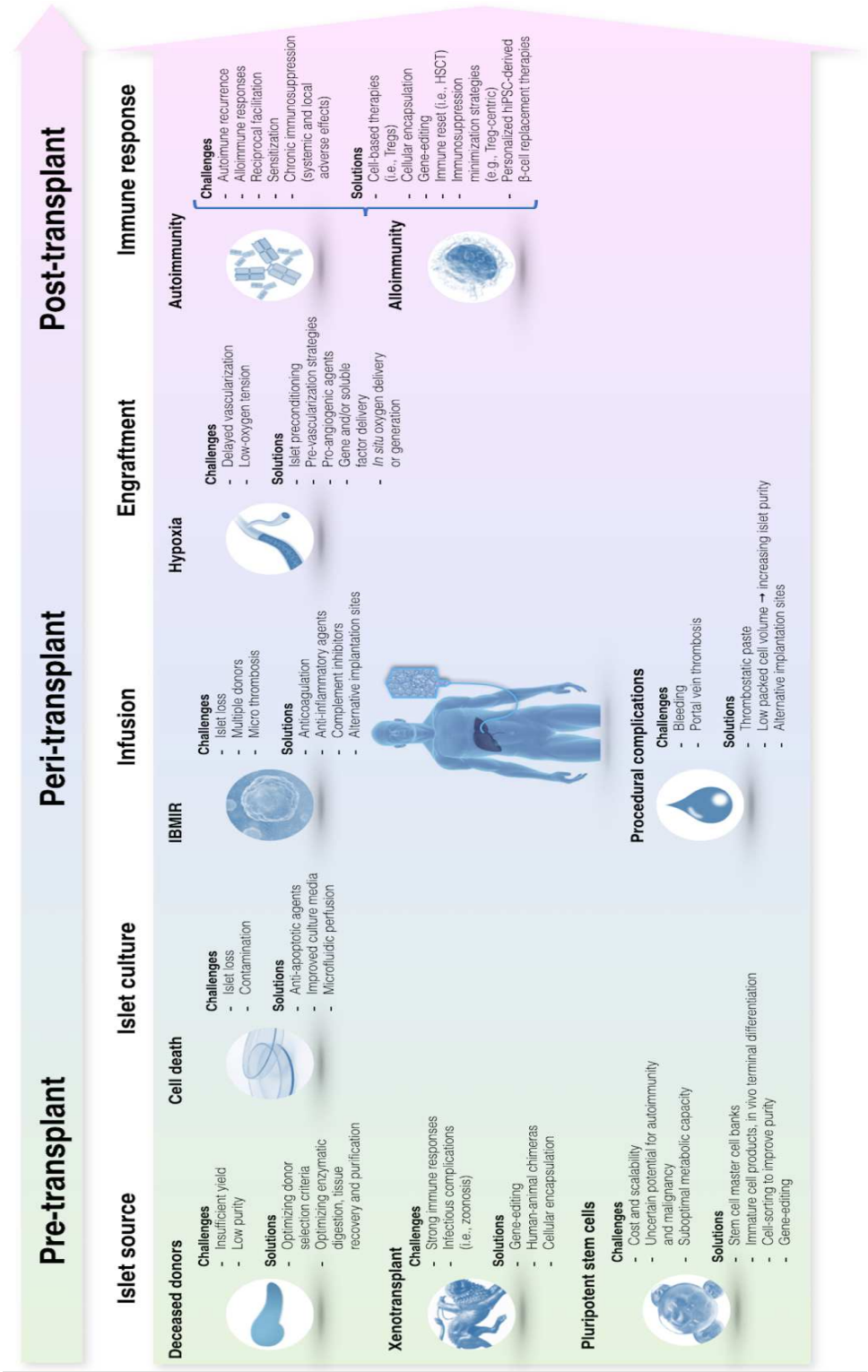
The main challenges to advance ITx as a potential cure for T1D are: limited islet source, significant cell death, suboptimal engraftment, and the need for lifelong immunosuppression. In

this section, we will discuss these and other issues preventing efficient clinical translation. We summarize these challenges and propose some potential solutions in **Figure 1.1.3**.

1.1.5.1 - Islet Source

Although human islet isolation has improved significantly over the last 20 years, the fact that ~ 30% of expenses associated with ITx are related to islet isolation[36] highlights the need to make the most efficient use of resources. One approach is to optimize donor selection to increase islet yield and quality, which would translate into fewer donor pancreata needed to attain a sufficient islet mass to support long-term graft survival and insulin independence. Several donor characteristics (age, weight, height, body mass index (BMI), body surface area, pancreas weight) and hospitalization and procurement characteristics (cause and type of death [brain or cardiac], length of hospitalization, use of vasopressors, glycemic control, cold ischemia time, pancreas physical properties) may predict the quantity and quality of the islet preparations and some of them correlate with clinical outcomes.[41, 42]

Figure 1.1.3. Challenges to advance clinical islet transplantation as a cure for type 1 diabetes and potential solutions



Weight, height, and BMI, appear to be the most consistent donor characteristics correlated with islet yield and quality,[42-44] which may be explained by a positive correlation between BMI and pancreas weight.[45] The first effort to standardize pancreatic donors for islet isolation came with the Islet Donor Score, by O'Gorman et al., in which characteristics of the donor, as well as physical properties of the pancreas, were shown to predict successful islet isolation.[46] This was followed by the North American Islet Donor Score, which was derived from a multicentric study in which donor characteristics of 1,056 isolations were analyzed to obtain predictor variables for successful isolation.[47] This scoring system showed a useful predictive capacity for successful isolations[47] and could be a useful tool to optimize donor selection. Although there is less information on donor selection aspects predicting islet function, age has been negatively correlated with stimulation indexes as well as insulin secretion as a proportion of total insulin content.[48] Similarly, cold-ischemia time may negatively correlate with insulin content.[48] Thus, expedite transport of donor pancreata to islet processing facilities to minimize cold ischemia should be promoted. More research looking at factors affecting the success of islet isolations is needed, however, a systematic approach to reporting these data would allow a comprehensive analysis of these aspects. Adequate reporting of the characteristics of human islet preparations used for preclinical research could also open research avenues for improvement of islet isolation processes and, ultimately, have a positive impact on clinical outcomes.[49]

Optimizing the isolation process itself is an alternative to increase islet yield and quality. In this regard, much of the research has focused on improving enzymatic digestion. The efficacy of tissue dissociating enzymes for islet isolation (i.e., collagenase and proteases) is hampered by variability in extracellular matrix and basement membrane components of each donor

pancreata. This complicates establishing standardized protocols for islet isolation. Digestion enzymes have undergone a process of refinement and purification to decrease contamination with endotoxins and lot-to-lot variability and efforts to better characterize the exact composition of these enzymes,[50] as well as incorporating other proteases that are synergistic with collagenase activity,[51] have significantly improved islet yield and quality. However, significant lot-to-lot variability and donor pancreas heterogeneity remains an issue.[52] Factors affecting enzymatic activity and digestion include age, cold ischemia time, preservation solutions and temperature.[53] In this regard, the use of recombinant and/or high-purity/enriched enzymes, as well as context-/donor-specific collagenase:protease ratios have improved islet isolation consistency and quality.[52] Importantly, these interventions should be combined with strategies limiting cell death during culture, a pivotal step in the process of ITx. During the first 24 hours of culture, ~15-20% of the islets are lost. Longer cold ischemia time, lower islet purity, two-layer preservation methods and higher islet indexes (big islets) increase the risk of islet loss.[54] Fortunately, many interventions can improve islet recovery after culture,[55] albeit a thorough revision is beyond the scope of this review. While providing flexibility to clinical ITx programs, islet culture is a major issue precluding FDA approval of clinical ITx in the US, since this step is perceived to violate the principle of minimal manipulation required for approval of cell-based products. Thus, interventions during islet culture will have to balance its positive effects while showing no relevant biologic alterations that could further complicate regulatory approval.

Islet xenotransplantation (Xeno-ITx), mainly using pig islets, has been moving into the clinical realm over the past decades. The principal reason to strive for Xeno-ITx using pig islets is that there is unlimited availability, however, other aspects such as these islets coming from an ethically acceptable source, the pig pancreas being similar to the human pancreas, their low

cost and the potential for Xeno-ITx to be an elective, rather than an urgent procedure further strengthen the case for Xeno-ITx.[56] The first successful Xeno-ITx was done by Groth et al. in 1994. In this report, ten patients with T1D were treated with fetal pig islets and, while no significant metabolic improvement was achieved, islet survival was documented up to 400 days post-transplant.[57] Islet survival for up to 9.5 years with the use of cellular encapsulation was later documented by Elliot et al..[58] Throughout its evolution, a number of reports have demonstrated a certain degree of efficacy with Xeno-ITx in terms of glycemic control.[59] A recent abstract presented by Xiaoqian et al. at the international congress of The Transplantation Society evaluating porcine Xeno-ITx coupled with immunosuppression and autologous Treg infusion showed significant metabolic improvement, with a 45% decrease in insulin requirements and a 22.5% decrease in HbA1c levels at a 1-year follow-up.[60] However, outcomes have not yet equated those observed with human ITx, and few cases of transient insulin independence have been reported.[59] This may be explained by lower doses as compared to human ITx, as well as stronger immune responses. The latter is one of the main limitations with Xeno-ITx, which, as previously mentioned, has been tackled using cell-based therapies and cellular encapsulation (see below). Another concern with Xeno-ITx is the risk of infection. Within the potential infectious pathogens, the porcine-endogenous retrovirus (PERV) is the most commonly recognized, albeit this has become a central dilemma in the field. Patience et al. initially described PERV transmission from porcine to human cells in vitro,[61] however, no cases of in vivo transmission of PERV have been documented in preclinical and clinical trials to date.[62] A recent report by Matsumoto et al. has shown no presence of PERV (or any other porcine viruses) in patients undergoing encapsulated porcine Xeno-ITx over a 5 to 7-year follow-up period;[63] which coincides with results from the previously mentioned pilot clinical

trial by Xiaoqian M et al..[60] These reports should alleviate any concerns around the microbial safety of porcine Xeno-ITx, which, coupled with efficient large-scale isolation of porcine islets should support more clinical trials in the near future.

A concise comment on stem cell therapies in T1D, mainly human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), is in order. The main principle with stem cell therapies in T1D is recapitulating the in vivo islet differentiation processes (i.e., those occurring during embryogenesis) to generate functional human islets in vitro. These therapies provide a potentially unlimited islet supply and a unique opportunity to study these structures at a patient-specific level, as well as to modify cell products to optimize their potency, but more importantly, to decrease or even eliminate their immunogenicity.[64] hESCs were first used as a "starting product" for islet and β-cell differentiation protocols and quickly showed similar potency as mature islets in terms of diabetes reversal in pre-clinical models.[65-67] Following the landmark report of Takahashi and Yamanaka in 2006 showing that differentiated human cells (i.e., skin fibroblasts) can be reprogrammed (induced) into a pluripotential state (i.e., hiPSCs) using forced gene expression (i.e., Oct4, Sox2, Klf4, c-Myc),[68] efforts to use this alternative source for cell therapies quickly permeated into the field.[69] First achieved by Alipio et al. in 2010,[70] diabetes reversal in pre-clinical models using hiPSC-derived islet- or β-like clusters is now frequently reported (for a detailed review, see Velazco-Cruz et al.[71]). Overall, the pluripotent state of both hESCs and hiPSCs endows them with similar properties, however, which "starting material" is better to generate functional human islets is unknown. Ethical issues dominate the debate, and naturally favor hiPSCs. However, beyond the ethical debate, aspects regarding genome integrity and regulation, abnormal developmental potential, as well as costs and scalability should also be considered. Briefly, current evidence suggests that hiPSCs have

a higher risk of development and accumulation of genomic mutations, partly due to unsilenced reprogramming factors.[72] hiPSCs have a variable yield in terms of the mature/terminal cell products following differentiation, which has been attributed to differences in transcriptomes and methylomes possibly originated from the epigenetic memory of hiPSCs, given their somatic origin as compared to the germ-line origin of hESCs.[73] This could promote difference in cellular composition between stem-cell derived islets and mature human islets, which is being tackled by β and α cell enrichment using fluorescence activated cell sorting (FACS) or magnetic beads sorting.[74] β- or α cell enrichment, however, poses a problem of "unphysiological" β or α cell ratios within islets, but whether this has any consequences in islet function remains an open question. hESCs have accumulated more evidence than hiPSCs in terms of differentiation efficiency and overall safety. In fact, only hESCs-derived β-like cells are currently being tested clinically in patients with T1D (NCT03162926, NCT03163511, NCT02239354 and NCT02939118). Finally, costs and scalability seem to also favor hESCs over hiPSC.[75] However, the unavoidable fact that hiPSCs provide the only path to truly personalized regenerative medicine supports a robust argument to drive future efforts to optimize its production and extend their use.

1.1.5.2 - Islet Survival and Engraftment

Over 25% of islets are immediately lost after infusion into the portal circulation.[76] The number of transplanted (and surviving) islets is a predictor of better graft function and insulin independence,[42] highlighting the importance of preventing islet loss in the peri-transplantation period. The central phenomenon explaining immediate islet loss is the instant blood-mediated inflammatory reaction (IBMIR). This is a complex response of the innate immune system

triggered by direct exposure of islets (and tissue factor) to the bloodstream. IBMIR is not restricted to the allo- or xenotransplantation setting, as evidence in autologous human ITx has been reported.[77] IBMIR consists of activation of the coagulation cascade, the complement pathway, cytokine secretion and acute cell-mediated injury.[78] Accordingly, interventions to prevent IBMIR act on these components. Hence, anticoagulation and antiplatelet therapies, as well as anti-inflammatory agents targeting cytokine responses, are now routinely used in clinical ITx. The addition of anticoagulants such as heparin[35] and anti-inflammatory agents such as TNF-α inhibitors (infliximab and, particularly, etanercept)[22, 30, 79] and interleukin-1 inhibitors (anakinra) has shown to improve clinical outcomes in ITx.[79] However, two important aspects potentially affecting patient management of IBMIR (e.g., obviating/minimizing anticoagulation and anti-inflammatory therapies) should be explored in the future. First, clinical Xeno-ITx trials demand investigation into potential similarities and differences in IBMIR when using human vs pig islets. A second aspect relates to the implantation site. Intraportal infusion and the liver microenvironment make IBMIR particularly relevant, however, it cannot be excluded that IBMIR will happen in extrahepatic sites of implantation. This should be explored in coming studies assessing these alternative implantation sites.

Hypoxia represents another relevant aspect impacting islet survival and engraftment following ITx. Islets, and particularly β-cells, are ill-equipped to handle hypoxia and reactive oxidative stress due to low expression of antioxidants.[80] Despite natural compensatory mechanisms to accelerate neovascularization, this process requires ~ 7-14 days,[81] with many islets (particularly larger ones) dying within this period. Low oxygen tension in many implantation sites, including the liver, further complicate this issue. Fortunately, strategies to increase vascularization and enhance islet survival, even in the most hostile environments (i.e.,

the subcutaneous) have shown promising results which have tremendous potential for clinical translation.[82, 83]

1.1.5.3 - Chronic immunosuppression

The use of lifelong immunosuppression has myriad detrimental implications, both systemically and for the transplanted islets. The holy grail of transplantation is achieving operational tolerance, that is, maintaining organ/graft function and survival without immunosuppression. However, beyond the liver, operational tolerance is rare in solid organ transplantation. The hope of achieving operational tolerance is even lower in clinical ITx, since patients mount auto and alloimmune responses after transplant, which may even facilitate each other (reciprocal facilitation/regulation).[84] The most straightforward approach to tackle this issue is thoughtful and prudent immunosuppression regimes. In this regard, there is evidence suggesting that certain immunosuppressants may control autoreactivity better than others. For example, induction immunosuppression with anti-thymocyte globulin, as compared to daclizumab, and maintenance immunosuppression with tacrolimus or mycophenolate mofetil, as compared to sirolimus, both showed to increase the risk of autoantibody recurrence in patients undergoing ITx.[84] These studies highlight the need to dissect the beneficial "off-target" effects of immunosuppressants, particularly on immunoregulatory mechanisms (e.g., regulatory T cells or Tregs). In this sense, a "Treg-centric" view on immunosuppression post-transplant has been recently advocated.[85] Tregs are central players in immune responses driving tolerance (or loss of tolerance) in autoimmune diseases, and have also gained astounding research momentum in the field of transplantation.[86] Emerging evidence suggests that immunosuppressants have different "Treg profiles", which can be used to foster a Treg-rich

environment following transplantation that could minimize or even eliminate the need for immunosuppression (i.e., operational tolerance). While this remains to be formally tested in randomized clinical trials, induction immunosuppression with alemtuzumab[87] and combined anti-thymocyte globulin + daclizumab[88)] has been associated with increased Treg percentages and higher Treg to effector T cell ratios following ITx. Regarding maintenance immunosuppression, sirolimus and mycophenolate mofetil have been shown to have a Treg-favoring effect, as compared to tacrolimus and cyclosporine A.[89] While additional aspects of immunosuppression, such as safety profiles and costs should be kept in mind, these preliminary considerations regarding the "off-target" effects of current immunosuppressants should motivate research involving other immunosuppressants and extend knowledge into specific effects on other immunoregulatory mechanisms beyond Tregs to proceed with more evidence-informed decisions in patient management.

Beyond minimizing chronic immunosuppression, efforts to eliminate it altogether are also under way. Three strategies dominate the field: adoptive cell transfer (ACT) therapies, cellular encapsulation and gene-editing approaches. Treg-based ACT therapies are the most widely studied, however, mesenchymal stem cells, dendritic cells and macrophages have been studied as well. Clinical trials using Treg-based ACT have demonstrated safety and efficacy in a vast number of diseases, including T1D.[90, 91] In the field of kidney transplantation, the recent multicenter ONE Study showed that ACT using Treg-based products enabled minimization of immunosuppression (i.e., tacrolimus monotherapy) in 40% of the patients treated with these therapies compared to 2% in those in the reference group.[92] Importantly, ACT-treated patients showed significantly lower rates of opportunistic infections compared to controls.[92] Whether multiple dosing at different time points would allow further reduction or elimination of

immunosuppression remains to be tested. Additionally, as enhanced cellular products, such as donor alloantigen-reactive Tregs (darTregs) or chimeric T-cell receptor Tregs (CAR-Tregs),[93] move forward into the clinic, the potential for ACT therapies to achieve operational tolerance will be more completely elucidated. Cellular encapsulation strategies, on the other hand, provide promising alternatives to abrogate the need for immunosuppression altogether. ITx has been the prototypical model to test cellular encapsulation technologies, since islets are amenable to macro and microencapsulation.[59] These strategies have advanced greatly in the last decades, with low-fouling, "immune friendly" biomaterials,[94] and composite bioscaffolds now allowing localized immunosuppression/immunoregulation at the implantation site,[83, 95] showing promising results in terms of graft acceptance and long-term diabetes reversal in preclinical models. These strategies are compatible with stem-cell therapies[96-98)] and xenotransplantation.[59] Thus, it is expected that future excursions into the clinic will yield positive results. Convincingly showing that cellular encapsulation is safe and effective in controlling hyperglycemia would open the door for β-cell replacement therapies to be used in many patients with T1D and other forms of diabetes. Even if metabolic exhaustion leads to graft attrition, encapsulation could allow safe retrieval and "refilling" of these cellular products. Combination with minimally-invasive implantation sites, such as the subcutaneous tissue, could even make these ambulatory procedures. Finally, the advent of safe and effective gene-editing has propelled many efforts to apply these techniques into the field of ITx. While many experiments using overexpression and underexpression of several molecules have shown promising results, specific efforts to prevent allo-[99-101] and autoimmune[102] destruction of islet could prove revolutionary. Viacyte Inc. has developed the cell product PEC-QT, which consists of edited clonal hESC line (CyT49) that lack the β2-microglobulin gene and express a transgene encoding programmed death-ligand 1

to protect cells from autoimmune attack.[103] It is expected that PEC-QT, in combination with their PEC-Direct macroencapsulation device, will move into clinical trials soon, which will have tremendous implications in the field.

1.1.5.4 - Clinical Translation and Outcome Assessment

Beyond the scientific aspects limiting ITx as a true cure for T1D, challenges concerning clinical study design, comprehensive reporting, and regulatory requirements also need to be addressed. Clinical study design has been inherently difficult in clinical ITx, which overall precludes high-quality evidence-informed clinical practice. Most clinical reports are retrospective, without entirely comparable control groups and limited by center-to-center variability in experience and expertise. To date, there is only one published multicenter randomized clinical trial comparing ITx with intensive insulin therapy, the TRIMECO trial.[21] In this trial, 50 patients with T1D and severe glycemic lability were randomized to either immediate ITx or delayed ITx (after 6 months of intensive insulin therapy). While this trial showed a clear advantage for achieving the primary outcome (a modified β-score > 6 points at 6 months after 1st infusion or randomization) in those treated with ITx vs insulin therapy (64% vs 0%, respectively), this is not surprising, given that two of the components of the β-score (C-peptide and insulin doses) were unlikely to be modified with intensive insulin therapy, precluding these patients from achieving the primary outcome. Beyond the limitations of this trial, it was a tremendous achievement since inclusion of appropriate controls (i.e., patients with an indication for ITx) had been a major barrier to contextualize the true benefits of early ITx.[21] Longer follow-up of these patients will undoubtedly provide more information on these issues, albeit previous experience has been favorable.[27] It should be emphasized that ITx is not an

alternative, but rather a complement for patients in which intensive insulin therapies are not tolerated or have failed. This should guide appropriate and context-specific outcome selection and reporting to evaluate the efficacy of ITx. A recent consensus report from the IPITA/EPITA opinion leaders workshop has focused on this issue.[33] Specific cut-offs and variables proposed to asses graft functional status can be consulted in the report, however, the key message within this document is that graft function should not be synonymous with clinical success, and the latter should prevail when considering clinical decision-making (i.e., continuing monitoring/immunosuppression and/or considering additional islet infusions). Hence, albeit insulin independence is highly desirable, this should not be attempted at the expense of suboptimal glycemic control. Finally, it should be remembered that the main indication for ITx relates to problematic hypoglycemia and impaired awareness of hypoglycemia, thus, through this lens, resolution of this highly-disabling clinical issues should be considered equally (if not more) important measures of success following ITx. This later aspect is also relevant to carry appropriate economic or safety assessments to support health coverage of clinical ITx by governmental or private insurance agencies.

1.1.6 - Conclusion

ITx is now a robust option for patients with T1D suffering from problematic hypoglycemia and impaired awareness of hypoglycemia. Substantial advances in the field have made ITx comparable to whole pancreas transplantation, however, many challenges remain. A systematic approach at tackling these challenges, coupled with an astounding display of ingenuity and a surge of recent discoveries challenging current paradigms around cell replacement therapies, are already showing promising results that bring tremendous hope to

clinicians and patients for a true cure for every patient with T1D and, perhaps, other forms of diabetes. Currently, the field of β-cell replacement therapies is relatively small, however, the magnitude of the problem and the consequences on public and private health systems are motivating researchers and institutions to migrate into the field. This has promoted a soar of clinical islet transplant programs all over the world which should inevitably lead to global collaboration in the field and research endeavors without borders. It is only by working together that the promise of curing diabetes will be fulfilled.

1.1.7 - References

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CHAPTER 1

PART 2 - PANCREATIC ISLET TRANSPLANTATION IN TYPE 1 DIABETES: 20-YEAR EXPERIENCE FROM A SINGLE CENTRE COHORT

CHAPTER 1, PART 2 - PANCREATIC ISLET TRANSPLANTATION IN TYPE 1 DIABETES: 20-YEAR EXPERIENCE FROM A SINGLE CENTRE COHORT

Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada

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Summary

Background Islet transplantation offers an effective treatment for selected people with type 1 diabetes and intractable hypoglycaemia. Long-term experience, however, remains limited. We report outcomes from a single-centre cohort up to 20 years after islet transplantation.

Methods This cohort study included patients older than 18 years with type 1 diabetes undergoing allogeneic islet transplantation between March 11, 1999, and Oct 1, 2019, at the University of Alberta Hospital (Edmonton, AB, Canada). Patients who underwent islet-after-kidney transplantation and islet transplantation alone or islet transplantation before whole-pancreas transplantation (follow-up was censored at the time of whole-pancreas transplantation) were included. Patient survival, graft survival (fasting plasma C-peptide >0·1 nmol/L), insulin independence, glycaemic control, and adverse events are reported. To identify factors associated with prolonged graft survival, recipients with sustained graft survival (≥90% of patient follow-up duration) were compared with those who had non-sustained graft survival (<90% of follow-up duration). Multivariate binary logistic regression analyses were done to determine predictors of sustained graft survival.

Findings Between March 11, 1999, and Oct 1, 2019, 255 patients underwent islet transplantation and were included in the analyses (149 [58%] were female and 218 [85%] were White). Over a median follow-up of 7·4 years (IQR 4·4–12·2), 230 (90%) patients survived. Median graft survival was 5·9 years (IQR 3·0–9·5), and graft failure occurred in 91 (36%) patients. 178 (70%) recipients had sustained graft survival, and 77 (30%) had non-sustained graft survival. At baseline, compared with patients with non-sustained graft survival, those with sustained graft survival had longer median type 1 diabetes duration (33·5 years [IQR 24·3–41·7] *vs* 26·2 years [17·0–35·5]; p=0·0003), median older age (49·4 years [43·5–56·1] *vs* 44·2 years [35·4–54·2]; p=0·0011), and lower median insulin requirements (0·53 units/kg per day [0·45–0·67] *vs* 0·59 units/kg per day [0·48–0·70]; p=0·032), but median HbA_{1c} concentrations were similar (8·2% [7·5–9·0] *vs* 8·5% [7·8–9·2]; p=0·23). 201 (79%) recipients had insulin independence, with a Kaplan-Meier estimate of 61% (95% CI 54–67) at 1 year, 32% (25–39) at 5 years, 20% (14–27) at 10 years, 11% (6–18) at 15 years, and 8% (2–17) at 20 years. Patients with sustained graft survival had significantly higher rates of insulin independence (160 [90%] of 178 *vs* 41 [53%] of 77; p<0·0001) and sustained improvements in glycaemic control mixed-main-effects model group effect, p<0·0001) compared with those with non-sustained graft survival. Multivariate analyses identified the combined use of anakinra plus etanercept (adjusted odds ratio 7·5 [95% CI 2·7–21·0], p<0·0001) and the BETA-2 score of 15 or higher (4·1 [1·5–11·4], p=0·0066) as factors associated with sustained graft survival. In recipients with sustained graft survival, the incidence of procedural complications was lower (23 [5%] of 443 infusions *vs* 17 [10%] of 167 infusions; p=0·027), whereas the incidence of cancer was higher (29 of [16%] of 178 *vs* four [5%] of 77; p=0·015) than in those with non-sustained graft survival; most were skin cancers (22 [67%] of 33). End-stage renal disease and severe infections were similar between groups.

Interpretation We present the largest single-centre cohort study of long-term outcomes following islet transplantation. Although some limitations with our study remain, such as the retrospective component, a relatively small sample size, and the absence of non-transplant controls, we found that the combined use of anakinra plus etanercept and the BETA-2 score were associated with improved outcomes, and therefore these factors could inform clinical practice.

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Introduction

Insulin therapies remain the cornerstone treatment of type 1 diabetes. Although life saving, intensive insulin regimens increase the risk of severe hypoglycaemic events,[1] which are associated with substantial morbidity and mortality (4–10% of total deaths).[2] Novel insulin delivery systems might reduce severe hypoglycaemic events, but a 2020 network meta-analysis suggests limited benefits.[3] The most physiological way to achieve and maintain normoglycaemia, while simultaneously ameliorating hypoglycaemia, is by restoring β-cell mass through pancreatic islet or whole-pancreas transplantation.

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1.2.1 - Abstract

Background

Islet transplantation (ITx) offers an effective treatment for selected people with type 1 diabetes (T1D) and intractable hypoglycaemia. Long-term experience, however, remains limited. Herein, we report outcomes from a single-centre cohort up to 20-years post-ITx.

Methods

Subjects with T1D undergoing allogeneic ITx were included (March/1999 – October/2019, n=255). Patient and graft survival, insulin independence, glycemic control, and adverse events are reported. To identify factors associated with prolonged graft survival, recipients with sustained graft survival (SGS, graft survival $\geq$90% of patient follow-up duration, n=178) were compared to those experiencing non-sustained graft survival (NSGS, graft survival <90% of follow-up duration, n=77).

Results

Over a median follow-up of 7.4 (IQR 4.4 - 12.2) years, patient survival was 90% (230/255). Median graft survival [fasting C-peptide >0.1 nmol/L] was 5.9 (IQR 3 – 9.5) years, graft failure occurred in 36% (91/255) of cases. Subjects with SGS had longer diabetes duration, older age, and lower insulin requirements at baseline, while HbA_{1c} levels were similar. Insulin independence was ever achieved in 79% (201/255) of recipients, with 1-, 5-, 10-, 15- and 20-year Kaplan-Meier estimates of 61%, 32%, 20%, 13% and 8%, respectively. SGS recipients achieved significantly higher rates of insulin independence and sustained improvements in glycemic control compared to NSGS. Multivariate analyses identified the combined use of anakinra plus etanercept and the BETA-2 score as factors associated with SGS. Procedural

complications, end-stage renal disease, cancer, and severe infections were similar between groups.

Conclusion

We present the largest single-centre cohort study of long-term outcomes following ITx. While some limitations with our study remain, such as the retrospective component, a relatively small sample size, and the absence of non-transplant controls, we identify relevant factors associated with improved outcomes that inform clinical practice.

1.2.2 - Research in Context

Current guidelines recommend ITx to treat intractable problematic hypoglycaemia, however, reports of long-term outcomes remain scarce. To assess current evidence, we executed a scoping review including available studies reporting long-term outcomes following allogeneic pancreatic islet transplantation (ITx). The search was completed on December 2021 and evaluated MEDLINE, EMBASE, and Scopus. Inclusion criteria were studies published since 2000, evaluating adult patients (≥18 years) and reporting ≥ 10-year follow-up data. Abstract presentations and case reports were excluded. No language restrictions were applied. Six publications were identified: retrospective cohort = 5, prospective observational = 1 (**Table 1.2.1**). The first study evaluated 7 patients from the NIS01 trial (EXIIST-Extended Immunosuppression in Islet Transplantation) and showed 100% patient survival and graft function at 10-years, coupled with substantial improvements in glycemic control. A study by Nakamura et al. including 7 patients showed benefits in glycemic control and hypoglycaemia compared to multiple daily insulin injections with a trend towards increased infectious and digestive complications secondary to immunosuppression, albeit no detectable serum C-peptide was found after 5-years post-first transplant. A prospective cohort study by Vantyghem et al. included patients receiving ITx alone (ITA, n=14) or ITx after kidney (IAK, n=14) and reported >90% patient survival, 78% graft survival, improved glycemic control and glycemic lability, as well as near complete eradication of severe hypoglycemic episodes at 10-years. These authors also reported no significant changes in renal function over time. A study by the GRAGIL network evaluated 10-year outcomes with ITA (n=24) and IAK (n=20), showing 86% patient survival and 52% graft survival. They also reported sustained improvement in glycemic control and lability, and found that >70% were free from severe hypoglycemic events. Most recently, a

cohort study of 49 subjects by Lemos et al. from the Miami group showed a 95% patient survival rate with data up to 20 years. Another recent publication from the same group including patients with up to 20-years of follow-up reported graft failure in 38% of the cases. Overall, our literature review demonstrates that 10-year outcomes after ITx while promising, remain scarce (~100 patients reported), with only two publications from one group reporting outcomes to 20-years follow-up post-transplant.

1.2.2.1 - Added value of this study

The current study represents the largest long-term report of a single-centre's cumulative and comprehensive outcomes following ITx. By reporting 20-year follow-up data, this study supports the notion that ITx is a safe procedure in terms of patient survival, but also in terms of major procedure- and immunosuppression-related adverse events. This work also shows that 10-year and 20-year graft survival (maintenance of a fasting plasma C-peptide >0.1 nmol/L throughout follow-up) post-ITx is achieved in 58% and 48% of recipients in our cohort, respectively. Moreover, we demonstrate that insulin independence is routinely achieved after ITx (79% of patients), although typically $\geq$2 islet infusions are required and occurs at a median time of 95 (IQR 30 - 196) days. This report incorporates a comprehensive follow-up of metabolic outcomes following ITx, including graft function, glycemic control and insulin requirements, and includes long-term data regarding the impact of ITx on measures of hypoglycaemia and glycemic lability. Finally, this study identified two factors, the combined use of anakinra plus etanercept (for $\geq$1 transplant), and the BETA-2 score within the first year, to be associated with sustained graft survival, a finding which may be used to optimize outcomes after ITx and resource allocation.

1.2.2.2 - Implications of all the available evidence

This study demonstrates a favorable profile of ITx in terms of patient safety and sustained metabolic control. Future research should involve comparative studies including control recipients with type 1 diabetes experiencing severe hypoglycaemia but not transplanted, patients undergoing whole pancreas transplantation, and those utilizing current or novel insulin delivery technologies. By providing a thorough description of patients undergoing ITx, we contribute to improving our understanding of optimal conditions in which β-cell replacement therapies can thrive and achieve maximum long-term therapeutic benefit.

Table 1.2.1. Studies describing long-term outcomes in patients undergoing pancreatic islet transplantation

Author, year, country, *Journal*	Patients and Methods	Main Results

Brennan et al., 2016, U.S.A., *Am. J Transplant*[1]	- Seven subjects
	  o 5 female / 2 male
	  o Median follow-up: 10.2 yr
	- Participants from a previous clinical trial (NIS01, NCT00014911)
	  o Peak C-peptide >0.3 ng/mL during a MMTT
	  o Absence of severe hypoglycaemia, creatinine levels <1.6 mg/dL and HbA_{1c} <12%.
	  o Median baseline HbA1c of 6.9 (IQR 6.7-7.2)
	  o Median insulin units/kg/day of 0.49 (IQR 0.45-0.50
	- Induction IS: daclizumab
	- Maintenance IS: any combination of calcineurin inhibitors, antimetabolites, and antiproliferative drugs
	- Median of 3 islet infusions
	  o Median IEQ/kg: 18,721
		- Graft function, defined as C-peptide >0.3 ng/mL at 90 min in response to a MMTT, was demonstrated in all subjects for at least 10 years.
		  o 1 patient experienced graft loss/failure at 10.9 years post-first transplant.
		- All subjects achieved insulin independence
		  o Median time of 54 months
		  o Two patients remained off exogenous insulin at the last visit
		- No deaths were reported.

Nakamura et al., 2020, Japan, *J Diabetes Investig*[2]	- Seven subjects
	  o 5 female / 2 male
	  o Median follow-up: 10.2 yr
	- Compared to 26 age-matched subjects on exogenous insulin therapies (multiple daily injections or continuous subcutaneous insulin infusion)
	- No inclusion or exclusion criteria reported
	- Median baseline HbA1c of 6.8 (IQR 6.1-8.5)
	- Induction IS: basiliximab
	- Maintenance IS: tacrolimus plus sirolimus, with substitution to MMF if side-effects associated with sirolimus emerged.
	- 2 patients received one infusion, 2 received two infusions, and 3 received three infusions
	  o Median of 408,000 IEQ/infusion
		- Graft loss/failure was defined as a stimulated serum C-peptide >0.3 ng/mL
		  o 57% (4/7) and 28.5% (2/7) patients showed graft survival at 2- and 5-years post-first infusion.
		- Improvements in HbA_{1c} levels compared to insulin therapies.
		  o Statistical significance was only shown for the 1-year time point.
		- Improvements in the incidence of severe hypoglycaemia
		  o 14% (1/7) in the ITx group vs 31% (8/26) in the insulin-treated group
		- A trend towards increased incidence of infections, with 43% (3/7) in the ITx group vs 12% (3/26) in the insulin-treated group

Vantyghem et al., 2018, France, ***Diabetes Care***[3]	- 28 subjects
	  o 14 IAK / 14 ITA
	  o 15 female / 13 male
	  o Median follow-up: 11.5 yr (IQR 8.9-12.9)
	- Patients enrolled in clinical trials (NCT00446264 and NCT01123187).
	- Inclusion criteria: age>18 years-old, type 1 diabetes for >5 years, arginine-stimulated C-peptide <0.3 ng/mL, hypoglycaemia unawareness (for ITA), stable renal function and contraindication for SPK (for IAK), albuminuria <300 mg/24 hr, daily insulin <1.2 units/kg, history of malignancy, lack of compliance.
	- Median baseline HbA1c of 8.2 (IQR 7.3-9.0)
	- Induction IS: daclizumab
	- Maintenance IS: tacrolimus plus sirolimus
	- 10 patients received two infusions, and 10 received three infusions
	  o Median IEQ/kg: 18,721
		- Patient survival was >90%
		  o An incidence rate for mortality of 0.3% per 100 patient-years was reported
		- Insulin independence was defined as the absence of exogenous insulin therapy associated with an HbA_{1c} of $\leq$6.5%.
		  o This outcome was achieved in all patients, at a median time of 91 days (IQR 61-115)
		  o 39% and 28% of patients remained insulin independent at 5- and 10-years post-first infusion, respectively.
		- Graft loss/failure was defined as a fasting serum C-peptide <0.3 ng/mL
		  o An 83% and 78% 5- and 10-year graft survival rate respectively was reported.
		- Optimal primary graft function (defined as a BETA-score* $\geq$7) identified patients with improved outcomes
		  o Median Insulin independence of 6 (1)-10) years vs 0.4 (0.2-1.1) years.
		  o Median graft survival of 10 (IQR 8-10) vs 4.% (IQR 0.8-10) years
		- No significant changes in renal function over time following ITx were observed

Lablanche et al., 2021, France and Switzerland, ***Am J Transplant***[4]	- 44 subjects, 31 completed a 10-year follow-up
	  o 15 IAK / 16 ITA
	- Patients enrolled in clinical trials (NCT00639600)
	- and NCT00321256)
	- Inclusion criteria: age 18-65 years-old, type 1 diabetes for >5 years, C-peptide negative, severe hypoglycaemia, eGFR<50 ml/min/1.73m^2 (ITA) or functional kidney graft (IAK) , proteinuria <500 mg/24 hr, daily insulin <0.7 units/kg or <50 IU/day, history of malignancy, lack of compliance.
	- Median baseline HbA1c of 8.0 (IQR 7.1-9.1)
	- Induction IS: daclizumab or basiliximab
	- Maintenance IS: tacrolimus plus sirolimus
		- Graft survival at 5- and 10-years post-first infusion was 95% and 89%, respectively (86% overall).
		  o An incidence rate for mortality of 0.8 and 2 per 100 patient-years for ITA and IAK, respectively.
		- Insulin independence was defined as the absence of exogenous insulin therapy or oral anti-diabetic agents associated with an HbA_{1c} of <7%, 2-h post-prandial glucose <10 mmol/L, and a basal plasma C-peptide $\geq$0.5 ng/mL.
		  o This outcome was achieved in 20% and 5% of patients at 5- and 10-years post-first infusion, respectively.
		- Graft loss/failure was defined as a stimulated serum C-peptide <0.3 ng/mL

	- All patients scheduled to receive up to three infusions within 3 months - Median IEQ/kg: 9,867 (IQR 7,410-11,890)	o A 79% and 52% graft survival rate at 5- and 10-years was reported, respectively. - A significant decrease in eGFR was observed at 10-years following both ITA and IAK.
Lemos et al. 2021, U.S.A., *Diabetes Care*[5]	- 49 subjects o 29 female / 20 male - Inclusion criteria: age 18-65 years-old, type 1 diabetes for >5 years, C-peptide negative, impaired awareness of, marked glycaemic lability, history of severe hypoglycaemia in the prior 12 months - IS not reported - Number of infusions of islet (e.g., IEQ/kg) was reported	- Patient survival 96% o >80% patient survival at 20-years o An incidence rate for mortality of 3.3 (2.1-5) per 1,000 patient-years was reported - Graft survival definition not reported o Median duration of graft function while on IS was 4.4 (1.3-12.2). - Insulin independence not reported
Lemos et al. 2021, 2021, *J Clin Endocrinol Metab*[6]	- 56 patients o 49 ITA / 7 IAK o 29 female / 27 male - Patients enrolled in clinical trials (NCT02000687, NCT01999361, NCT01999374, NCT00306098 - Induction IS: not reported - Maintenance IS: tacrolimus and sirolimus, with MMF in four patients, and all three combined in three patients. - Median IEQ/kg: 13,185 (IQR 10,685-16,415) for group 1 (≥1 female donor) and 11,219 (IQR 8,029-16509) for group 2 (only male donors)(p=0.222)	- Patient survival not reported o Median patient follow-up was 4.% (1.4-11.6) years - Graft loss/failure was defined as a stimulated C-peptide <0.3 ng/mL following a MMTT o Subanalyses showed that female recipients, as well as recipients receiving islets obtained from female donors had a significantly improved graft survival - Insulin independence not reported - Durable improvements in glycaemic control (HbA_{1c}% levels) were reported

MMTT: mixed-meal tolerance test, IQR: interquartile range, IS: immunosuppression, MMF: mycophenolate mofetil, ITx: islet transplantation, IAK: islet-after-kidney transplant, ITA: islet transplant alone, SPK: simultaneous pancreas-and-kidney transplant, eGFR: estimated glomerular filtration rate,

*BETA score includes: ranges from 0 (no graft function) to 8 (excellent graft function). This score gives two points for normal fasting glucose (≤5.5 mmol/L), HbA_{1c} ≤6.1% (43 mmol/mol), stimulated and/or basal C-peptide (≥0.3 nmol/L), and absence of insulin or oral hypoglycaemic agent use. No point is awarded if fasting glucose is in the diabetic range (≥7 mmol/L), $HbA1_c$ is ≥7% (53 mmol/mol), C-peptide secretion is undetectable on stimulation, or daily insulin use is ≥0.25 units/kg. One point is given for intermediate values.

1.2.3 - Background

Insulin therapies remain the cornerstone treatment of type 1 diabetes (T1D). Although life-saving, intensive insulin regimens increase risk of severe hypoglycemic events (SHEs)[7], which are associated with substantial morbidity and mortality (4-10% of total deaths).[8] Novel insulin delivery systems may reduce SHEs, however, a recent network meta-analysis suggests limited benefits.[9] The most physiologic way to achieve and maintain normoglycaemia, while simultaneously ameliorating hypoglycaemia, is by restoring β-cell mass through pancreatic islet (ITx) or whole pancreas transplantation (PTx).

Outcomes following ITx and PTx have improved over the last two decades. Both therapies can lead to insulin independence, near complete elimination of SHEs and sustained improvements in glycemic control.[10] Large long-term reports are available for PTx, but not for ITx. Outcomes beyond 10-years post-transplant have only been reported for ~100 patients (**Table 1.2.1**).[1-6] While ITx is limited to selected people with T1D, the lack of long-term data limits optimal clinical practice and evidence-informed shared-decision making. Herein, we report a large single-centre experience with ITx across a 20-year period.

1.2.4 - Methods

1.2.4.1 - Study design and Patient Selection

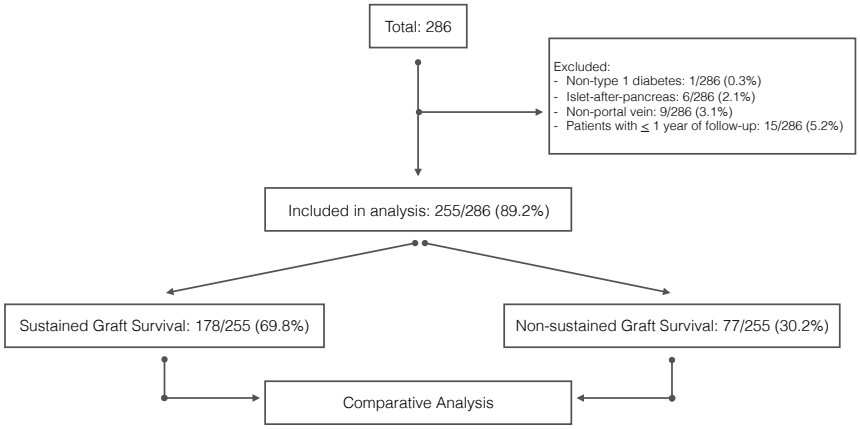
This cohort includes people with T1D undergoing allogeneic ITx at the University of Alberta Hospital between March 11, 1999 and October 1st 2019. Candidates were aged >18, with T1D duration >5 years, and a negative stimulated C-peptide, measured following mixed-meal tolerance tests where possible, or post-prandial if hyperglycemic. Primary indications for ITx are described in **Table 1.2.2**. Data was obtained retrospectively and prospectively from local and provincial databases. Islet-after-kidney transplantation (IAK, n=16) and islet transplant alone (ITA, n=239) were included. Patients having extrahepatic infusions, <1-year follow-up, or undergoing PTx before ITx were excluded. Patients undergoing ITx before PTx (6/255, 2%) were included, however, follow-up was censored at the time of PTx (**Figure 1.2.1**). This study was approved by our institutional health research ethics board (PRO000001120 and PRO00087040). Patient consent for the use of health data for research purposes was obtained for all subjects.

Table 1.2.2. Primary indications for pancreatic islet transplantation

	Thresholds*	Total population (%)
Severe recurrent hypoglycaemia, n (%)	HYPO score ≥1,047	33/255 (13)
Glycaemic lability, n (%)	Lability index ≥433	24/255 (9)
Combination of severe hypoglycaemia and glycaemic lability, n (%)	HYPO score ≥423 and lability index ≥329	117/255 (46)
Hypoglycaemia unawareness, n (%)	Clarke score ≥4	26/255 (10)
Other indications (progressive secondary complications), n (%)	..	30/255 (12)
Missing data, n (%)	..	25/255 (10)

*Thresholds were established from Ryan et al., 2005 (*Diabetes Care*)[11] and Geddes et al., 2007 (*Diabetes Care*)[12]. Thresholds were used as guidelines and evaluation for pancreatic islet transplantation was individualized according to patient profile and preferences.

Figure 1.2.1. Flow diagram for patient selection



Note: this figure is included as a supplementary figure in the published paper.

1.2.4.2. - Transplant procedures

Islet isolation was performed as previously reported.[13] Suitable islet preparations were loaded into a gravity infusion bag with heparin (70 IU/kg). Percutaneous cannulation of a peripheral branch of the portal vein was done by interventional radiologists using ultrasound and fluoroscopy. Islets were infused with sequential portal pressure monitoring. Since 2005, Avitene™ (microfibrillar collagen hemostatic powder) was used to obliterate the liver tract to prevent bleeding.[14] Insulin and heparin infusions post-transplant were used from 2005 onwards.[15] Heparin use was targeted to a partial thromboplastin time of 60-80s for 48-hours post-transplant, followed by enoxaparin 30 mg qd and aspirin 81 mg qd for 7 and 14 days, respectively.

Two islet infusions were anticipated with the aim of achieving insulin independence. A second infusion was decided at 2-4 weeks post-first transplant in subjects not achieving insulin independence or following loss of insulin independence, dependent on initial function and islet mass. A third infusion was considered where islet mass was inadequate, or engraftment poor.

Supplemental infusions were administered to restore insulin independence (typically recipients achieving ≥1-year of insulin independence) or eliminate recurrent hypoglycaemia. These were not recommended for recipients unlikely to further maintain durable graft function (i.e., not tolerating adequate immunosuppression or experiencing recurrent rapid graft loss).

1.2.4.3 - Immunosuppression and Anti-inflammatories

Use of induction and maintenance immunosuppression, and anti-inflammatory therapies is detailed in **Table 1.2.3**.

Table 1.2.3. Immunosuppression regimes used for patients undergoing pancreatic islet transplantation at the University of Alberta

	Sustained graft survival	Non-sustained graft survival	*P* values*
Induction immunosuppression	**Total infusions=443**	**Total infusions=167**	
Basiliximab, n (%)	61 (13.8)	20 (11.0)	0.41
Anti-thymocyte globulin, n (%)	60 (13.5)	16 (9.6)	0.19
Daclizumab, n (%)	105 (23.7)	64 (38.3)	<0.0001
Alemtuzumab, n (%)	218 (49.2)	70 (41.9)	0.11
Belatacept, n (%)	6 (0.9)	5 (2.9)	0.18
Anti-inflammatories			
Anakinra alone, n (%)	1 (0.002)	1 (0.005)	0.47
Etanercept alone, n (%)	67 (15.1)	30 (18.0)	0.39
Anakinra + etanercept, n (%)	238 (53.6)	49 (29.3)	<0.0001
Infliximab, n (%)	8 (1.8)	23 (13.8)	<0.0001
None, n (%)	129 (29.1)	64 (37.2)	0.029
Maintenance immunosuppression	**Patients=178**	**Patients=77**	
Tacrolimus, n (%)	178 (100)	77 (100)	-
Mofetil mycophenolate, n (%)	168 (94.4)	66 (85.7)	0.021
Sirolimus, n (%)	67 (37.4)	43 (55.8)	0.007

Note: Maintenance immunosuppression was with two immunosuppressant agents at a time. The Edmonton protocol involved maintenance immunosuppression using high-dose sirolimus (target 12-15 ng/mL during the first year, 7-10 ng/mL onwards) and tacrolimus (3-6 ng/ml), with substitution of MMF (up to 1g bid) and increased tacrolimus levels (8-10 ng/mL) in recipients experiencing side effects from sirolimus. Modifications to our protocols included increased tacrolimus levels (10-12 ng/mL during the first 3 months, 8-10 ng/mL onwards) and MMF (up to 1g bid). Tacrolimus targets were adjusted (6-8 ng/mL or lower) in case of adverse effects or renal dysfunction. *X^2 was used for univariate comparisons of categorical variables. *P* values correspond to sustained

graft survival vs non-sustained graft survival. This figure is included as a supplementary figure in the published paper.

1.2.4.4 - Follow-up

Recipients were followed by endocrinologists and transplant surgeons with expertise in ITx, with weekly visits for the first month, and every 3-6 months thereafter. At each visit, graft function, glycemic control, use of insulin or other glucose-lowering therapies, safety and tolerability of immunosuppression was assessed. Resumption of exogenous insulin or initiation other glucose-lowering therapies was at the clinicians' discretion, considering patient wishes and access to different therapies. Immunosuppression was balanced to minimize toxicity, considering graft, renal function and side effects.

1.2.4.5 - Study Outcomes

Operational definitions are described in **Table 1.2.4.** Primary outcomes include patient survival and death-censored graft survival, and insulin independence[11, 16] rates and total duration. Complete graft failure was defined as persistent ($\geq$2 measurements) fasting plasma C-peptide $\leq$0.1 nmol/L ($\leq$0.3 ng/mL) without recovery or subsequent infusion.[16] To evaluate factors associated with continued graft function, sub-analyses stratified by percentage of graft survival (fasting C-peptide >0.1 nmol/L) throughout patient follow-up were done ([graft survival/patient survival]*100). Two groups were analyzed: sustained graft survival (SGS, graft survival $\geq$90% of patient follow-up) and non-sustained graft survival (NSGS, graft survival <90% of patient follow-up). Other outcomes include severity of hypoglycaemia (HYPO score),[17] glycaemic lability (lability index),[17] hypoglycaemia unawareness (Clarke Score),[12, 18] fasting C-peptide levels, insulin requirements, glycemic control (HbA_{1c} and fasting plasma glucose) and BETA-2 scores.[19] These are presented as medians of available values at specific

time intervals. We report adverse events post-ITx: life-threatening procedure-related complications, chronic kidney disease (CKD), end-stage renal disease (ESRD), cancer, and life-threatening infections (**Table 1.2.4**).

Table 1.2.4. Operational definitions

	Definition
Complete graft failure	- Persistent (≥ 2 measurements) C-peptide level <0.1 nmol/L/0.3 ng/mL without recovery - No subsequent infusions - Patients dying with a fucntioning graft were censored (death-censored graft survival analysis)
Insulin independence	- No exogenous insulin use for ≥14 days with: - Fasting plasma glucose ≤8 mmol/L - 2-hr post-prandial glucose < 10 mmol/L - HbA1c <7%
Total duration of insulin independence	- A sum of all episodes of insulin independence throughout patient follow-up
BETA-2 score	$\text{BETA-2 score} = \frac{\left(\sqrt{\text{fasting C-peptide (nmol/L)}} \times (1 - \text{insulin dose [units/kg]})\right)}{\text{Fasting plasma glucose (mmol/L)} \times \text{HbA1c(\%)}} \times 1000$ - Data imputation: o Fasting C-peptide levels: 1) patients with complete graft failure had fasting C-peptide levels imputed as the last value (i.e., last value carried forward) for the rest of their follow-up; 2) in patients with a C-peptide level <0.1 nmol/L, subsequent values were assumed to be equal up until they received a subsequent transplant, and 3) averages were used to impute for missing data between time points, only if no transplants were performed during this period. o Beta-2 scores: imputation of Beta-2 scores was done when calculation was not possible due to missing data. In this scenario, the following strategies were followed: 1) use of imputed fasting C-peptide values (see above), 2) Beta-2 scores were considered equal to 1 if fasting C-peptide levels were <0.05 nmol/L, and equal to 3 if C-peptide levels were between 0.05 and 0.1 nmol/L, when other information was missing (e.g., insulin requirements).
Dyslipidemia	- Diagnosis on record - Treatment - LDL >100 mg/dL
Hypertension	- Diagnosis on record - Treatment

Macrovascular disease	-	Myocardial infarction - Coronary artery disease - Cerebrovascular disease - Peripheral arterial disease
Stage 3 chronic kidney disease	-	Diagnosis on record - Persistent eGFR <60 ml/min/1.73m^2 using CKD-EPI - Time to stage 3 CKD: considered at the moment of first recorded eGFR<60 ml/min/1.73m^2 coupled with evidence of persistence ($\geq$ 3 months)
Stage 4 chronic kidney disease	-	Diagnosis on record - Persistent eGFR <30 ml/min/1.73m^2 using CKD-EPI - Time to stage 4 CKD: considered at the moment of first recorded eGFR<30 ml/min/1.73m^2 coupled with evidence of persistence ($\geq$ 3 months)
Stage 5 chronic kidney disease - End-stage renal disease (ESRD)	-	Diagnosis on record o Persistent eGFR <15 ml/min/1.73m^2 using CKD-EPI o Dialysis o Kidney transplant - Time to ESRD: considered at the moment of first recorded eGFR<15 ml/min/1.73m^2 coupled with evidence of persistence ($\geq$ 3 months)
Procedure-related, life-threatening complications	-	Any complication occurring during hospitalization classified as Clavien-Dindo grade >3 (requiring surgical, endoscopic or radiological intervention) - Any complication requiring admission and related to the procedure or immunosuppression occurring within 90 days of discharge from the transplant/infusion hospitalization episode.
Life-threatening infections	-	Any infection requiring hospitalization
Cancer	-	Diagnosis on record - Cancers diagnosed within 6 months of 1st infusion were excluded (1 breast cancer diagnosed 3 months post-1st infusion, 1 germ cell tumor diagnosed 21 days post-1st infusion)

Note: This table is included as supplementary material in the published version of this manuscript.

1.2.4.6 - Statistical Analysis

An intention-to-treat analysis was conducted. Continuous variables are reported as median and interquartile ranges (IQR), and compared using Mann-Whitney tests. Categorical variables are presented as frequencies and percentages, and compared using X^2 tests. Mixed main effects models using the maximum-likelihood method were used to analyze outcomes over time; time and group effects are reported. Correlations between BETA-2 scores at 6-12 months (i.e., within 1 year post-first ITx) and graft survival or total duration of insulin independence

were assessed using Spearman's rank correlation tests and univariate median regression (coefficients with 95%CI). Imputations to calculate BETA-2 scores are described in **Table 1.2.4.** Percentages of imputed values are shown in **Table 1.2.5.** Patient and graft survival, and achievement and total duration of insulin independence were analyzed using Kaplan-Meier estimates. Hazard ratios (and 95%CI) were derived using Cox proportional hazard regression; proportional hazard assumptions were verified using Schoenfeld's residuals test. Incidence rates (and 95%CI) are also reported. Multivariate binary logistic regression analyses were conducted to determine predictors of SGS. Independent predictors were based on clinical relevancy and statistically significant differences between groups (i.e., $p<0.05$) in univariate analyses. Statistical analyses were performed using Stata® (Version 12.0, StataCorp, College Station, Texas) and GraphPad Prism (Version 9, GraphPad Software, LLC, San Diego, California).

Table 1.2.5. Percentages of imputed values for fasting C-peptide levels (nmol/L) and BETA-2 scores

Time of follow-up after 1st transplant	Beta-2 score, missing values (imputed values)	Fasting C-peptide, missing values (imputed values)
1-6 months	1/255 (0/2)	1/255 (0/1)
6 - 12 months	78/255 (28/78)	5/255 (1/5)
12 – 24 months	79/255 (22/79)	11/255 (2/11)
24 – 36 months	96/236 (25/96)	19/236 (12/19)
36 – 48 months	105/223 (27/105)	27/223 (12/27)
48 – 60 months	92/196 (29/92)	30/196 (17/30)
60 – 72 months	92/184 (28/92)	34/184 (18/34)
72 – 84 months	81/162 (27/81)	34/162 (23/34)
84 – 96 months	71/135 (26/71)	35/135 (26/35)
96 – 108 months	63/117 (24/63)	32/117 (22/32)
108 – 120 months	51/99 (21/51)	28/99 (20/28)
120 – 132 months	43/86 (20/43)	29/86 (21/29)
132 – 144 months	40/77 (20/40)	28/77 (23/28)
144 – 156 months	33/66 (13/33)	19/66 (12/19)
156 – 168 months	30/57 (9/30)	20/57 (12/20)
168 – 180 months	28/47 (10/28)	19/47 (12/19)
180 – 192 months	23/36 (8/23)	12/36 (8/12)

192 – 204 months	15/25 (6/15)	10/25 (8/10)
204 – 216 months	11/18 (3/11)	7/18 (3/7)
216 – 228 months	5/10 (1/5)	2/10 (2/2)
228 to 240 months	4/9 (0/4)	2/9 (2/2)

Note: This table is included as supplementary material in the published version of this manuscript.

1.2.5 - Results

1.2.5.1 - Patient and Islet Infusion Characteristics

A total of 255 patients were included. **Table 1.2.6** shows baseline patient characteristics**.** Overall, 70% (178/255) of recipients achieved SGS. The median (IQR) duration of diabetes and age at baseline was 30.6 years (22.6–40.2) and 48.8 (41.3–55.8), respectively. Recipients with SGS had longer T1D duration and were older at baseline ($p \leq 0.001$ for both). There were no differences in baseline HYPO scores, lability indexes or Clarke scores between groups. While recipients with SGS had lower insulin requirements (0.53 [0.45–0.67] vs 0.59 [0.48–0.70] units/kg/day, $p=0.032$), baseline stimulated C-peptide and HbA_{1c} levels were similar. Overall, 88% (225/255) of subjects received ≥2 islet infusions, 37% (94/255) ≥3 infusions, 13% (32/255) ≥4 infusions, and 2% (4/255) received 5 infusions. The proportion of patients receiving ≥3 islet infusions was higher in recipients with SGS (42% [75/178] vs 25% [19/77], $p=0.008$) (**Table 1.2.6**). In our cohort, 51% (131/255) received all infusions <12-months post-first ITx. Median time to last infusion was 11.6 (2.4 – 47.6) months, however, 7% (18/255) received infusions ≥10-years (one as late as 203.7 months). Subjects with SGS had a significantly longer time to last infusion (**Table 1.2.6)**. Additionally, recipients with SGS had more infusions ($p=0.01$) and greater infused islet mass ($p<0.0001$) (**Table 1.2.6**)

Table 1.2.6. Demographic and baseline characteristics of patients undergoing pancreatic islet transplantation

	Total population (n=255)	Sustained graft survival (n=178)	Non-sustained graft survival (n=77)	*P**
Type of transplant, n (%)				
ITA	239/255 (93.7)	163/178 (91.6)	76/77 (98.7)	0.031
IAK	16/255 (6.3)	15/178 (8.4)	1/77 (1.3)	
Patient characteristics at first transplant				
Sex				
Male, n (%)	106/255 (41.6)	77/178 (43.3)	29/77 (37.7)	0.41
Female, n (%)	149/255 (58.4)	101/178 (56.7)	48/77 (62.3)	
Race				
White, n (%)	218/255 (85.5)	154/178 (86.5)	64/77 (83.1)	
Black, n (%)	32/255 (12.6)	20/178 (11.2)	12/77 (15.6)	
Asian Canadian, n (%)	2/255 (0.8)	2/178 (0.6)	0/77 (0)	0.64 for all
Indigenous Peoples, n (%)	2/255 (0.8)	1/178 (0.005)	1/77 (0.1)	
Unknown, n (%)	1/255 (0.4)	1/178 (0.005)	0/77 (0)	
Age at diagnosis, yr (IQR)	14 (9 - 23)	14 (9.3 - 22)	16 (8 - 27)	0.53
Year of diagnosis (range)	1978 (1948 - 2007)	1977 (1948 - 2006)	1980 (1957 - 2007)	0.046
Duration of DM, yr (IQR)	30.6 (22.6 - 40.2)	33.5 (24.3 - 41.7)	26.2 (17.0 - 35.5)	0.0003
Age at transplant, yr (IQR)	48.8 (41.3 – 55.8)	49.4 (43.5 – 56.1)	44.2 (35.4 – 54.2)	0.001
Body-mass index, kg/m^2 (IQR)	25 (22.8 - 27.8)	24.9 (22.7 – 27.7)	25 (23 - 27.9)	0.72
Hypertension (%)	173/255 (67.8)	128/178 (71.9)	45/77 (58.4)	0.035
Dyslipidemia (%)	144/255 (56.5)	104/178 (58.4)	40/77 (52)	0.34
Stage 3 CKD - eGFR <60 ml/min/1.73 m^2 (%)	38/255 (14.9)	26/178 (14.6)	12/77 (15.6)	0.84
Macrovascular disease (%)	52/255 (20.4)	43/178 (24.2)	9/77 (11.7)	0.023
HYPO score (IQR)	1,207 (531 - 2,422)	1,289 (531 - 2,760)	966 (534 - 1,856)	0.20
Lability index (IQR)	449 (296 - 699)	447 (280 - 670)	452 (322 - 725)	0.58
Clarke score (IQR)	5 (4 – 7)	5 (5 – 6)	5 (4 – 7)	0.90
Stimulated C-peptide (nmol/L) (IQR)	0.02 (0.02 - 0.05)	0.02 (0.02 - 0.03)	0.02 (0.02 - 0.05)	0.06
HbA_{1c}%, mmol/mol (IQR)	8.2, 66.1 (7.5 – 9, 58.7 - 74.9)	8.2, 66.1 (7.5 – 9, 58.7 - 74.9)	8.5, 69.4 (7.8 – 9.2, 61.7 - 77.1)	0.23
Insulin units/kg/day (IQR)	0.54 (0.46 - 0.68)	0.53 (0.45 - 0.67)	0.59 (0.48 - 0.70)	0.032

eGFR, ml/min/1.73 m^2 (IQR)	85.9 (70.5 – 97)	84.8 (69.8 – 95.6)	88.2 (71.1 - 102.4)	0.32
Infusion characteristics				
Number of infusions per patient (IQR)	2 (2 – 3)	2 (2 – 3)	2 (2 – 3)	0.01
One infusion, n (%)	30/255 (11.8)	21/178 (11.8)	9/77 (11.7)	0.032
Two infusions, n (%)	131/255 (51.4)	82/178 (46.1)	49/77 (63.6)	for all
Three infusions, n (%)	62/255 (24.3)	46/178 (25.8)	16/77 (20.8)	
Four infusions, n (%)	28/255 (10.9)	25/178 (14)	3/77 (3.9)	
Five infusions, n (%)	4/255 (1.6)	4/178 (2.3)	0/77 (0)	
Time between infusions, mo (IQR)				
Time to 2nd infusion	5 (2.1 – 11.1)	4.9 (2.6 - 10.5)	5.8 (2.6 – 12.1)	0.86
Time to 3rd infusion	40.4 (16.6 – 70.9)	41.4 (19.6 – 73.9)	34.6 (8.7 – 66.9)	0.19
Time to 4th infusion	91 (68.5 – 140.5)	97.6 (68.5 – 143.7)	69.3 (67.8 – 83.2)	0.26
Time to 5th infusion	165.4 (143.6– 181.5)	165.4 (143.6– 181.5)	..	..
Time to last infusion	11.6 (2.4 - 47.6)	14.3 (2.6 - 57.7)	7.0 (1.6 - 16.6)	0.005
Recipients with all infusions ≤6 months, n (%)	98/255 (38.4)	63/178 (35.4)	35/77 (45.5)	0.13
Recipients with all infusions ≤12 months, n (%)	131/255 (51.4)	83/178 (46.6)	48/77 (62.3)	0.021
Total IEQs/kg of body weight, x1,000 (IQR)	14.3 (11.1 – 18.6)	15.9 (11.9 – 18.6)	11.9 (10.6 – 15.5)	<0.0001
Recipients with >11,000 IEQ/kg, n (%)	196 (76.9)	144 (80.9)	52 (67.5)	0.02
IEQs/kg of body weight for first infusion, x1,000 (IQR)	6.04 (5.19 – 7.06)	6.20 (5.23 – 7.27)	5.71 (5.04 – 6.48)	0.02
Weighted purity per patient†, % (IQR)	59.9 (51.4 - 68.5)	59.9 (51.6 - 67.7)	59.8 (59.9 - 67.7)	0.99
Weighted islet size index per patient† (IQR)	1.14 (0.96 - 1.27)	1.14 (1 – 1.28)	1.05 (0.91 – 1.16)	0.002
At least one female donor, n (%)	168/255 (66.4)	120/178 (68.2)	48/77 (62.3)	0.37

Data are n (%) and median (IQR). ITA: islet transplantation alone, IAK: islet-after-kidney transplantation, M: male, F: female, IEQ: islet equivalent, CKD: chronic kidney disease.

*X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables. *P* values correspond to sustained graft survival vs non-sustained graft survival. †Weighted averages were calculated as follows: weighted average= sum of weighted terms/total number of terms. For example, weighted average= purity^{infusion1}(islet number^{infusion1}) + purity^{infusion2}(islet number^{infusion2}) +…/total number of islets infused. ‡ Islet size index is defined as the number of islet equivalents divided by the number of islet particles (i.e., IEQ/islet number).

1.2.5.2 - Patient Survival

Over a median follow-up of 7.4 (4.4–12.2) years, and 2,161.9 patient-years, crude mortality was 10% (**Table 1.2.7**), with an incidence rate of 11.6 per 1,000 person-years (95%CI, 7.8-17.1). The estimated patient survival probability was 74% at 20-years (**Figure 1.2.2**). Median age at death was 62.3 years (range: 39.2–78.2). Causes of death are presented in **Table 1.2.8**. Patient survival was not impacted by graft survival or insulin independence outcomes (**Figure 1.2.3)**.

Table 1.2.7. Patient and graft survival following pancreatic islet transplantation

	Total population (n=255)	**Sustained graft survival (n=178)**	**Non-sustained graft survival (n=77)**	***P****
Patient and Graft Survival				
Median patient follow-up post-1st transplant, yr (IQR)	7.5 (4.4 – 12.2)	7.4 (4.4 -12.8)	7.4 (4.8 - 11.9)	0.82
Mortality, n (%)	25/255 (9.8)	18/178 (10.1)	7/77 (9.1)	0.80
Median age at death, yr (IQR)	62.3 (45.4 – 68)	62.0 (45.4 – 68.4)	62.1 (45.5 - 66.3)	0.86
Median graft survival post 1st transplant, yr (IQR)	5.9 (3 – 9.5)	7.4 (4.1 – 12.8)	3.1 (1.5 - 5.2)	<0.0001
Graft failure, n (%)	91/255 (35.7)	14/178 (7.8)	77/77 (100)	<0.0001
1-yr graft failure, n (%)	17/255 (6.7)	0/178 (0)	17/77 (22.1)	..
Median percentage of total follow-up with a surviving graft (IQR)	100 (69 - 100)	100 (100)	45.4 (20.9 - 68)	<0.0001

Data are n (%) and median (IQR).

*X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables. *P* values correspond to sustained graft survival vs non-sustained graft survival.

Figure 1.2.2. Kaplan-Meier estimates (95% CI) for patient survival after first transplant.

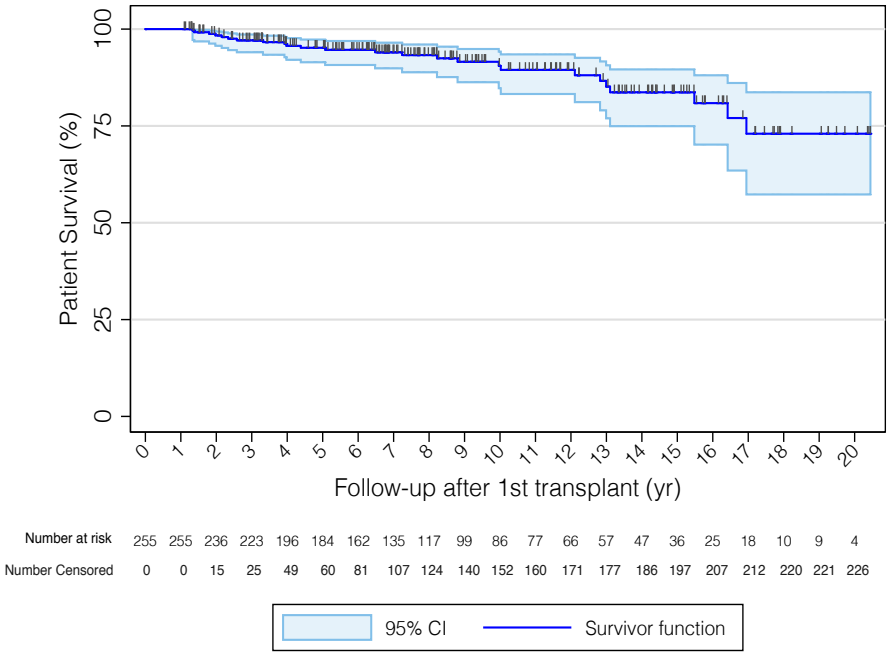
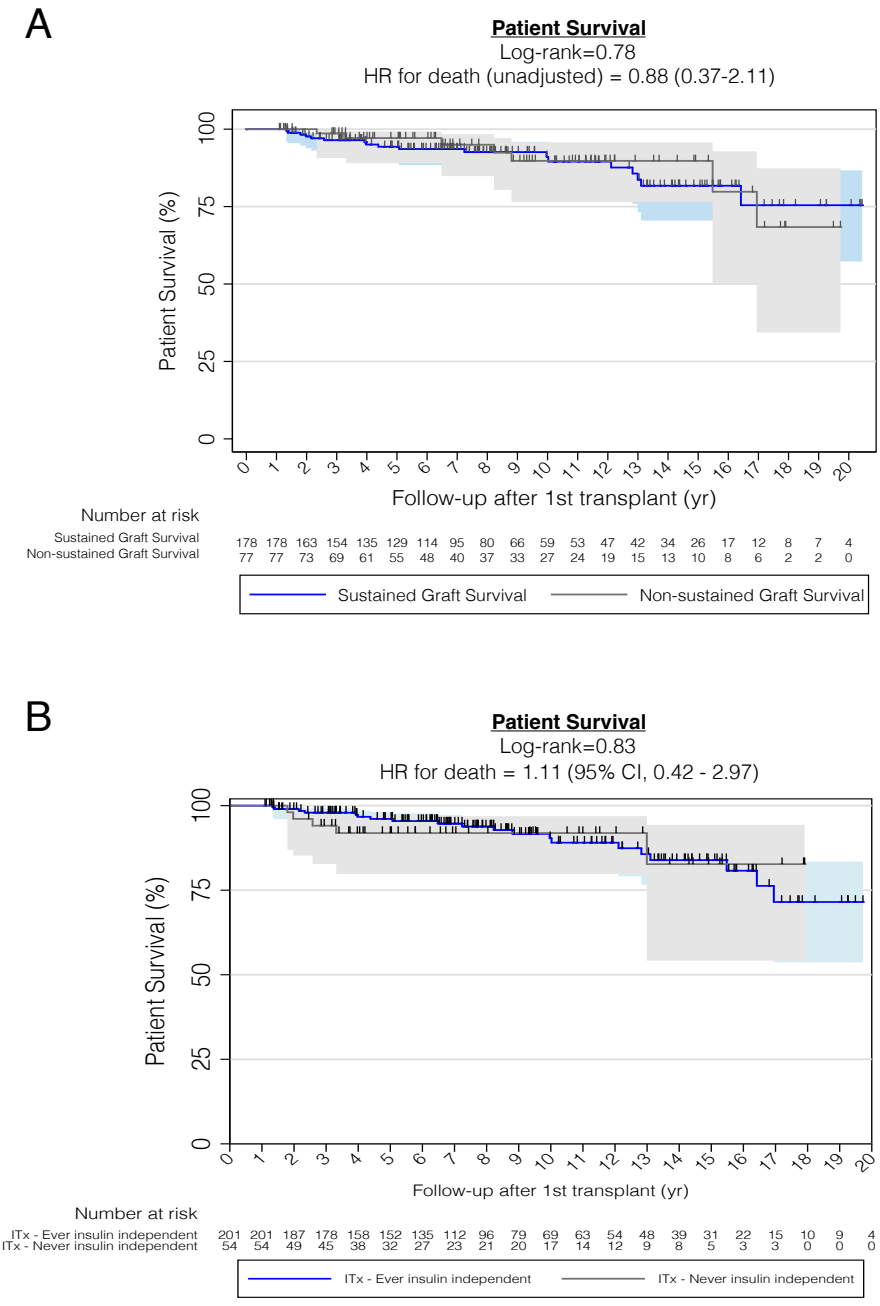


Table 1.2.8. Causes of death

	Total population (n=25/255)	Sustained graft survival (n=18/178)	Non-sustained graft survival (n=7/77)	*P**
Cardiovascular/cerebrovascular (%)	9 (36)	5 (27.8)	4 (57.1)	
Infection-related, n (%)	4 (16)	3 (16.7)	1 (14.3)	0.37
Malignancy-related, n (%)	3 (12)	3 (16.7)	0 (0)	for
Suicide/overdose, n (%)	4 (16)	4 (22.2)	0 (0)	all
Unknown/other, n (%)	5 (20)	3 (16.7)	2 (28.6)	

*X^2 was used to compare categorical variables. *P* values correspond to sustained graft survival vs non-sustained graft survival. This table is included as supplementary material in the published version of this manuscript.

Figure 1.2.3. Patient survival according to graft survival outcome during follow-up and achievement of insulin independence.

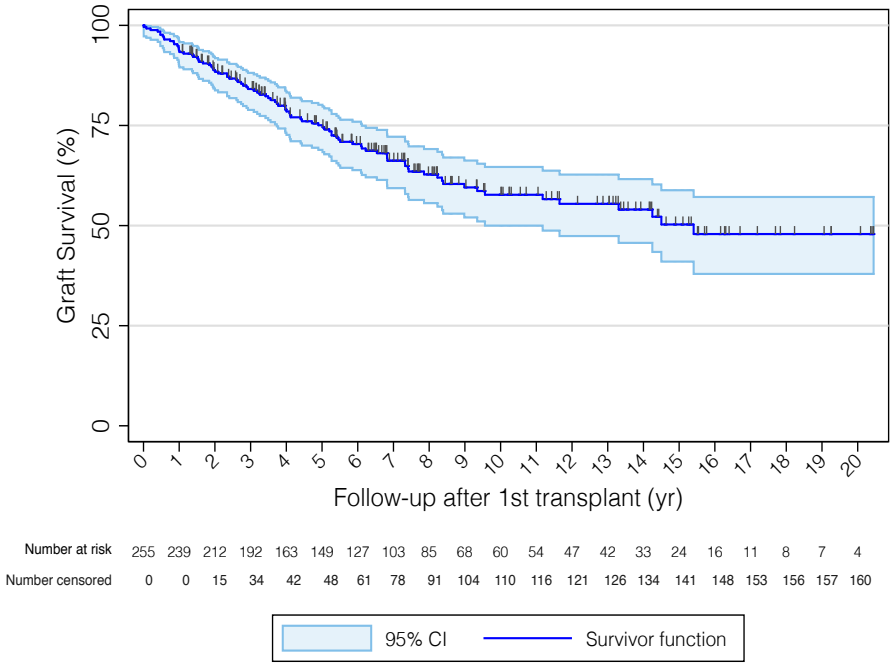


Note: Panel A shows Kaplan-Meier patient survival estimates stratified by graft survival outcome during follow-up. Panel B shows Kaplan-Meier patient survival estimates stratified by achievement of insulin independence. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). This figure is included as supplementary material in the published version of this manuscript.

1.2.5.3 - Graft Survival and Function

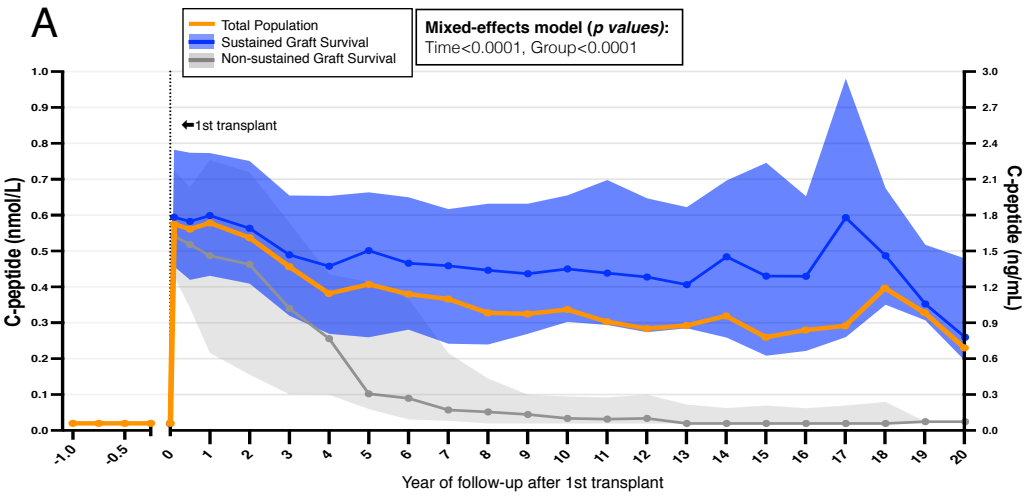
Median graft survival was 5.9 (3–9.5) years, and graft failure occurred in 36% (**Table 1.2.7**); 19% (17/91) of these happened <1-year after first infusion. The incidence rate for graft failure was 51.6 per 1,000 person-years (95%CI, 42–63.4). Kaplan-Meier estimates for graft survival at 1-, 5-, 10-, 15- and 20-years were 94%, 75%, 58%, 50% and 48%, respectively (**Figure 1.2.4**).

Figure 1.2.4. Kaplan-Meier estimates (95% CI) for graft survival after first transplant.

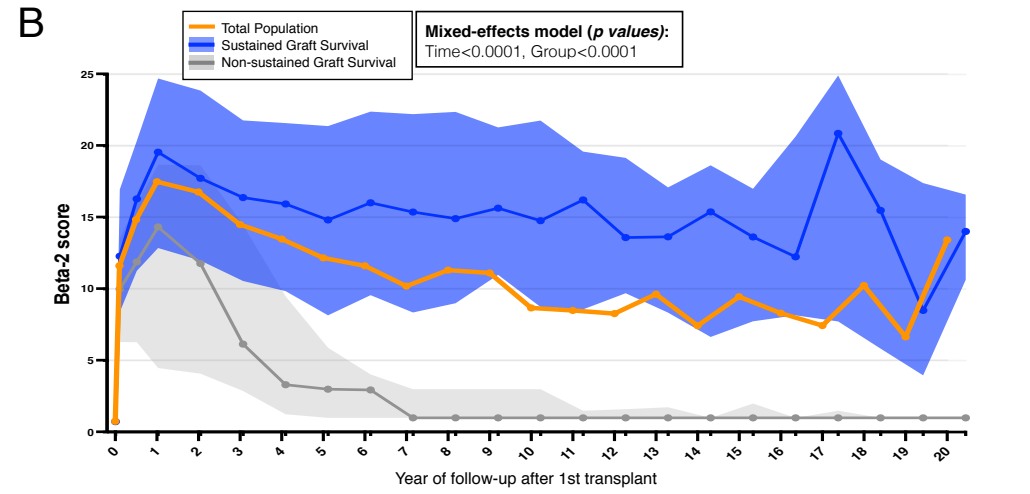


Fasting C-peptide levels increased substantially post-ITx and remained so throughout follow-up (**Figure 1.2.5A**). Moreover, differences in graft function between groups, as measured by the BETA-2 score, were observed as early as 1-month post-ITx (**Figure 1.2.5B**).

Figure 1.2.5. Fasting C-peptide levels and BETA-2 scores after first transplant.



Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	145	177	177	175	160	149	132	123	110	89	73	62	52	49	42	36	29	24	16	10	8	7
NSGS	57	77	74	71	69	59	51	45	41	37	34	29	26	23	17	13	11	8	7	4	2	2
Missing data(%)	20.8	0.4	1.6	3.5	3.0	6.7	6.6	8.7	6.8	6.7	8.5	8.1	9.3	6.5	10.6	14.0	14.9	11.1	8.0	22.2	0.0	0.0



Year	1 mo	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	177	154	148	122	107	99	88	78	62	51	44	41	36	32	25	20	14	10	7	5	4
NSGS	77	76	51	50	43	38	34	32	30	28	27	25	22	21	14	11	9	7	6	3	1	1
Missing data (%)	0.4	0.8	19.6	22.4	30.1	35.0	32.1	34.8	33.3	33.3	33.3	30.3	26.7	26.0	30.3	36.8	38.3	41.7	36.0	44.4	40.0	44.4

Note: Panel A shows fasting C-peptide serum levels before and after first transplant. Panel B shows BETA-2 scores during follow-up, starting at 1-month post 1st-transplant. Data are presented as median (solid line) and interquartile ranges (shaded area) for the total population (orange), and those with sustained graft survival (dark blue) and non-sustained graft survival (gray). A table describing available values in each group, as well as the percentage of missing data at every time point is included below each graph. Statistics using a mixed-effects model comparing SGS vs NSGS are shown

We observed significant correlations with graft survival and BETA-2 scores calculated within 1-year post-first infusion, which persisted when only patients having all infusions within 6- and 12-months were analyzed (**Table 1.2.9**).

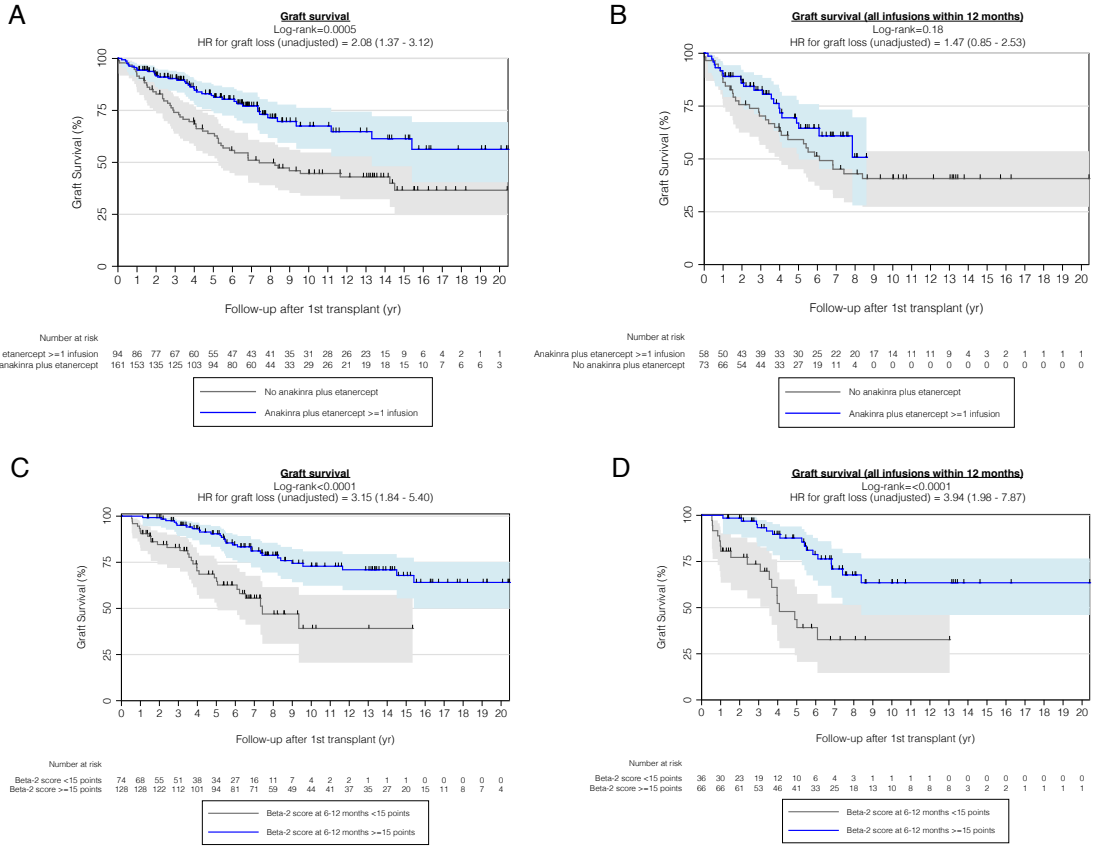
In multivariate logistic regression analyses, the combined use of anakinra plus etanercept (≥1 infusions) and a BETA-2 score ≥15 within 1-year post-first infusion significantly increased the odds of achieving SGS in univariate (**Figure 1.2.6**) and multivariate (**Table 1.2.10)** analyses. For the use of anakinra plus etanercept, the adjusted odds ratio (aOR) for SGS was 7.5 (95%CI, 2.7–21.0, $p<0.0001$), while the aOR for a BETA-2 score ≥15 was 4.1 (95%CI, 1.5–11.4, $p=0.007$, **Table 1.2.10**). Similar findings persisted in multivariate analyses restricted to recipients having all infusions within 6- and 12-months (**Table 1.2.11**). Sub-analyses stratified by the need of infusions beyond 6- and 12-months post-first transplant showed that graft survival was longer in patients having supplementary infusions (**Figure 1.2.7**).

Table 1.2.9. Correlation of BETA-2 scores with graft survival and total duration of insulin independence following pancreatic islet transplantation

	Spearman's Rho, *p* value	β-coefficient (95%CI), Pseudo R^2
Graft survival	0.49, <0.0001	0.29 (0.19-0.40), 0.11
Graft survival (patients with all infusions within 6 months)	0.59, <0.0001	0.26 (0.15-0.37), 0.16
Graft survival (patients with all infusions within 12 months)	0.51, <0.0001	0.26 (0.16-0.35), 0.16
Total duration of Insulin Independence	0.68, <0.0001	0.19 (0.14-0.24), 0.22
Total duration of Insulin Independence (patients with all infusions within 6 months)	0.79, <0.0001	0.16 (0.07-0.25), 0.23
Total duration of Insulin Independence (patients with all infusions within 12 months)	0.73, <0.0001	0.18 (0.09-0.25), 0.22

Note: This table is included as supplementary material in the published version of this manuscript.

Figure 1.2.6. Kaplan-Meier (KM) estimates (with 95%CI) for graft survival stratified by the combined use of anakinra plus etanercept or BETA-2 scores.



Note: Panel A shows KM estimates stratified by the combined use of anakinra plus etanercept (≥ 1 infusion). Panel B shows KM estimates stratified by the combined use of anakinra plus etanercept (≥ 1 infusion); this analysis is restricted to patients having all infusions within 12 months post-1st transplant. Panel C shows KM estimates stratified by a BETA-2 score cut-off of 15 points. Panel D shows KM estimates stratified by a BETA-2 score cut-off of 15 points; this analysis is restricted to patients having all infusions within 12 months post-1st transplant. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

Table 1.2.10. Multivariate binary logistic regression model for sustained graft survival

Sustained graft survival (n=194/255, 76.1%)	Adjusted* Odds Ratios	95%CI, p value
Age at 1st transplant in years	1.02	0.97 – 1.06, 0.52
Hypertension at 1st transplant	1.43	0.59 – 3.47, 0.43
Macrovascular disease at 1st transplant	1.20	0.41 – 3.51, 0.73
IEQs/kg of body weight (per 1,000 IEQs)	1.01	0.94 – 1.10, 0.64
Weighted average of islet size index†‡	2.94	0.57 – 15.08, 0.19
Insulin units/kg/day	0.84	0.06 – 11.34, 0.91
Use of anakinra plus etanercept (≥1 infusion)	7.51	2.67 – 21.02, <0.0001
BETA-2 score ≥15 points at 6-12 months post-1st transplant	4.11	1.48 – 11.43, 0.0066

*Logistic regression models were adjusted for age at 1st transplant, hypertension at baseline, macrovascular disease at baseline, total IEQs/kg of body weight (per 1,000 IEQs), weighted average of islet index, insulin units/kg/day, use of anakinra plus etanercept (≥1 infusion), as well as BETA-2 scores at 6-12 months. Only patients with graft survival ≥ 12 months were included in the model.

†Weighted averages were calculated as follows: weighted average= sum of weighted terms/total number of terms. For example, weighted average= islet size index*islets infusion1 + islet size index*islets infusion2 +…/total number of islets infused.

‡ Islet size index is defined as the number of islet equivalents divided by the number of islet particles (i.e., IEQ/islet number).

This table is included as supplementary material in the published version of this manuscript.

Table 1.2.11. Multivariable binary logistic regression models for sustained graft survival, restricted to patients having all their infusions within 12 months

	Adjusted* Odds ratios	95%CI, *p* value
Sustained graft survival, all infusions within 6 months (n=70/98, 71.4%)		
Age at 1st transplant in years	1.08	0.989 – 1.18, 0.09
Hypertension at baseline	0.60	0.13 – 2.76, 0.51
Macrovascular disease at baseline	1.22	0.11 – 14.01, 0.88
IEQs/kg of body weight (per 1,000 IEQs)	1.07	0.86 – 1.34, 0.54
Weighted average of islet size index†‡	3.80	0.22 – 66.00, 0.36
Insulin units/kg/day	0.18	0.002 – 19.66, 0.46
Use of anakinra + etanercept (≥1 infusion)	**7.02**	**1.16 – 42.56, 0.0340**
BETA-2 score ≥15 points at 6-12 months post-1st transplant	**11.74**	**1.71 – 80.48, 0.0121**
Sustained graft survival, all infusions within 12 months (n=95/131, 77.1%)		
Age at 1st transplant in years	1.04	0.98 – 1.11, 0.18
Hypertension at baseline	0.74	0.23 – 2.36, 0.61
Macrovascular disease at baseline	1.68	0.30 – 9.37, 0.55
IEQs/kg of body weight (per 1,000 IEQs)	1.04	0.89 – 1.20, 0.60

Weighted average of islet size index†‡	2.34	0.34 – 16.11, 0.39
Insulin units/kg/day	0.73	0.02 – 22.61, 0.86
Use of anakinra + etanercept (≥1 infusion)	**3.88**	**1.02 – 14.71, 0.0461**
BETA-2 score ≥15 points at 6-12 months post-1st transplant	**2.88**	**0.76 – 10.89, 0.12**

*Logistic regression models were adjusted for age at 1st transplant, hypertension at baseline, macrovascular disease at baseline, total IEQs/kg of body weight (per 1,000 IEQs), weighted average of islet size index insulin units/kg/day, use of anakinra plus etanercept (≥1 infusion), as well as BETA-2 scores at 6-12 months. Only patients with graft survival ≥ 6 and 12 months were included in the models, respectively.

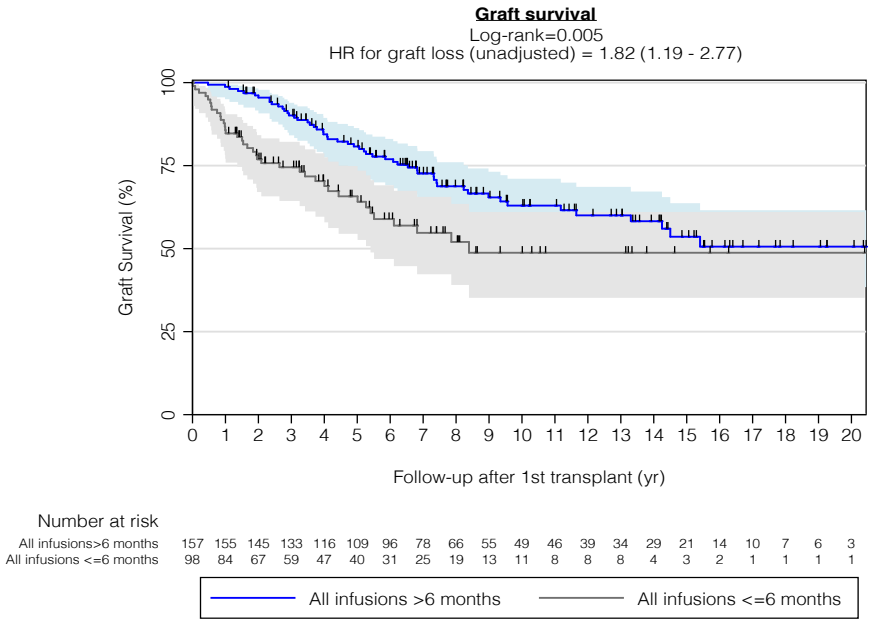
† Weighted averages were calculated as follows: weighted average= sum of weighted terms/total number of terms. For example, weighted average= islet size index*islets infusion1 + islet size index*islets infusion2 +…/total number of islets infused.

‡ Islet size index is defined as the number of islet equivalents divided by the number of islet particles (i.e., IEQ/islet number).

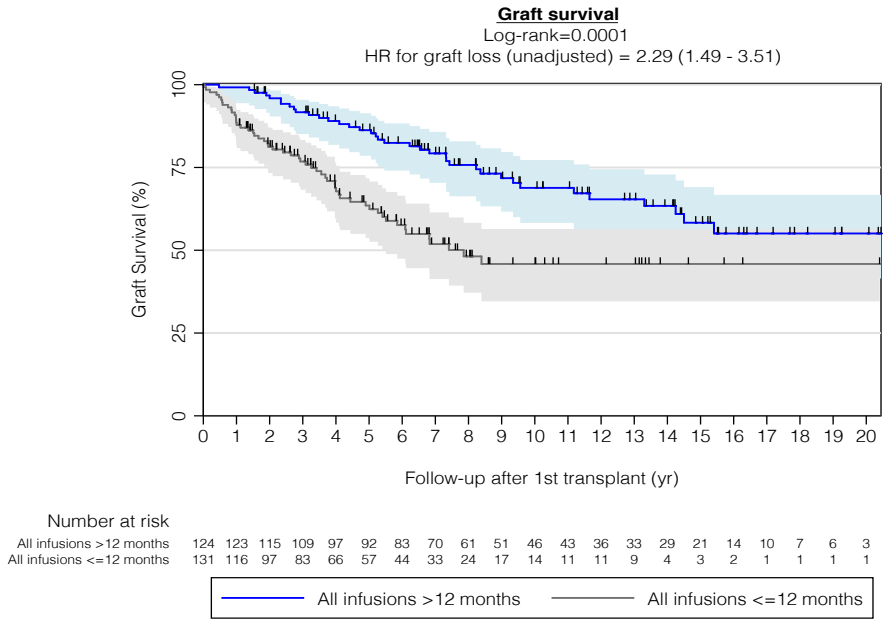
This table is included as supplementary material in the published version of this manuscript.

Figure 1.2.7. Kaplan-Meier (KM) estimates (with 95%CI) for graft survival by period of islet infusions.

A



B

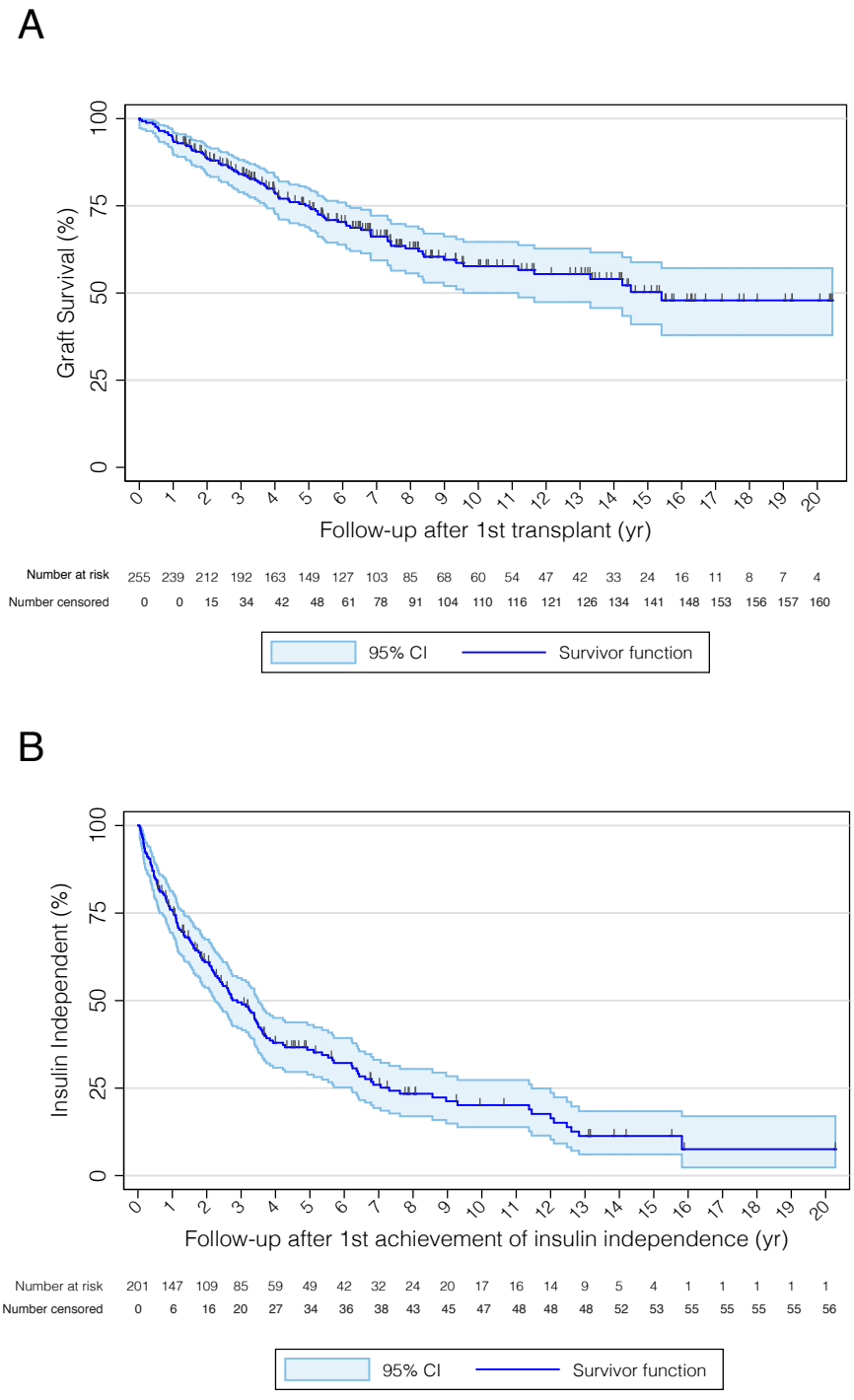


Note: Panel A shows KM estimates for graft survival and a comparison based on whether patients had all their infusions within 6 months. Panel B shows KM estimates for graft survival and a comparison based on whether patients had all their infusions within 12 months. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

1.2.5.4 - Insulin Independence

Insulin independence was ever achieved in 79% (201/255) (**Figure 1.2.8A)**, and more frequently in recipients with SGS (90% vs 53%, p<0.0001). Estimated of total duration of insulin independence estimates at 1-, 5-, 10-, 15- and 20-years were 61%, 32%, 20%, 13% and 8%, respectively (**Figure 1.2.8B**). Median time to insulin independence was 95 (30–196) days, while the median number of infusions to insulin independence was 2 (1–2). No differences were observed between groups (**Table 1.2.12**). Median total duration of insulin independence was 2.3 (IQR 0.9–4.9) years, which was significantly higher in recipients with SGS (**Table 1.2.12** and **Figure 1.2.9)**.

Figure 1.2.8. Kaplan-Meier (KM) estimates (with 95%CI) for graft survival (A) and total duration (B) of insulin independence



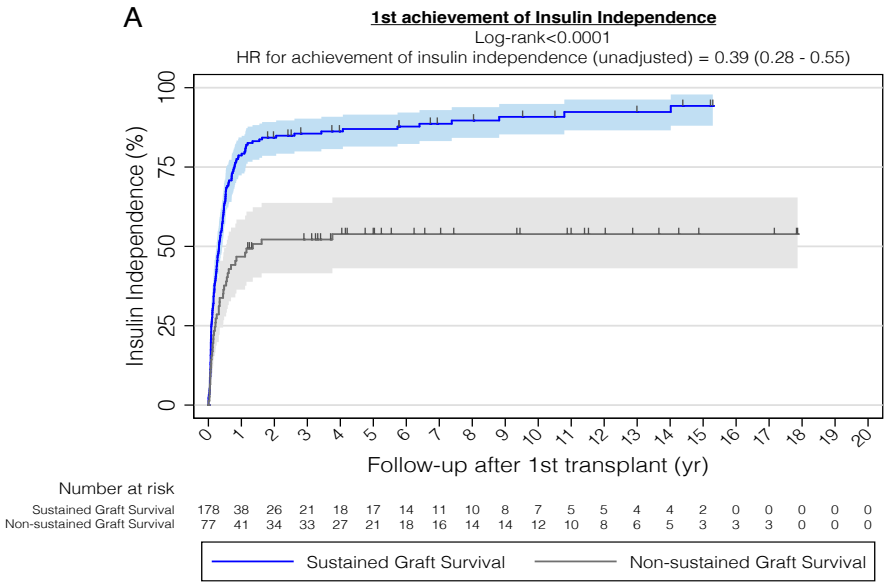
Note: Panel A shows KM estimates for graft survival. Panel B shows KM estimates for total duration of insulin independence. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel.

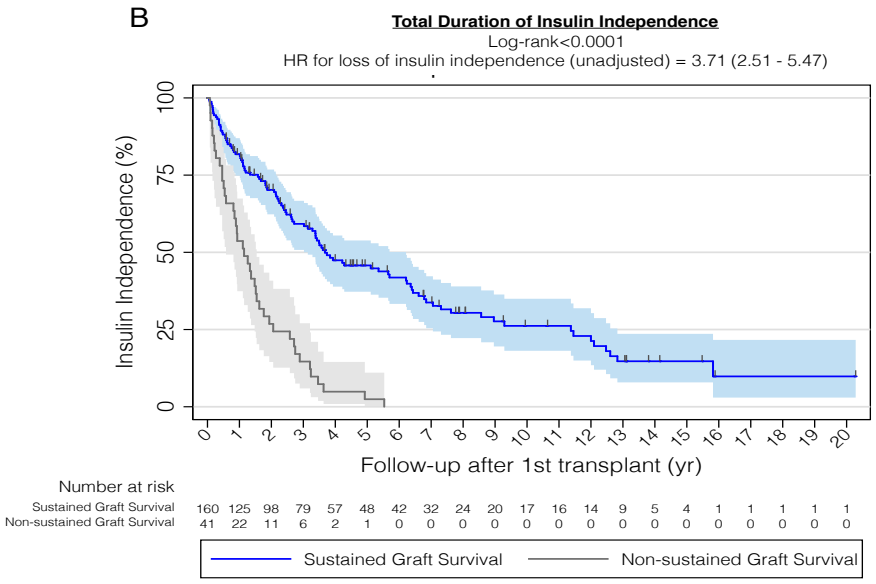
Table 1.2.12. Insulin independence outcomes following pancreatic islet transplantation

	Total population (n=255)	Sustained graft survival (n=178)	Non-sustained graft survival (n=77)	*P**
Insulin independence				
Insulin independence ever achieved, n (%)	201/255 (78.8)	160 (89.9)	41/77 (53.3)	<0.0001
Median time to insulin independence, days (IQR) †	95 (30 - 196)	96.5 (33 - 193.5)	81 (31 - 204)	0.75
Median number of infusions required for insulin independence (IQR) †	2 (1 - 2)	2 (1 - 2)	2 (1 - 2)	0.45
Median total duration of insulin independence, yr (IQR)†	2.3 (0.9 - 4.9)	2.7 (1.1 - 6.3)	1.1 (0.5 - 2)	<0.0001
Median percentage of total follow-up with insulin independence (IQR)	22 (2.5 - 63.5)	39.2 (11.2 - 77.7)	1.3 (0 - 17)	<0.0001

Data are n (%) and median (IQR).
*X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables.
P values correspond to sustained graft survival vs non-sustained graft survival.
† Only patients achieving insulin independence were included for this analysis.

Figure 1.2.9. Kaplan-Meier (KM) estimates (with 95%CI) for achievement and total duration of insulin independence stratified by graft survival outcome.

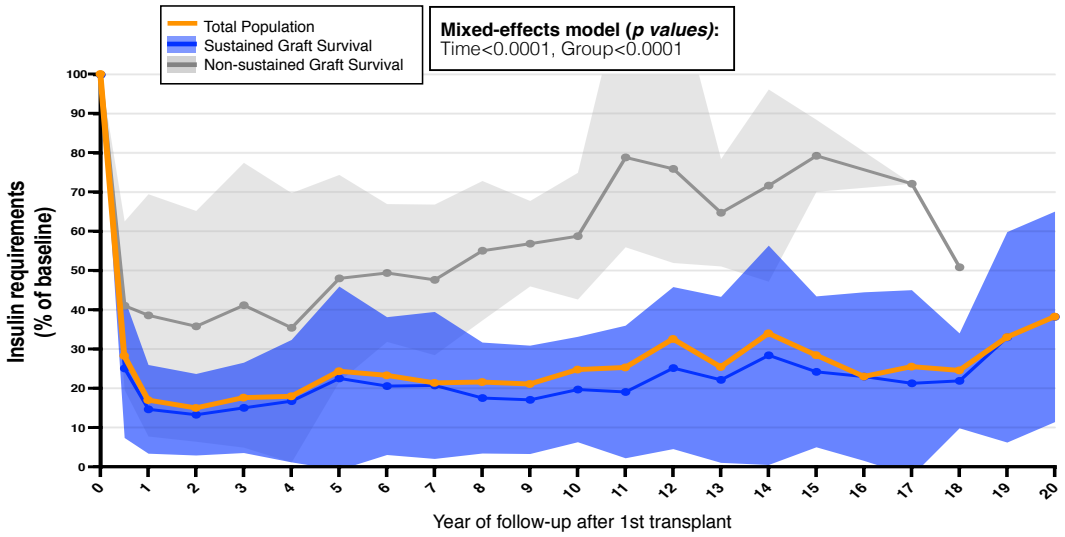




Note: Panel A shows KM estimates for achievement of insulin independence. Panel B shows KM estimates for total duration of insulin independence. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

A substantial decrease in insulin requirements was documented in recipients with SGS (**Figure 1.2.10)**. Similar to graft survival, we found significant correlations with BETA-2 scores measured within 1-year post-first infusion and total duration of insulin independence, which persisted in analyses restricted to patients having all infusions within 6- and 12-months (**Table 1.2.11)**. In contrast to graft survival, supplementary infusions (**Figure 1.2.11)** and use of anakinra plus etanercept (**Figure 1.2.11**) did not positively impact total duration of insulin independence. Conversely, a BETA-2 score $\geq$15 within 1-year post-first infusion was still associated with a longer total duration of insulin independence (**Figure 1.2.11)**.

Figure 1.2.10. Insulin requirements (as a percentage of baseline) after first transplant.

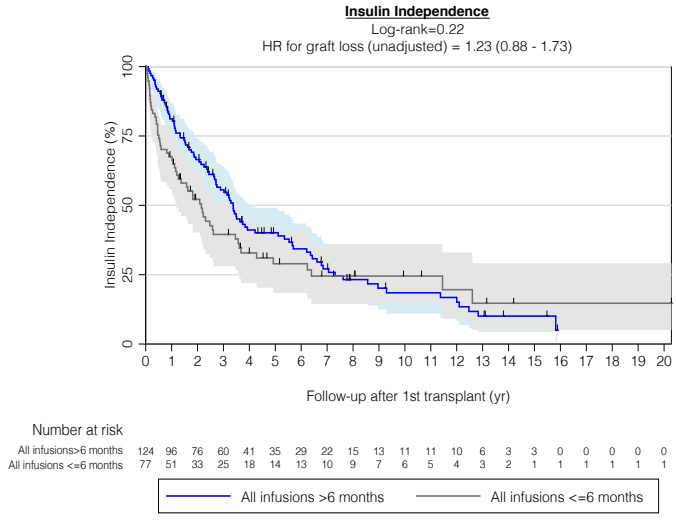


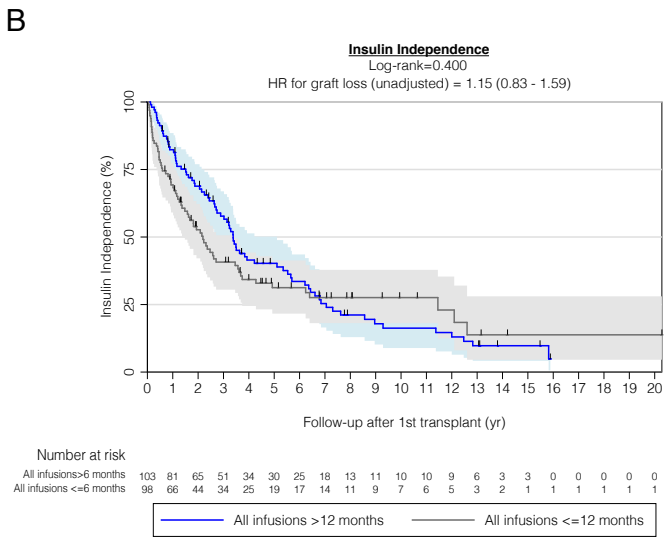
Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	177	154	149	125	111	98	86	78	63	53	47	43	41	36	27	24	14	11	10	5	6
NSGS	77	77	51	45	35	23	15	11	9	8	6	7	5	7	3	4	2	0	1	1	0	0
Missing data (%)	0.4	0.4	19.6	23.9	32.2	39.9	42.3	47.3	46.3	47.4	49.6	45.5	44.2	37.7	40.9	45.6	44.7	61.1	52.0	38.9	50.0	33.3

Note: Data are presented as median (solid line) and interquartile ranges (shaded area) for the total population (orange), and those with sustained graft survival (dark blue) and non-sustained graft survival (gray). A table describing available values in each group, as well as the percentage of missing data at every time point is included below each graph. Statistics using a mixed-effects model comparing SGS vs NSGS are shown

Figure 1.2.11. Kaplan-Meier (KM) estimates (with 95%CI) for total duration of insulin independence by period of islet infusions.

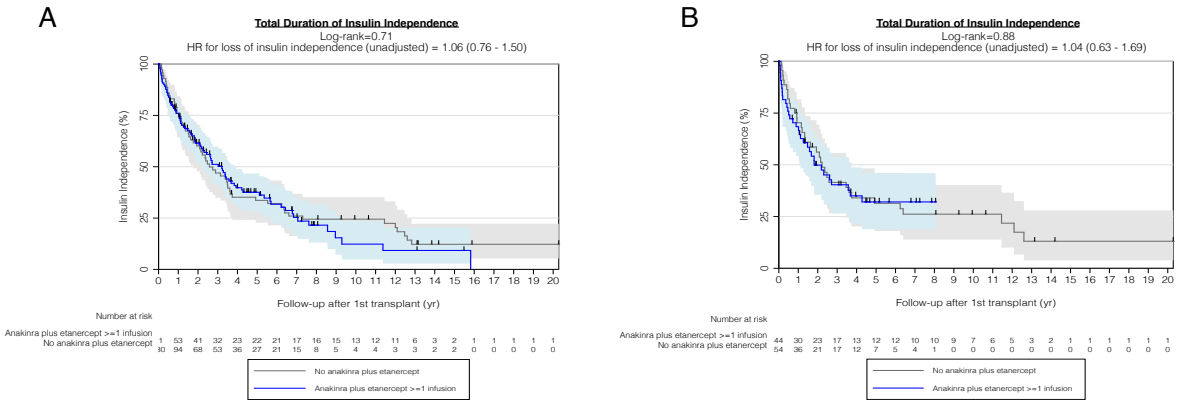
A

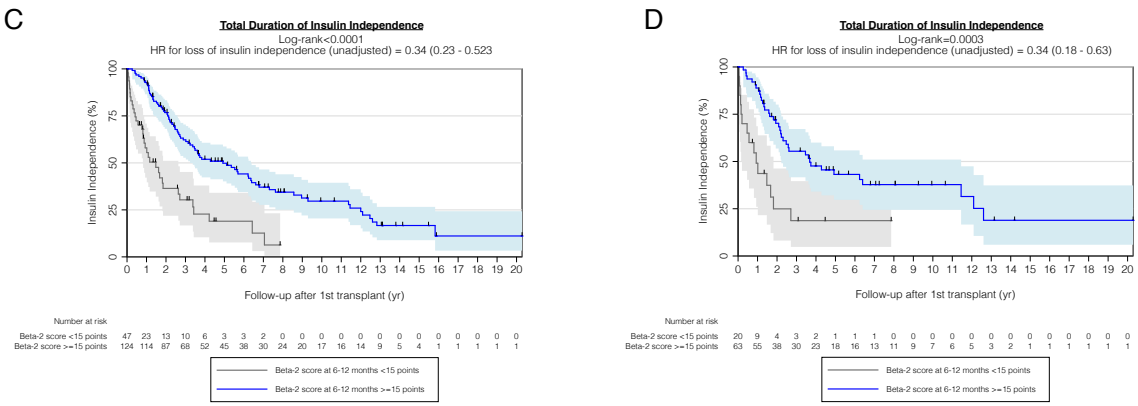




Note: Panel A shows KM estimates for insulin independence and a comparison based on whether patients had all their infusions within 6 months. Panel B shows KM estimates for insulin independence and a comparison based on whether patients had all their infusions within12 months. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

Figure 1.2.12. Kaplan-Meier (KM) estimates (with 95%CI) for total duration of insulin independence stratified by the combined use of anakinra plus etanercept and BETA-2 scores.



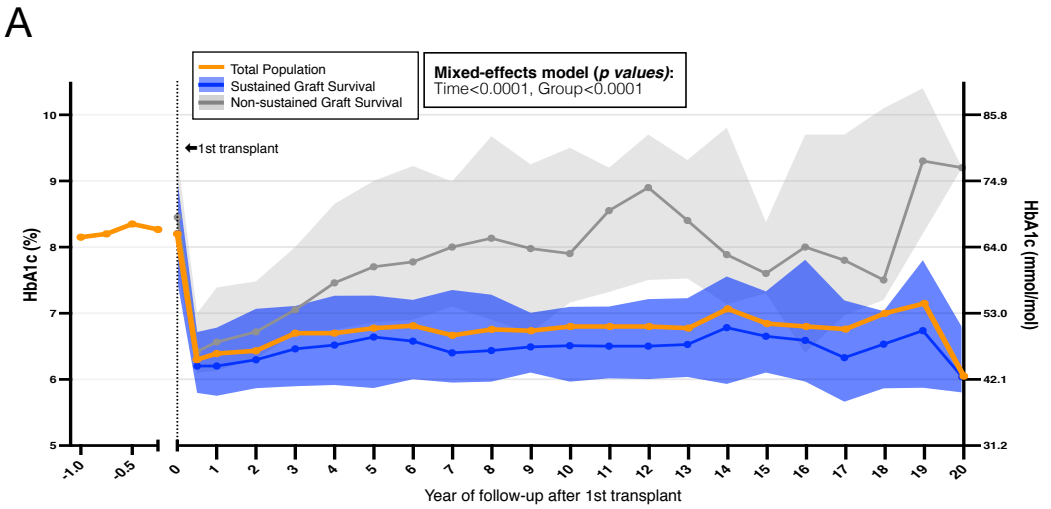


Note: Panel A shows KM estimates stratified by the combined use of anakinra plus etanercept (≥ 1 infusion). Panel B shows KM estimates stratified by the combined use of anakinra plus etanercept (≥ 1 infusion); this analysis is restricted to patients having all infusions within 12 months post-1st transplant. Panel C shows KM estimates stratified by a BETA-2 score cut-off of 15 points. Panel D shows KM estimates stratified by a BETA-2 score cut-off of 15 points; this analysis is restricted to patients having all infusions within 12 months post-1st transplant. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

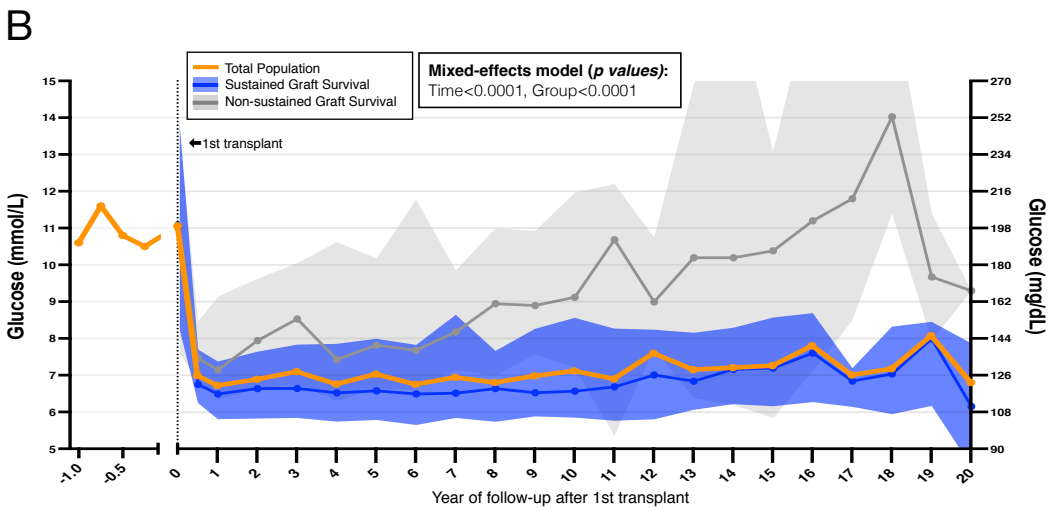
1.2.4.5 - Glycemic control and Measures of Hypoglycemia and Glycemic Lability

Marked improvements in glycemic control were evident following ITx. As expected, both HbA_{1c} levels (**Figure 1.2.13A**) and fasting plasma glucose (**Figure 1.2.13B**) showed sustained improvements in recipients achieving SGS. Additionally, HbA_{1c} levels <7% during follow-up were significantly more prevalent in these subjects (**Table 1.2.13)**. Non-insulin glucose-lowering agents were used at any point in 50% (127/255), and more commonly in recipients achieving SGS (p=0.045, **Table 1.2.14).** Measures of hypoglycaemia, such as HYPO scores, lability indexes and Clarke scores also improved significantly throughout follow-up, particularly in patients achieving SGS (**Figure 1.2.14**).

Figure 1.2.13. HbA_{1c} (A) and fasting plasma glucose (B) levels after first transplant.



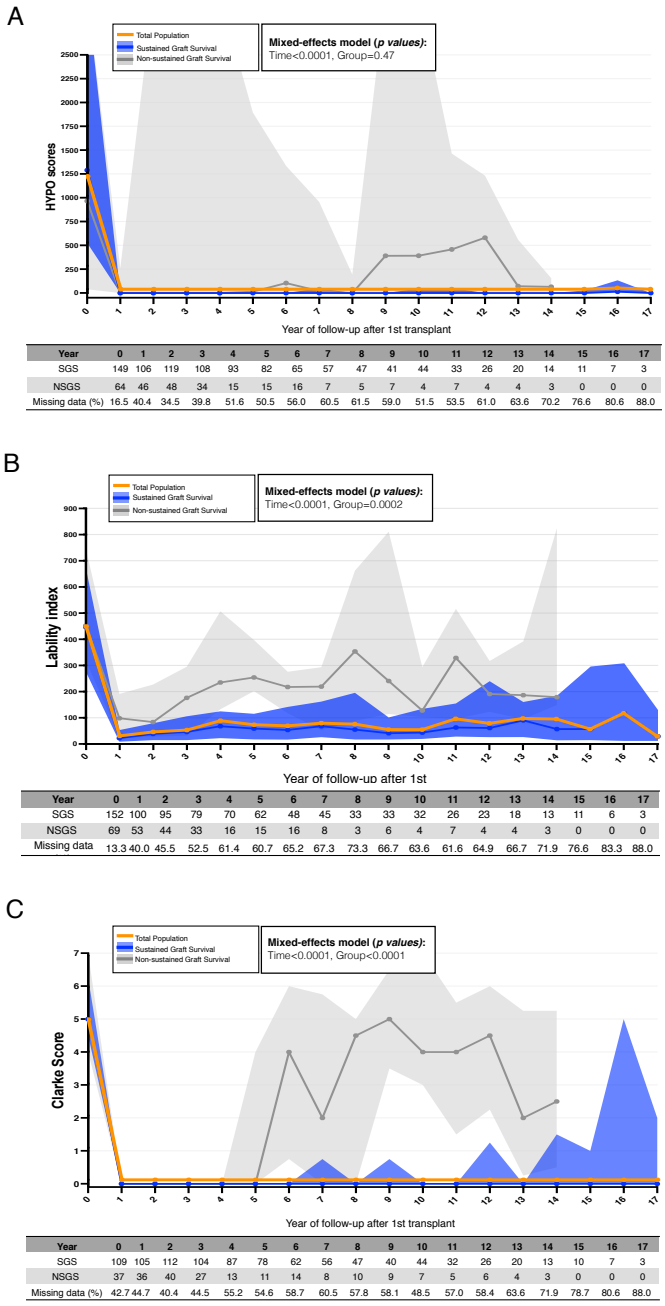
Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	177	177	175	163	147	131	122	110	88	77	61	54	48	44	40	31	26	17	10	8	6
NSGS	77	76	75	73	71	55	43	43	35	32	31	27	22	19	13	12	11	7	7	3	2	1
Missing data (%)	0.4	0.8	1.2	2.7	0.8	9.4	11.2	10.3	10.5	11.1	7.7	11.1	11.6	13.0	13.6	8.8	10.6	8.3	4.0	27.8	0.0	22.2



Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	148	177	177	174	157	147	129	119	107	91	74	63	54	46	42	39	29	24	15	9	8	6
NSGS	60	77	75	71	60	55	42	38	27	21	24	18	11	13	12	10	4	4	3	2	2	1
Missing data (%)	18.4	0.4	1.2	3.9	8.1	9.4	12.8	14.7	17.3	17.0	16.2	18.2	24.4	23.4	18.2	14.0	29.8	22.2	28.0	38.9	0.0	22.2

Note: Panel A shows HbA_{1c} levels before and after first transplant. Panel B shows fasting plasma glucose level before and after first transplant. Data are presented as median (solid line) and interquartile ranges (shaded area) for the total population (orange), and those with sustained graft survival (dark blue) and non-sustained graft survival (gray). A table describing available values in each group, as well as the percentage of missing data at every time point is included below each graph. Statistics using a mixed-effects model comparing SGS vs NSGS are shown in each panel.

Figure 1.2.14. Measures of burden of hypoglycemia, glycemic lability and hypoglycemia unawareness after first transplant



Year	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SGS	149	106	119	108	93	82	65	57	47	41	44	33	26	20	14	11	7	3
NSGS	64	46	48	34	15	15	16	7	5	7	4	7	4	4	3	0	0	0
Missing data (%)	16.5	40.4	34.5	39.8	51.6	50.5	56.0	60.5	61.5	59.0	51.5	53.5	61.0	63.6	70.2	76.6	80.6	88.0

Year	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SGS	152	100	95	79	70	62	48	45	33	33	32	26	23	18	13	11	6	3
NSGS	69	53	44	33	16	15	16	8	3	6	4	7	4	4	3	0	0	0
Missing data	13.3	40.0	45.5	52.5	61.4	60.7	65.2	67.3	73.3	66.7	63.6	61.6	64.9	66.7	71.9	76.6	83.3	88.0

Year	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SGS	109	105	112	104	87	78	62	56	47	40	44	32	26	20	13	10	7	3
NSGS	37	36	40	27	13	11	14	8	10	9	7	5	6	4	3	0	0	0
Missing data (%)	42.7	44.7	40.4	44.5	55.2	54.6	58.7	60.5	57.8	58.1	48.5	57.0	58.4	63.6	71.9	78.7	80.6	88.0

Note: Panel A shows HYPO scores during follow-up. Panel B shows lability indexes during follow-up. Panel C shows Clarke scores during follow-up. Data are presented as median (solid line) and interquartile ranges (shaded area) for the total population (orange), and those with sustained graft survival (dark blue) and non-sustained graft survival (gray). A table describing available values in each group, as well as the percentage of missing data at every time point is included below each graph. Statistics using a mixed-effects model comparing SGS vs NSGS are shown in each panel.

Table 1.2.13. Median difference in HbA_{1c} (%) levels over time following pancreatic islet transplantation

Follow-up after 1st transplant	Total population (N=255)	Sustained Graft Survival (n=178)	Non-sustained Graft Survival (n=77)
Baseline (n:255, SGS: 178, NSGS:77)			
Median (IQR)	8.32 (1.2)	8.3 (1.2)	8.4 (1.2)
1 to 6 months (n: 255, SGS: 178, NSGS: 77)			
Median difference vs baseline* (IQR)	1.86 (1.15-2.65)	1.86 (1.1 – 2.67)	1.86 (1.29 – 2.58)
Patients with HbA_{1c} <7%, n (%)	210 (82.4)	153 (86.0)	57 (74.0)
6 to 12 months (n: 255, SGS: 178, NSGS: 77)			
Median difference vs baseline (IQR)	1.7 (0.88-2.5)	1.81 (1.03 – 2.52)	1.5 (0.70 – 2.38)
Patients with HbA_{1c} <7%, n (%)	188 (73.7)	138 (77.5)	50 (64.9)
12 to 24 months (n: 255, SGS: 178, NSGS: 77)			
Median difference vs baseline (IQR)	1.50 (0.76-2.3)	1.61 (0.82 – 2.53)	1.28 (0.53 – 2.1)
Patients with HbA_{1c} <7%, n (%)	170 (66.7)	129 (72.5)	41 (53. 2)
24 to 36 months (n: 236, SGS: 163, NSGS: 73)			
Median difference vs baseline (IQR)	1.41 (0.64-2.28)	1.6 (0.84 – 2.4)	1.18 (0.11 – 1.41)
Patients with HbA_{1c} <7%, n (%)	151 (64.0)	117 (71.8)	34 (46.6)
36 to 48 months (n: 223, SGS: 154, NSGS: 69)			
Median difference vs baseline (IQR)	1.29 (0.56-2.13)	1.54 (0.72 – 2.35)	0.60 (-0.15 – 1.31)
Patients with HbA_{1c} <7%, n (%)	121 (54.3)	102 (66.2)	18 (26.1)
48 to 60 months (n: 196, SGS: 135, NSGS: 61)			
Median difference vs baseline (IQR)	1.23 (0.54-2.17)	1.47 (0.80 – 2.3)	0.54 (-0.4 – 1.05)
Patients with HbA_{1c} <7%, n (%)	105 (53.2)	105 (77.8)	13 (21.3)
60 to 72 months (n: 184, SGS: 129, NSGS: 55)			
Median difference vs baseline (IQR)	1.03 (0.41-1.89)	1.33 (0.6 – 2.3)	0.45 (-0.45 – 0.95)
Patients with HbA_{1c} <7%, n (%)	90 (48.9)	79 (61.2)	11 (20.0)
72 to 84 months (n: 162, SGS: 114, NSGS: 48)			
Median difference vs baseline (IQR)	1.21 (0.31-2.12)	1.43 (0.79 – 2.27)	0.21 (-0.17 – 0.85)
Patients with HbA_{1c} <7%, n (%)	80 (49.4)	80 (70.2)	8 (16.7)

84 to 96 months (n: 135, SGS: 95, NSGS: 40)			
Median difference vs baseline (IQR)	1.0 (0.10-1.86)	1.39 (0.72 – 2.0)	0.01 (-0.68 – 0.44)
Patients with HbA_{1c} <7%, n (%)	68 (50.4)	60 (63.2)	8 (20.0)
96 to 108 months (n: 117, SGS: 80, NSGS: 37)			
Median difference vs baseline (IQR)	1.0 (0.22-2.0)	1.5 (0.71 – 2.08)	0.15 (-0.75 – 1.18)
Patients with HbA_{1c} <7%, n (%)	65 (55.5)	59 (73.8)	8 (21.6)
108 to 120 months (n: 99, SGS: 66, NSGS: 33)			
Median difference vs baseline (IQR)	0.8 (-0.10-1.9)	1.36 (0.31 – 2.32)	0 (-1.0 – 0.98)
Patients with HbA_{1c} <7%, n (%)	47 (47.5)	41 (62.1)	6 (18.2)
120 to 132 months (n: 86, SGS: 59, NSGS: 27)			
Median difference vs baseline (IQR)	1.0 (0.1-1.95)	1.34 (0.5 – 2.39)	0.18 (-1.15 – 0.6)
Patients with HbA_{1c} <7%, n (%)	41 (47.7)	38 (64.4)	3 (11.1)
132 to 144 months (n: 77, SGS: 53, NSGS: 24)			
Median difference vs baseline (IQR)	0.96 (0.20-1.86)	1.35 (0.5 – 2.34)	-0.20 (-0.9 – 0.6)
Patients with HbA_{1c} <7%, n (%)	35 (45.5)	33 (62.3)	2 (8.3)
144 to 156 months (n: 66, SGS: 47, NSGS: 19)			
Median difference vs baseline (IQR)	0.96 (0.44-1.95)	1.18 (0.63 – 2.45)	0.45 (-0.15 – 0.83)
Patients with HbA_{1c} <7%, n (%)	30 (45.5)	30 (63.8)	2 (10.5)
156 to 168 months (n: 57, SGS: 42, NSGS: 15)			
Median difference vs baseline (IQR)	0.72 (0.09-1.96)	1.11 (0.37 – 2.09)	0.16 (-0.09 – 0.80)
Patients with HbA_{1c} <7%, n (%)	24 (42.1)	24 (57.1)	1 (6.7)
168 to 180 months (n: 47, SGS:34, NSGS: 13)			
Median difference vs baseline (IQR)	0.76 (0.12-2.26)	0.77 (0.12 – 2.29)	0.45 (0.10 – 1.23)
Patients with HbA_{1c} <7%, n (%)	23 (48.9)	21 (61.7)	2 (15.4)
180 to 192 months (n: 36, SGS: 26, NSGS: 10)			
Median difference vs baseline (IQR)	0.66 (0.17-1.8)	0.72 (0.1 – 2.3)	0.6 (0.17 – 0.7)
Patients with HbA_{1c} <7%, n (%)	17 (47.2)	17 (65.4)	2 (20.0)
192 to 204 months (n: 25, SGS: 17, NSGS: 8)			
Median difference vs baseline (IQR)	0.8 (0.41-2.48)	1.2 (0.45 – 2.88)	0.4 (-0.7 – 0.8)
Patients with HbA_{1c} <7%, n (%)	14 (56.0)	14 (82.4)	2 (25)

204 to 216 months (n: 18, SGS: 12, NSGS: 6)			
Median difference vs baseline (IQR)	1.4 (0.730-2.25)	1.78 (0.30 – 2.83)	1.1 (0.3 – 2.5)
Patients with HbA_{1c} <7%, n (%)	6 (33.3)	6 (50.0)	0 (0)
216 to 228 months (n: 10, SGS: 8, NSGS: 2)			
Median difference vs baseline (IQR)	1.92 (0.3-2.5)	1.54 (0.73 – 2.34)	-0.45 (-2.4 – 1.5)
Patients with HbA_{1c} <7%, n (%)	4 (40)	4 (50.0)	0 (0)
228 to 240 months (n: 9, SGS: 7, NSGS: 2)			
Median difference vs baseline (IQR)	2.1 (-0.7-2.47)	2.08 (0.93 – 2.47)	-1.2 (..)
Patients with HbA_{1c} <7%, n (%) (%)	5 (55.5)	5 (71.4)	0 (0)

SGS: sustained graft survival, NSGS: non-sustained graft survival, IQR: interquartile range

* Median differences (IQR) are calculated between baseline and follow-up values. This table is included as supplementary material in the published version of this manuscript.

Table 1.2.14. Use of non-insulin glucose-lowering agents post-transplantation

	Total population (n=255)	Sustained graft survival (n=178)	Non-sustained graft survival (n=77)	*P**
Use of non-insulin glucose-lowering agents, n (%)	127/255 (49.8)	96/178 (53.9)	31/77 (40.3)	0.045
SGLT-2 inhibitors, n (%)	29/127 (22.8)	25/96 (27.1)	4/31 (12.9)	0.13
GLP-1 agonists, n (%)	24/127 (18.9)	18/96 (18.8)	6/31 (19.4)	0.94
DPP-4 inhibitors, n (%)	84/127 (66.1)	77/96 (80.0)	7/31 (22.6)	<0.0001
Thiazolidinediones (glitazones), n (%)	27/127 (21.3)	13/96 (13.5)	14/31 (45.2)	<0.0001
Metformin, n (%)	43/127 (33.9)	26/96 (27.0)	17/31 (54.8)	0.005
Other (acarbose, glyburide, repaglinide), n (%)	22/127 (17.3)	11/96 (11.5)	11/31 (35.5)	0.002

*X^2 was used to compare categorical variables. *P* values correspond to sustained graft survival vs non-sustained graft survival. SGLT-2: sodium-glucose transporter-2, GLP-1: glucagon-like peptide-1, DPP-4: dipeptidyl peptidase-4. This table is included as supplementary material in the published version of this manuscript

1.2.5.6 - Adverse Events

Procedure-related life-threatening complications were uncommon (7%, 40/610 infusions); 48% (19/40) related to bleeding (4/19 requiring only readmission/transfusion, 7/19 embolization and 8/19 surgery). A lower rate in subjects achieving SGS was observed (**Table 1.2.15)**. Regarding adverse effects of long-term immunosuppression, we investigated CKD and ESRD, malignant neoplasms, and life-threatening infections (**Table 1.2.15**).

Table 1.2.15. Adverse events following pancreatic islet transplantation

	Total population (n=255)	Sustained graft survival (n=178)	Non-sustained Graft Survival (n=77)	*P**
Procedural complications, n infusions (%)	40/610 (2.9)	23/443 (5.2)	17/167 (10.2)	0.026
Stage 3 CKD†, n (%)	132/217 (60.8)	104/152 (68.4)	28/65 (43.1)	<0.0001
Stage 4 CKD, n (%)	43/255 (16.9)	30/178 (16.9)	13/77 (16.9)	0.99
Baseline eGFR>60 ml/min/1.73m^2	25/217 (11.5)	19/152 (12.5)	6/65 (9.2)	0.49
Baseline eGFR<60 ml/min/1.73m^2	18/38 (47.4)	11/26 (42.3)	7/12 (58.3)	0.36
IAK	2/16 (12.5)	2/15 (13.3)	0/1 (0)	0.70
Stage 5 CKD, n (%)	18/255 (7.1)	11/178 (6.2)	7/77 (9.1)	0.41
Baseline eGFR>60 ml/min/1.73m^2	8/217 (3.7)	6/152 (4)	2/65 (3.1)	0.76
Baseline eGFR<60 ml/min/1.73m^2	10/38 (26.3)	5/26 (19.2)	5/12 (41.7)	0.14
IAK	0/16 (0)	0/15 (0)	0/1 (0)	..
Dialysis/Kidney Transplant, n (%)	14/255 (5.5)	8/178 (4.5)	6/77 (7.8)	0.29
Baseline eGFR>60 ml/min/1.73m^2	6/217 (2.8)	5/152 (3.3)	1/65 (1.5)	0.47
Baseline eGFR<60 ml/min/1.73m^2	8/38 (21.1)	3/26 (11.5)	5/12 (41.7)	0.034
IAK	0/16 (0)	0/15 (0)	0/1 (0)	..
Life-threatening infections, n (%)‡	29/224 (12.9)	19/162 (11.7)	10/62 (16.1)	0.38
Cancer, n (%)	33/255 (12.9)	29/178 (16.3)	4/77 (5.2)	0.015
Non-skin cancer, n (%)	11/33 (33.3)	7/29 (24.1)	4/4 (100)	0.003

Data are n (%) and median (IQR). Stage 3 CKD, Stage 4 CKD and Stage 5 CKD are defined as a persistent estimated glomerular filtration rate <60 mL/min/1.73m^2, <30 mL/min/1.73m^2, and <15 mL/min/1.73m^2, respectively (see table 1.2.3).

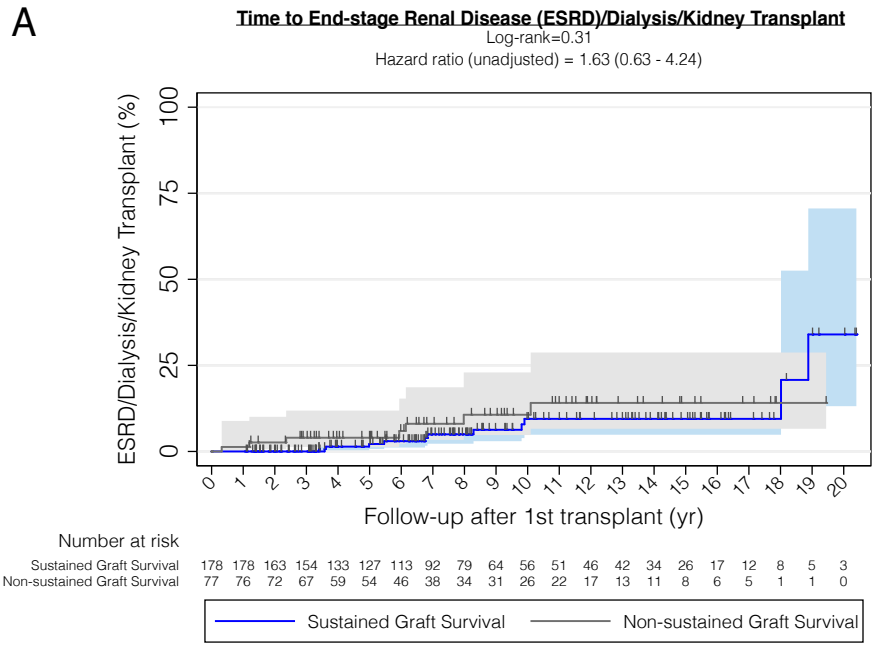
*X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables. *P* values correspond to sustained graft survival vs non-sustained graft survival.

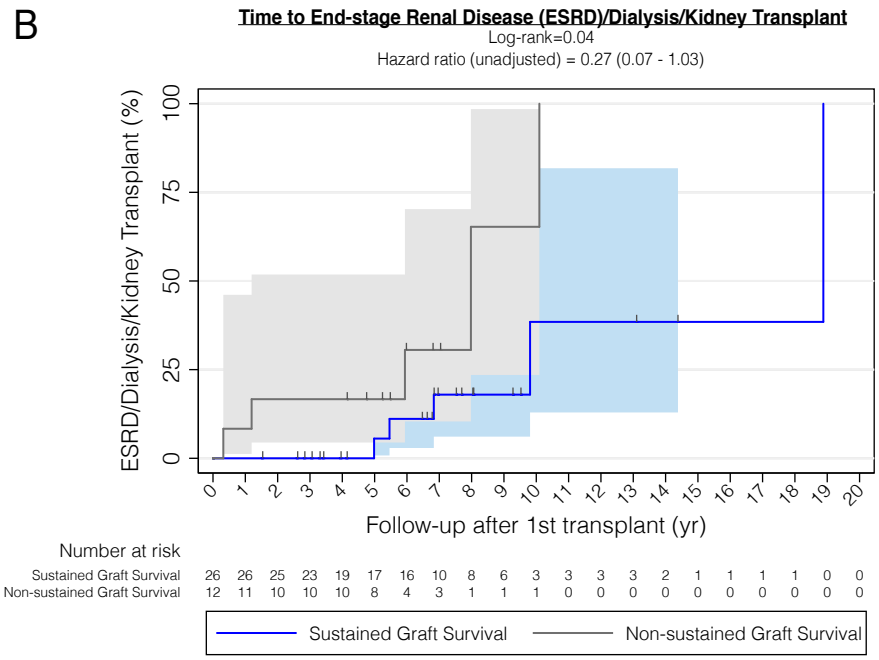
† For this analysis, only patients with eGFR≥60 ml/min/1.73m^2 at baseline were included (n=217/255, 85.1%).

‡ For this analysis, only patients undergoing ITx after March 1st, 2002 were included in the analysis (n=224/255, 87.8%)

The continued use of immunosuppression (recipients with SGS) did not increase risk of ESRD (**Figure 1.2.15A**). In fact, recipients with baseline stage 3 CKD (eGFR<60 ml/min/1.73m^2) achieving SGS had delayed progression to ESRD compared to those with NSGS (**Figure 1.2.15B**). Both groups experienced a significant fall in eGFR following ITx, which was more pronounced in recipients with SGS (**Figure 1.2.16A**). This might be explained by the continued use of immunosuppression, as evidenced by higher tacrolimus levels in subjects with SGS (**Figure 1.2.16B**), but also by the older age in these subjects, since serum creatinine levels during follow-up were similar between groups (**Figure 1.2.16C).**

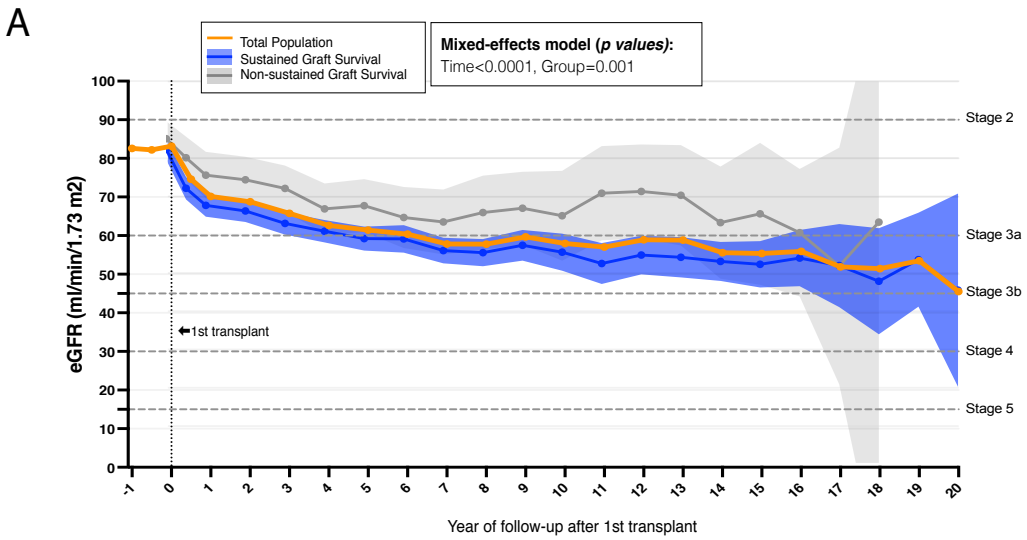
Figure 1.2.15. Incidence of end-stage renal disease/dialysis or kidney transplant after first islet transplant





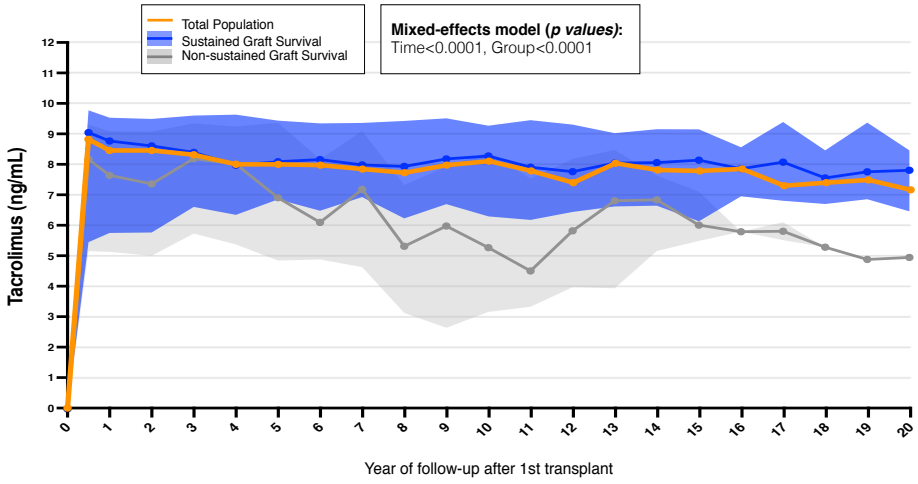
Note: Panel A shows KM estimates for onset of ESRD post-1st ITx in the total population stratified by graft survival outcome. Panel B shows KM estimates for onset of ESRD post-1st ITx in patients with baseline eGFR<60 ml/min/1.73m2 (stage 3 CKD), stratified by graft survival outcome. Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

Figure 1.2.16. Renal function and tacrolimus levels after first islet transplant



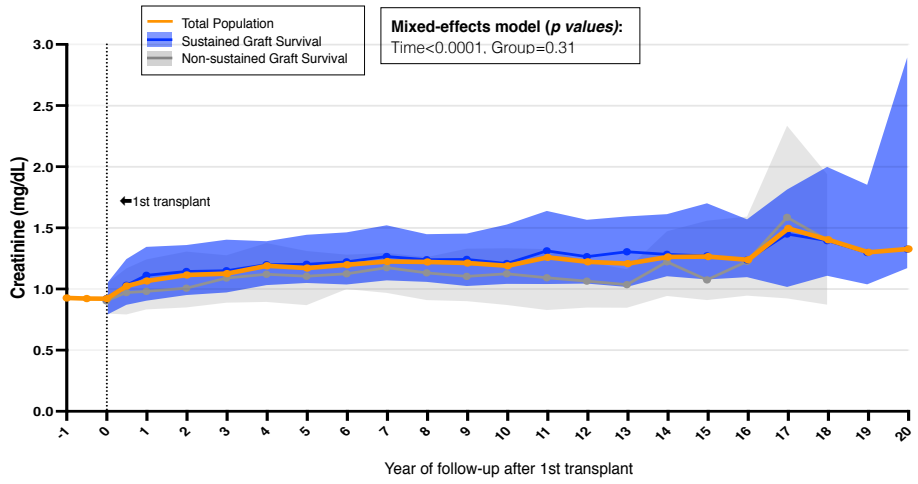
Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	177	178	173	162	149	132	122	108	91	75	62	55	48	40	36	33	23	16	10	8	5
NSGS	77	77	77	76	69	59	50	41	37	27	24	21	18	16	15	12	10	10	6	3	0	0
Missing data(%)	0.4	0.4	0.0	2.4	2.1	6.7	7.1	11.4	10.5	12.6	15.4	16.2	15.1	16.9	16.7	15.8	8.5	8.3	12.0	27.8	20.0	44.4

B



Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	173	175	173	157	146	129	119	107	89	74	62	56	49	44	41	30	25	17	11	8	7
NSGS	78	67	64	66	52	41	32	20	17	12	12	8	5	4	4	3	3	2	2	2	1	1
Missing data (%)	0.0	5.9	6.3	6.3	11.4	16.1	17.9	24.5	23.5	25.2	26.5	29.3	29.1	31.2	27.3	22.8	29.8	25.0	24.0	27.8	10.0	11.1

C

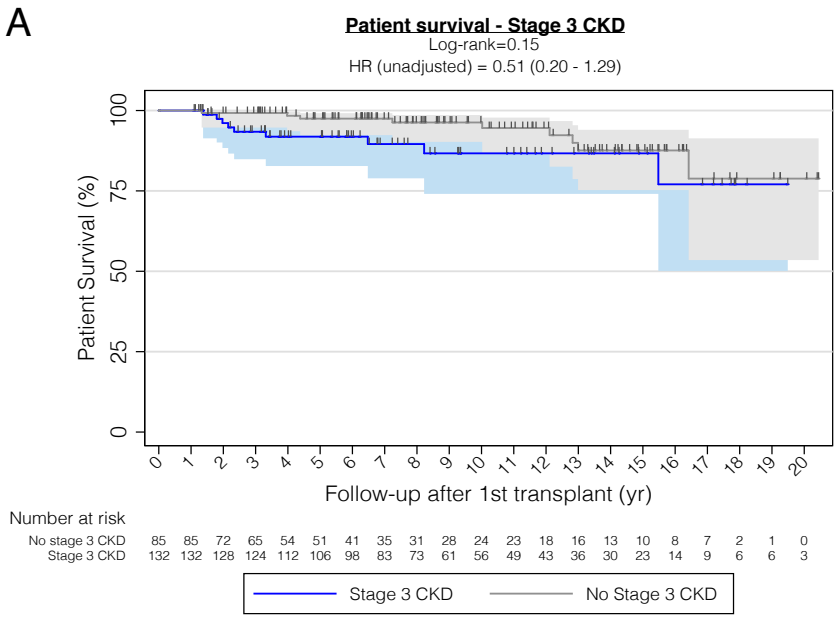


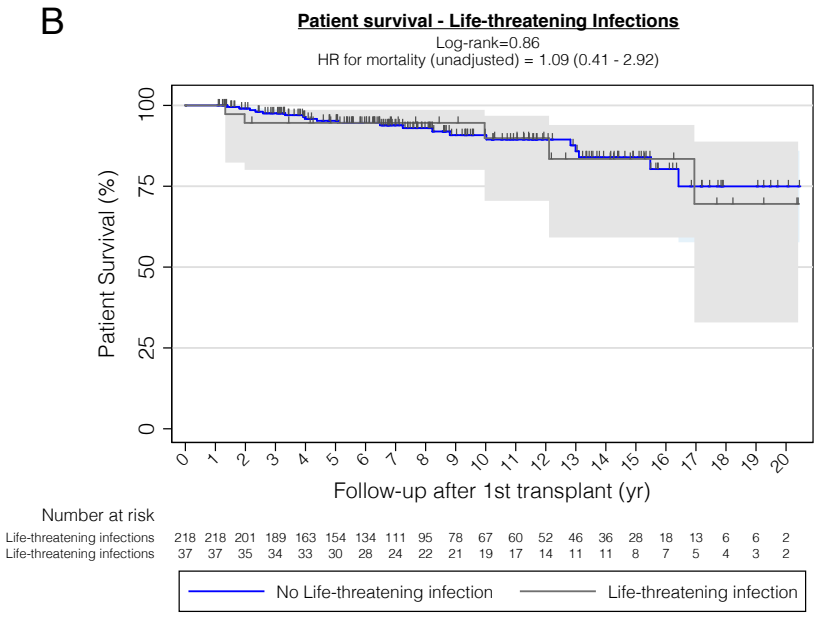
Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SGS	177	177	178	173	162	149	132	122	108	91	75	62	55	48	40	36	33	23	16	10	8	5
NSGS	77	77	77	76	69	59	50	41	37	27	24	21	18	16	15	12	10	10	6	3	0	0
Missing data (%)	0.4	0.4	0.0	2.4	2.1	6.7	7.1	11.4	10.5	12.6	15.4	16.2	15.1	16.9	16.7	15.8	8.5	8.3	12.0	27.8	20.0	44.4

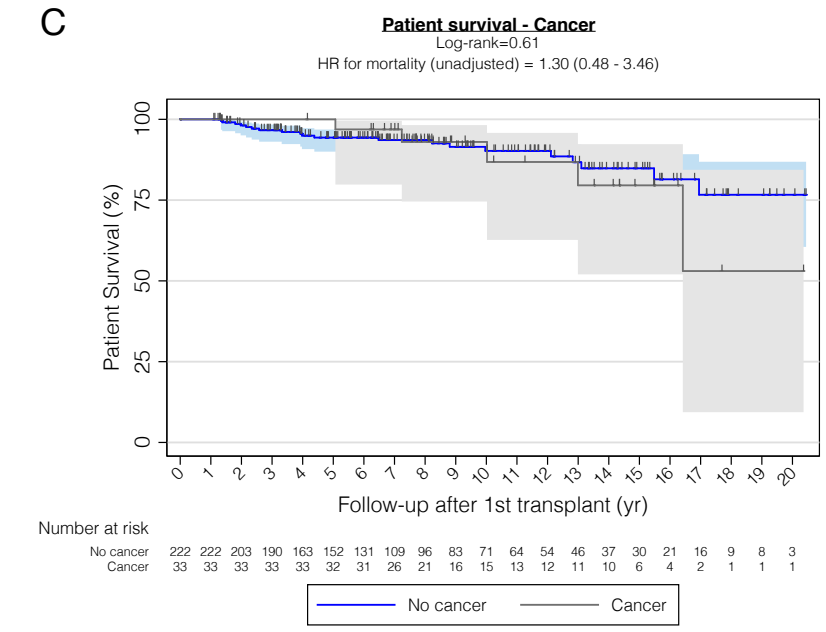
Note: Panel A shows estimated glomerular filtration rate during follow-up. Panel B shows tacrolimus levels during follow-up. Panel C shows serum creatinine levels during follow-up. Data are presented as median (solid line) and interquartile ranges (shaded area) for the total population (orange), and those with sustained graft survival (dark blue) and non-sustained graft survival (gray). A table describing available values in each group, as well as the percentage of missing data at every time point is included below each graph. Statistics using a mixed-effects model comparing SGS vs NSGS are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

While a higher incidence of stage 3 CKD amongst recipients with SGS as compared to NSGS was observed (**Table 1.2.15**), developing stage 3 CKD did not increase mortality (**Figure 1.2.17A).** The incidence of life-threatening infections was 13% (29/224) and malignant neoplasms occurred in 13% (33/255) (mostly skin cancer [67%, 22/33)]). There was a higher incidence of malignant neoplasms in recipients with SGS, although a lower proportion of non-skin cancers was observed (**Table 1.2.15**). Life-threatening infections and malignant neoplasms did not increase mortality (**Figure 1.2.17B-C)**.

Figure 1.2.17. Impact of stage 3 chronic kidney disease, life-threatening infections and cancer on patient survival







Note: Panel A shows KM estimates for patient survival stratified by the onset of stage 3 chronic kidney disease. Panel B shows KM estimates for patient survival stratified by the occurrence of life-threatening infections. Panel C shows KM estimates for patient survival stratified by the occurrence of cancer.
Data are presented as survivor function (solid line) and 95%CI (shaded area). Censored cases are represented by hash marks in the survivor function line (solid line). Statistics are shown in each panel. This figure is included as supplementary material in the published version of this manuscript.

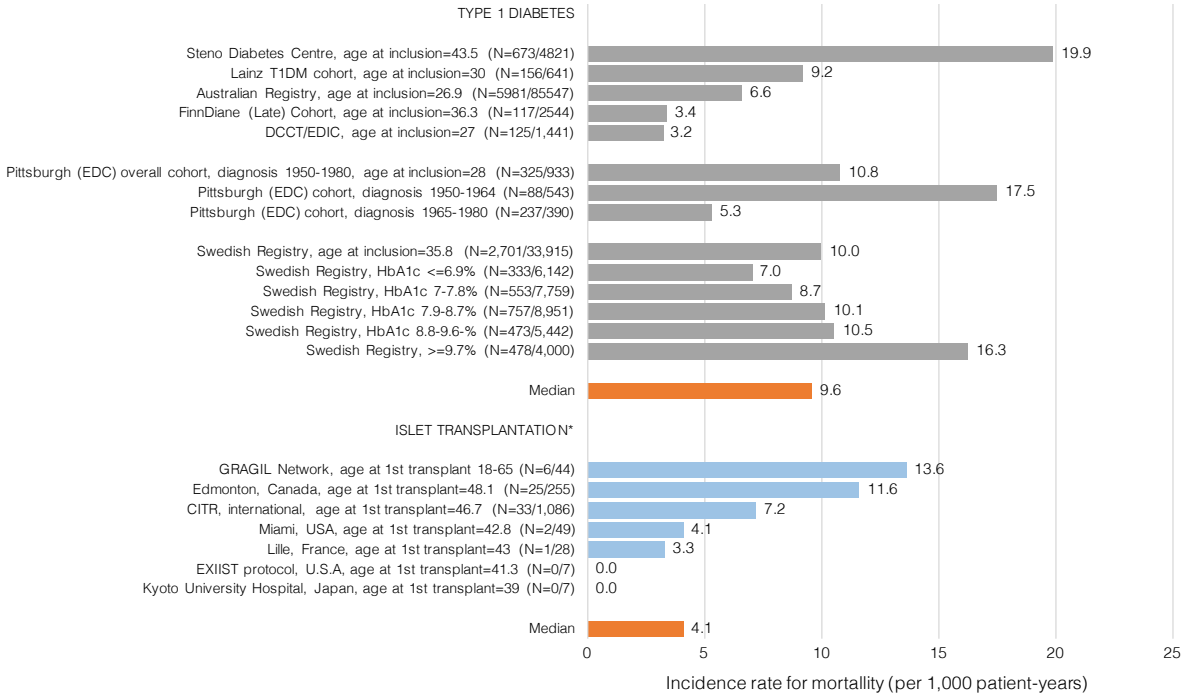
1.2.6 - Discussion

Pancreatic islet transplantation provides a valuable therapeutic option for people with T1D and problematic hypoglycaemia. While clinical evidence worldwide is becoming available, long-term outcomes remain scarce. Herein, we provide a comprehensive report from the largest programme globally.

Our experience supports the safety of ITx regarding long-term patient survival, despite the risks of chronic immunosuppression. Mortality rates (11.6 per 1,000 patient-years), however, differ from those reported in 10- and 20-year follow-up studies by the Lille (3 per 1,000 patient-years) and Miami (3.28 per 1,000 patient-years) groups.[5, 6] Our experience resembles that of the Swiss-French GRAGIL Network (20 per 1,000 patient-years for IAK and 8 per 1,000 patient years for ITA).[4] Of relevance, our population was older compared to the Lille and Miami cohorts, with 11% (29/255) >60 years-old at baseline. These subjects showed higher mortality compared to younger recipients (9.7 vs 29.1 per 1,000 patient-years, p=0.034) and comprised 25% of deaths. Differences in sample size, patient selection and follow-up, and accessibility to ITx may have also contributed to higher mortality rates. Regardless, mortality post-ITx should be contextualized by factoring chronic immunosuppression and its risks. Accordingly, while malignant neoplasms were more frequent in patients with SGS (with longer exposure to immunosuppression), these did not increase mortality. The same occurred with life-threatening infections and stage 3 CKD. A similar incidence of advanced CKD between groups suggests that immunosuppression may not be the principal determinant for progression of CKD. Moreover, SGS seems to confer protection for progression to ESRD in subjects with baseline impaired renal function. Comparative analyses with similar cohorts (i.e., T1D with problematic hypoglycaemia) could also help contextualize mortality post-ITx.[20, 21] **Figure 1.2.18** shows

mortality rates from cohorts including adults with T1D and those undergoing ITx. While there is heterogeneity among studies and few long-term ITx reports, it appears this therapy improves patient survival.

Figure 1.2.18. All-cause mortality rates among different cohorts of adult patients living with type 1 diabetes and comparison with long-term cohorts of patients undergoing islet transplantation



Note: Only long-term reports including incidence rates for mortality were included. The following reports were not included due to lack of reporting of incidence rates for mortality (event per patient-years); 1) Lehman et al. (*Diabetes Care, 2015*) including patients with simultaneous islet-kidney (SIK) and islet-after-kidney (IAK) transplantation reported a crude 10-year mortality rate of 44.6%; 2) Nijhoff et al. (*IPITA abstract, 2019*) including patients with IAK reported a crude mortality rate of 20.1%; 3) Leguier et al. (*IPITA abstract, 2021*) including patients with ITA and IAK reported a crude mortality rate of 8.2% (all deaths occurred in patients with IAK, 4/14, 28.6%). This figure is included as supplementary material in the published version of this manuscript.

Other reports have suggested ITx decreases mortality in patients experiencing severe hypoglycaemia,[22, 23] however, multicentre comparative studies are required. Lastly, it is

noteworthy that 16% of deaths in our cohort were due to suicide. This has been documented previously in people with T1D experiencing marked glycemic lability,[22, 24] and highlights the need for continued psychological support and interventions post-ITx.

In this study, we observed excellent graft survival and function over time. While the Collaborative Islet Transplant Registry (CITR) report[16] and other studies show similar patient characteristics,[1, 3, 4, 6, 23, 25] our cohort received greater islet mass, which may have positively impacted graft survival.[16, 25] A large islet mass may have also favored achievement of insulin independence. Importantly, achievement of insulin independence ("ever-achieved") does not imply permanence, and indeed most subjects returned to fractional insulin over time. Other favorable factors[16], such as recipient age $\geq$35 years, induction immunosuppression with T-cell depletion and/or TNF-α inhibition, and maintenance immunosuppression with both mTOR and calcineurin inhibitors were highly prevalent, albeit only 23% (57/255) of patients had all present. This is similar to the CITR data.[16] The Miami group recently reported female sex (for patients and donors) as a favorable factor for prolonged graft survival.[6] Although we documented high proportions of female recipients and donors, these were similar between groups. Finally, it should be emphasized that the use of supplementary infusions confounds interpretation of graft survival, and contrasts with centres limiting infusions to a certain number[25] or time period,[3, 4] representing differences in regulations, funding, and philosophy of practice. The fact that a greater proportion of patients with SGS received $\geq$3 infusions compared to those with NSGS suggests that more infusions are required long-term to sustain graft function and glycemic control. Conversely, supplementary infusions did not impact total duration of insulin independence, which demands more detailed cost-benefit analyses of maintaining graft function with supplementary infusions, both at patient and provider levels.

Using multivariate logistic regression, we found that the short-term (i.e., peri-transplant) combined use of anakinra plus etanercept and a BETA-2 score ≥15 within 1-year post-first infusion increased the probability of SGS. Our findings with anakinra plus etanercept resonate with evidence from a recent systematic review[26], suggesting that dual anti-inflammatory therapies be considered for routine use in ITx, while the BETA-2 score may help evaluate engraftment. This score, and its previous iteration, the BETA score, may identify patients with optimal engraftment who are likely to achieve prolonged graft survival and insulin independence,[19, 27] however, prediction of long-term outcomes is limited to scarce reports with few patients,[3, 4, 27] most within research settings. Accuracy of prognostic scores is affected by follow-up time and context (e.g., research protocols), and sample size. While we found that the BETA-2 score correlated with graft survival and total insulin independence duration, correlations were modest and demands further refinement. A larger sample might help obtain more robust estimates and measures of precision, which could improve the prognostic accuracy of the BETA-2 score and other predictors. Regarding islet mass, we observed that, after adjusting for different factors, total islet mass was not associated with SGS. This contrasts with previous reports[16, 25], suggesting that recipient factors, and measures of engraftment, play a more dominant role in predicting long-term graft survival. Validating our findings using multicentre cohorts, and incorporating immunological markers (e.g., auto-antibodies, regulatory T cells, immunosuppression levels) and/or markers of β-cell death into composite prognostic tools could prove transformational in predicting outcomes following ITx and may help further optimize resource allocation.

Beyond high graft survival and insulin independence rates, we document durable improvements in measures of hypoglycaemia and glycemic control. The potential of ITx to

ameliorate chronic diabetes-related complications and improve quality of life demands further exploration. Moreover, the prognostic significance of proteinuria, progression of retinopathy, autonomic neuropathy, represent areas of opportunity for future studies. While these potential benefits must be balanced against incidence of adverse events, our study suggests these are relatively infrequent (e.g., procedure-related), have no impact in patient survival (e.g., stage 3 CKD, cancer, life-threatening infections), and may resemble those of the general population living with T1D (e.g., ESRD).[28]

Our study is limited by its long-term and retrospective component, and lack of appropriate controls. First, outcomes might have been influenced by variability over time in islet isolation and clinical care. We previously reported on how these factors may impact outcomes following ITx.[15, 29] Second, we observed substantial missing data, which introduces bias (e.g., recipients with graft failure are less prone to continue follow-up) and precludes a granular evaluation of graft function, for example using the Igls criteria. Introduced in 2018, these criteria propose a C-peptide cut-off of >0.17 nmol/L to establish graft function, in contrast to the more traditional >0.1nmol/L used herein, in previous CITR reports, and in long-term studies (**Table 1.2.1**).[30] Comparisons between these and other cut-offs to establish clinically relevant graft function remains an area of opportunity. Missing data was particularly relevant for insulin requirements, and measures of hypoglycaemia and glycemic lability, which highlights a need for robust approaches to collect this information, for example, using patient-friendly glycemic monitoring mobile applications. The issue of missing data might be also exacerbated by the few patients having 15- or 20-years follow-up data, which makes some findings at extreme time points less reliable. Finally, mortality rates remain to be compared to those of patients living with T1D and severe hypoglycaemia (e.g., on the waiting list for an

ITx), and to those having a PTx. This is also relevant to contextualize the incidence of adverse events reported in this study, particularly CKD, cancer, and life-threatening infections.

In conclusion, we present an in-depth long-term analysis from the largest islet transplant programme globally. A comparative analysis between recipients with sustained and non-sustained graft survival identified favorable factors associated with improved outcomes, and revealed a comprehensive picture of the robust metabolic impact that may be achieved and maintained with sustained graft survival. Description of long-term outcomes following ITx contributes to optimize shared-decision making in clinical practice and to improve conditions in which β-cell replacement therapies can thrive and achieve maximum therapeutic benefit.

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CHAPTER 2

PART 1 – WHOLE PANCREAS AND ISLET CELL TRANSPLANTATION

CHAPTER 2, PART 1 – WHOLE PANCREAS AND ISLET CELL TRANSPLANTATION

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Title: Whole Pancreas and Islet Cell Transplantation

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2.1.1 - Introduction: What is the Problem?

Diabetes care has undergone one of the most notable revolutions in the history of medicine. Closing into the 100th year anniversary of the discovery of insulin, our paradigm has evolved from saving lives to optimizing treatment for patients with diabetes. Realization that intensive glycemic control ameliorates progression of diabetes-related complications both in patients with type 1 diabetes (DCCT and EDIC cohorts)[1, 2] and type 2 diabetes (UKPDS)[3] prompted efforts to establish rigorous targets of glycemic control (HbA1c levels $\leq$ 7%), which are now advocated for most nonpregnant adults with diabetes.[4, 5] While the implications of these targets have been debated, particularly in relation to patient-important outcomes (e.g., end-stage renal disease (ESRD)),[6] optimal glycemic control remains one of the maxims in diabetes care. This fact has changed the natural history of diabetes and transformed its epidemiological picture. Consequently, chronic manifestations of the disease such as nephropathy, neuropathy or vascular disease have become more common and now occupy a more prominent space in clinical guidelines.

Unfortunately, striving for optimal glycemic control is challenging, particularly in type 1 diabetes (T1D). Iatrogenic hypoglycemia is particularly relevant and one of the most consistently reported adverse effects with intensive insulin treatment. Early clinical trials reported a two-to-sixfold increase in the incidence of severe hypoglycemic episodes (SHEs)[2, 3, 7] with more than half of these occurring during sleep.[7] An incidence of ~ 50% in patients with T1D[8] supports the generalized perception that severe hypoglycemia is limited to T1D. However, a meta-analysis reporting a prevalence of SHEs of 23% in patients with type 2 diabetes (T2D) treated with insulin[9] highlights the relevance of this issue in both populations. The global burden of hypoglycemia-related mortality is equally worrisome. While catastrophic scenarios such as

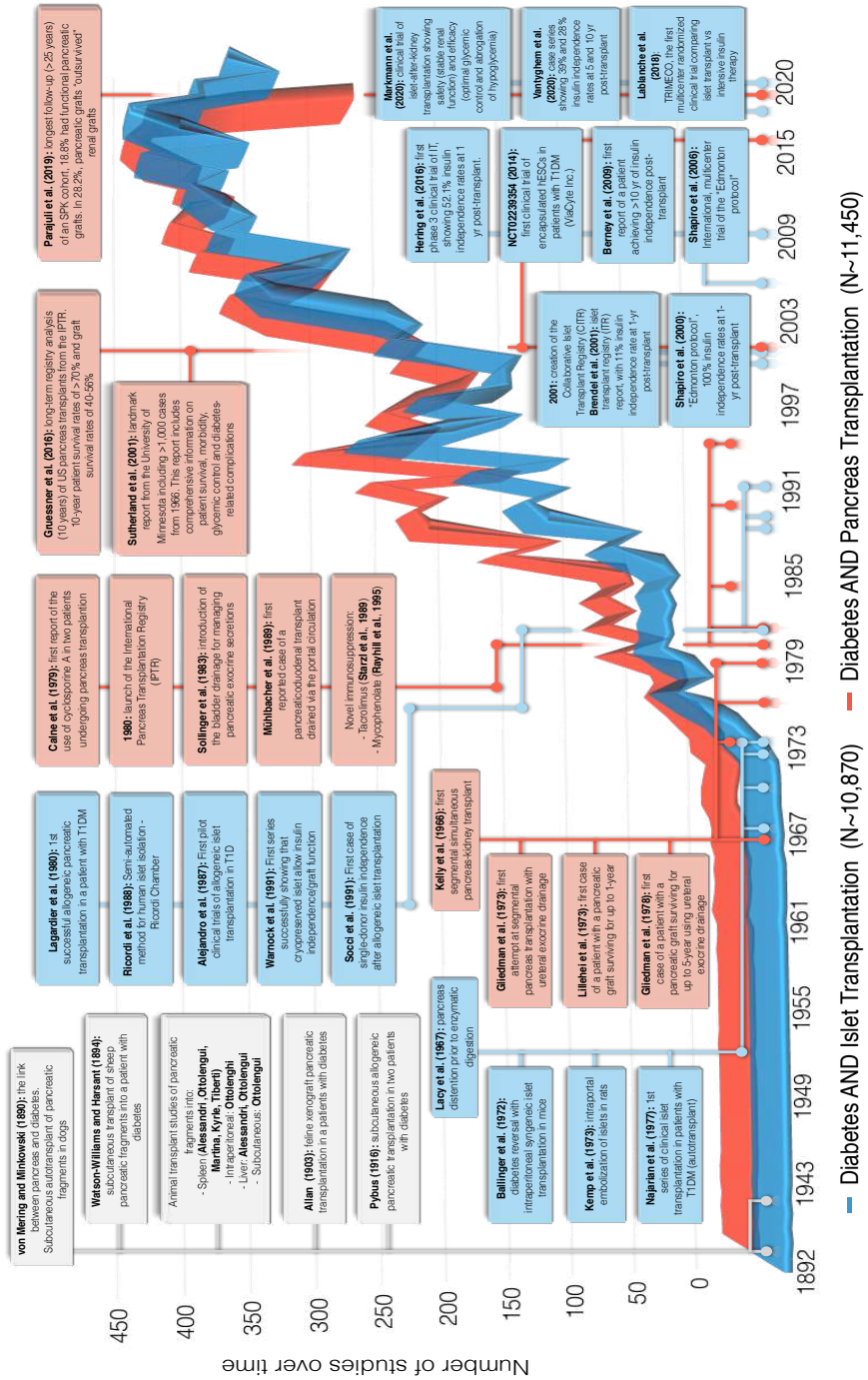
the "death-in-bed syndrome" remain uncommon,[10] a study using the World Health Organization mortality database reports a standardized overall proportion of 4.49 deaths due to hypoglycemia per 1000 total diabetes deaths (range: 0.11-283.1).[11] This makes hypoglycemia a key barrier for optimal glycemic control.[12] Despite these numbers, hypoglycemia remains a relatively neglected complication.[13] It is noteworthy that the 2021 American Diabetes Association's Standards of Care now includes a time in range of >70% and below range of <4% when using ambulatory glucose profiles as a key glycemic goal.[5] This will raise awareness of the importance of addressing glycemic variability. However, problematic hypoglycemia (defined as $\geq$ 2 episodes of SHE per year or 1 episode + hypoglycemia unawareness/extreme glycemic lability/maladaptive behavior) demands more individualized management. Current recommendations propose a four-stage tiered algorithm: 1) structured or hypoglycemic-specific education programs, 2) continuous subcutaneous insulin infusion or continuous glucose monitoring, 3) sensor-augmented insulin pumps and/or very frequent contact with a specialized hypoglycemia service and 4) β-cell replacement therapies with either whole pancreas (PTx) or islet cell transplantation (ITx).[14] While β-cell replacement therapies are the last resource, they effectively address the problem; both abrogate problematic hypoglycemia in nearly all patients while providing optimal glycemic control. In this chapter, we will describe PTx and ITx, dissect the current clinical evidence, explore the main challenges ahead, and present some potential solutions.

2.1.2 - β-cell Replacement Therapies: A Potential Solution for Diabetes

β-cell replacement therapies are robust alternatives to treat brittle diabetes and problematic hypoglycemia that persists despite conventional therapies. They have undergone

significant refinements, and outcomes have improved substantially since their inception. **Figure 2.1.1** depicts the growth of research in the field and provides historical context.

Figure 2.1.1. Growth of islet transplantation research and a timeline to clinical success



Note: A non-systematic search in PubMed for publications using the terms (islet transplant* OR pancreas transplant*) AND diabet* up to January 1, 2021. Additionally, we included several key historical studies in the figure, despite not appearing in the original search, due to its relevance. Used with permission from with permission from Marfil-Garza BA, Senior PA and Shapiro AMJ. Whole Pancreas and Islet Cell Transplantation. In: Holt RIG, Cockram C, Flyvbjerg A, Goldstein BJ, editors. Textbook of Diabetes 6th Edition (In press)

Before the discovery of insulin, a relationship between the pancreas and diabetes, which was first identified by Von Mering and Minkowski in 1892, had already driven several attempts to reverse diabetes through pancreatic tissue transplantation.[15] British surgeons Watson-Williams and Harsant performed the first transplant of fragmented sheep pancreatic tissue to a 13-year old patient with diabetic ketoacidosis in 1894; James Allan followed with xenografts composed of feline pancreatic fragments in another patient with diabetes.[16] Charles Pybus was the first to transplant human cadaveric fragmented pancreatic tissue into the subcutaneous space of two male patients with diabetes in 1916.[17] Repeated failure to achieve normoglycemia, likely due to prompt immune rejection (the basis of transplantation immunology was not established until the 1950s), coupled with the success with contemporary insulin therapies, led to abandonment of β-cell replacement therapies for a few decades. It was not until de 1960s, when the use of steroids and new immunosuppressants allowed successful renal transplantation, that the interest in both PTx and ITx was reinvigorated.

2.1.3 - Whole pancreas transplantation

2.1.3.1 - History

PTx was first conceived as a complementary procedure to improve the clinical success of kidney transplantation in patients with ESRD secondary to diabetes. In 1966, Drs. Kelly and Lillehei performed the first simultaneous segmental pancreas-kidney transplant on a patient with T1D at the University of Minnesota. This first attempt allowed complete insulin independence for 6 days before insulin resistance (probably due to high doses of steroids) and graft pancreatitis (probably due to pancreatic duct ligation) ensued. The patient died 2 weeks after pancreatectomy from a pulmonary embolism.[18] The second simultaneous pancreas-kidney

(SPK) transplant, performed by Dr. Lillehei, consisted of a pancreaticoduodenal transplant (whole pancreas) with a cutaneous duodenostomy to manage exocrine drainage.[19] Subsequently, iterations of the surgical technique by Drs. Lillehei, Lárgiader and Idezuki, such as enteric drainage through a Roux-en-Y duodenal jejunostomy, enabled graft survival for up to 1-year post-transplant.[19] Other attempts at PTx, mostly SPK, were done in USA, Europe, and even South America, however, success was minimal (only one patient from Dr. Lillehei's series, out of 25 reported up to 1970s, maintained function for 1-year post-transplant).[20] Ureteral exocrine drainage, pioneered by Gliedman and colleagues in the early 1970s, improved graft survival (up to 50 months), however, this was ultimately abandoned due to anastomotic leakage.[21] In the 1970-1980s, segmental PTx was revisited, with two techniques to manage exocrine drainage showing improved outcomes, the open drainage (into the peritoneal cavity) and duct polymer injection. The former, as reported by Dr. Sutherland *et al.* from the University of Minnesota, allowed insulin independence for 18 years, however, some cases suffered from peritonitis and/or pancreatic ascites.[22] Segmental PTx was later coupled with bladder drainage, pioneered by Dr. Sollinger from the University of Wisconsin, which became a very common technique during the 1980s due to the low incidence of complications and the added benefit of offering a non-invasive method to monitor for rejection.[23] Later modifications of this technique, such as the duodeno-cystostomy, implemented as part of a whole PTx procedure became popular in the late 1980s; nearly 90% of PTx had bladder drainage during that period. However, a high incidence of urinary tract infections, reflux pancreatitis, and metabolic abnormalities (e.g., acidosis) demanded surgical conversion to enteric drainage in many cases, which led to a relative abandonment of this technique.[20] Enteric drainage is now the preferred approach, with as many as 90% of PTx having enteric exocrine drainage.[24] Venous drainage is an interesting

issue. To replicate physiology, portal drainage was initially explored, however, it didn't became widely adopted, and because of technical complexity and no clear metabolic benefits, only ~ 8-20% of the cases are managed with this approach currently;[24] most patients undergo systemic venous drainage to the common and/or external iliac vessels or the distal vena cava.

Following relative standardization of the surgical techniques, the issue of immunosuppression became central for long-term clinical success. Professor Sir Roy Calne and colleagues first reported the use of cyclosporine A (CsA) as a successful agent for immunosuppression following PTx (he was also the first to describe portal vein drainage in PTx). Combination with steroids was advocated by Dr. Starzl's group to reduce CsA-induced nephrotoxicity; triple therapy with CsA, steroids and azathioprine was later introduced by Dr. Sutherland's group.[20] Successful clinical trials with tacrolimus by Dr. Starzl led to its widespread use as maintenance immunosuppression.[20] In the mid-1990s, mycophenolate mofetil (MMF) was then introduced.[24] Induction immunosuppression regimes involving T cell depletion, such as anti-thymocyte globulin (ATG), and steroids were later introduced in the late 1990s to prevent early rejection.

2.1.3.2 - Procedural considerations

Today, the typical PTx occurs in a patient with T1D (~ 90%), older than 30 years-old (~ 90%), with ESRD, and consists of an SPK (~ 84%), with enteric exocrine (91-92%), and systemic venous (~ 80%) drainage.[24] PTx is a major surgery demanding expertise in perioperative management, thus, >70% of the cases are done in high-volume centers. Donor selection is a key consideration for a successful PTx. The "ideal" pancreatic graft would come from a young (<50 years old) and lean (normal BMI) donor. A detailed description of the

surgical technique is beyond the scope of this chapter, however, several considerations are worth highlighting. PTx carries the greatest risk of graft thrombosis among abdominal transplants, which is the most common cause of early graft loss (5-10%). Thus, careful consideration of surgical aspects such as optimal vessel mobilization (i.e., use of atraumatic, vascular clamps, proper length venotomy and arteriotomy, proper suture technique) and flexibility to choose the optimal site of vascular anastomosis are all important details. Importantly, while systemic anastomosis is the preferred method for venous drainage (largely due to less complex dissection being required), the jury is still out on the superior metabolic and potential immunological benefits of the portal venous drainage approach.[25]

A final comment concerns immunosuppression. As compared to ITx, T-cell depleting agents (i.e., ATG) are the most commonly used agent for induction immunosuppression in PTx (68% of the cases).[24] Stronger, more specific, and more expensive immunosuppressants, such as basiliximab or alemtuzumab, have not been able to consistently show a clinical benefit when compared (even in a randomized fashion) with ATG.[26] For maintenance immunosuppression, the preferred regimen consists of tacrolimus, MMF and prednisone (± tapering/withdrawal), however, a shift towards steroid-free immunosuppression has been advocated recently, particularly by high-volume centers.[24] This practice remains controversial as a consistent clinical benefit has not been observed.[27]

2.1.3.3 - Indications

Most recipients of a PTx have T1D, however, this procedure has also been undertaken in patients with T2D. There are three main types of PTx: simultaneous pancreas-kidney (SPK),

pancreas-after-kidney (PAK), and pancreas-alone (PTA) transplants. Hence, some of the indications, although overlapping, may differ with each of these procedures (**Table 2.1.1**).

Table 2.1.1. Indications and contraindications for β-cell replacement therapies

A.	**Indications**

	Patients with T1D (typically with a duration >5 years)
	• Patients with T2D may be candidates for a PTx if they have the following conditions:
	  o Low exogenous insulin requirement
	  o BMI <30 kg/m^2

	HbA1c > 7.5-8.0% despite expert diabetes management (including Endocrinologist/Diabetologist and diabetes educators)
	Problematic hypoglycemia (≥ 2 episodes/year of severe hypoglycemia) despite optimal diabetes management with insulin pump and adequate monitoring by an Endocrinologist/Diabetes and diabetes educators • Recurrent episodes of diabetic ketoacidosis and/or severe, rapidly progressing complications of diabetes may also be considered
	At least one episode of severe hypoglycemia in the past year, defined as a blood glucose <54 mg/dL plus one of the following symptoms: memory loss; confusion; behavioral changes; impaired consciousness; seizure; or visual symptoms, in which the subject was unable to treat him/herself and that resolved after carbohydrate intake, or glucagon administration.
	Evidence of impaired awareness of hypoglycemia and/or extreme glycemic lability using objective scores, such as the Clark score (≥ 4), HYPO score (≥ 1000) or lability index (≥400), among others. • A composite of Clarke score ≥4 + HYPO score ≥75th percentile (≥423) + lability index ≥75th percentile (≥329) may also be used • Major fear or maladaptive behavior related to hypoglycemia may also be considered
	Candidates for either a simultaneous pancreas-kidney or islet-kidney transplant should meet criteria for a kidney transplant alone
B.	**Contraindications**
	Age >60 years • This is an absolute contraindication for PTx and a relative contraindication for ITx
	BMI >30 kg/m^2 (28 kg/m^2 may be preferred for PTx)
	Insulin requirements >1.0 U/kg/day or HbA1c >10.0% • High insulin requirements and/or HbA1c levels are not a contraindication for PTx
	Untreated proliferative retinopathy
	High cardiovascular risk (threshold for a prohibitive cardiovascular risk may be lower for PTx) • Uncontrolled hypertension • Myocardial infarction within 6 months • Evidence of ischemia on functional cardiac testing in the previous year • Left ventricular ejection fraction <30%
	History of malignancy • Completely resected squamous or basal cell carcinoma of the skin are not a contraindication
	Untreated infection (including viral infections, such as hepatitis B or C and HIV)

	• History of opportunistic infections such as aspergillus, histoplasmosis, or coccidioidomycosis in the previous year.
	Inability to comply with immunosuppression and proper follow-up
	Any medical (including psychiatric) condition that could interfere with safe participation and follow-up post-transplant

*Adapted from Samoylova et al., 2019[28], Wojtusciszyn A et al., 2018[59], Rickels MR et al., 2018[60], and Dajani KZ and Shapiro AM, 2019[181].

In broad terms, SPK is recommended for patients with severe diabetes and ESRD (estimated glomerular filtration rate [eGFR] <20 ml/min/1.73 m^2); PAK is usually recommended as a sequential procedure when recipients of a kidney transplant have a viable living kidney donor identified, but a deceased pancreas has not been identified; PTA is recommended for patients that have frequent, acute and severe complications of diabetes, such as ketoacidosis and hypoglycemia with or without hypoglycemia unawareness.[28]

2.1.4 - Islet cell transplantation

2.1.4.1 - History

In the early 1960s, Dr. Paul Lacy and his team at the Washington University School of Medicine inaugurated the modern era of islet transplant research by championing a paradigm shift in the field: separation of the endocrine and exocrine components of the pancreas and islet purification. Building on early ideas from Leonid W. Ssobolew and the pioneering works of Drs. Hellesträm and Moskalewski using manual pancreatic dissection[29] and the use of collagenase,[30] respectively, Dr. Lacy's team introduced the two-step islet isolation process using intraductal pancreatic distention to dissociate the tissue and islet purification using discontinuous density gradients (e.g., Ficoll®).[16] This became the gold standard for rodent islet isolation. Having access to pure and functional islets led to successful transplantation studies and diabetes reversal in murine models.[31] The conditions for the first clinical trials were met

when Drs. Kemp and Lacy established the superiority of intraportal infusion (as compared to intraperitoneal) for ITx;[32] this remains the gold standard in the clinic.[33]

In the late 1970s, Dr. John Najarian and colleagues, at the University of Minnesota, were the first to demonstrate that insulin independence can be achieved with intraportal islet infusion (autotransplantation).[34, 35] In 1980, Drs. Largiadèr, Kolb and Binswanger, at Zurich University, reported the first case of insulin independence (~ 10 months) in a patient with diabetes following allotransplantation of pancreatic microfragments (~200,000 islets) into the spleen.[36] Subsequent clinical success was hampered by impure islet preparations and suboptimal immunosuppression, which led to infusion of low islet masses and complications (e.g., thrombosis) and prompt immune rejection.[37] Thus, islet isolation techniques were revisited to continue moving forward. Intraductal collagenase perfusion of the pancreas, coupled with gentle mechanical dissociation and density gradient purification was introduced by Drs. Warnock, Rajotte and colleagues at the University of Alberta.[38, 39] This approach yielded high purity islet preparations (>90%) and enabled high rates of post-transplant normoglycemia after a single-donor islet transplant in dogs.[38] Shortly thereafter, these researchers reported on a 36-year-old patient with T1D that achieved sustained (at least 5 months) insulin independence after receiving an islet graft composed of both fresh and cryopreserved islets with a 75% purity; such purity enabled high doses (>10,000 islets/kg) without any complications.[40, 41] The biggest breakthrough came with the introduction of the "automated" method for islet isolation, designed and implemented by Dr. Camillo Ricordi, while working in Dr. Lacy's laboratory. As stated in his 1988 paper,[42] the automated method met the following requirements "1) minimal traumatic action on the islets, 2) continuous digestion in which the islets that are progressively liberated can be saved from further enzymatic action, 3) minimal human intervention in the digestion

process, and 4) high yield and purity of the isolated islets". This method swiftly became the standard for human islet isolation all over the world. A few years later, Dr. Scharp and colleagues reported on the first case of a patient with T1D achieving transient insulin independence using the automated method.[43] The same year, a case series of six patients with T1D treated with ITx using the automated method for islet isolation was reported by Dr. Socci and colleagues from the San Raffaele Institute of Milan, Italy. Notably, this series included the first patient to achieve transient insulin independence after ITx from a single donor.[44] Further additions to islet isolation protocols included semiautomated density gradient separation using the Cobe® IBM 2991 cell separator by Lake et al.,[45] cold preservation solutions by Olack et al.,[46] and techniques for islet staining by Latif et al.,[47] that ultimately led to a standardization of reporting on islet preparations (i.e., numbers, mass, viability, etc.) which was much required to allow meaningful comparisons of results between centers.[48] While optimization of the islet isolation process remains an ongoing effort in the field, advances up to now have enabled infusion of pure and large islet masses (up to 1,000,000 islets). However, the lack of long-term clinical success in the late 1990s shifted the focus towards improving immunosuppression and peritransplant care.

In 1997, Secchi *et al.*, reported that induction immunosuppression with steroids and ATG, coupled with maintenance immunosuppression using azathioprine, CsA and steroids, as well as peritransplant use of insulin allowed insulin independence rates of 35% after intraportal ITx in patients with "insulin-dependent diabetes mellitus".[49] Researchers at the University of Miami, using OKT3 (anti-CD3) as induction agent (instead of ATG), reported graft survival rates of up to 6 years' post-transplant in 2/5 patients.[50] Unfortunately, by the end of the 1990s, the global clinical experience was not encouraging. By 2000, the Islet Transplant Registry,

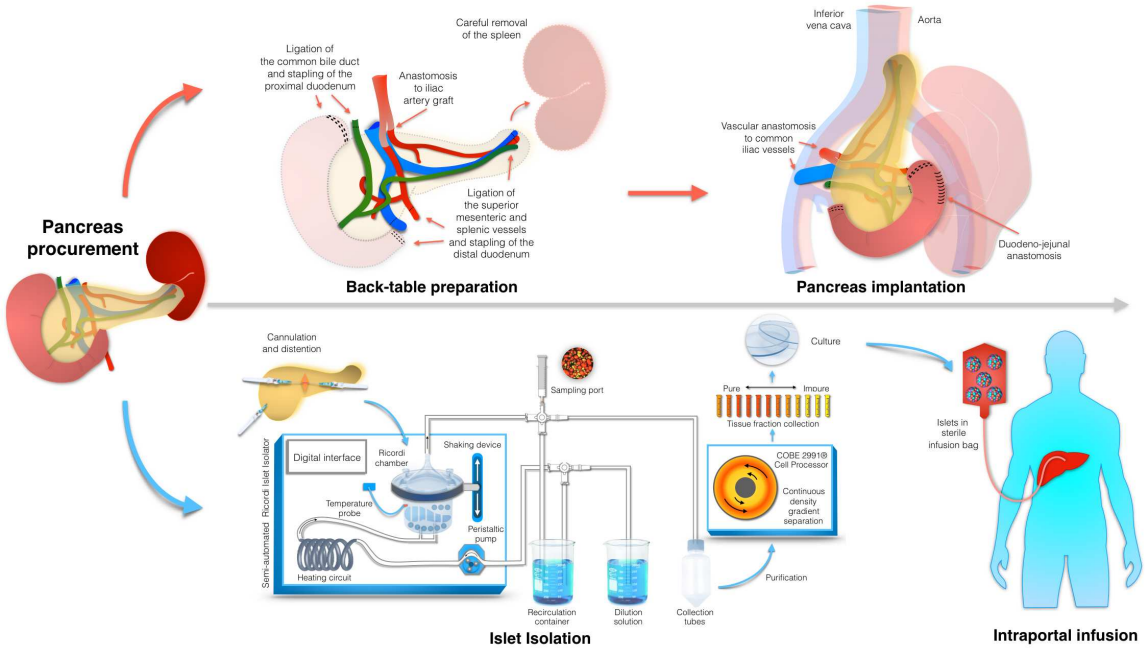
which included information on 237 allotransplants, reported a 1-year insulin independence rate of 11%.[51] Importantly, a milestone in the field was achieved that year with the "Edmonton Protocol", carried forward by a team led by James Shapiro at the University of Alberta. By using large numbers of fresh islets (>11,000 IEQ/kg) and a steroid-free immunosuppression regimen based on daclizumab (anti-IL-2 antibody), sirolimus and tacrolimus, this protocol was the first to achieve 100% 1-year insulin independence rates in 7 consecutive non-uremic patients with T1D.[52] The "Edmonton Protocol" reinvigorated research in the field and inaugurated the new era of ITx and motivated many countries to start ITx programs. Clinical outcomes after the "Edmonton Protocol" will be discussed below.

2.1.4.2 - Procedural considerations

The pathway toward a successful ITx differs when compared to PTx and other organs. In addition to obtaining a viable organ, ITx requires pancreas processing and islet isolation. This process is done by tissue specialists at dedicated facilities, following good-manufacturing practices (**Figure 2.1.2**).[53] Importantly, the efficiency and success of islet isolation (adequate number, quality, purity, viability) is variable and heavily dependent on the skills of the isolation team. Following islet isolation, suitable preparations are infused into the intraportal circulation of the recipient through percutaneous transhepatic access. This procedure is typically done using procedural sedation and through fluoroscopic guidance by interventional radiologists. After confirming proper positioning of the catheter with a portal venogram, the islets are infused using a closed gravity-fed bag.[54] The use of heparin to prevent the instant blood-mediated inflammatory response (IBMIR) and portal vein thrombosis together with a peritransplant insulin infusion to maintain euglycemia and promote β-cell "rest" following transplantation is

a key component of most ITx protocols.[55] These interventions are modified to subcutaneous low molecular-weight heparin and aspirin, as well as subcutaneous insulin, 48 hours after transplant.

Figure 2.1.2. Pancreas (A) and islet cell transplantation (B) procedures



Note:

Pancreas transplantation

(A) The pancreas graft is removed *en bloc* with the duodenum and spleen to prevent injury to the proximal and distal pancreas. During cold phase dissection, the portal and superior mesenteric veins, as well as the gastroduodenal, splenic and superior mesenteric arteries, are carefully identified, ligated and divided individually. During back-table preparation, the spleen, peripancreatic tissue and fat are removed carefully to avoid parenchymal injury to the pancreas. The duodenal segment is shortened by stapling proximally and distally. The splenic and superior mesenteric vessels are ligated, as well as the common bile duct. Vascular reconstruction is performed with the donor iliac artery bifurcation as a "Y"-graft to provide single inflow to the splenic and superior mesenteric arteries. No extension graft is used for the portal vein due to a higher risk of thrombosis. Integrity of the vascular arcade is tested with fluid flush. The prepared graft is implanted in the right iliac fossa. The diagram shows vascular anastomosis to the common iliac vessels and a duodeno-jejunal anastomosis for exocrine drainage, but these may vary by center.

Islet cell transplantation

(B) Islet isolation starts with processing of the resected pancreas (i.e., resection of the spleen, duodenum and the peripancreatic fat). Following exposure through an incision of the pancreas mid-body, the main pancreatic duct is cannulated with two catheters, directed at the head and the tail of the pancreas; flow through the major papilla is blocked with a third catheter. Distention of the pancreas is achieved by infusing cold collagenase through the catheters using a perfusion machine. After distention, the pancreas sliced and introduced in the Ricordi chamber

for enzymatic and mechanical digestion. Enzymatic digestion is achieved by activating the collagenase (warming to ~ 36ºC); mechanical digestion occurs by introducing silicon nitride/metal marbles inside the chamber and shaking of the chamber. The solution is recirculated until the pancreas is appropriately digested, which is assessed by taking samples at different time points and staining them with the zinc-binding dye dithizone. When deemed appropriate, the solution is diluted to stop enzymatic activity and the tissue is then purified using a cell processor and continuous density gradient centrifugation. The purest tissue fractions are harvested for culture and, subsequently, transplantation (the islet isolation diagram has been adapted from Marfil-Garza BA, Shapiro AMJ, Kin T. Clinical islet transplantation: Current progress and new frontiers. ***J Hepatobiliary Pancreat Sci***. 2021;28(3):243-54). Figure used with permission from with permission from Marfil-Garza BA, Senior PA and Shapiro AMJ. Whole Pancreas and Islet Cell Transplantation. In: Holt RIG, Cockram C, Flyvbjerg A, Goldstein BJ, editors. Textbook of Diabetes 6th Edition (In press).

In terms of immunosuppression, the original "Edmonton Protocol" has been modified in many centers around the world, including the Edmonton site itself. Current induction immunosuppression either uses agents to prevent activation of lymphocytes (anti-CD25 (IL-2 receptor α-chain) blockers such as daclizumab, basiliximab)) or lymphodepletion agents (ATG (i.e., horse- or rabbit-derived), anti-CD52 blockers (i.e., alemtuzumab)), or anti-CD3 blockers (i.e., OKT3, teplizumab); some centers include steroids into their induction immunosuppression regimens. These are usually combined with anti-inflammatory agents such as TNF-α (i.e., etanercept, infliximab) and/or IL-1 (i.e., anakinra) inhibitors[56] Current maintenance immunosuppression regimens include combinations of calcineurin inhibitors (i.e., tacrolimus, CsA), mTOR inhibitors (i.e., sirolimus, everolimus), and/or MMF. Strategies to improve islet survival, engraftment, and avoid immune rejection will be discussed below.

2.1.4.3 - Indications

Lifelong immunosuppression post-transplant has been associated with toxicity and adverse effects, such as opportunistic infections and neoplasms. Arguably, this issue represents the major limitation for the generalized use of ITx in every patient with diabetes. Thus, in its current state, ITx is limited to patients with T1D suffering from refractory problematic hypoglycemia and/or impaired awareness of hypoglycemia,[14] in which the perceived benefits

on quality of life[57] and survival,[58] as compared to no transplant, outweigh the risks related to the procedure and lifelong immunosuppression. For those with functioning renal transplants, already committed to lifelong immunosuppression the risk-benefit ratio is simpler. Regarding patient selection, recent recommendations by the TREPID working group and the IPITA/EPITA opinion leader workshop include accounting for physiological age, weight, cardiovascular risk, the presence of diabetes-related chronic complications, previous organ transplantation (and sensitization state), current use of immunosuppression, and importantly, the patient's capacity to deal with hypoglycemia.[59, 60] A synthesis of current indications and contraindications for ITx is presented in **Table 2.1.1.** It should be emphasized that specific instruments to assess severity of hypoglycemia, glycemic lability and impaired awareness of hypoglycemia, such as the HYPO score,[61] Lability Index, the glucose coefficient of variation, and the Clarke and Gold scores,[62] may help to complement clinical assessment and decide on eligibility for ITx (or PTx) and to define appropriate patient-centered outcomes and realistic expectations,[59] however, they should not represent the main and only strategy for patient selection.

2.1.5 - Clinical Outcomes – State-of-the-art

2.1.5.1 - Whole pancreas transplantation

2.1.5.1.1 - Patient Survival and Morbidity

Advances in surgical techniques, peritransplant care and immunosuppression have improved patient survival and decreased morbidity following PTx. Currently, the 3-year patient survival rate of SPK, PAK and PTA is 95%, 93% and 96%, respectively.[24] Reports with longer follow-up show a 10-year patient survival rate of ~ 76%, ~ 72% and ~ 82% for SPK, PAK and PTA, respectively.[63] Interestingly, while it is clear that SPK confers a patient survival benefit

when compared to remaining on the waitlist[64-67] or to kidney transplant alone (living or deceased donor),[68] there is controversy for PAK and PTA. Studies looking at the UNOS and the international pancreas transplant registry (IPTR) databases, found no overall benefit on patient survival following PAK as compared to remaining on the waiting list, however, restricting the analysis to follow-up beyond 1-year showed a benefit on patient survival (HR 0.18 (95%CI 0.13-0.25).[66] In contrast, a more recent study found that PAK conferred no patient survival benefit at 5 - 10 years (HR 1.07, 95% 0.84-1.37).[65] For PTA, the same report with UNOS/IPTR data found no overall benefit on patient survival, however, restricting the analysis to follow-up beyond 1-year showed a benefit on patient survival (mortality hazard ratio 0.15 (95%CI 0.08-0.29).[66] Another study, later showed that PTA added 2.4 life-years compared to remaining on the waiting list.[67] Conversely, more recent studies have shown no patient survival benefit with PTA.[69] Importantly, specific analysis considering pancreatic graft status in both PAK and PTA patient population were not done with these studies and are needed as there is evidence from the SPK patient population that benefits on patient survival seem to be conditional on pancreatic and kidney graft survival;[24, 68]; naturally, kidney graft survival may be more strongly associated with patient survival.[24, 70] Studies showing improvement in average life years saved and quality-adjusted life years using probabilistic simulation models[71] strengthen the case for SPK in patients with severe diabetes and ESRD as a more cost-effective strategy compared to kidney transplant alone. Finally, the decision to move forward with PTx should be weighed against the higher 90-day post-transplant mortality rates for all PTx categories, as compared to patients on the waiting list.[65]

2.1.5.1.2 - Glycemic Control and Graft Survival

Currently, the 3-year pancreatic graft survival rates are 86.9%, 78.8% and 74.0% for SPK, PAK and PAT, respectively.[24] Reports with longer follow-up show a 10-year pancreatic graft survival of ~ 55%, ~ 38% and ~ 35% for SPK, PAK and PTA, respectively.[63] These numbers somewhat represent the rates of insulin independence post-transplant given that one of the definitions for graft failure in PTx includes return to insulin. Thus, considering that patients are insulin independent, optimal glycemic control as well as complete abrogation of SHEs is expected as long as the pancreatic graft is surviving. Indeed, patients with a failed pancreatic graft seem to "lose" all benefit in terms of glycemic control and incidence of symptomatic hypoglycemia.[72] Interestingly, impaired glucose tolerance may be observed after 10-years of follow-up in ~ 50% of the cases, even when only evaluating "functional" pancreatic grafts;[73] the clinical impact of these findings (i.e., diabetes-related complications) remains unknown. Several risk factors may correlate with graft failure, such as higher-volume center, older age, BMI >30 kg/m^2, use of dialysis pre-transplant, enteric drainage, immunological status pre-transplant (PRA>20%) non-depleting induction immunosuppression, and the use of maintenance immunosuppression regimens other than tacrolimus + MMF + steroids.[24, 74] The duration of diabetes has also been shown to be a determinant of graft survival, with slightly better rates of graft survival reported for patients with long-standing (>20 years) T1D, particularly for patients undergoing an SPK.[74] Interestingly, rates of graft survival, as well as glycemic control, are similar between both patients with T1D and T2D.[75] This, coupled with the fact that PTx seems to be associated with a low rate of T1D recurrence (~ 7%),[76] suggests that alloimmunity is the predominant determinant of graft survival. However, conversion from a negative to a positive autoantibody status has been recently suggested to confer a lower

probability of graft survival over time.[77] Currently, the benefits of monitoring either allo- and autoantibody status post-transplant are not clearly established.

2.1.5.1.2 - Diabetes-Related Complications

2.1.5.1.2.1 - Retinopathy

Early reports comparing successful vs failed PTx showed no difference in progression of retinopathy, both for PTA[78] and SPK.[79, 80] Importantly, most patients had previous laser photocoagulation and their degree of retinopathy remained stable. Later, a study comparing successful SPK vs failed SPK/kidney transplant alone showed improvement in visual acuity and stabilization of retinopathy in the former group for up to 3-years of follow-up.[81] Although ~ 80% of patients had laser photocoagulation before transplant, the need for further laser therapy was decreased in the successful SPK group vs the failed SPK/kidney transplant alone group.[81] When compared to conventional insulin therapy, PTA seems to ameliorate progression and/or improve retinopathy in patients with non-proliferative retinopathy and stabilize progression in those with proliferative retinopathy;[82] however, the benefits on stabilization of proliferative retinopathy seem to be more pronounced in the SPK population.[83] A recent and large study looking at retinopathy after PTx (mean follow-up of 4.2 years) suggests that, in almost 80% of the cases, retinopathy remains stable. Interestingly, 92% of the cases that progressed did so within 1-year post-transplant (early worsening).[84] These authors identified baseline degree of retinopathy, recent (< 1-year) treatment with photocoagulation, and PTA as risk factors for early worsening.[84] Another recent study looking at patients that underwent SPK and had a surviving graft for >25 years reported stabilization of retinopathy in 90% of the patients; comparative analysis with patients having a failed graft were lacking, unfortunately.[85] Importantly, most

patients undergo PTx late in the course of their disease, most have some degree of retinopathy at the time of transplant (~ 90-100%) and the majority have laser-treated and/or proliferative retinopathy.[82, 84] Initially close and then ongoing monitoring of retinopathy after transplant seems prudent since the benefit of PTx may be more related to stabilization rather than improvement or reversal of retinopathy, particularly for patients with proliferative retinopathy.

2.1.5.1.2.2 - Neuropathy

Up to 80-100% of patients undergoing PTx have signs of peripheral neuropathy.[22, 86, 87] However, neuropathy after PTx has been relatively understudied and, overall, there is a lack of recent reports.[22] A study comparing patients with successful vs failed SPK showed rapid and long-lasting recovery of neurophysiological parameters such as nerve conduction velocity and amplitude, however, no significant differences were observed compared to control subjects.[86] The classic reports from the University of Minnesota group, which compared successful PTx with control groups (including failed PTx and patients on the waiting list), showed improvement and/or stabilization in nerve conduction studies in the PTx group. More importantly, these findings also translated into a "clinical benefit", which was mainly driven by a lack of deterioration in neurological examination scores as compared to controls.[87, 88] Studies including patients undergoing PTA also showed improvement in neurophysiological parameters and neurological examination scores.[89] Correlation of this latter outcome with patient-important outcomes, such as decreased pain, numbness or complete sensory loss remains undetermined and should be explored in the future.

Autonomic neuropathy, which significantly impacts quality of life has also been understudied. Several measurements of autonomic neuropathy, such as vasomotor function

(e.g., capillary vasoconstriction responses), cardiac function (e.g., heart rate variability) and gastric function (e.g., gastric emptying) have been reported to improve in PTx as compared to kidney transplant alone.[90, 91] A recent study on SPK long-term survivors, reported improvement/stabilization of gastroparesis in 75% of the patients.[85] Importantly, improvements in autonomic neuropathy have been correlated with improvement in quality of life,[90] and hypoglycemia symptom recognition,[92] albeit more studies would strengthen these notions.

2.1.5.1.2.3 - Nephropathy

The combined percentages of SPK (84%) and PAK (8%) somewhat reflect the prevalence of kidney disease in candidates for a PTx.[24] As such, two potential "renal" outcomes post-transplant emerge: 1) kidney graft survival/function and 2) native kidney function. For the first outcome, studies have shown that in patients undergoing SPK, a surviving pancreatic graft confers a survival benefit for the kidney graft,[93] however, this seems to be only observed when compared to patients receiving a deceased-donor kidney graft.[94] For patients undergoing PAK, a surviving pancreatic graft confers a survival benefit for the kidney graft, even when compared to patients receiving a living-donor kidney graft. Interestingly, a living-donor kidney graft also improved the rates of pancreatic graft survival as compared to deceased-donor kidney graft.[65]

As for the second outcome, early studies showed that normoglycemia after successful PTA could reverse histological lesions in established diabetic nephropathy after 10 years of follow-up.[95] Later, studies comparing PTA with intensive insulin therapy showed improvement of proteinuria and no significant changes in creatinine clearance,[96] which, in the context of immunosuppression constitutes a favorable outcome. A large case series by Boggi et al., confirmed that proteinuria improved after a PTA, with 54% of patients with macroalbuminuria

at baseline reversing to either microalbuminuria (18%) or normal (36%).[89] These authors reported that patients with an eGFR >90 mL/min had a faster and more pronounced deterioration in renal function (~ -4.9 mL/min/year vs ~ -2 ml/min/yr in those with <90 mL/min at baseline, $p<0.05$); possibly due to correction of hyperfiltration.[97] Regarding onset of ESRD after a PTA (i.e., need for dialysis or kidney transplantation), large studies have shown a 5-year cumulative incidence of 3.5, 12.2 and 26.0 and a 10-year cumulative incidence of 21.8, 29.9 and 52.2% in patients with a baseline eGFR (mL/min/1.73 m^2) of >90, 89-60, and <60, respectively.[98] A recent report looking at different patient cohorts (Joslin Clinic, Finnish Diabetic Nephropathy Study, Steno Diabetes Center Copenhagen, and INSERM) shows a similar 10-year cumulative incidence of ESRD in non-transplant patients with T1D with earlier stages of chronic kidney disease (16.5-31.1%); higher incidence rates with more advanced disease were also observed.[99] However, the effects of PTA on renal function are still debated, and nephrotoxicity secondary to immunosuppression should be accounted for. Diabetes is also a risk factor for ESRD post-transplant,[100] thus, the combination of immunosuppression and diabetes may be particularly harmful. Nevertheless, this remains controversial, as large studies in nonrenal solid organ transplants have shown an overall 5-year cumulative incidence rate of ESRD as high as 18.1% and 21.3% for liver and intestine transplantation, respectively,[100] which compares similarly to PTA. Overall, the decision to move forward with a PTA should be weighed against the adverse effects of lifelong immunosuppression, among which nephrotoxicity is central, particularly with calcineurin inhibitors.

2.1.5.1.2.4 - Cardiovascular disease

Cardiovascular disease and risk factors is highly prevalent in candidates for a PTx. In a large study, 51.8-79.5% had hypertension, 4.1-4.9% had peripheral vascular disease, 1.9-4.1% had a prior coronary bypass, 3.8-5.4% had a prior coronary intervention, 1.8-2.1% had valvular disease, and 0.8-1.1% had a pulmonary circulation disorder at baseline.[101] SPK has the highest prevalence, followed by PAK and PTA.[22] The prevalence of coronary artery disease observed with coronary angiography is substantially higher (~50-70%).[102-105] The presence of cardiovascular disease at baseline confers worse prognosis in terms of patient and graft survival,[22] however, studies have shown that PTx stabilizes and/or improves disease markers such as carotid intima-media thickness,[106, 107] left ventricular function,[108, 109] LDL-cholesterol,[89, 97] triglycerides,[107, 109] inflammatory and prothrombotic factors (i.e., homocysteine, Von-Willebrand factor, D-dimer, etc.),[107] and endothelial dysfunction.[107] More importantly, the incidence of patient-important outcomes, such as cardiovascular death and/or major adverse cardiovascular events (i.e., fatal/nonfatal myocardial infarction or stroke), is lower in patients undergoing PTx when compared to kidney transplant alone, both deceased-donor[109-111] and living-donor,[112] as well as patients on the waiting list.[109, 111] The benefit seems to be mostly observed for SPK, and somewhat dependent on pancreatic graft survival.[112] In contrast, the evidence for PAK and PTA in terms of patient-important cardiovascular outcomes is inconclusive.[22, 112] Analyses including graft status in these two latter patient populations could shed more light into these important matters.

2.1.5.2 - Islet cell transplantation

2.1.5.2.1 - Patient Survival and Morbidity

ITx is one of the safest transplant in terms of patient survival. The latest report from the Collaborative Islet Transplant Registry (CITR), which gathers clinical data from allogeneic ITx from centers all over the world, reports a 5-year survival rate of 98.4%.[113] Single-center studies have reported a mortality rate of 0.3-1.0% per 100-patient-years.[114, 115] While patient morbidity and transplant-related adverse effects are fairly common, a substantial decrease in serious events related to infusion or immunosuppression from ~ 20.0% in the early eras (1999-2002) to ~ 7% in most recent eras (2011-2014) has been observed.[113] Similar to SPK, studies suggest that a simultaneous islet-kidney transplant (SIK) confers a survival benefit as compared to both patients having a kidney transplant alone and patients on the waiting list.[58] Importantly, ITx has also been shown to substantially improve health-related quality of life.[57, 116] However, samples have been typically small and more studies including appropriate control groups and sufficient follow-up are needed.

2.1.5.2.2 - Glycemic Control and Graft Survival

Initial excitement with publication of the "Edmonton Protocol" was subsequently tempered by reports showing that insulin independence was not usually sustainable over time, with only ~ 10% of patients maintaining insulin independence beyond 5 years.[117] Later, an international, multicenter trial of the "Edmonton Protocol" reported 1-year insulin independence rates of only 44%,[118] which highlighted the importance of center experience in islet isolation and peritransplant care. However, over the last two decades, a steady improvement in outcomes related to insulin independence and graft survival has been

observed.[119] The most recent CITR report shows 5-year insulin independence rates of ~ 30%,[113] and single center studies have recently reported 10-year insulin independence rates of 18-28%.[115, 120] More consistent has been the change in insulin use following ITx, with a decrease of ~ 70% at 5-years and ~ 50% at 10-years.[113, 115] While insulin independence and reductions in insulin use are highly desirable, evaluating the success of ITx around this outcome may underestimate clinical benefit since the primary goal of ITx is to address problematic and recurrent hypoglycemia. Hence, an appropriate measure of its success would be abrogation or minimization of severe hypoglycemic episodes (SHEs). This view coincides with a patient-centered approach, and recent position statements and workshops have advocated for this outcome to take an equally central place when evaluating the success of ITx.[59, 60] In this regard, >90% of patients have complete abrogation of SHEs at 5-years post-transplant,[113] but rates of 80-100% at 10-years post-transplant have also been reported.[115, 121] Similarly, restoration of awareness of hypoglycemia is an important outcome after ITx. This is a key phenomenon occurring in candidates for ITx. Unfortunately, there is controversy regarding the extent of recovery in terms of the counter-regulatory hormonal responses and symptom recognition during hypoglycemia following ITx. Early experience showed no improvement in both parameters,[122] however, more recent studies have been shown to significantly improve awareness of hypoglycemia after ITx.[123-126] This should be further studied in the future. Finally, abrogation of hypoglycemia cannot occur at the expense of glycemic control. In this regard, ITx also has also shown to be effective, with composite outcomes, such as absence of SHEs + HbA1c <6.5 or 7.0% (depending on the study), being reported in 62.5-87.5%, 58.3-71.0 %, 35-55%, and 21.9-36.3% at 1-, 2-, 5- and 10-years, respectively.[113, 121, 126-128] Finally, most studies show a substantial and sustained improvement in glycemic control.[129, 130]

2.1.5.2.3 - Diabetes-Related Complications

2.1.5.2.3.1 - Retinopathy

Retinopathy is common in patients undergoing ITx, ~60% have some degree of retinopathy.[113] Most studies report stabilization and/or improvement of retinopathy after transplant. Two studies comparing ITx to intensive insulin therapy have reported no progression of retinopathy; patients with more severe retinopathy were more protected.[131, 132] A case series reports similar outcomes, with one patient demonstrating improvement of retinopathy 1 year post-transplant.[133] Finally, a before vs after study showed an increase in blood flow velocities of central retinal arteries and veins 1-year post-transplant, however, no clinical or patient-important outcomes (e.g., the use of laser photocoagulation) were reported.[134] We recommend close ophthalmologic follow-up post-transplant since some patients may suffer from vitreous hemorrhage and/or need laser photocoagulation/vitrectomy after an ITx.[117]

2.1.5.2.3.1 - Neuropathy

On average, ~ 30% of patients undergoing ITx are reported to have peripheral neuropathy.[113] Whether ITx has an impact in this outcome remains controversial. Two studies comparing ITx vs intensive insulin therapy found no signification deterioration of nerve conduction velocities following ITx,[131, 132] while two other studies have shown improvement in nerve conduction velocities through time.[133, 135] One of these latter studies showed decreased expression of advanced glycation end products and their specific receptor in nerves and perineural vessels from skin biopsies of patients undergoing islet-after-kidney transplantation (IAK) vs kidney transplantation alone.[135] Finally, a study including 21 patients undergoing IAK showed no deterioration of motor parameters, as well as improvement in sensory parameters at

5-years post-transplant.[136] Autonomic neuropathy is present in ~ 20% of patients' pre-transplant, however, the evidence on the impact of ITx on this condition is scarce.

2.1.5.2.3.2 - Nephropathy

Evaluation of renal function is common in many clinical reports concerning ITx. It should be emphasized that, as compared to PTx, most (~ 80%) patients undergoing ITx do not have ESRD at baseline.[113] Despite optimal glycemic control during follow-up, some studies have reported a decline in eGFR following ITx. In a previous report from our group, we observed a median rate of decline of 0.39 mL/min/1.73 m^2/month, however, with wide inter-patient variability. Additionally, the proportion of patients with micro- and macroalbuminuria also increased post-transplant.[137] However, more recent studies have reported lower rates of renal function decline (GFR measured by ^{99m}Tc-DTPA) following an ITx as compared to medically-treated patients.[131, 132, 138] A recent study has reported no statistically significant reduction of eGFR, even after 10 years of follow-up.[115] These conflicting findings may be explained by differences in immunosuppression regimens and baseline renal function. In this regard, there is evidence suggesting that the combination of tacrolimus plus sirolimus might be more nephrotoxic than tacrolimus plus MMF,[131, 132, 138-140] unfortunately, reports with longer follow-up have introduced some controversy into this notion.[115] Studies directly comparing these two regimens are lacking and further research is needed. Renal function status should be considered in the patient selection process for ITx and, particularly, in decisions regarding immunosuppression regimens and post-transplant care.

2.1.5.2.3.3 - Cardiovascular disease

While ITx may be the preferred treatment modality in patients with a high burden of cardiovascular disease, the prevalence of coronary artery disease (CAD), cerebrovascular disease and peripheral vascular disease according to the latest CITR report is <10%, <3% and <5%, respectively.[113] These low numbers might be due to underreporting, since there are studies reporting that ~ 43% of asymptomatic ITx candidates have evidence of CAD on angiography.[141] Additionally, these low numbers may be driven by the fact that most patients undergo islet-transplant-alone (ITA), as other studies including SIK patients show a higher prevalence of coronary heart disease.[142, 143] In general, cardiovascular death rates seem to be lower in patients with a successful ITx as compared to those with unsuccessful ITx.[144] There is also evidence suggesting that following ITx, the incidence of CAD events does not substantially increase compared to non-transplanted patients with T1D, with a rate of 11 events per 1000 patient-years, which is slightly higher than that of the general T1D population (8.9 events per 1000 patient-years).[145] Although more studies are needed to assess patient-important cardiovascular outcomes following ITx, there are reports showing improvements in echocardiographic parameters (e.g., ventricular ejection fraction), vascular parameters (e.g., intima media thickness), cardiovascular biomarkers (e.g., atrial natriuretic peptide, triglycerides, low-density lipoproteins), as well as hemostatic parameters (e.g., prothrombotic factors, platelet function/ultrastructure).[58, 144, 146, 147]

2.1.6 - Whole Pancreas and Islet Cell Transplantation: Competing or Complementary Therapies?

It is tempting to compare PTx and ITx, however, this might not be appropriate. Overall, there are few studies directly comparing these two therapies (**Table 2.1.2**). Importantly,

although some indications overlap, patients undergoing PTx and ITx are inherently different. Current reports consistently show that as compared to PTx, patients undergoing ITx are older and with a longer duration of disease.[142, 143, 148-151] Additionally, studies have found that patients undergoing SIK have a higher prevalence of CAD compared to SPK,[142, 143] and lower patient survival rates.[143] These differences might be confounded by indications and contraindications for each treatment (e.g., older patients might not be eligible for PTx, SIK might be too sick for SPK). In contrast, studies focusing on PTA vs ITA have not reported differences in cardiovascular status and/or patient survival.[148, 151]

In terms of patent morbidity, there is consistency regarding early mortality (<1-year) and post-transplant complications (e.g., relaparotomy), where PTx has been associated with a higher frequency of these outcomes (**Table 2.1.2**). Regarding insulin independence, there is also controversy. Two studies from a single center comparing SPK vs simultaneous islet-kidney (SIK) or IAK report higher rates and longer duration of insulin independence.[142, 143] Conversely, studies evaluating PTA vs ITA show similar rates and duration of insulin independence.[148, 150] Glycemic control (i.e., HbA1c levels), on the other hand, seems to be "better" with PTx as compared to ITx, however the clinical impact remains unknown.

Finally, there is evidence that a PTx after a failed ITx has similar benefits as a primary PTx, despite the potential for sensitization.[153] Similarly, a small case series suggest that ITx after a failed PTx may be a feasible alternative given the complexity of a 2nd major surgical procedure.[154] Accordingly, PTx and ITx should be looked as complementary, rather than competing therapies.

Table 2.1.2. Studies describing patients undergoing whole pancreas and islet cell transplantation

Study	Patients and Methods	Main Results

Frank et al., 2004, United States	• Retrospective cohort
	• T1D
	• SPK/PAK: 25/5, total=30
	  • Induction IS: thymoglobulin
	  • Maintenance IS: TAC + MMF + steroids
	  • Mean age (range): 40 (24-55)
	  • Mean duration of DM (range): 27 (11-42)
	• ITA/IAK: 9/4, total=13 (one ITA excluded from analysis
	  • Induction IS: daclizumab
	  • Maintenance IS: TAC + SRL
	  • Mean age (range): 42 (28-56)
	  • Mean duration of DM (range): 28 (9-41)
	  • Mean total IEQs/patient: 15,475 IEQ/kg
		• SPK/PAK
		- Insulin independence: 26/30 patients (86.7%)
		- Rejection: 6/30 (20%)
		- HbA1c: 5.0% at 1-year post-transplant
		- Complications:
		  o Overall: 24 complications occurring in 30 patients, including 1 death.
		  o Post-transplant surgery: 7/30 patients (23.3%)
		- Costs: ~ 50,000 USD
		- Median duration of hospitalization: ~ 13 days
		• ITA/IAK
		- Insulin independence: 11/12 patients (91.7%) for at least 2 months
		- Rejection: 3/12 (33.3%), 1 patient had to stop IS due to a non-healing wound
		- HbA1c: 6.3% at 1-year post-transplant
		- Complications:
		  o Overall: 1 patient requiring surgery post-transplant and transfusion
		  o Post-transplant surgery: 1/13 patients (7.7%), due to hemothorax
		  o IS-related: 10/13 had mouth ulcers related to SRL
		  o eGFR (mL/min/1.73m^2): loss of 16.5 ml/min in ITA recipients
		- Costs: ~ 90,000 dollars
		• Median duration of hospitalization: ~ 4.5 days

Gerber et al., 2008, Switzerland	• Retrospective cohort
	• T1D with ESRD
	• SPK: 25
	  • Induction IS: basiliximab
	  • Maintenance IS: TAC + MMF
	  • Mean age (SD): 39.9 (6.0)
	  • Mean duration of DM (SD): 30.3 (7.1)
	• SIK: 13
	  • Induction IS: basiliximab
	  • Maintenance IS: TAC + MMF
		• SPK
		- Insulin independence: 24 patients (96%) at 1-year post-transplant
		- Change in HbA1c: 8.7% → 5.8% at 3-years post-transplant (3 patients had a mean HbA1c of 5.3 at 5-years post-transplant)
		- Complications:
		  o Overall: 12 patients (48%) had complications related to the pancreas
		  o Laparotomy post-transplant: 10 patients (40%)
		  o eGFR (mL/min/1.73m^2): 10.4 $\pm$ 4.1 at baseline → 67.3 $\pm$ 12.5 at 3-years post-transplant
		- Costs: 57,772 $\pm$ 30,649 euros (2008)

	• Mean age (SD): 52.6 (9.5)
	• Mean duration of DM (SD): 41.7 (9.1)
	• Mean total IEQs/patient (SD): 345,070 (137,511)
	  ○ Mean number of infusions (SD): 2.2 (1.3)
		- Median duration of hospitalization (SD): 22 (12) days
		• SIK
		- Insulin independence: 4 patients (31%) at 1-year post-transplant
		- Change in HbA1c: 8.1 % → 5.8% at 3-years post-transplant (5 patients had a mean HbA1c of 6.2 at 4-years post-transplant)
		- Complications:
		  ○ Overall: 2 patients (15%) had complications related to the islets
		  ○ Laparotomy post-transplant: 0 patients
		  ○ eGFR (mL/min/1.73m^2): 11.8 $\pm$ 6.7 at baseline → 49.6 $\pm$ 24.0 at 3-years post-transplant
		- Costs: 76,227 $\pm$ 8,966 euros (2008)
		- Median duration of hospitalization (SD): 18 (7) days (compiled)

Maffi et al., 2011, Italy	• Retrospective cohort
	• T1D without ESRD
	• PTA: 33
	- Induction IS: ATG + MPDN
	- Maintenance IS: TAC + MMF, MMF + CsA
	- Mean age (SD): 37 (8.4)
	- Mean duration of DM (SD): 20 (8.6)
	• ITA: 33
	- Induction IS: Daclizumab or ATG
	- Maintenance IS: TAC + SRL or SRL + MMF
	- Mean age (SD): 36 (8.6)
	- Mean duration of DM (SD): 23 (9.9)
	- Mean total IEQ/kg (SD): not reported
	  ○ One infusion: 9 (27.3%)
	  ○ Two infusions: 16 (48.4%)
	  ○ Three infusions: 8 (24.2%)
		• PTA
		- Insulin independence: 25 patients (75.7%)
		- Change in HbA1c: not reported
		- Complications:
		  ○ Laparotomy post-transplant: 18 patients (54.5%)
		  ○ Bleeding: 5 patients (15.5%)
		  ○ CMV reactivation: 21 patients (63.6%)
		  ○ Deterioration of renal function: 4 patients (12.1%), 1 required hemodialysis
		- Median duration of hospitalization (IQR): 19 (16-24) days
		• ITA
		- Insulin independence: 19 patients (57%)
		- Change in HbA1c: not reported
		- Complications:
		  ○ Laparotomy post-transplant: 0 patients (0%)
		  ○ Bleeding: 12 patients (36.6%)
		  ○ CMV reactivation: 2 patients (6.0%)
		  ○ Deterioration of renal function: 5 patients (15.1%), 2 required hemodialysis
		- Median duration of hospitalization (IQR): 16 (9-19) days (compiled)

Bellin et al., 2012,	• Retrospective cohort	• PTA - Insulin independence: 52% at 5-years

United States (data from CITR was included)	○ Different cohorts, indirect comparisons with PTA • T1D without ESRD • PTA: 677 - Induction IS: ATG or alemtuzumab or anti-CD3 or IL-2 receptor antagonists - Maintenance IS: TAC or CsA or SRL or MMF - Mean age (SD): 33.3 (7.1) • ITA: 269 - Induction IS: ATG or alemtuzumab or anti-CD3 or IL-2 receptor antagonists - Maintenance IS: TAC or CsA or SRL or MMF or efalizumab - Mean age (SD): 40.6 (1.4) – 45.1 (1.5) - Cumulative IEQ x 1000 (SD): 614 (46) – 908 (87) ○ One infusion: 79 (29.4) ○ Two infusions: 114 (42.3) ○ Three infusions: 72 (26.8) ○ $\geq$ four infusions: 4 (1.5)	- Change in HbA1c levels: not reported - Complications: not reported • ITA - Insulin independence: 0-50% at 5-years ○ 50% in patients receiving induction IS with T-cell depleting antibodies + TNF-α inhibitors. - Change in HbA1c levels: not reported - Complications: not reported
Lehman et al., 2015, Switzerland	• Prospective cohort • T1D with ESRD • SPK/PAK: 93/1, total=94 - Induction IS: ATG (SPK) or Basiliximab (PAK) - Maintenance IS: TAC + MMF - Mean age (SD): 44.2 (7.6) - Mean duration of DM (SD): 32.1 (8.2) • SIK/IAK: 23/15, total=38 - Induction IS: ATG (SIK) or Basiliximab (IAK and reinfusions)	• SPK/PAK - Insulin independence: 73.6% at 5-years - Mean decrease in HbA1c levels: 7.8% → 5.9% - Patient survival at 10 years: 88.5% - Complications: ○ 9/94 (9.6%) graft explants, 39/94 (41.5%) patients with early laparotomy (45 total laparotomies, only 4 not related to the pancreas transplantation) ○ GFR decline of 9.5 $\pm$ 23.3 mL/min/1.73m^2 at 13-years post-transplant • SIK/IAK - Insulin independence: 9.3% at 5-years ○ 20% mean decrease of insulin dosing (in those without insulin independence) - Mean decrease in HbA1c levels: 8.0% → 6.5%

	- Maintenance IS: TAC + SRL (later changed to MMF)
	- Mean age (SD): 51.8 (9.0)
	- Mean duration of DM (SD): 37.0 (11.0)
	- Mean total IEQ/kg (SD): 11,408 (10,380)
	  o Mean number of infusions (SD): 2.1 (1.3)
		- Drop in severe hypoglycemia: 346 ± 445 per 100 patient-years to 11.1 ± 15.2
		- Patient survival at 10 years: 65.4%
		- Complications:
		  o 4/38 (10.5%) early laparotomy (only 2 related to the islet transplant)
		  o 1 death (accidental puncture of an intercostal artery)
		  o GFR decline of 13.3 ± 13.8 mL/min/1.73m^2 at 13-years post-transplant

Moassesfar et al., 2016, United States	• Retrospective cohort
	• T1D patients without ESRD
	• PTA: 15
	- Induction IS: ATG + MPDN
	- Maintenance IS: MMF + TAC + PDN (tapering)
	- 6 females (40%)
	- Mean age (SD): 42.5 (10.45)
	- Mean duration of DM (SD): 29.9 (8.1)
	• ITA: 10
	- Induction IS: ATG + MPDN. 2nd infusion: basiliximab
	- Maintenance IS: belatacept (n=5) or efalizumab (n=5) + SRL ± MMF
	- 1 female (10%)
	- Mean age (SD): 51.8 ± 8.3
	- Mean duration of DM (SD): 40.3 (11.1)
	- Mean total IEQ/kg (SD): 12,952 (NR)
	  o One infusion: 6 (60%)
	  o Two infusions: 4 (40%)
		• PTA
		- Mean duration of insulin independence: 55 months, 93% at 1 year, 64% at 3 years
		- Mean decrease in HbA1c levels: 7.3% → 5.5%
		- Complications:
		  o Surgical: 9/15 (4 requiring pancreatectomy), Medical: 3/15, Vascular: 1/15, Infectious: 3/15 (1 readmission for surgical site infection), Renal: 7/15 (1 patient needing dialysis)
		  ▪ Change in GFR: 86.3 ± 18 mL/min/1.73 m^2 → 67.9 ± 25.4 mL/min/1.73 m^2 (p=0.025 vs baseline)
		- Costs: $134,748 USD
		- Mean duration of hospitalization: 12 days
		• ITA
		- Mean duration of insulin independence: 35 months, 90% at 1 year, 70% at 3 years
		- Mean decrease in HbA1c levels: 7.2% → 5.7%
		- Complications:
		  o Surgical: 0/10, Medical: 1/10, Vascular: 2/10, Infectious: 1/10, Renal: 4/10
		  ▪ Change in GFR: 79 ± 13.7 mL/min/1.73 m^2 → 72.9 ± 20.4 mL/min/1.73 m^2 (p=0.5 vs baseline)
		- Costs: $138,872 USD
		- Mean duration of hospitalization: 5.75

Voglová et al., 2017, Czech Republic	• Retrospective cohort
	• T1D without ESRD
	• PTA/PAK: 36/13, total=49
	- Induction IS: ATG + MPDN + basiliximab (PAK)
	- Maintenance IS: TAC + MMF + PDN (tapering)
		• PTA/PAK
		- Insulin independence: 73% at 1 year, 68% at 2 years, 55% at 5 years
		- Mean decrease in HbA1c levels: 7.4% → 4.1%
		- Complications:
		  o 11 patients (22.2%) had a graftectomy
		  o Surgical revision had to be performed in 23 patients (47%)

	- Median age (IQR): 39 (33-50) - Median duration of DM (IQR): 24 (16.5-31) • ITA/IAK/SIK: 24/4/2, total=30 - Induction IS: ATG + MPDN + etanercept - Maintenance IS: TAC + SRL - Median age (IQR): 48.5 yo (37-57) - Median duration of DM (IQR): 27.5 (19.5-34) - Median total IEQ/kg (IQR): 12,349 (6,387-15,331) o One infusion: 11 (36.6%) o Two infusions: 9 (27.3%) o Three infusions: 10 (33.3%)	o GFR decreased at 2 and 5 year from 78.6 (63.6-97.8) mL/min/1.73m^2 to 61.2 (39.6-76.8) and 58.8 (41.4-77.4) mL/min/1.73m^2, respectively • ITA/IAK/SIK - Insulin independence: 5 patients (17%) temporal insulin independence 10 patients (42%) with >30% insulin dose reduction - Mean decrease in HbA1c levels: 7.35% → 5.8% - Complications: o Bleeding in 10 patients (33%), 8 (27%) required urgent operation ▪ 4 patients (13%) with an intrahepatic hematoma o 1 patient (3.3%) with portal vein thrombosis o No significant change in GFR at 2 and 5-year follow-up
Nordheim E et al., 2021, Norway	• Retrospective cohort • T1D • PTA: 74 • Induction IS: ATG • Maintenance IS: TAC + MMF + steroids • Mean age (SD): 38.2 (9.6) • Mean duration of DM (SD): 24.9 (11) • ITA: 12 • Induction IS: ATG + etanercept • Maintenance IS: TAC + SRL or MMF • Mean age (SD): 46.3 (9.5) • Mean duration of DM (SD): 35.8 (10.7) • Mean total IEQs/patient (SD): not reported	• PTA - Insulin independence: 54/74 (73%) at 1-year post-transplant - Graft function: 8% had partial graft function, 19% had graft failure at 1-year post-transplant - HbA1c: 5.0% at 1-year post-transplant - Complications: NR - Costs: ~ 50,000 USD - Median duration of hospitalization: ~ 13 days • ITA - Insulin independence: 0/12 (0%) at 1-year post-transplant - Graft function: 90% had partial graft function, 10% had graft failure at 1-year post-transplant - HbA1c: 6.3% at 1-year post-transplant - Complications: not reported - Costs: ~ 90,000 dollars • Median duration of hospitalization: ~ 4.5 days

T1D: type 1 diabetes, PTA: pancreas transplantation alone, ITA: islet transplantation alone, IS: immunosuppression, ATG: antithymocyte globulin, MPDN: methylprednisolone, MMF: mycophenolate mofetil, TAC: tacrolimus, SRL: sirolimus, IEQ: islet equivalents, GFR: glomerular filtration rate, SPK: simultaneous pancreas-kidney transplantation, PAK: pancreas-after-kidney transplantation, SIK: simultaneous islet-kidney transplantation, IAK: islet-after-kidney transplantation, IQR: interquartile range, CITR: Collaborative Islet Transplant Registry.

2.1.7 - Whole Pancreas and Islet Cell Transplantation: Challenges and Potential Solutions

The main challenges for β-cell replacement therapies concern with: 1) organ/tissue source and preservation, 2) periprocedural management (including postoperative care/complications and engraftment), and 3) chronic immunosuppression. These challenges predominate at different moments of the transplantation process (**Figure 2.1.3**). While out of the scope of this chapter, several interesting aspects regarding potential solutions to these challenges will be discussed.

2.1.7.1 - Organ/tissue source and preservation

The lack of organs/tissues affects both PTx and ITx. Currently, only ~ 17% of donors have pancreas graft recovery with the intention to transplant.[155] It appears that multidisciplinary strategies including changes in legislation (i.e., "opt-out" policies for organ donation), together with national transplant coordination networks and coordinators (i.e., organ donation specialists in every hospital) and promotion of a culture of donation (physician- and public-driven) could substantially increase the pool of donors.[156] Other strategies to increase the quality (and quantity) of organs for transplantation involves organ perfusion and preservation technologies. These have evolved over the years, however, while there is preclinical evidence of improved outcomes with both PTx[157, 158] and ITx,[159] research is scarce in comparison to the kidney, liver and lung grafts.[160] These technologies, however, could expand the donor pool to include marginal donors (e.g., donation after cardiac death), and should be further explored in the future.

Figure 2.1.3. Challenges to advance β-cell replacement therapies and potential solutions

Note: The relative importance of these challenges within the context of each type of transplant is highlighted using different colors; red for whole pancreas and blue for islet cell transplantation. Figure Used with permission from with permission from Marfil-Garza BA, Senior PA and Shapiro AMJ. Whole Pancreas and Islet Cell Transplantation. In: Holt RIG, Cockram C, Flyvbjerg A, Goldstein BJ, editors. Textbook of Diabetes 6th Edition (In press)

Regarding ITx, islet isolation consumes a significant proportion of the costs,[161] which demands resource optimization. Many of these are fixed costs, so central or regional isolation hubs might be a strategy. Another approach is standardization of donor selection. Scores like the North American Islet Donor Score that have been derived by studying donor characteristics associated with successful isolations (>400,000 IEQ per pancreas) could be useful to optimize donor selection for islet isolation.[162] Optimization of enzymatic digestion[163] and post-isolation islet culture/preservation (~ 15-20% of the isolated islets are lost during culture)[164] are also actively being investigated. Increasing islet yield and quality to reduce the number of pancreata needed to support long-term glycemic control after ITx is a vital research avenue that should continue to be explored in the future.

Perhaps the most exciting developments concerning tissue source for β-cell replacement therapies relate to xenotransplantation and stem cell-based therapies. Islet xenotransplantation (Xeno-ITx) has been steadily moving towards the clinic over the past decades. Preliminary clinical experience with Xeno-ITx, although limited, has demonstrated safety, albeit moderate efficacy. This might be explained by the overall lower doses used and the stronger immune responses compared to human ITx. However, recent results are promising, showing a 45% decrease in insulin requirements and a 22.5% decrease in HbA1c levels at 1-year post-transplant of neonatal pig islets.[165] Immune responses following Xeno-ITx have been also tackled by using cellular encapsulation (*see below*), with documented survival of encapsulated neonatal porcine islet for up to 9.5 years.[166] The risk of zoonosis, particularly with the porcine-endogenous retrovirus, has been proven to be mostly theoretical, as no cases of *in vivo* transmission in preclinical and clinical trials have been documented.[167] Considering the advances in large-scale isolation of porcine islets, more clinical trials with Xeno-ITx in the near future are expected.

Regarding stem-cell therapies in T1D, there are two sources for cellular products: human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Stem-cell therapies build on recapitulating the *in situ* islet differentiation processes (i.e., embryogenesis) to generate functional human islets *in vitro*. Stem-cell therapies provide a potentially unlimited islet supply, but also the capacity to modify cell products to optimize their potency and decrease or even eliminate their immunogenicity.[168] Additionally, hiPSCs offer the possibility of individualized regenerative therapies. Islet-like structures differentiated from hESCs were the first to show similar potency as mature islets in terms of diabetes reversal in mouse models.[169-171] After Takahashi and Yamanaka first generated hiPSCs in 2006,[172] efforts permeated into the field of diabetes and hiPSC-derived islet-like structures were quickly generated from patients with T1D;[173] demonstration of diabetes reversal in mouse models promptly followed[174] and has been increasingly reported in recent years.[175] Whether hESCs or hiPSCs are similar in their capacity to generate functional human islets and in their translational potential is debated. Ethical considerations favor hiPSCs, however, aspects regarding genome integrity and regulation, abnormal developmental potential, as well as costs and scalability should also be considered. Currently, hESCs have accumulated more evidence than hiPSCs in terms of differentiation efficiency and overall safety. In fact, clinical trials in patients with T1D are limited to hESCs-derived islet-like cells (NCT03162926, NCT03163511, NCT02239354 and NCT02939118). However, the unavoidable fact that hiPSCs represents the only option for a truly personalized regenerative medicine is a strong argument driving ongoing research efforts.

2.1.7.2 - Periprocedural care

There are distinct challenges associated with periprocedural care in PTx and ITx. Optimal donor and patient selection, as well as pre-transplant care are essential to decrease the incidence of peri- and post-transplant complications. To optimize donor selection in PTx, there are identified risk factors and tools, such as the Pancreas Donor Risk Index (PDRI) and the pre-procurement pancreas allocation suitability score (P-PASS), that have been used to assess the quality of the graft, the risk of allograft failure, and complications, such as graft thrombosis.[176] However, they have not been widely implemented and, since donors are still relatively scarce, rejecting organs based on any score may not be cost-efficient. Aspects related to surgery, such as optimal back-table preparation, the use of IV heparin at the moment of implantation, avoidance of venous extension grafts, as well as avoidance of intraoperative hypotension should be considered to decrease the risk of major complications after PTx such as bleeding and thrombosis[177] Another issue is acute graft pancreatitis. While "physiological" pancreatitis occurs in most cases, this condition has uncertain clinical implications. Mannitol, furosemide and octreotide have been recommended prior to completion of the vascular anastomosis to prevent this condition.[177] Conversely, early pancreatitis (<3 months post-transplant) occurs in 3-38% of the cases, while late acute pancreatitis (>3 months post-transplant) occurs in 14-25% of the cases.[177] Risk factors for pancreatitis are related to the donor (i.e., age >50, cardiovascular death, hemodynamic instability prior to procurement), graft procurement and preparation, surgery (i.e., bladder exocrine drainage), and infections (i.e., cytomegalovirus). It is important to prevent this outcome since it decreases graft and patient survival.[178] Importantly, the only way of decreasing the incidence of postoperative complications is by gaining experience with surgical techniques and periprocedural care. Unfortunately, the number of PTx has decreased

in recent years, which raises concerns for a potential surge in technical complications related to a lack of exposure and inexperience with this procedure.[155] The reasons for this decrease are multifactorial and include: a lack of patient referral, the "competition" with novel insulin therapies and ITx, as well as the excitement with novel therapies (e.g., immunotherapies, stem cell-based therapies), the lack of dissemination of improving outcomes, among others.[179] A potential approach is to conduct outreach sessions involving engaged physicians (e.g., transplant surgeons, endocrinologists) and patients. Expanding the recipient pool (e.g., older, non-T1D patients) which ultimately drives the need for pancreatic grafts is another alternative.[179] This would increase experience and positively impact the quality of the surgical procedures involved in PTx.

There are two major complications following ITx, bleeding and portal thrombosis. The overall incidence of bleeding after ITx is 7%, the main risk factors being high-dose heparin (>45 IU/kg) and the number of infusions.[180] The use of coils or hemostatic agents (e.g., Avitene™ or D-Stat™) to obliterate the percutaneous tract has shown to substantially decrease the risk of major bleeding.[181] This allows safer initiation of systemic anticoagulation with IV heparin,[55, 182] which ameliorates the instant blood-mediated inflammatory response (IBMIR) and contributes to preventing portal vein thrombosis. This latter complication presents in 3-10% of the cases, and typically as a partial thrombosis. Risk factors include a portal pressure during infusion of >22-25 mmHg, a large packed-cell volume (>5.5 mL or 0.25 mL/kg) and thrombophilic disorders,[182] thus, these scenarios should be avoided.[181, 182] Early cell death is another major concern in ITx, and one of the main explanations behind the need for repeated infusions. The surviving islet mass directly correlates with graft survival,[183] unfortunately, at least 25% of islets are lost within minutes of infusion into the portal circulation;[184] the central

phenomenon believed to explain this is IBMIR, an innate immune response triggered by direct exposure of islets (and tissue factor) to the blood. It consists of activation of the coagulation cascade, the complement pathway, cytokine secretion and cell-mediated injury.[185] Blocking this response with anticoagulation[55] and anti-inflammatory agents such as TNF-α inhibitors (etanercept) and interleukin-1 inhibitors (anakinra) has been shown to improve clinical outcomes in ITx.[56, 119, 150] Finally, hypoxia represents another relevant factor affecting islet survival. β-cells are ill-equipped to handle hypoxia and reactive oxidative stress due to low expression of antioxidants.[186] The process of neovascularization after infusion requires ~ 7-14 days,[187] and many islets die within this period. Strategies to decrease hypoxia and enhance vascularization, even in the most hostile implantation sites (i.e., the subcutaneous space), using prevascularization,[188] extracellular matrix-based scaffolding, proangiogenic factors, co-culture/co-transplantation with pro-angiogenic supporting cells show promise and great potential for clinical translation.[189]

2.1.7.3 - Chronic immunosuppression

The need for lifelong immunosuppression is perhaps the greatest obstacle for β-cell replacement therapies to become a true cure for diabetes. The holy grail of transplantation is operational tolerance, that is, maintaining organ/graft function and survival without immunosuppression. Unfortunately, the probability of achieving operational tolerance after PTx or ITx is minimal, as compared to other organs (e.g., the liver). This may be explained by the underlying autoimmune process in most patients, which may potentiate alloimmune responses after transplant, and *vice versa* (reciprocal facilitation/regulation).[190] Despite the hostile immunological milieu, there are potential approaches to minimize or even eliminate the need

for lifelong immunosuppression. First, there is evidence suggesting that certain immunosuppressants may work better than others at controlling autoreactivity. For example, ATG (compared to daclizumab) and tacrolimus or MMF (compared to sirolimus), have been associated with an increased risk of autoantibody recurrence in patients undergoing ITx.[190] This has motivated an interest in the "off-target" immunoregulatory effects of pharmacologic immunosuppression, such as those involving regulatory T cells (Tregs). In this sense, a "Treg-centric" view on immunosuppression post-transplant has been contemplated.[191] Tregs are central in immunological tolerance, and key players in autoimmune diseases. In transplantation, these cells have gained astounding research momentum.[192] There are recent studies suggesting that different immunosuppressants evoke distinct Treg responses, and that these can potentially be used to foster a Treg-rich environment post-transplant. Alemtuzumab [193] and ATG plus daclizumab[194] have both been associated with increased Treg percentages and higher Treg to effector T cell ratios following ITx. Sirolimus and MMF have also been shown to have a Treg-favoring effect, when compared to tacrolimus and CsA.[195] These preliminary findings should be deepened and extended to other immunosuppressants, as well as to other immunoregulatory mechanisms beyond Tregs.

Other interesting research avenues include adoptive cell transfer (ACT) therapies, cellular encapsulation and cellular gene-editing. Clinical trials using Treg-based ACT therapies have demonstrated safety and efficacy in delaying disease progression in patients with T1D.[196, 197] In transplantation, the multicenter ONE Study showed that Treg-based ACT following kidney transplantation enabled minimization of immunosuppression (i.e., tacrolimus monotherapy) in 40% of the patients compared to 2% in those in the standard care group [198]. Additionally, ACT-treated patients showed significantly lower rates of opportunistic infections

compared to controls,[198] which suggests that these therapies provide a more nuanced regulation of immune responses than pharmacological immunosuppression. Enhanced and more targeted cellular products, such as donor alloantigen-reactive Tregs (darTregs) or chimeric T-cell receptor Tregs (CAR-Tregs),[199] are entering the clinical realm, which will further elucidate the potential of ACT therapies to enable operational tolerance. Cellular encapsulation, which involves providing a physical barrier made of different biomaterials to protect cells from the immune responses, represents a promising alternative to abrogate immunosuppression altogether. Naturally, these technologies apply only to ITx and stem cell-based therapies, as you cannot encapsulate a whole pancreas. Overall, the clinical and preclinical experience is encouraging. The introduction of low-fouling, "immune friendly" biomaterials,[200] and composite bioscaffolds enabling localized immunosuppression/immunoregulation[201, 202] has resulted in improved graft acceptance and longer duration of diabetes reversal in preclinical models of ITx. Moreover, there is evidence that these strategies are compatible with Xeno-ITx [203] and stem-cell therapies.[204-206] Clinical trials with encapsulated hESC-derived pancreatic endocrine progenitors (ViaCyte Inc.) that differentiate into fully mature islet-like structures *in vivo* are ongoing, but preliminary results suggest that these cells can survive for up to 2-years within macroencapsulation devices.[207] Achieving safe and effective cellular encapsulation could broaden indications for β-cell replacement therapies to patients with other forms of diabetes. Finally, the advent of efficient gene-editing techniques to prevent allo-[208, 209] and autoimmune[210] destruction of stem cell-derived islets could revolutionize the field. In this regard, Viacyte Inc. has developed the cellular product PEC-QT, an edited clonal hESC line that lacks the β2-microglobulin gene and express a transgene encoding programmed death-

ligand 1 to protect cells from immune attack.[211] It is expected that many of these potentially game-changing strategies will move into clinical trials soon.

2.1.8 - Summary and Concluding Remarks

β-cell replacement therapies are now consolidated options in the therapeutic arsenal of physicians caring for patients with diabetes and persistent problematic hypoglycemia. The path to clinical success has been paved by brilliant researchers that have carried the field forward with unwavering perseverance and uninterrupted innovation. Today, both PTx and ITx have shown to be safe and effective. Additionally, there is evidence that both therapies improve patient survival and positively impact the natural history of diabetes by ameliorating progression of chronic complications. For these notable achievements, they have earned the well-deserved title of potential cures for diabetes. While tempting to compare them to each other, PTx and ITx seem to benefit different populations and have their specific niches. In this regard, they can perfectly function as complementary therapies and succeed when the other one has failed.

Currently, β-cell replacement therapies are limited to a very selected group of patients in which the benefits outweigh the risks associated with these procedures. The main challenges include expanding the supply of organs/tissues to treat as many patients as needed, optimizing periprocedural care to decrease procedural risks, and minimizing or eliminating the need for lifelong immunosuppression to avoid long-term adverse effects. Fortunately, the legacy of those brilliant, perseverant, and innovative researchers endures and continues to thrive in today's graduate students, scientists and clinicians involved in the field of β-cell replacement therapies. The paradigm in diabetes care has changed from saving to treating patients, the moment has come to change our paradigm once again from treating to curing patients.

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CHAPTER 2

PART 2 - WHOLE PANCREAS AND PANCREATIC ISLET TRANSPLANTATION: COMPARATIVE OUTCOMES OF A SINGLE-CENTRE COHORT OVER 20-YEARS

CHAPTER 2, PART 2 - WHOLE PANCREAS AND PANCREATIC ISLET TRANSPLANTATION: COMPARATIVE OUTCOMES OF A SINGLE-CENTRE COHORT OVER 20-YEARS

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2.2.1 - Abstract

Background

Whole pancreas (PTx) and islet (ITx) transplantation are effective therapies for people with type 1 diabetes and problematic hypoglycemia. However, comparative long-term studies are scarce. Herein, we present the largest single-centre comparative analysis of both therapies with up to 20-years of follow-up.

Research Design and Methods

We included patients undergoing PTx (n=146) and ITx (n=266) at the University of Alberta from January 1999-October 2019. Primary outcome was patient survival. Other outcomes include graft survival, insulin independence, glycemic control, procedure-related complications, and hospital readmissions.

Results

Crude mortality was 14.4% and 9.4% after PTx and ITx, respectively (p=0.124). Age-adjusted hazard-ratio for mortality was 2.3 (95% CI, 1.2-4.5, p=0.01) for PTx vs ITx. Graft failure ensued in 19.9% and 34.2% after PTx and ITx, respectively (p=0.002). Insulin independence was achieved in 92.5% and 78.6% of PTx and ITx patients, respectively (p<0.001), while the median total duration of insulin independence was 6.7 (IQR 2.9 - 12.4) and 2.1 (IQR 0.8 - 4.6) yr for PTx and ITx group, respectively (p<0.001). Improved glycemic control was sustained for up to 20-years post-transplant in both groups. Procedure-related complications and hospital readmissions were higher after PTx (p<0.001 for both).

Conclusions

PTx has a higher age-adjusted mortality, incidence of procedure-related complications and hospital readmissions compared to ITx. Conversely, graft survival, insulin independence and

glycemic control are significantly better with PTx. This study provides data to balance risks and benefits with current β-cell replacement therapies, which could improve shared-decision making.

2.2.2 - Background

Exogenous insulin therapy remains the cornerstone life-sustaining treatment for people with type 1 diabetes. Unfortunately, achieving optimal glycemic targets with insulin increases the risk of severe hypoglycemic episodes (SHEs).[1] While contemporary technologies for insulin delivery and glucose monitoring show promise,[2, 3] some patients remain recalcitrant to the most advanced strategies and experience persistent SHEs,[4] which promotes progression to hypoglycemia unawareness.[5, 6] This scenario substantially impacts quality of life and can lead to death in up to 10% of patients.[7] In selected cases, an effective means to ameliorate the burden of hypoglycemia, while maintaining normoglycemia, is through whole pancreas (PTx) or pancreatic islet transplantation (ITx).[8]

PTx and ITx are established therapies that substantially improve the lives of people with type 1 diabetes experiencing problematic hypoglycemia by preventing SHEs almost completely. In the US, PTx is an insured treatment, while ITx remains experimental. However, ITx is insured in several countries including Canada, the UK, Switzerland, France, Italy and Australia. Both procedures can also enable insulin independence and sustained improvements in glycemic control in a large proportion of recipients. While there are well-defined indications for each procedure[9, 10] and directed policies for organ allocation in some countries,[11] overlap of indications between ITx and PTx remains, mostly so for pancreas-alone and islet-alone transplantation. Despite growing literature, few studies have directly compared outcomes between ITx and PTx therapies, most including small samples and/or short-term follow-up (**Table 2.2.1**). These limitations preclude evidence-informed clinical decision-making, and underscore a need for evidence that can contribute to optimizing clinical practice. Herein, we

report a single-centre experience with ITx and PTx over a 20-year period and provide an exploratory comparative analysis.

Table 2.2.1. Studies comparing patients undergoing whole pancreas and pancreatic islet transplantation

Author, year, country	Patients and Methods	Main Results

Frank et al., 2004, United States	• Retrospective cohort
	• T1D
	• SPK/PAK: 25/5, total=30
	  • Induction IS: thymoglobulin
	  • Maintenance IS: TAC + MMF + steroids
	  • Mean age (range): 40 (24-55)
	  • Mean duration of DM (range): 27 (11-42)
	• ITA/IAK: 9/4, total=13 (one ITA excluded from analysis
	  • Induction IS: daclizumab
	  • Maintenance IS: TAC + SRL
	  • Mean age (range): 42 (28-56)
	  • Mean duration of DM (range): 28 (9-41)
	  • Mean total IEQs/patient: 15,475 IEQ/kg
		• SPK/PAK
		- Insulin independence: 26/30 patients (86.7%)
		- Rejection: 6/30 (20%)
		- HbA1c: 5.0% at 1-year post-transplant
		- Complications:
		  o Overall: 24 complications occurring in 30 patients, including 1 death.
		  o Post-transplant surgery: 7/30 patients (23.3%)
		- Costs: ~ 50,000 USD
		- Median duration of hospitalization: ~ 13 days
		• ITA/IAK
		- Insulin independence: 11/12 patients (91.7%) for at least 2 months
		- Rejection: 3/12 (33.3%), 1 patient had to stop IS due to a non-healing wound
		- HbA1c: 6.3% at 1-year post-transplant
		- Complications:
		  o Overall: 1 patient requiring surgery post-transplant and transfusion
		  o Post-transplant surgery: 1/13 patients (7.7%), due to hemothorax
		  o IS-related: 10/13 had mouth ulcers related to SRL
		  o eGFR (mL/min/1.73m^2): loss of 16.5 ml/min in ITA recipients
		- Costs: ~ 90,000 dollars
		• Median duration of hospitalization: ~ 4.5 days

Gerber et al., 2008, Switzerland	• Retrospective cohort
	• T1D with ESRD
	• SPK: 25
	  • Induction IS: basiliximab
	  • Maintenance IS: TAC + MMF
	  • Mean age (SD): 39.9 (6.0)
	  • Mean duration of DM (SD): 30.3 (7.1)
	• SIK: 13
	  • Induction IS: basiliximab
	  • Maintenance IS: TAC + MMF
	  • Mean age (SD): 52.6 (9.5)
		• SPK
		- Insulin independence: 24 patients (96%) at 1-year post-transplant
		- Change in HbA1c: 8.7% → 5.8% at 3-years post-transplant (3 patients had a mean HbA1c of 5.3 at 5-years post-transplant)
		- Complications:
		  o Overall: 12 patients (48%) had complications related to the pancreas
		  o Laparotomy post-transplant: 10 patients (40%)
		  o eGFR (mL/min/1.73m^2): 10.4 $\pm$ 4.1 at baseline → 67.3 $\pm$ 12.5 at 3-years post-transplant
		- Costs: 57,772 $\pm$ 30,649 euros (2008)
		- Median duration of hospitalization (SD): 22 (12) days
		• SIK

	• Mean duration of DM (SD): 41.7 (9.1)
	• Mean total IEQs/patient (SD): 345,070 (137,511)
	  o Mean number of infusions (SD): 2.2 (1.3)
		- Insulin independence: 4 patients (31%) at 1-year post-transplant
		- Change in HbA1c: 8.1 % → 5.8% at 3-years post-transplant (5 patients had a mean HbA1c of 6.2 at 4-years post-transplant)
		- Complications:
		  o Overall: 2 patients (15%) had complications related to the islets
		  o Laparotomy post-transplant: 0 patients
		  o eGFR (mL/min/1.73m^2): 11.8 $\pm$ 6.7 at baseline → 49.6 $\pm$ 24.0 at 3-years post-transplant
		- Costs: 76,227 $\pm$ 8,966 euros (2008)
		- Median duration of hospitalization (SD): 18 (7) days (compiled)

Maffi et al., 2011, Italy	• Retrospective cohort
	• T1D without ESRD
	• PTA: 33
	  - Induction IS: ATG + MPDN
	  - Maintenance IS: TAC + MMF, MMF + CsA
	  - Mean age (SD): 37 (8.4)
	  - Mean duration of DM (SD): 20 (8.6)
	• ITA: 33
	  - Induction IS: Daclizumab or ATG
	  - Maintenance IS: TAC + SRL or SRL + MMF
	  - Mean age (SD): 36 (8.6)
	  - Mean duration of DM (SD): 23 (9.9)
	  - Mean total IEQ/kg (SD): not reported
	  o One infusion: 9 (27.3%)
	  o Two infusions: 16 (48.4%)
	  o Three infusions: 8 (24.2%)
		• PTA
		- Insulin independence: 25 patients (75.7%)
		- Change in HbA1c: not reported
		- Complications:
		  o Laparotomy post-transplant: 18 patients (54.5%)
		  o Bleeding: 5 patients (15.5%)
		  o CMV reactivation: 21 patients (63.6%)
		  o Deterioration of renal function: 4 patients (12.1%), 1 required hemodialysis
		- Median duration of hospitalization (IQR): 19 (16-24) days
		• ITA
		- Insulin independence: 19 patients (57%)
		- Change in HbA1c: not reported
		- Complications:
		  o Laparotomy post-transplant: 0 patients (0%)
		  o Bleeding: 12 patients (36.6%)
		  o CMV reactivation: 2 patients (6.0%)
		  o Deterioration of renal function: 5 patients (15.1%), 2 required hemodialysis
		- Median duration of hospitalization (IQR): 16 (9-19) days (compiled)

Bellin et al., 2012, United States (data from CITR was included)	• Retrospective cohort
	  o Different cohorts, indirect comparisons with PTA
	• T1D without ESRD
	• PTA: 677
	  - Induction IS: ATG or alemtuzumab or anti-CD3 or IL-2 receptor antagonists
	  - Maintenance IS: TAC or CsA or SRL or MMF
	  - Mean age (SD): 33.3 (7.1)
		• PTA
		- Insulin independence: 52% at 5-years
		- Change in HbA1c levels: not reported
		- Complications: not reported
		• ITA
		- Insulin independence: 0-50% at 5-years
		  o 50% in patients receiving induction IS with T-cell depleting antibodies + TNF-α inhibitors.
		- Change in HbA1c levels: not reported

	• ITA: 269 - Induction IS: ATG or alemtuzumab or anti-CD3 or IL-2 receptor antagonists - Maintenance IS: TAC or CsA or SRL or MMF or efalizumab - Mean age (SD): 40.6 (1.4) – 45.1 (1.5) - Cumulative IEQ x 1000 (SD): 614 (46) – 908 (87) o One infusion: 79 (29.4) o Two infusions: 114 (42.3) o Three infusions: 72 (26.8) o ≥ four infusions: 4 (1.5)	- Complications: not reported
Lehman et al., 2015, Switzerland	• Prospective cohort • T1D with ESRD • SPK/PAK: 93/1, total=94 - Induction IS: ATG (SPK) or Basiliximab (PAK) - Maintenance IS: TAC + MMF - Mean age (SD): 44.2 (7.6) - Mean duration of DM (SD): 32.1 (8.2) • SIK/IAK: 23/15, total=38 - Induction IS: ATG (SIK) or Basiliximab (IAK and reinfusions) - Maintenance IS: TAC + SRL (later changed to MMF) - Mean age (SD): 51.8 (9.0) - Mean duration of DM (SD): 37.0 (11.0) - Mean total IEQ/kg (SD): 11,408 (10,380) o Mean number of infusions (SD): 2.1 (1.3)	• SPK/PAK - Insulin independence: 73.6% at 5-years - Mean decrease in HbA1c levels: 7.8% → 5.9% - Patient survival at 10 years: 88.5% - Complications: o 9/94 (9.6%) graft explants, 39/94 (41.5%) patients with early laparotomy (45 total laparotomies, only 4 not related to the pancreas transplantation) o GFR decline of 9.5 ± 23.3 mL/min/1.73m^2 at 13-years post-transplant • SIK/IAK - Insulin independence: 9.3% at 5-years o 20% mean decrease of insulin dosing (in those without insulin independence) - Mean decrease in HbA1c levels: 8.0% → 6.5% - Drop in severe hypoglycemia: 346 ± 445 per 100 patient-years to 11.1 ± 12.2 - Patient survival at 10 years: 65.4% - Complications: o 4/38 (10.5%) early laparotomy (only 2 related to the islet transplant) o 1 death (accidental puncture of an intercostal artery) o GFR decline of 13.3 ± 13.8 mL/min/1.73m^2 at 13-years post-transplant
Moassesfar et al., 2016, United States	• Retrospective cohort • T1D patients without ESRD • PTA: 15 - Induction IS: ATG + MPDN - Maintenance IS: MMF + TAC + PDN (tapering) - 6 females (40%)	• PTA - Mean duration of insulin independence: 55 months, 93% at 1 year, 64% at 3 years - Mean decrease in HbA1c levels: 7.3% → 5.5% - Complications:

	- Mean age (SD): 42.5 (10.45) - Mean duration of DM (SD): 29.9 (8.1) • ITA: 10 - Induction IS: ATG + MPDN. 2nd infusion: basiliximab - Maintenance IS: belatacept (n=5) or efalizumab (n=5) + SRL ± MMF - 1 female (10%) - Mean age (SD): 51.8 ± 8.3 - Mean duration of DM (SD): 40.3 (11.1) - Mean total IEQ/kg (SD): 12,952 (NR) o One infusion: 6 (60%) o Two infusions: 4 (40%)	o Surgical: 9/15 (4 requiring pancreatectomy), Medical: 3/15, Vascular: 1/15, Infectious: 3/15 (1 readmission for surgical site infection), Renal: 7/15 (1 patient needing dialysis) ▪ Change in GFR: 86.3 ± 18 mL/min/1.73 m^2 → 67.9 ± 25.4 mL/min/1.73 m^2 (p=0.025 vs baseline) - Costs: $134,748 USD - Mean duration of hospitalization: 12 days • ITA - Mean duration of insulin independence: 35 months, 90% at 1 year, 70% at 3 years - Mean decrease in HbA1c levels: 7.2% → 5.7% - Complications: o Surgical: 0/10, Medical: 1/10, Vascular: 2/10, Infectious: 1/10, Renal: 4/10 ▪ Change in GFR: 79 ± 13.7 mL/min/1.73 m^2 → 72.9 ± 20.4 mL/min/1.73 m^2 (p=0.5 vs baseline) - Costs: $138,872 USD - Mean duration of hospitalization: 5.75
Voglová et al., 2017, Czech Republic	• Retrospective cohort • T1D without ESRD • PTA/PAK: 36/13, total=49 - Induction IS: ATG + MPDN + basiliximab (PAK) - Maintenance IS: TAC + MMF + PDN (tapering) - Median age (IQR): 39 (33-50) - Median duration of DM (IQR): 24 (16.5-31) • ITA/IAK/SIK: 24/4/2, total=30 - Induction IS: ATG + MPDN + etanercept - Maintenance IS: TAC + SRL - Median age (IQR): 48.5 yo (37-57) - Median duration of DM (IQR): 27.5 (19.5-34) - Median total IEQ/kg (IQR): 12,349 (6,387-15,331) o One infusion: 11 (36.6%) o Two infusions: 9 (27.3%) o Three infusions: 10 (33.3%)	• PTA/PAK - Insulin independence: 73% at 1 year, 68% at 2 years, 55% at 5 years - Mean decrease in HbA1c levels: 7.4% → 4.1% - Complications: o 11 patients (22.2%) had a graftectomy o Surgical revision had to be performed in 23 patients (47%) o GFR decreased at 2 and 5 year from 78.6 (63.6-97.8) mL/min/1.73m^2 to 61.2 (39.6-76.8) and 58.8 (41.4-77.4) mL/min/1.73m^2, respectively • ITA/IAK/SIK - Insulin independence: 5 patients (17%) temporal insulin independence 10 patients (42%) with >30% insulin dose reduction - Mean decrease in HbA1c levels: 7.35% → 5.8% - Complications: o Bleeding in 10 patients (33%), 8 (27%) required urgent operation ▪ 4 patients (13%) with an intrahepatic hematoma o 1 patient (3.3%) with portal vein thrombosis o No significant change in GFR at 2 and 5-year follow-up
Nordheim E et al.,	• Retrospective cohort • T1D	• PTA - Insulin independence: 54/74 (73%) at 1-year post-transplant

2021, Norway	• PTA: 74
	  • Induction IS: ATG
	  • Maintenance IS: TAC + MMF + steroids
	  • Mean age (SD): 38.2 (9.6)
	  • Mean duration of DM (SD): 24.9 (11)
	• ITA: 12
	  • Induction IS: ATG + etanercept
	  • Maintenance IS: TAC + SRL or MMF
	  • Mean age (SD): 46.3 (9.5)
	  • Mean duration of DM (SD): 35.8 (10.7)
	  • Mean total IEQs/patient (SD): not reported
		- Graft function: 8% had partial graft function, 19% had graft failure at 1-year post-transplant
		- Complications: not reported
		• ITA
		- Insulin independence: 0/12 (0%) at 1-year post-transplant
		- Graft function: 90% had partial graft function, 10% had graft failure at 1-year post-transplant
		- Complications: not reported

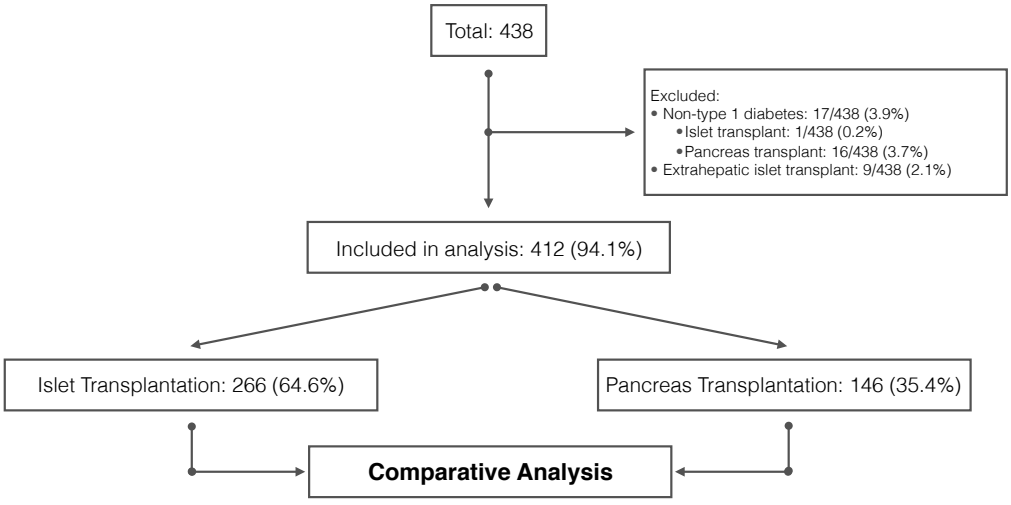
T1D: type 1 diabetes, PTA: pancreas transplantation alone, ITA: islet transplantation alone, IS: immunosuppression, ATG: antithymocyte globulin, MPDN: methylprednisolone, MMF: mycophenolate mofetil, TAC: tacrolimus, SRL: sirolimus, IEQ: islet equivalents, GFR: glomerular filtration rate, SPK: simultaneous pancreas-kidney transplantation, PAK: pancreas-after-kidney transplantation, SIK: simultaneous islet-kidney transplantation, IAK: islet-after-kidney transplantation, IQR: interquartile range, CITR: Collaborative Islet Transplant Registry. This table is included as supplementary material in the submitted manuscript.

2.2.3 - Research Design and Methods

2.2.3.1 - Study design and Patient Selection

We include people with type 1 diabetes undergoing allogeneic PTx or ITx at the University of Alberta Hospital between January 1st 1999 and October 1st 2019. Simultaneous pancreas-kidney transplantation (SPK), pancreas-after-kidney transplantation (PAK), pancreas transplant alone (PTA), islet-after-kidney transplantation (IAK) and islet transplant alone (ITA) were included. Pediatric patients, those without type 1 diabetes, or with at least one extrahepatic/extraportal islet infusion were excluded (**Figure 2.2.1**). Patients that underwent both types of transplants (pancreas-after-failed ITx or islet-after-failed PTx) were classified as either PTx (6/146) or ITx (6/266) patients based on their first transplant. Follow-up for this subset of patients was censored once the alternative transplant occurred. This study was approved by our institutional health research ethics board (PRO00001120 and PRO00087040). Patient consent for the use of health data for research purposes was obtained for all subjects.

Figure 2.2.1. Flow diagram for patient selection



Note: this figure is included as supplementary material in the submitted manuscript

2.2.3.2 - Organ Procurement and Transplant procedures

For PTx, the pancreas was procured after neurological determination of death and following flushing with cold preservation solution via the infrarenal aorta. Preparation of the pancreas, including splenectomy and arterial reconstruction with iliac Y-graft was completed on the back table. The pancreas graft was placed in the mid-abdomen, with anastomosis of the Y-graft to the patient's common iliac artery, portal vein to the superior mesenteric vein or inferior vena cava, and duodenum to an appropriate proximal segment of small bowel for enteric drainage. Systemic heparin infusions were initiated in pancreas transplant recipients in selected cases based on surgeon preference provided the operative field remained dry. Aspirin (81 mg qd) was started once eating.

Islet isolation was performed as previously reported [12]. Suitable islet preparations were loaded into a gravity infusion bag. After 2005, heparin (70 IU/kg of body weight) was added to the infusion bag, previously a dose of 35 IU/kg was used. Percutaneous cannulation of a peripheral branch of the portal vein was carried out by interventional radiologists under ultrasound and fluoroscopic guidance. The use of Avitene™ (microfibrillar collagen hemostat) replaced Tisseel™ (fibrin) in 2005 to obliterate the liver tract and prevent major bleeding. Islets were infused with periodic portal pressure monitoring. Insulin and heparin infusions were initiated post-ITx (2005 onwards). Heparin use was targeted to a partial thromboplastin time of 60-80s for 48-hours post-transplant, followed by enoxaparin 30 mg qd for 7 days and aspirin 81 mg qd for 14 days.

2.2.3.3 - Immunosuppression

For patients undergoing PTx, induction consisted of basiliximab (101/146, 69.2%), daclizumab (22/146, 15%), or anti-thymocyte globulin (15/146, 10.3%) together with tapering methylprednisolone (146/146, 100%). Muromonab/OKT3 (8/146, 5.5%) was used in early cases. During follow-up, PTx patients received tacrolimus plus mycophenolate mofetil (MMF) and prednisone taper with indefinite maintenance dose of 2.5-5 mg qd. For patients undergoing ITx, induction immunosuppression also varied. Of 626 infusions, the following induction immunosuppression medications were used: alemtuzumab (299/626, 47.7%), daclizumab (169/626, 27.0%), basiliximab (80/626, 12.8%), belatacept (11/266, 1.7%, used with basiliximab) anti-thymocyte globulin (78/626, 12.5%). Anti-inflammatory therapies were used as follows: none (195/626, 31.2%) anakinra alone (2/626, 0.3%), etanercept alone (95/626, 12.2%), anakinra and etanercept (303/626, 48.4%), infliximab alone (31/626, 4.9%). In ITx, the following maintenance immunosuppression medications were used: tacrolimus (266/266, 100%), MMF (249/266, 93.6%), sirolimus (113/266, 42.5%).

2.2.3.4 - Follow-up

PTx recipients were followed by transplant surgeons with expertise in PTx, and by transplant nephrologists (for recipients of SPK/PAK), weekly for the first month post-transplant, then every 3 months until 1 year, and the yearly, thereafter. ITx recipients were followed by endocrinologist and transplant surgeons with expertise in ITx, weekly for the first month after any islet infusion, and every 3-6 months, thereafter.

For both transplants, graft function, glycemic control and use of insulin or other glucose lowering therapies, as well as safety and tolerability of immunosuppression was assessed at

each visit. Reintroduction of exogenous insulin and/or initiation of other glucose lowering therapies was at the clinicians' discretion to maintain optimal glycemic control, while considering patient preferences, and accessibility and affordability of different options. Immunosuppression targets were individualized and optimized to minimize toxicity and side effects. Supplementary infusions were considered to restore insulin independence or eliminate recurrent hypoglycemia. These were not recommended for subjects where maintenance of durable graft function was unlikely (i.e., not tolerating adequate immunosuppression or experiencing rapid graft loss).

2.2.3.5 - Study Outcomes

The primary outcome in this study was patient survival. Secondary outcomes include: death-censored graft survival, insulin independence, glycemic control, procedure-related major complications, and hospital readmissions. Operational definitions are described in **Table 2.2.2.** To provide a fair and equivalent endpoint for comparison of graft survival between groups, we used a common definition of complete graft failure in both PTx and ITx recipients. For PTx, complete graft failure was defined as graft pancreatectomy or loss of C-peptide ($\leq$0.1 nmol/L [$\leq$0.3 ng/mL]). While return to injected insulin has been used historically as a standard measure of pancreas graft failure,[13] including this criterion precluded a head-to-head comparison of both transplant modalities. For ITx, complete graft failure was defined as a persistent ($\geq$2 measurements) C-peptide level $\leq$0.1 nmol/L (0.3 ng/mL) without recovery or subsequent infusions.[14] Additionally, graft function following ITx was assessed using the BETA-2 score at 1-month post-first infusion;[15] early optimal graft function was defined as a BETA-2 score $\geq$15 points. Additional outcomes include insulin independence rates and total duration, the incidence

of procedure-related major complications, life-threatening infections, and cancer, as well as the frequency of hospital readmissions (**Table 2.2.2**). For hospital readmissions, we limited our analysis to March 1st, 2002 to October 1st, 2019 due to restrictions with provincial databases.

Table 2.2.2. Operational definitions

	Definition
Complete graft failure – Islet Transplantation	- Persistent (≥ 2 measurements) C-peptide level <0.1 nmol/L/<0.3 ng/mL without recovery or subsequent infusions - Patients dying with a functioning graft were censored (death-censored graft survival analysis)
Complete graft failure – Pancreas Transplantation	- Graft loss (i.e., thrombosis, graftectomy, etc.) - Persistent (≥ 2 measurements) C-peptide level <0.1 nmol/L/<0.3 ng/mL without recovery - Patients dying with a functioning graft were censored (death-censored graft survival analysis)
Insulin independence	- No exogenous insulin use for ≥14 days with: o Fasting plasma glucose ≤8 mmol/L o 2-hr post-prandial glucose < 10 mmol/L o HbA1c <7%
Total duration of insulin independence	- A sum of all episodes of insulin independence throughout patient follow-up
BETA-2 score	$\text{BETA-2 score} = \frac{\left(\sqrt{\text{fasting C-peptide (nmol/L)}} \times (1 - \text{insulin dose [units/kg]})\right)}{\text{Fasting plasma glucose (mmol/L)} \times \text{HbA1c(\%)}} \times 1000$
Stage 3 chronic kidney disease	- Diagnosis on record - Persistent eGFR <60 ml/min/1.73m^2 using CKD-EPI - Time to stage 3 CKD: considered at the moment of first recorded eGFR<60 ml/min/1.73m^2 coupled with evidence of persistence (≥ 3 months)
Stage 4 chronic kidney disease	- Diagnosis on record - Persistent eGFR <30 ml/min/1.73m^2 using CKD-EPI - Time to stage 4 CKD: considered at the moment of first recorded eGFR<30 ml/min/1.73m^2 coupled with evidence of persistence (≥ 3 months)
Stage 5 chronic kidney disease – End-stage renal disease (ESRD)	- Diagnosis on record o Persistent eGFR <15 ml/min/1.73m^2 using CKD-EPI o Dialysis o Kidney transplant - Time to ESRD: considered at the moment of first recorded eGFR<15 ml/min/1.73m^2 coupled with evidence of persistence (≥ 3 months)
Procedure-related, life-threatening complications	- Any complication occurring during hospitalization classified as Clavien-Dindo grade >3 (requiring surgical, endoscopic or radiological intervention) - Any complication requiring admission and related to the procedure or immunosuppression occurring within 90 days of discharge from the transplant/infusion hospitalization episode. o Causality was evaluated through manual clinical record revision
Life-threatening infections	- Any infection requiring hospitalization
Cancer	- Diagnosis on record

	- Cancers diagnosed within 6 months of 1st infusion were excluded o For ITx this includes: 1 breast cancer diagnosed 3 months post-1st infusion, 1 germ cell tumor diagnosed 21 days post-1st infusion o For PTx, no patients were excluded
Hospital readmission	- Every hospital admission was included, except for o Readmissions ≤ 1 day of duration unless it culminated in death o Readmissions for repeated islet infusions o In patients having pancreas-after-islet or islet-after-pancreas, admissions occurring from/after the second type of transplant were excluded from analysis. o Admissions related to mental disorders, orthopedic procedures and/or fractures were excluded from analysis.
Causes of Hospital Readmission	- Procedure-related: readmissions due to complications secondary to the surgery or infusion (e.g., bleeding, abdominal sepsis, post-procedural pain). Causality for these admissions was established through manual record revision. - Diabetes-related: readmissions due to acute or chronic complications related to diabetes (e.g., hypo-/hyperglycemia, gastroparesis, amputation, etc.) - Infectious: readmissions due to infections (e.g., pneumonia, urinary tract infection). Opportunistic infections are included in this category. - Malignant neoplasms: readmissions due to cancer and/or chemotherapy. - Cardiovascular: readmission due to cardiovascular complications (e.g., myocardial infarction, stroke, etc.) - Renal: readmissions due to renal-related conditions (e.g., renal transplant rejection, renal transplant complications, acute renal failure, etc.) - Other surgical procedures: readmissions due to surgical procedures not directly related to transplant/infusion (e.g., hernia repair, cholecystectomy, appendectomy, etc.) - Immune/Immunosuppression-related: readmission due to immunological causes affecting the pancreas/islet graft or to side effects of immunosuppression (e.g., neutropenia) - Other diagnoses: readmission due to other diagnoses not directly related to any of the previously described causes (e.g., seizures, unspecified signs/symptoms)

Note: this table is included as supplementary material in the submitted manuscript

2.2.3.6 - Statistical Analysis

We conducted an intention-to-treat analysis. Results are expressed as medians and interquartile ranges (IQR) for continuous variables, unless otherwise indicated, and as frequencies and percentages for categorical variables. Continuous variables were compared using Mann-Whitney tests. Categorical variables were compared using X^2 tests. A mixed main effects model using the maximum-likelihood method was used to analyze glycemic control over time, effects of time and group are reported. Patient and graft survival, as well as total duration of insulin independence were analyzed using Kaplan-Meier estimates.

Hazard ratios were derived using a Cox proportional hazard regression models. Proportional hazard assumptions between groups were verified using the Schoenfeld's residuals test. Unadjusted and age-adjusted hazard ratios (and 95% confidence intervals [95% CI]) are reported in the graphs. Incidence rates and ratios (and 95% CI) were calculated and reported in the text. All statistical analyses were performed using Stata® (Version 12.0, StataCorp, College Station, Texas) and GraphPad Prism (Version 9, GraphPad Software, LLC, San Diego, California).

2.2.4 - Results

2.2.4.1 - Patient and Transplant Characteristics

We included 266 (64.6%) and 146 (35.4%) in the ITx and PTx groups, respectively. Baseline patient demographics and characteristics are shown in **Table 2.2.3.** Most patients in the ITx group had an ITA (93.2%), while most PTx recipients had an SPK (87.7%). ITx recipients included more females, were older at diagnosis and transplant, and had longer duration of type 1 diabetes ($p<0.001$). Our ITx cohort received a median of 2 (IQR 2-3) infusions; 13.5% received one, 51.3% received two, 23.2% received three, 10.5% received four, and 1.5% received five. At baseline, median C-peptide and HbA1c (%) levels, and insulin requirements were similar between groups. As expected, baseline serum creatinine levels were higher for PTx, reflecting that most patients had concomitant end-stage renal disease (ESRD).

Table 2.2.3. Demographic and baseline characteristics

Variable	Islet Transplantation (n=266)	Pancreas Transplantation (n=146)	*P* value*
Type of transplant, n (%)			
ITA	248 (93.2)	-	-
IAK	18 (6.8)	-	
SPK	-	128 (87.7)	
PTA	-	12 (8.2)	
PAK	-	6 (4.1)	
Demographics and clinical data at 1st transplant			
Gender, M/F, n (%)	114 (42.9) / 152 (57.1)	93 (64.4) / 52 (35.6)	<0.001
Age at diagnosis, yr (IQR)	14 (9.3 - 23)	11 (8 - 16)	<0.001
Duration of DM, yr (IQR)	30.8 (22.7 - 41)	28 (23 - 35)	0.014
Age at transplant, yr (IQR)	49.1 (41.4 - 56.1)	41 (36 - 47)	<0.001
Body-mass index (IQR)	24.9 (22.9 - 27.8)	24.7 (23 - 27.5)	0.85
Number of transplant/infusions per patient (IQR)	2 (2 - 3)	1 (0)	<0.001
IEQs/kg per infusion x 1,000 (IQR)	5.9 (5.3 - 6.9)	-	-
Total IEQs/kg x 1,000 (IQR)	14.1 (11 - 18.4)	-	-
Laboratory values at 1st transplant			
C-peptide (nmol/L) (IQR)	0.02 (0.02 - 0.03)	0.02 (0.01 - 0.09)	0.23

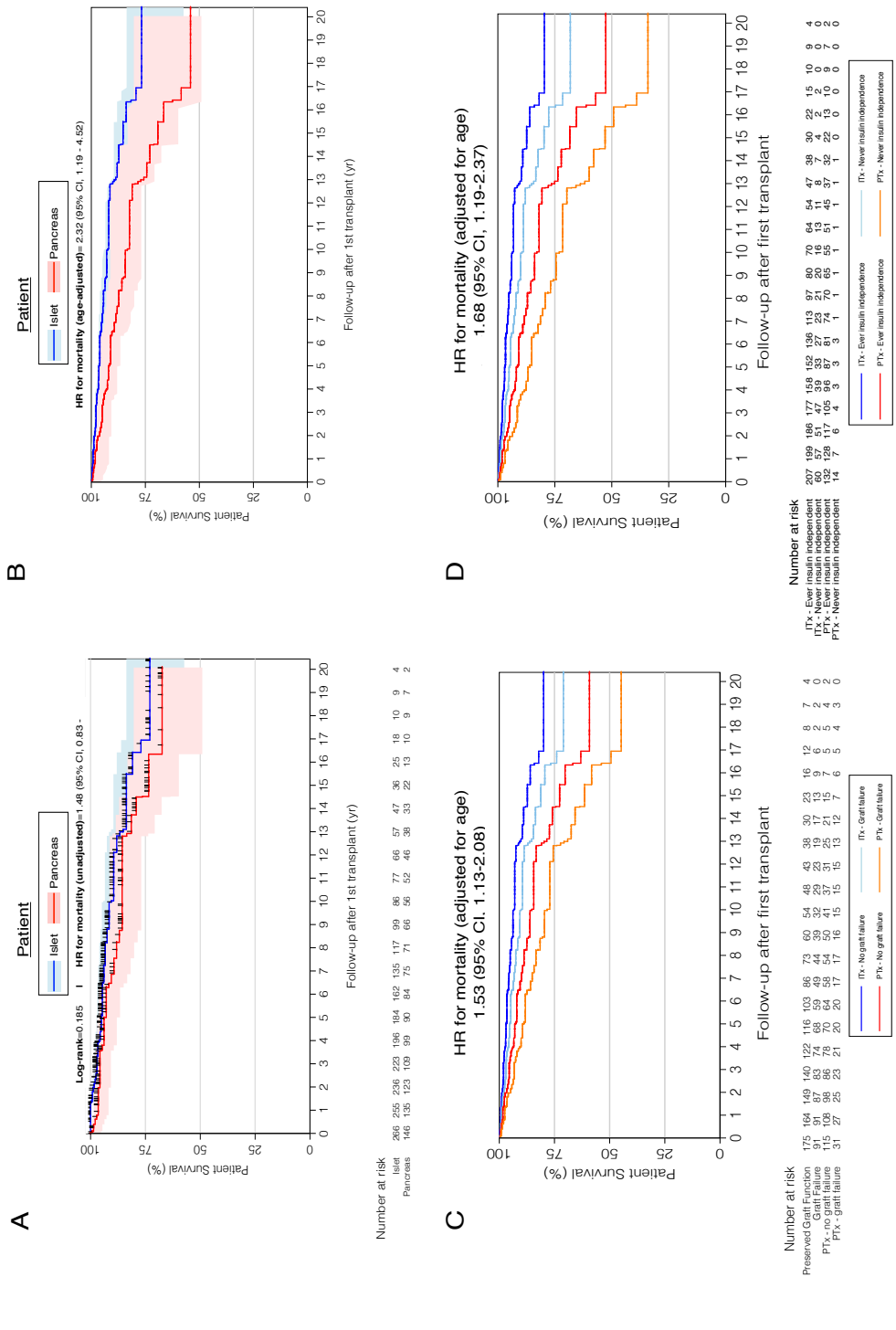
HbA1c % (IQR)	8.2 (7.5 - 9)	8.2 (7.3 - 9.3)	0.86
Insulin units/kg (IQR)	0.54 (0.46 - 0.69)	0.51 (0.42 - 0.67)	0.25
Creatinine, mg/dl (IQR)	0.9 (0.8 - 1)	7.6 (5.5 - 9.3)	<0.001

Data are presented as n (%) and median (IQR). ITA: islet transplantation alone, IAK: islet-after-kidney transplantation, SPK: simultaneous pancreas-kidney transplantation, PAK: pancreas-after-kidney, PTA: pancreas transplantation alone. M: male, F: female, IEQ: islet equivalent. *X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables.

2.2.4.2 - Patient Survival

The total follow-up was 2,170.2 and 1,214.1 patient-years for the ITx and PTx groups, respectively. Median patient follow-up was similar between groups (**Table 2.2.4)**. Patient survival analyses are shown in **Figure 2.2.2A.** Overall, there were no differences in mortality rates between groups. The mortality rate was 11.5 (95%CI 7.8-17.0) and 17.3 (95%CI 11.3-26.5) per 1,000 person-years in ITx and PTx patients, respectively (incidence rate ratio of 1.5 [95%CI 0.8-2.8, *p*=0.174]). However, we observed a higher 1-year mortality in the PTx group (0% ITx vs 3.6% PTx, *p*=0.002, **Table 2.2.4**). Additionally, when adjusting for age, we observed a higher hazard ratio for mortality in PTx vs ITx recipients (**Figure 2.2.2B**). Age-adjusted risk for mortality was also higher in patients experiencing graft failure and not achieving insulin independence, particularly for PTx (**Figure 2.2.2C and D**). Causes of death are summarized in **Table 2.2.5.**

Figure 2.2.2. Patient survival



Note: Panel A shows Kaplan-Meier estimates for patient survival after first transplant stratified by type of transplant. Panel B shows age-adjusted patient survival after first transplant using a Cox proportional-hazard model stratified by type of transplant. Panel C shows patient survival adjusted for age and occurrence of graft failure using a Cox Proportional Hazard Model. Panel D shows patient survival adjusted for age and achievement of insulin independence using a Cox Proportional Hazard Model

Table 2.2.4. Patient survival

Variable	Islet Transplantation (n=266)	Pancreas Transplantation (n=146)	*P* value*
Patient Survival			
Patient follow-up post-1st transplant, yr (IQR)	7.1 (3.9 - 11.9)	7.4 (3 - 13.7)	0.93
Mortality, n (%)	25 (9.4)	21 (14.4)	0.124
Mortality 1-yr post-1st transplant (%)	0 (0.0)	5 (3.6)	0.002
Age at death, yr (IQR)	62.3 (45.5 - 68.1)	50.6 (42.8 - 54.5)	<0.001

Data are presented as n (%) and median (IQR). *X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables.

Table 2.2.5. Causes of Death

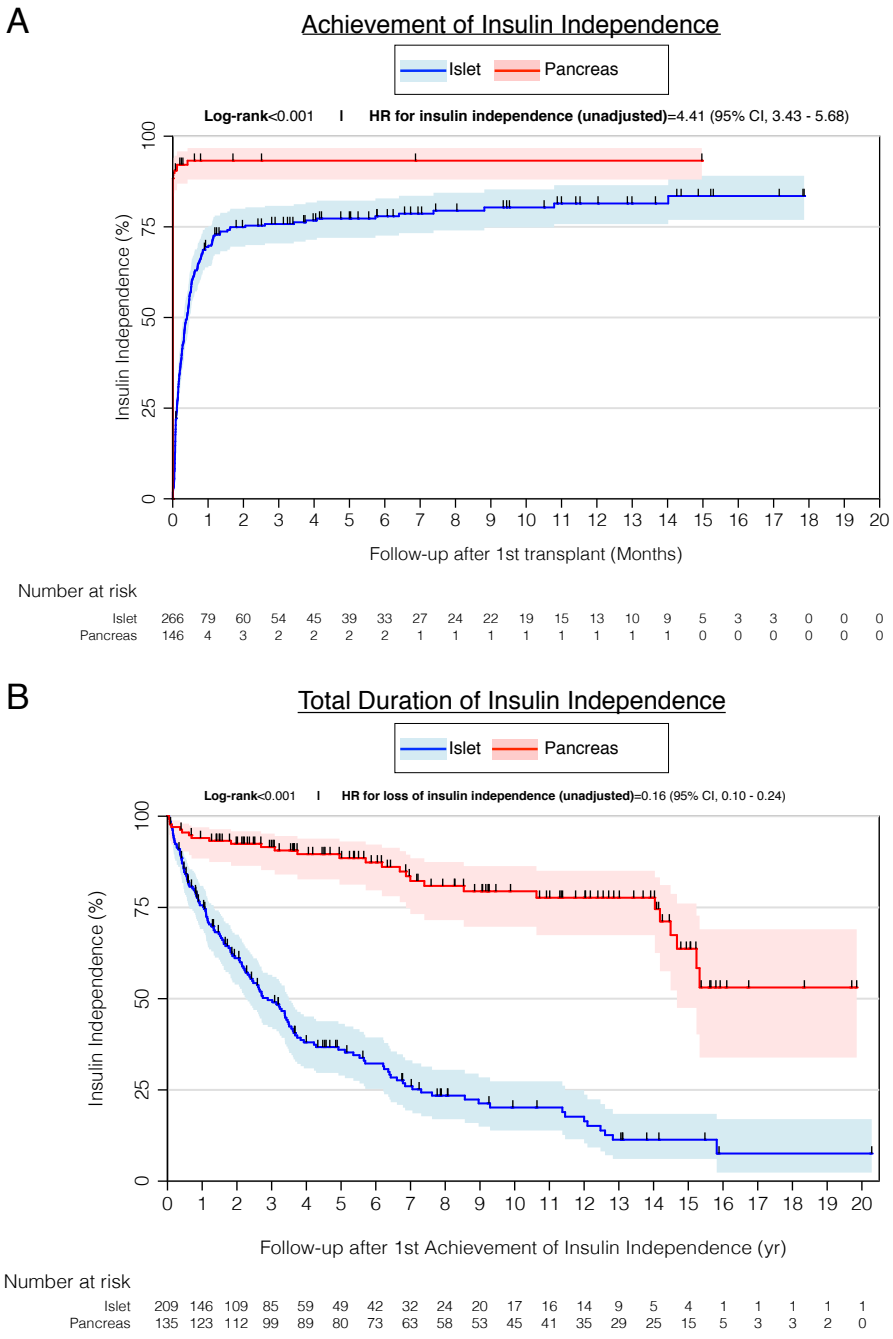
Cause of Death	Islet Transplantation (n=25/266, 9.4%)	Pancreas Transplantation (n=21/146, 14.4%)	*P**
Cardiovascular/ cerebrovascular (%)	9 (36)	5 (23.81)	0.189 for all
Infection-related, n (%)	4 (16)	3 (14.3)	
Malignancy-related, n (%)	3 (12)	2 (9.5)	
Suicide/overdose, n (%)	4 (16)	0 (0)	
Unknown, n (%)	4 (16)	9 (42.9)	
Other, n (%)	1 (4)	2 (9.5)	

*X^2 was used to compare categorical variables. *P* values correspond to Islet vs Whole Pancreas Transplantation. This table is included as supplementary material in the submitted manuscript.

2.2.4.3 - Insulin Independence, Graft Survival and Glycemic Control

Insulin independence (ever achieved) rates were significantly lower in the ITx group compared to the PTx group (78.6% vs 92.5%, p<0.001). The incidence rate for achievement of insulin independence per 1,000 patient-years was 381.4 (95%CI 333.0-436.7) for the ITx group and 4,598.7 (95%CI 3,884.9-5,443.7) for the PTx group, with an incidence rate ratio of 12.0 (95%CI 9.6-15.0, *p*<0.001). This difference reflects time to supplementary infusions that could enable insulin independence in ITx recipients as time to achievement of insulin independence was longer in this population (**Figure 2.2.3**). Finally, median total duration of insulin independence was shorter for the ITx group compared to PTx (2.1 [IQR 0.8 – 4.6] years vs 6.7 [IQR 2.9 – 12.4] years, *p*<0.001, **Figure 2.2.3** and **Table 2.2.4**).

Figure 2.2.3. Insulin independence



Note: **Note:** Panel A shows Kaplan-Meier estimates for first achievement of insulin independence after first transplant stratified by type of transplant. Panel A shows Kaplan-Meier estimates for total duration of insulin independence after first achievement of insulin independence stratified by type of transplant.

Table 2.2.6. Insulin independence and graft survival outcomes

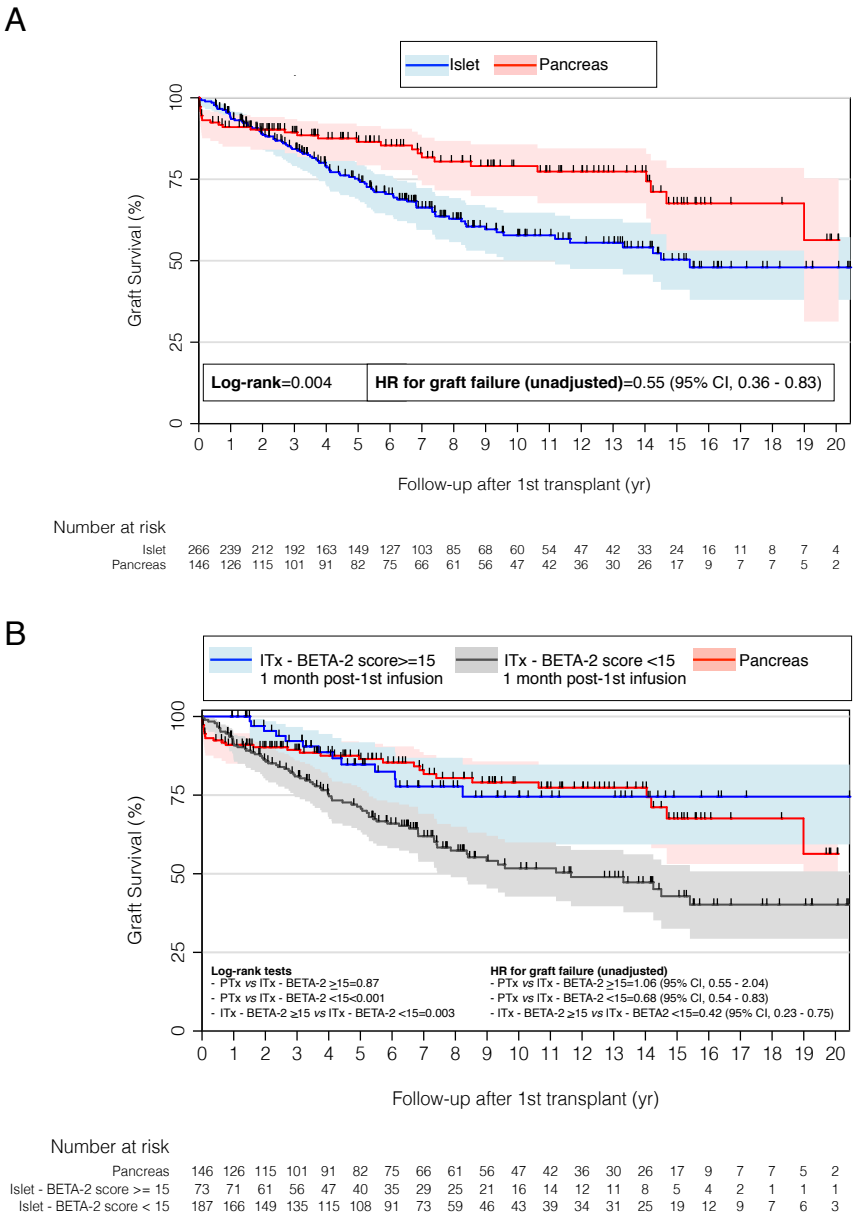
Variable	Islet Transplantation (n=266)	Pancreas Transplantation (n=146)	*P* value*
Insulin independence†			
Insulin independence ever achieved, n (%)	209 (78.6)	135 (92.5)	<0.001
Time to insulin independence, days (IQR)	91 (30 - 192)	1 (1)	<0.001
Total duration of insulin independence, yr (IQR)	2.1 (0.8 - 4.6)	6.7 (2.9 - 12.4)	<0.001
Percentage of follow-up off-insulin (IQR)	22.7 (2.7 - 65.3)	100 (97.7 - 100)	<0.001
Graft Survival			
Graft failure‡, n (%)	91 (34.2)	29 (19.9)	0.002
1-yr graft failure, n (% of total graft failures, ITx: 91, PTx: 29)	16 (17.6)	13 (44.8)	0.003
Graft survival post-1st transplant, yr (IQR)	5.5 (2.6 - 9.3)	6.2 (2.4 - 12)	0.45
Percentage of follow-up with a surviving graft (IQR)	100 (73 - 100)	100 (100)	0.012

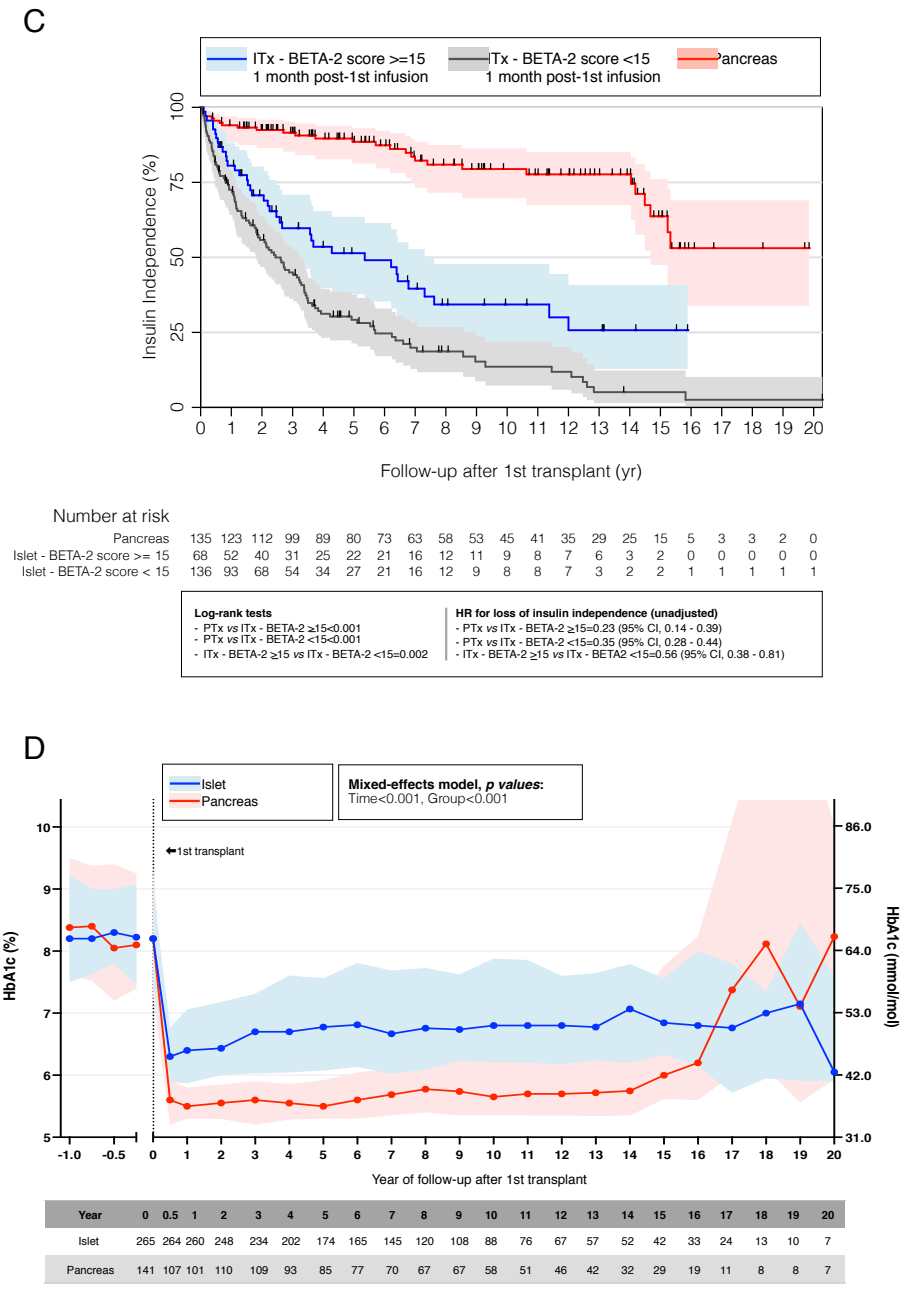
Data are presented as n (%) and median (IQR). *X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables. †Insulin independence is defined as ≥ 14 days without insulin coupled with a fasting plasma glucose level <8 mmol/L, a 2-hour post-prandial glucose <10 mmol/L and HbA1c<7%. ‡Graft failure was defined as C-peptide negativity (≤0.1 nmol/L or 0.3 ng/mL) for ITx and PTx, as well as pancreatectomy in PTx patients. Both were death-censored. § All infusions were accounted to calculate the total duration of hospitalization related to transplants.

Data on graft survival are presented in **Table 2.2.6** and **Figure 2.2.4A.** The incidence rate for death-censored graft failure was 51.4 (95%CI 41.8-63.1) and 27.1 (95%CI 18.8-39.0) per 1,000 patient-years in the ITx and PTx group, respectively, with an incidence rate ratio of 1.9 (95%CI 1.23-3.0, p=0.002). We observed that patients with early optimal graft function (BETA-2 score ≥15 points at 1-month post-first infusion) showed similar graft survival to PTx patients (**Figure 2.2.4B**). Similarly, a BETA-2 score ≥15 identified ITx recipients with longer total duration of insulin independence, however, PTx still compared favorably in this outcome (**Figure 2.2.4C**). Notably, patients with early optimal graft function had similar total infused islet equivalents per kg of body weight (IEQ/kg) compared to those not achieving this outcome (14,253.5 [IQR 11,348 - 18,820] IEQs/kg for BETA-2 score ≥15 points vs 13,371.7 [IQR 10,170 - 16,908] IEQs/kg for BETA-2 score <15 points, p=0.13).

Finally, improvements in glycemic control were marked in both groups, though lower levels of HbA1c were observed in the PTx group (**Figure 2.2.4D**). In both groups, recipients maintaining graft function had sustained improvements in HbA1c levels for up to 20 years post-first transplant (p<0.001 for time and group effects, **Figure 2.2.5**).

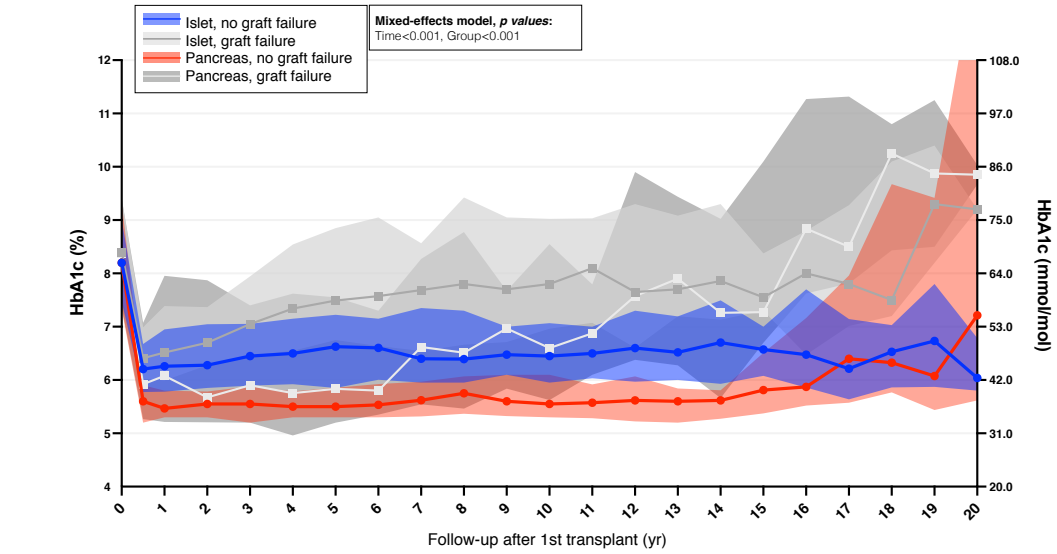
Figure 2.2.4. Graft survival, total duration of insulin independence and glycemic control





Note: Panel A shows Kaplan-Meier estimates for the graft survival after first transplant stratified by type of transplant. Panel B shows Kaplan-Meier estimates for graft survival after first transplant stratified by type of transplant and islet graft functional status at 1-month post-first infusion (assessed using the BETA-2 score). Panel C shows Kaplan-Meier estimates for total duration of insulin independence stratified by type of transplant and islet graft functional status at 1 month post-first infusion (assessed using the BETA-2 score). Panel D shows HbA1c (% and mmol/mol) levels after first transplant and presents a comparative analysis between PTx and ITx. The graph presents medians (solid lines) and interquartile range (shaded area). A mixed-effects models was calculated for the post-transplant period, time and group effects are reported in Panel D.

Figure 2.2.5. Glycemic control post-transplantation stratified by graft failure outcomes



Available values

Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ITx, no graft failure	174	174	171	162	149	134	119	110	99	79	71	55	49	43	40	36	27	23	16	10	8	6
ITx, graft failure	91	90	89	86	85	68	55	55	46	41	37	33	27	24	17	16	15	10	8	3	2	1
PTx, no graft failure	113	95	91	97	90	77	70	62	54	53	53	45	39	34	29	22	18	14	6	5	6	5
PTx, graft failure	28	12	10	13	19	16	15	15	16	14	14	13	12	12	13	10	11	5	5	3	2	2

Note: The graph represents medians (solid lines) and interquartile ranges (shaded area). A mixed–effects models was calculated for the post–transplant period, time and group effects are reported in the figure. This figure is included as supplementary material in the submitted manuscript.

2.2.4.4 - Procedure-Related Complications and Hospital Readmissions

Total length-of-stay for transplantation was longer for patients in the PTx group, even after accounting for the cumulative length-of-stay for all islet infusions (6.6 [IQR 5 - 9) vs 15 [IQR 11 - 25] days, $p<0.001$, **Table 2.2.7)**. The incidence of major procedure-related complications was significantly higher in the PTx group (7.2% ITx vs 51.4% PTx, $p<0.001$). Additionally, hospital readmissions were more common and occurred earlier in the PTx group (unadjusted HR 2.65, 95%CI 1.98-3.54, **Figure 2.2.6**). Although PTx was associated with a significantly higher number of readmissions per patient, the length-of-stay per readmission was similar between groups (**Table 2.2.7**). Admissions related to the transplant/infusion, as well as

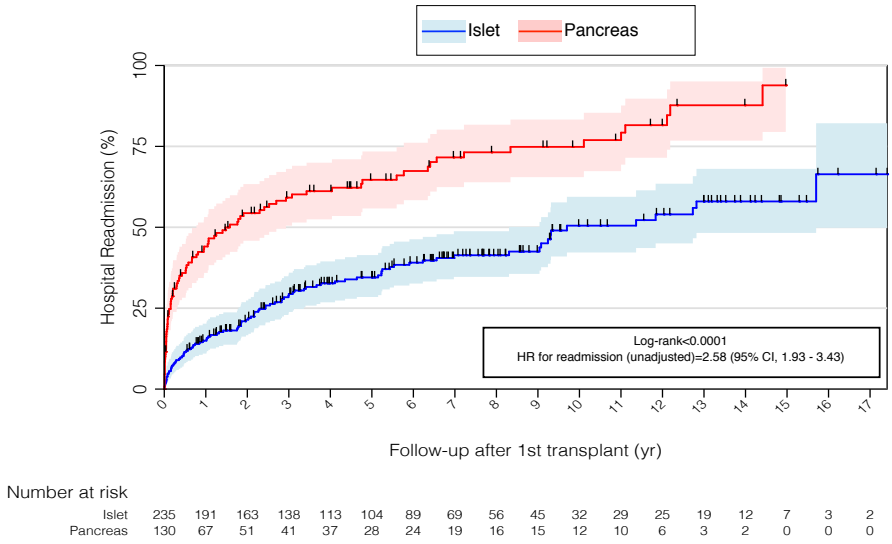
those related to renal conditions (including renal transplant rejection) and those due to infections were more frequent in patients undergoing PTx (**Table 2.2.7)**. Of note, tacrolimus levels were significant higher in the first-year post-transplant but lower thereafter in the PTx group, compared to the ITx group (**Figure 2.2.7**). The remaining causes of hospital readmission were similar between groups (**Table 2.2.7**).

Table 2.2.7. Procedure-related complications and hospital readmissions

Variable	Islet Transplantation (n=266)	Pancreas Transplantation (n=146)	*P* value*
Procedure-related complications and Hospital Readmission†			
Length-of-stay for transplant/infusions (IQR)‡	6.5 (5 - 9)	15 (11 - 25)	<0.001
Major procedure-related complications§, n (% of total procedures, IT; 626, PTx: 146)	45 (7.2)	75 (51.4)	<0.001
Patients readmitted‖ post-1st transplant, total follow-up, n (%)	96 (40.9)	91 (70.0)	<0.001
Number of readmissions per patient (IQR)	0 (0 - 1)	2 (0 - 3)	<0.001
Length-of-stay per readmission, days (IQR)	5 (3 - 9)	5 (3 - 10)	0.35
Causes of Hospital Readmission†			
Related to pancreas transplant or islet infusion, n (%)	18 (18.8)	36 (39.6)	0.002
Related to immune rejection and/or immunosuppression, n (%)	7 (7.3)	14 (15.4)	0.08
Diabetes-related (e.g., hypo-/hyperglycemia, chronic complications), n (%)	21 (21.9)	22 (24.2)	0.709
Infectious, n (%)	27 (28.1)	40 (44.0)	0.024
Malignant neoplasm, n (%)	8 (8.3)	5 (5.5)	0.446
Cardiovascular, n (%)	13 (13.5)	10 (11.0)	0.595
Renal (e.g., transplant rejection, acute kidney injury), n (%)	9 (9.4)	33 (36.3)	<0.001
Other surgical procedures (e.g., hernia repair, appendectomy), n (%)	6 (6.3)	11 (12.1)	0.165
Other diagnoses, n (%)	40 (41.7)	36 (39.6)	0.769

Data are presented as n (%) and median (IQR). *X^2 was used to compare categorical variables, Mann-Whitney tests were used to compare continuous variables. † Only admissions occurring from March 1st 2002 to October 1st 2019 and patients first transplanted during this time period were included for analysis (Islet: 235, Pancreas: 130). Admissions related to repeated infusions are excluded from this analysis. ‡ All infusions were accounted to calculate the total duration of hospitalization related to transplants. § Procedure-related complications include: 1) In-hospital Clavien-Dindo grade $\geq$3 complications, or 2) Readmissions occurring within 90 days of discharge related to transplant/infusion.

Figure 2.2.6. Hospital readmission after first transplant

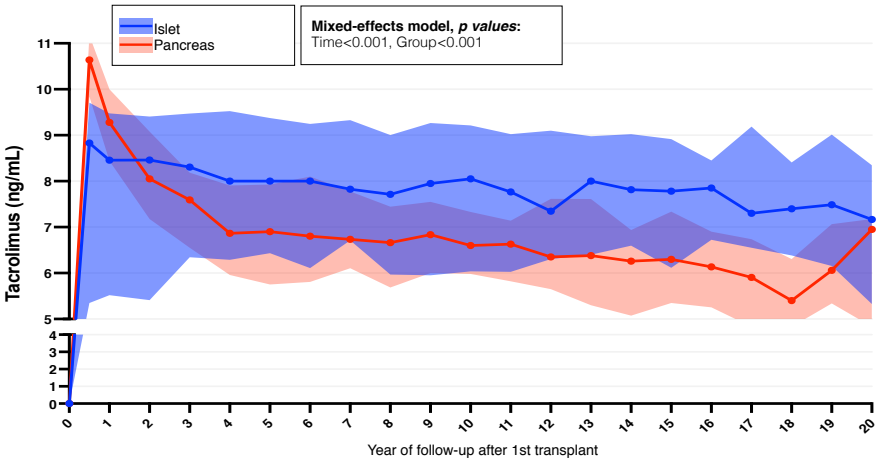


Number at risk

Islet	235	191	163	138	113	104	89	69	56	45	32	29	25	19	12	7	3	2
Pancreas	130	67	51	41	37	28	24	19	16	15	12	10	6	3	2	0	0	0

Note: Kaplan-Meier estimates for hospital readmission after first transplant stratified by type of transplant. For this analysis, only patients having their first transplant after March 1st, 2002 were included. This figure is included as supplementary material in the submitted manuscript.

Figure 2.2.7. Tacrolimus levels over time following whole pancreas and pancreatic islet transplantation



Year	0	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Islet	266	251	248	239	209	187	161	139	124	101	86	70	61	53	48	44	33	27	19	13	9	8
Pancreas	146	121	114	117	109	99	95	83	76	69	68	62	52	49	46	38	33	22	12	9	7	7

Note: The graph represents medians (solid lines) and interquartile ranges (shaded area). A mixed–effects models was calculated for the post–transplant period, time and group effects are reported in the figure. This figure is included as supplementary material in the submitted manuscript.

2.2.5 - Conclusions

Herein, we report long-term outcomes with whole pancreas and pancreatic islet transplantation in a large single-centre cohort with up to 20-year follow-up, which represents a unique opportunity to identify similarities and differences with both therapies. We describe relevant outcomes, such as patient and graft survival, insulin independence rates, glycemic control, and major procedure-related complications and hospital readmissions.

Our experience supports the safety of either ITx or PTx in terms of patient survival. Notably, though unadjusted mortality was similar between groups, early (<1 yr after 1st transplant) and age-adjusted mortality was higher after PTx. Lehman *et al*., in 2015, reported differences in 10-year mortality rates in a cohort of 94 and 38 patients undergoing SPK/PAK (10-year mortality rate of 11.5%) or simultaneous islet-kidney (SIK)/IAK (10-year mortality rate of 34.6%), respectively.[16] Our mortality rates following PTx resemble those of Lehman *et al.*, and similarly, are lower to those from the International Pancreas Transplant Registry (10-year mortality rate of 23.9% for SPK and ~30% for PAK).[17] Conversely, mortality in our ITx cohort was lower than that reported by Lehman *et al.* Admittedly, our population predominantly consisted of patients undergoing ITA, which may well explain these differences. Data from the Collaborative Islet Transplant Registry report supports this notion, where SIK and IAK transplant recipients were found to be disproportionally represented among fatalities, comprising 57.6% of deaths, but only 2.2% and 16.9% of the total population, respectively.[14] This suggests that people with type 1 diabetes and ESRD might obtain a greater benefit from whole pancreas transplantation, either SPK or PAK, although many of these individuals may not fulfil the more stringent age, cardiovascular reserve, body mass index and other comorbidity

restrictions applied in PTx practice. This is in line with current evidence-informed clinical practice recommendations.[5]

Mortality rates following ITx and PTx at our center resemble those reported from large registries that include similar non-transplanted people with type 1 diabetes, regarding baseline age and glycemic control.[18, 19] Previous studies have shown that PTx, and particularly SPK, confers a survival benefit compared to remaining on the waiting list,[20] or receiving a kidney transplant alone.[21] Similarly, although with less evidence, early reports suggested that SIK transplantation improved patient survival compared to continuing dialysis or receiving a kidney transplant alone [22]. Arguably, it can be inferred that because severe hypoglycemia substantially increases (~3.4-fold) mortality,[23] ITA may also offer a survival benefit by abrogating SHEs, irrespective of achievement or maintenance of insulin independence. However, comparison of mortality rates following ITx or PTx with those of the general population of people with type 1 diabetes remains challenging. Indeed, mortality rates always need to be contextualized by comparisons with similar cohorts (i.e., similar prevalence of ESRD or problematic hypoglycemia). This is a key area of opportunity that remains to be addressed in future studies.

Our cohorts were similar to previous reports with respect to male:female ratios, age at diagnosis, duration of diabetes, body mass index, number of infusions, HbA1c levels and insulin use pre-transplant.[17, 24-26] Additionally, our ITx patients received similar islet mass (i.e., IEQ/kg and total infused IEQ) compared to other studies.[25-27] Islet mass has been positively associated with sustained graft survival and insulin independence,[14] particularly after single-donor ITx.[28] In this study, while sufficient islet mass was transplanted, graft survival was significantly lower with ITx, and only recipients achieving early optimal graft function (1-month BETA-2 score $\geq$15), perhaps reflecting superior engraftment, compared similarly to PTx. This contrasts with

previous studies reporting similar graft survival rates following ITx and PTx.[27, 29-31] Improved graft survival in ITx recipients with early optimal graft function are not explained entirely by differences in transplanted islet mass, and may reflect inherent differences in potency between islet preparations or biological factors affecting engraftment, such as innate and alloimmune responses. Regarding insulin independence, our findings resonate with an early comparative report by Frank *et al*., in which ITx recipients had significantly shorter duration of insulin independence compared to PTx, despite receiving an average of ~15,500 IEQs/kg,[27] which is similar to our cohort. Insulin independence rates in recipients achieving early optimal islet graft function (1-month BETA 2 score ≥15) were still lower compared to PTx. Overall, these findings suggest that, while islet mass is associated with better outcomes after ITx (and might be an intrinsic contributing factor to graft survival after PTx), other factors might play important roles in sustaining graft function, and particularly, insulin independence. For instance, maintenance immunosuppression is different between ITx and PTx, with higher overall exposure to tacrolimus and no corticosteroid use in ITx, which could alter susceptibility to either recurrent autoimmunity or alloimmune rejection. Differences in early and chronic immune responses between ITx and PTx might also be a determinant of long-term graft survival. These differences could be driven by the nature and degree of cell death pre- and peri-transplant (i.e., after isolation and during culture vs warm/cold ischemia), as well as post-transplant (i.e., hypoxia due to delayed revascularization vs ischemia-reperfusion injury). Quantifying islet/β-cell death post-ITx has been evaluated as a predictor of graft function, although most correlations have been low-to-moderate and long-term outcomes have not been sufficiently studied.[32-34] Further studies will focus on identifying favorable patient-, isolation/procurement-, and immune-related

factors to integrate them into composite predictive tools that will help establish more realistic expectations with current β-cell replacement therapies.

The potential benefits obtained from prolonged insulin independence, improved glycemic control, and abrogation of severe hypoglycemia following ITx or PTx should be balanced with possible procedure- and immunosuppression-related morbidities. In this study, we found that PTx was associated with higher major procedure-related complications and hospital readmissions compared to ITx. Additionally, the causes of readmission reflected the differences in the type of procedure, as evidenced by a higher frequency of readmissions related to the transplant surgery and to renal conditions (including renal rejection). Finally, we also found that readmissions related to infections were more common in the PTx group as compared to ITx, which is likely related to the more invasive surgical approach, end-stage nature of the dialysis-dependent pancreas recipient with prior peritoneal or hemodialysis catheters, and the relatively high and prolonged exposure to corticosteroids, which does not occur in most ITx recipients (except for IAK). Overall, the information provided herein substantially contributes to informing the clinical shared decision-making process in this complex patient population by enabling a more evidence-informed balance of the risks and benefits with these therapies.

The main limitations of this study include its retrospective and single centre nature, the absence of adequate control groups, and the heterogeneity of the types of patients included in both groups. First, while retrospective studies are valuable, particularly when dealing with uncommon diseases and/or procedures, inherent biases should be considered. For example, we acknowledge that there may be missing data for some patients in terms of HbA1c and C-peptide levels especially in the pancreas transplant group, as well as hospital readmissions. The amount of missing data could be greater for patients experiencing graft failure, as these would be more

prone to discontinue their follow-up. Additionally, although most laboratory and hospitalizations data was obtained from centralized province-wide databases, we acknowledge that some hospital readmissions might have been missed if these occurred out-of-province. Second, patient survival and hospitalization rates following both ITx and PTx remain to be compared to those observed in two relevant populations: 1) people with type 1 diabetes treated with optimal insulin therapies, and 2) people with type 1 diabetes on the waiting list for an ITx or PTx. Efforts to address these limitations are underway in future studies at our centre. Third, by acknowledging that >90% of patients in the ITx received an ITA and nearly 90% in the PTx group received an SPK, we fully recognize that a comparison between ITx and PTx in our study remains inherently biased by differences in organ donor and patient characteristics, as well as clinical contexts, particularly regarding the presence of ESRD. Exploratory analyses of ITA and PTA in our cohort were limited by the small number of PTA recipients. This remains a key area of opportunity in the field, as there are only a few studies reporting outcomes with ITA and PTA. The largest, a comparison between a cohort of PTA (i.e., from the Scientific Registry of Transplant Recipients) and ITA recipients from different centres by Bellin *et al.* in 2012,[29] found similar between-group 5-year insulin independence rate of ~50%, although these were only observed when comparing PTA with ITA recipients treated with T cell-depleting induction immunosuppression and anti-inflammatory therapies. Maffi et al. also found similar 5-year insulin independence rates between PTA and ITA.[30] Finally, Moassesfar et al. reported, in 2016, similar 5-year insulin independence rates with PTA and ITA, as well as a higher incidence of procedure-related complications following PTA.[31] Overall, insulin independence rates in these studies are higher compared to our ITx cohort, and resemble more of those patients achieving early optimal graft function (1-month BETA-2 score $\geq$15). Patient selection criteria,

immunosuppression regimes, the inclusion of predominantly SPK recipients, and conditions related to a more stringent follow-up within a clinical research context might explain these discrepancies. Similar to our experience, previous comparative studies have also reported a higher incidence of procedure-related complications with PTA compared to ITA.[30, 31] Larger studies including multi-centre cohorts of ITA and PTA recipients could shed more light on these issues and strengthen recommendations on the risk and benefits with both therapies. It should be acknowledged that historically, standard definitions for graft failure differ in PTx vs ITx. In this study, we followed recent recommendations by Stratta et al. by implementing a definition for pancreas graft failure that reflects the context in which it is used,[35] in this case, a comparative study with ITx, and chose a common definition (loss of C-peptide or total graft pancreatectomy). However, we recognize that our attempts to generate a head-to-head comparison using other definitions (e.g., accounting for insulin use or requirements)[13] might be limited.

In conclusion, we provide a comprehensive report of long-term outcomes after ITx or PTx. This study includes the largest single-centre comparative analysis of ITx and PTx and shows results that demonstrate strengths and areas of opportunity with each of these therapies. Having robust data on long-term outcomes following ITx and PTx undoubtedly contributes to enhancing the shared-decision making process, and empowering people with type 1 diabetes and problematic hypoglycemia, but also physicians involved in the care of this unique patient population.

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CHAPTER 3

OUTCOMES FOLLOWING EXTRAHEPATIC AND INTRAPORTAL PANCREATIC ISLET TRANSPLANTATION: A COMPARATIVE COHORT STUDY

CHAPTER 3 - OUTCOMES FOLLOWING EXTRAHEPATIC AND INTRAPORTAL PANCREATIC ISLET TRANSPLANTATION: A COMPARATIVE COHORT STUDY

Original Clinical Science—General

Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study

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Background. Preliminary studies show promise for extrahepatic islet transplantation (ITx). However, clinical comparisons with intraportal ITx outcomes remain limited. **Methods.** This single-center cohort study evaluates patients receiving extrahepatic or intraportal ITx between 1999 and 2018. Primary outcome was stimulated C-peptide level. Secondary outcomes were fasting plasma glucose, BETA-2 scores, and fasting C-peptide level. Multivariable logistic modeling evaluated factors independently associated with a composite variable of early graft failure and primary nonfunction within 60 d of ITx. **Results.** Of 264 patients, 9 (3.5%) received extrahepatic ITx (gastric submucosal = 2, subcutaneous = 3, omental = 4). Group demographics were similar at baseline (age, body mass index, diabetes duration, and glycemic control). At 1–3 mo post–first infusion, patients receiving extrahepatic ITx had significantly lower stimulated C-peptide (0.05 nmol/L versus 1.2 nmol/L, $P < 0.001$), higher fasting plasma glucose (9.3 mmol/L versus 7.3 mmol/L, $P < 0.001$), and lower BETA-2 scores (0 versus 11.6, $P < 0.001$) and SUITO indices (1.5 versus 39.6, $P < 0.001$) compared with those receiving intraportal ITx. Subjects receiving extrahepatic grafts failed to produce median C-peptide ≥0.2 nmol/L within the first 60 d after transplant. Subsequent intraportal infusion following extrahepatic transplants achieved equivalent outcomes compared with patients receiving intraportal transplant alone. Extrahepatic ITx was independently associated with early graft failure/primary nonfunction (odds ratio 1.709, confidence interval 73.8-39 616.0, $P < 0.001$), whereas no other factors were independently predictive. **Conclusions.** Using current techniques, intraportal islet infusion remains the gold standard for clinical ITx, with superior engraftment, graft function, and glycemic outcomes compared with extrahepatic transplantation of human islets.

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3.1.1 - Abstract

Background: Preliminary studies show promise for extrahepatic islet transplantation (ITx). However, clinical comparisons with intraportal ITx outcomes remain limited.

Methods: This single-center cohort study evaluates patients receiving extrahepatic or intraportal ITx between 1999 - 2018. Primary outcome was stimulated C-peptide level. Secondary outcomes were fasting plasma glucose (FPG), BETA-2 scores, and fasting C-peptide level. Multivariable logistic modelling evaluated factors independently associated with a composite variable of early graft failure and primary non-function within 60-days of ITx.

Results: Of 264 patients, 9 (3.5%) received extrahepatic ITx (gastric submucosal = 2, subcutaneous = 3, omental = 4). Group demographics were similar at baseline (age, BMI, diabetes duration, and glycemic control). At 1-3 months post-first infusion, patients receiving extrahepatic ITx had significantly lower stimulated C-peptide (0.05 nmol/L vs 1.2 nmol/L, $p<0.001$), higher FPG (9.3 mmol/L vs 7.3 mmol/L, $p<0.001$), and lower BETA 2 scores (0 vs 11.6, $p<0.001$) and SUITO indices (1.5 vs 39.6, $p<0.001$) compared to those receiving intraportal ITx. Subjects receiving extrahepatic grafts failed to produce median C-peptide ≥ 0.2 nmol/L within the first 60-days after transplant. Subsequent intraportal infusion following extrahepatic transplants achieved equivalent outcomes compared to patients receiving intraportal-transplant alone. Extrahepatic ITx was independently associated with early graft failure/primary non-function (OR 1,709, CI 73.8-39,616.0, $p<0.001$), while no other factors were independently predictive.

Conclusions: Using current techniques, intraportal islet infusion remains the gold-standard for clinical ITx, with superior engraftment, graft function and glycemic outcomes compared to extrahepatic transplantation of human islets.

3.1.2 - Introduction

Clinical pancreatic islet transplantation (ITx) has evolved considerably since the groundbreaking Edmonton Protocol established 22-years ago.[1] Optimization of isolation techniques and clinical care has led to 10-year graft survival rates of nearly 80%, coupled with near complete abrogation from severe hypoglycemia, sustained improvements in glycemic control, and substantial reductions in insulin requirements.[2-8] Islet infusion into the intraportal hepatic circulation remains the gold-standard for clinical ITx. However, evaluation of alternative implantation sites continues to be explored, with promising preliminary experimental data supporting the gastric submucosa,[9, 10] omentum,[11-14] and subcutaneous space.[15-17] As ongoing research with stem cell-derived β cell replacement progresses, recent interest into extrahepatic transplant sites has expanded due to its increased accessibility for monitoring of potential off-target growth, that simultaneously facilitates graft recovery, if required. Understanding comparative outcomes after extrahepatic ITx in humans is valuable before extra hepatic sites can be considered for use with stem cell therapies. While preliminary case reports suggest a degree of success,[11] evidence remains scarce. Moreover, no comparative studies with intraportal ITx have been published to date.

Benefits from intraportal ITx include direct blood contact, which maximizes graft oxygenation, and insulin release into the portal circulation, which may facilitate a more physiologic glycemic response. However, caveats include islet damage from the instant blood mediated immune reaction, restrictions in packed cell volume, and rarely, procedural complications including portal venous thrombosis and bleeding.[18-22] Conversely, access for limited graft biopsy in more localized sites such as the gastric submucosa or skin, and the ease of complete graft retrieval at least in the subcutaneous site may have some advantages over the

liver. The omentum has been proposed as an attractive site due to easy operative accessibility, lack of volume restriction, and dense vascular supply with portal drainage, although it still involves a surgical (minimally invasive) procedure and its own unique risks including adhesive small bowel obstruction.[11, 13, 18] Similarly, gastric submucosal implantation allows for graft portal venous drainage, a large capacity for implantation, and offers the possibility to biopsy islet grafts endoscopically but has the least evidence evaluating efficacy in patients to date.[9, 10] Finally, the subcutaneous space offers procedural safety, technically easy graft implantation, and facilitates ongoing graft monitoring;[15-17] unfortunately, this space releases insulin systemically and is substantially more hypoxic, which requires prevascularization strategies in order to support islet engraftment.[15-17]

Herein, we report a large single-center experience with extrahepatic ITx and compare outcomes compared to intraportal ITx. We aim to evaluate graft survival, and glycemic outcomes for patients receiving extrahepatic ITx, including gastric submucosal, omental, and subcutaneous implants within devices, as compared to patients receiving intraportal ITx.

3.1.3 - Materials and Methods

3.1.3.1 - Study Design and Patient Selection

This is a single-center retrospective cohort study comparing individuals with type 1 diabetes (T1D) receiving allogeneic extrahepatic ITx with intraportal ITx between March 1999 and October 2018. The study protocol has been approved by the University of Alberta Health Research Ethics Board (PRO00001120) and all patients have consented to use of their data for research purposes. All adult (≥18 years old) patients diagnosed with T1D undergoing allogeneic ITx were included. Patients receiving pancreas transplants, autologous ITx, stem cell-based ITx, and with type 2 diabetes were excluded. Both patients receiving islet alone and islet after kidney transplantation were included; these were grouped because only short term outcomes were evaluated and prior kidney transplantation was deemed unlikely to be a substantial contributing factor.

Patients in the extrahepatic ITx group included those receiving gastric submucosal (n=2), omental (n=4), and subcutaneous device islet implantation (n=3).[23] Demographics, primary, and secondary outcomes were compared between groups to determine any differences. Patients receiving intraportal or extrahepatic ITx were analyzed as 'intention-to-treat' from their first procedure. Patients in the extrahepatic ITx group were further analyzed after they received subsequent intraportal ITx to assess the effect of extrahepatic grafts on the effectiveness of subsequent intraportal islet infusions. Data for patients receiving prevascularized subcutaneous ITx has previously been reported by our group and included in aggregate form in the current study.[23] Additionally, a secondary analysis comparing extrahepatic ITx to intraportal ITx occurring between January 2012-October 2018 was completed to enable evaluation of outcomes from contemporary groups. This was done to ensure any effects seen were not due to changes

in treatment over time including changes in immunosuppression, transplant technique, or patient selection. All extrahepatic transplants were completed during the January 2012-October 2018 timeline.

Patient demographics were collected at time of first transplant and included sex, age at T1D diagnosis, T1D duration, age, and body mass index (BMI). Measures of pre-transplant diabetes control including HbA1c, insulin dose (units/kg/per day), and fasting C-peptide levels (nmol/L) were also collected, as were markers of glycemic lability (Lability Index), and hypoglycemia awareness (Clarke score).[24] Transplant characteristics were also evaluated including number of islet infusions, timing of infusions, and total islet equivalents (IEQ)/kg of body weight received.

3.1.3.2 - Outcome Variables

The primary outcome of this study was stimulated C-peptide levels 1-3 months after first ITx measured at 90 mins after a mixed meal tolerance test.[1, 25] Secondary outcomes include fasting plasma glucose (FPG), and BETA-2 score. The BETA-2 score incorporates insulin dose (insulin units/kg/day), FPG (mmol/L), HbA1c (%), and fasting C-peptide levels (nmol/L) and has been validated as a predictive tool for glycemic control and insulin independence.[26, 27]

Additionally, we evaluated graft survival measured by fasting C-peptide levels over time. In the immediate post-infusion period, fasting C-peptides levels were measured every 2-5 days for the first 60-days and reported as 10-day medians and interquartile ranges. Subsequent C-peptide values for 5-years after first infusion were collected over 6-month intervals, and reported as medians with IQR. Continuous data are described as medians and IQR, with discrete data reported as absolute frequencies and percentages. To further assess the cohorts, rate of

primary non-function or early graft failure was determined for each group. Primary non-function was defined as C-peptide >0.1 nmol/L, and early graft failure was defined as a return to C-peptide values <0.1 nmol/L (or baseline) prior to a subsequent infusion or within 60 days of first infusion. Multivariable logistic regression analyzed the entire cohort for patient and transplant factors independently associated with a composite variable of primary non-function and early graft failure. Finally, we evaluated allosensitization to extrahepatic grafts defined as any calculated panel reactive antibodies (cPRA) increase or any *de novo* donor specific antibody development following transplant.

3.1.3.3 - Transplant Procedures

Intraportal transplantation involved ultrasound and fluoroscopy-guided percutaneous cannulation of portal venous circulation and islet infusion as described previously.[28] Islet isolation procedures and release criteria have also been described. Omental transplant was completed via the biological scaffold "sandwich" technique previously described by researchers from the Diabetes Research Institute in Miami, Florida.[11, 12] Patients were brought to the operating room, underwent general anesthesia and laparoscopy. The omentum was laid out flat islets suspended with the recipient's own plasma were dripped on to the omentum. Recombinant thrombin was then used to cover each of the islet droplets. The omentum was folded over to cover the implantation site and secured in place with ligaclips. Gastric submucosal transplants were completed as previously described by Echeverri et al.[10] Patients underwent conscious sedation with subsequent gastroscopy to evaluate the stomach; islets were then infused through a 19-gauge Boston Scientific Expect Slimline needle in eight submucosal locations throughout the stomach under direct vision. Procedures were performed by a

gastroenterologist with advanced training in therapeutic endoscopy. Finally, prevascularized subcutaneous space ITx was completed with islet implantation into a prevascularized non-immunoisolating polymer chamber device as previously described by Gala-Lopez et al.[23]

All extrahepatic transplants were completed as clinical trials intended to evaluate the potential of novel transplant sites. These sites were selected due to promising preliminary outcomes from others. Registered clinical trial protocols can be reviewed as follows: omental NCT02821026, gastric submucosal NCT02402439, and subcutaneous NCT01652911. In all cases, we collaborated with investigators who initially reported promising outcomes to optimally replicate their technique.

3.1.3.4 - Immunosuppression

Patients receiving intraportal ITx received various induction, anti-inflammatory, and maintenance immunosuppression regimens. Induction was primarily alemtuzumab (n = 288 infusions, 47.1%), followed by daclizumab (n = 170 infusions, 27.8%), basiliximab (n = 77 infusions, 12.6%), and anti-thymocyte globulin (n = 77 infusions, 12.6%). A total of 287 infusions (47.0%) used etanercept and anakinra. All patients received tacrolimus (100%) during follow up and most had mycophenolate (n = 234, 91.4%) as a secondary maintenance immunosuppressant with the remainder receiving sirolimus combined with tacrolimus. Comparatively, induction immunosuppression for infusions into extrahepatic sites was primarily alemtuzumab (n = 6, 60%) and the others received anti-thymocyte globulin (n = 4, 40%). Most infusions were accompanied by both etanercept and anakinra (n = 6, 60%), and 4 (40%) received only etanercept. All patients with extrahepatic ITx had tacrolimus and mycophenolate for maintenance immunosuppression.

3.1.3.5 - Statistical Analysis

For analysis of longitudinal C-peptide data, a mixed effects model using the maximum-likelihood method was fitted to determine differences over time and between groups, while accounting for missing data. For the analysis of categorical data, the X^2 tests were applied. To compare continuous variables in two independent groups, Mann-Whitney U tests were used. A value of $p<0.05$ is considered statistically significant.

To evaluate the independent effect of patient and transplant factors on early graft failure and primary non-function, a non-parsimonious multivariable logistic regression model was developed using hypothesis-driven selection methods. Variables with statistical significance in the multivariable model (Wald test $p<0.05$) were evaluated for multi-collinearity using the variance inflation factors (VIF). Variables with VIF >10 were further explored for collinearity diagnostic tests and excluded if deemed collinear.

3.1.4 - Results

Overall, 264 patients were included in this study. Of these, 9 (3.4%) patients received extrahepatic ITx for initial islet transplant before undergoing intraportal ITx. These patients were compared to 255 (96.6%) control patients receiving intraportal ITx. At baseline, patients were similar with regards to age at T1D diagnosis, BMI, and T1D duration (**Table 3.1**). Patients receiving extrahepatic ITx were more likely to be male (41.6% intraportal vs 77.8% extrahepatic, p=0.032) and were older at time of first transplant (48.8 intraportal vs 59.8 extrahepatic, p=0.025). Median fasting C-peptide levels, HbA1c levels and insulin requirements pre-ITx were similar between groups (**Table 3.1**).

Regarding infusion characteristics of the first ITx, both groups received a similar islet preparation purity (60% intraportal vs 55% extrahepatic, p=0.499) but patients receiving extrahepatic grafts received more islet equivalents (IEQs) per kg of body weight (6,100 IEQ/kg intraportal vs 7,000 IEQ/kg extrahepatic, p=0.018). Overall, after groups received all of their ITxs, both groups received a similar number of infusions, and there was no difference in total infused IEQs/kg of body weight (14,300 IEQ/kg intraportal vs 22,500 IEQ/kg extrahepatic, p=0.096). However, patients receiving extrahepatic infusions had a shorter delay between their third and fourth islet infusions (**Table 1**). Eight patients in the extrahepatic group received one extrahepatic implantation and one patient received two; patients receiving extrahepatic ITx were switched to the intraportal route if they failed to achieve clinical benefit from their initial graft including insulin reduction, improved glycemic lability, or reduced hypoglycemia. The decision to re-list patients for transplant was made following review by the ITx team and determination that late onset graft function was unlikely.

Table 3.1. Demographic and baseline characteristics of patients undergoing pancreatic islet transplantation according to implantation site

Variable	Intraportal (n=255)	Extrahepatic (n=9)	*P* value*
Demographics and clinical data at first transplant (baseline)			
Sex, M/F, n (%)	106 (41.6) / 149 (58.4)	7 (77.8) / 2 (22.2)	0.031
Age at diagnosis, yr (IQR)	14.0 (9 - 23)	15 (12 – 26)	0.417
Duration of DM, yr (IQR)	30.6 (22.6 – 40.2)	35.4 (25.3 – 46.1)	0.374
Age at transplant, yr (IQR)	48.8 (41.3 – 55.8)	59.8 (54.3 – 60.4)	0.025
Body-mass index (IQR)	25.0 (22.9 – 27.8)	25.4 (25.0 – 27.3)	0.751
Number of infusions per patient (IQR)	2 (2 – 3)	2 (2 – 4)	0.672
Number of extrahepatic infusions per patient (IQR)	-	1 (1-2)	-
Time between infusions, mo (IQR)			
Time to 2nd infusion	5.0 (2.1 – 11.1)	4.3 (3.0 – 8.5)	0.823
Time to 3rd infusion	40.4 (16.6 – 70.9)	5.5 (4.3 – 16.8)	0.014
Time to 4th infusion	91.0 (68.5 – 140.5)	15.1 (5.5 – 37.7)	0.007
Time to 5th infusion	165.4 (143.6 – 181.5)	14.6 (-)	0.157
Total IEQs/kg of body weight, x 1,000 (IQR)	14.3 (11.2 – 18.6)	22.5 (13.3 – 27.2)	0.097
1st infusion, x 1,000 (IQR)	6.1 (4.8 – 7.0)	7.0 (6.5 – 9.1)	0.018
Purity (1st infusion)	60 (50 - 70)	55 (45 – 65)	0.477
Lability index (IQR)	449 (296 – 699)	566 (374 – 608)	0.520
Clarke score (IQR)	5 (4 - 7)	5 (3 – 5)	0.052
Laboratory values at first transplant (baseline)			
C-peptide (nmol/L) (IQR)	0.02 (0.02 – 0.03)	0.02 (0.02)	0.055
HbA1c % (IQR)	8.2 (7.5 – 9.0)	8.1 (7.5 – 8.1)	0.333
Insulin units/kg/day (IQR)	0.54 (0.46 – 0.68)	0.51 (0.46 – 0.60)	0.588

M: male, F: female, IEQ: islet equivalent, LDL: low-density lipoprotein, HDL: high-density lipoprotein. Data are n (%) and median (IQR)

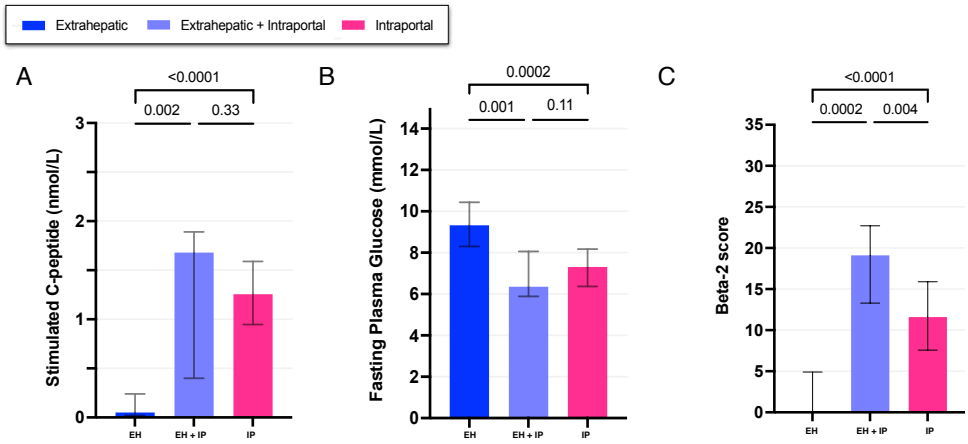
*X^2 was used to compare categorical variables, Mann-Whitney's test was used to compare continuous variables.

†Weighted averages were calculated as follows: weighted average= sum of weighted terms/total number of terms. For example, weighted average= $purity^{infusion1}$+ $purity^{infusion2}$ +…/total number of islets infused.

Primary outcome assessment demonstrated that patients receiving extrahepatic ITx had significantly lower stimulated C-peptide levels 1-3 months after first ITx compared to patients receiving only intraportal infusions (0.05 nmol/L, IQR 0.02-0.24 extrahepatic vs 1.26 nmol/L, IQR 0.95-1.59 intraportal; $p<0.001$; **Figure 3.1A**). Secondary outcomes showed statistically higher FPG, and lower BETA-2 scores (**Figure 3.1B-C**) early after extrahepatic ITx compared to intraportal (**Table 3.2**). Once patients with initial extrahepatic ITx received subsequent

intraportal islet infusions, they achieved similar stimulated C-peptide levels and FPG compared to those who initially received intraportal infusions (**Figure 3.1A-B**). Notably, BETA-2 scores were higher after intraportal transplant in the patients who initially received extrahepatic grafts (19.1, IQR 13.3-22.7; p=0.004; **Figure 3.1C**).

Figure 3.1. Stimulated C-peptide, fasting plasma glucose levels and BETA-2 scores in patients undergoing extrahepatic and intraportal pancreatic islet transplantation, as well as second (intraportal) islet transplantation in patients who initially received extrahepatic implantation



Note: Panel A shows stimulated C-peptide. Panel B shows fasting plasma glucose. Panel C shows BETA-2 scores. *EH: Extrahepatic transplant (n=9); EH + IP: Extrahepatic undergoing subsequent intraportal transplant (n=7); IP: intraportal transplant (n=93). Data is presented as medians and IQR. All stimulated C-peptide measures are taken 1-3 months after transplantation, except for one patient in the EH group in which stimulated C-peptide was measured 5-months, since this patient had a 2nd infusion in the gastric mucosa.

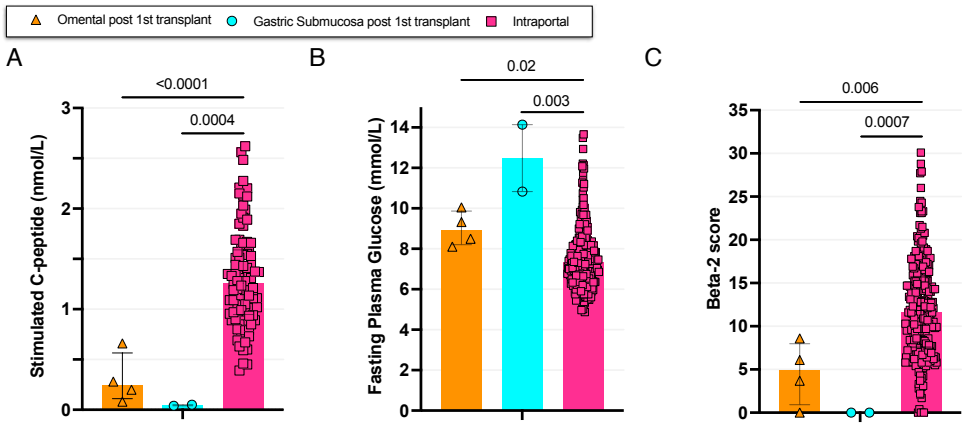
Table 3.2. Primary outcomes following extrahepatic and intraportal pancreatic islet transplantation

Variable	Extrahepatic	Extrahepatic + intraportal	Intraportal
Primary outcome			
Stimulated C-peptide (nmol/L)*	0.05 (0.02 – 0.24)	1.68 (0.4 – 1.89)	1.26 (0.95 – 1.59)
Secondary outcomes			
Fasting plasma glucose (mmol/L)	9.33 (8.3 – 10.44)	6.35 (5.89 – 8.06)	7.32 (6.39 – 8.18)
BETA-2 score	0 (0 – 4.9)	19.1 (13.3 – 22.7)	11.6 (7.55 – 15.7)

Note: Data is presented as medians and IQR. *All stimulated C-peptide measures are taken 1-3 months after transplantation, except for one patient in the EH group in which stimulated C-peptide was measured 5-months, since this patient had a 2nd infusion in the gastric mucosa.

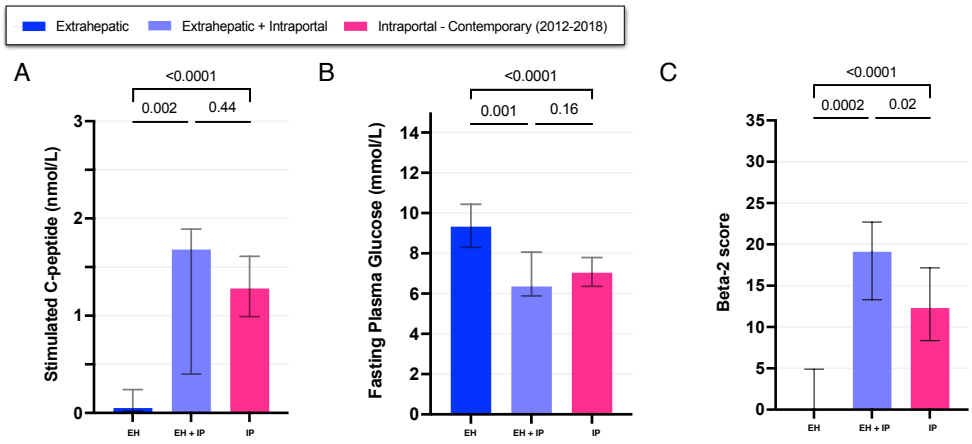
Similar differences in primary and secondary outcomes were observed in sub-analyses according to specific extrahepatic implantation sites compared to intraportal ITx with a suggestion that best outcomes may have been observed with the omental site (**Figure 3.2**). Secondary analysis of patients receiving extrahepatic ITx to contemporary intraportal ITx (n = 106) also showed similar outcomes (**Figure 3.3**).

Figure 3.2. Stimulated C-peptide, fasting plasma glucose levels and BETA-2 scores in patients undergoing omental, gastric submucosa and intraportal pancreatic islet transplantation



Note: Panel A shows stimulated C-peptide. Panel B shows fasting plasma glucose. Panel C shows BETA-2 scores. All stimulated C-peptide measures are taken 1-3 months after transplantation, except for one patient in the EH group in which stimulated C-peptide was measured 5-months, since this patient had a 2nd infusion in the gastric mucosa. Data are presented as median and IQR.

Figure 3.3. Stimulated C-peptide, fasting plasma glucose levels and BETA-2 scores in patients undergoing extrahepatic and intraportal pancreatic islet transplantation, as well as second (intraportal) islet transplantation in patients who initially received extrahepatic implantation

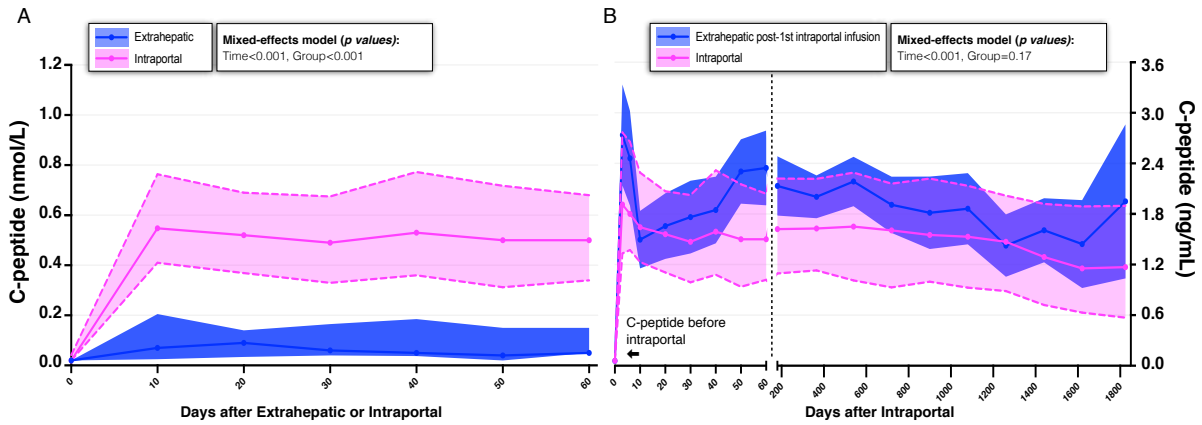


Note: Panel A shows stimulated C-peptide. Panel B shows fasting plasma glucose. Panel C shows BETA-2 scores. *EH: Extrahepatic transplant (n=9); EH + IP: Extrahepatic undergoing subsequent intraportal transplant (n=7); IP: intraportal transplant (n=55). Only patients undergoing islet transplantation between Jan 1st 2012 and October 1st 2018 were included in these graphs. All stimulated C-peptide measures are taken 1-3 months after transplantation, except for one patient in the EH group in which stimulated C-peptide was measured 5-months, since this patient had a 2nd infusion in the gastric mucosa. Data are presented as medians and IQR.

Assessment of fasting C-peptide levels over time showed that patients receiving extrahepatic transplants had significantly lower levels after their initial implants compared to those with intraportal infusions (mixed-effect model, group effect: $p<0.001$; **Figure 3.4A**). Of the 9 patients undergoing extrahepatic ITx, 7 (77.8%) elected to proceed with subsequent ITx following failed extrahepatic graft. Only one patient proceeded with subsequent extrahepatic

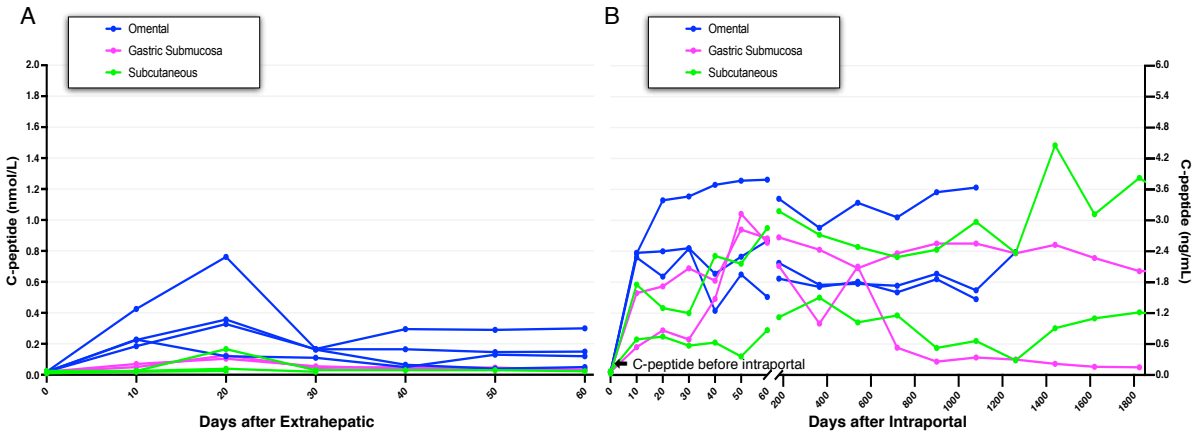
ITx, had similar early graft failure, and then proceeded with intraportal ITx. Patients receiving gastric submucosal, omental, or prevascularized subcutaneous transplant failed to produce a median fasting C-peptide level ≥0.2nmol/L in the first 60 days when compared to intraportal infusion, however, 3 of 4 subjects in the omental group had measurable C-peptide ≥0.2nmol/L at some point post extrahepatic transplant. Fasting C-peptide levels following intraportal ITx in patients who initially received extrahepatic implants was similar to those patients receiving whose initial transplants were via intraportal infusions (mixed effect model group effect: p=0.17; **Figure 3.4B**). All patients with extrahepatic ITx responded similarly after receiving subsequent intraportal transplant (**Figure 3.5**). One subject receiving gastric submucosal ITx developed *de novo* donor specific antibody, but no other patient had any cPRA increase after extrahepatic ITx.

Figure 3.4. Fasting C-peptide levels following extrahepatic and intraportal pancreatic islet cell transplantation



Note: Panel A shows fasting C-peptide 10-day medians for the first 60-days after extrahepatic or intraportal islet infusions. Panel B shows fasting C-peptide after intraportal transplant and after intraportal transplant in patients who initially received extrahepatic implantation (extrahepatic group). Data are presented as median (solid lines) and interquartile ranges (shaded area).

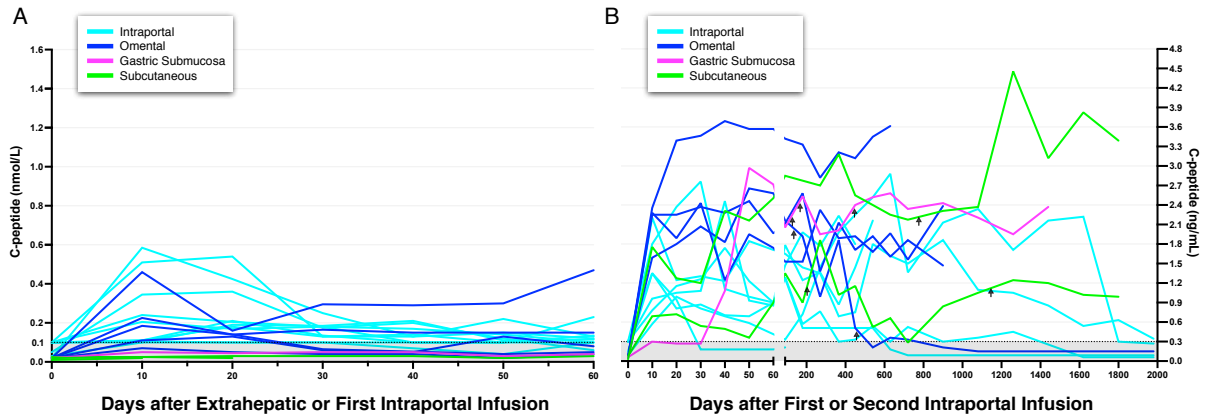
Figure 3.5. Fasting C-peptide levels following omental, gastric submucosa and subcutaneous pancreatic islet transplantation



Note: Panel A shows fasting C-peptide 10-day medians for the first 60-days after extrahepatic islet infusions. Panel B shows fasting C-peptide after intraportal transplant in patients who initially received extrahepatic implantation (extrahepatic group). Data are presented as individual values.

Primary non-function and/or early graft failure occurred significantly more following extrahepatic ITx than following initial intraportal transplant (88.9%, n = 8/9 extrahepatic vs 2.0%, n = 5/255 for intraportal, p<0.001, **Figure 3.6).** Extrahepatic ITx was independently associated with graft primary non-function (OR 1,709, CI 73.8-39,616.0, p<0.001). No other patient, transplant, or immunosuppression factors were independently predictive of primary non-function **(Table 3.3).** Evaluating patients with either early graft failure or primary non-function we see that only 14% (n = 1/7) with extrahepatic grafts experienced early graft failure after subsequent intraportal ITx; comparatively, in patients receiving initial intraportal ITx who experienced either graft primary non-function, 80% (n = 4/5) experienced similar early graft loss following second intraportal transplant.

Figure 3.6. Fasting C-peptide levels in patients having primary non-function or early graft failure following extrahepatic and intraportal islet transplantation



Note: Panel A shows fasting C-peptide 10-day medians for the first 60-days after extrahepatic or first islet infusion. Panel B shows fasting C-peptide after first (in patients with previous extrahepatic) or second intraportal infusion (in patients with primary non-function or early graft failure. Data are presented as individual values.

Table 3.3. Outcomes from multivariable logistic modelling evaluating factors independently associated with graft primary non-function (median C-peptide ≤0.1 nmol/L).

Risk Factor	Odds Ratio	95% confidence interval	*P* value
Age	0.97	0.89 - 1.06	0.549
Male gender	1.23	0.21 - 7.22	0.816
IEQ per kg of body weight	1.00	1.00 - 1.00	0.292
Tacrolimus levels (months 0-3 post-first infusion)	0.76	0.48 - 1.20	0.236
Body mass index (kg/m^2)	1.01	0.98 - 1.02	0.108
Extrahepatic transplant site	1,709	73.80 - 39,616.00	<0.001

3.1.5 - Discussion

This study demonstrates that administration of large numbers of high quality islets by an extrahepatic route failed to result in significant production of basal or stimulated C-peptide within the first three months post-transplant. Conversely, recipients of primary intraportal ITx demonstrated markedly superior C-peptide production in the first three months, as well as demonstrating sustained graft survival, and improved glycemic-related outcomes compared to extrahepatic ITx. However, individuals who had received extrahepatic ITx were able to achieve similar stimulated and fasting C-peptide levels, and similar glycemic outcomes once they subsequently received intraportal ITx, compared to those receiving initial intraportal ITx. Overall, when compared to intraportal ITx, extrahepatic implantation failed to show islet engraftment or improved diabetes outcomes in patients who subsequently received successful intraportal grafts, suggesting that the extrahepatic site and not patient or graft factors, was the cause of these outcomes. Prior to future clinical evaluation of these extrahepatic sites, ongoing optimization of these innovative techniques is required.

The study's primary outcome analysis showed that patients with intraportal ITx had significantly higher stimulated C-peptide over the first three months after implantation compared to those receiving extrahepatic islet grafts. Notably, extrahepatic grafts produced a median stimulated C-peptide < 0.1 nmol/L despite receiving a greater islet mass. C-peptide production remains a primary outcome measure that correlates with glycemic control, insulin independence and resolution of glycemic lability, particularly hypoglycemia.[29-32] Differences in secondary outcomes were also evident, with significantly higher FPG after extrahepatic ITx, and worse graft function measured by BETA-2 scores. This is in contrast to some cases reported previously where substantial C-peptide production was observed in 2 of 3 patients undergoing

omental ITx;[11, 14] and pre-clinical models demonstrating successful gastric submucosal ITx in large animal models,[10] and promising results for the prevascularized subcutaneous approach in mice.[15-17] However, while not clinically significant, C-peptide levels appeared higher in the omental when compared to the two other extrahepatic sites. Together, our data raise concerns about the feasibility of extrahepatic ITx, and emphasize a need to further optimize oxygenation, neovascularization and protection from fibrosis or other deleterious processes in extrahepatic sites to achieve clinical outcomes equivalent to intraportal ITx.

Importantly, patients who initially received extrahepatic transplant without success, and who subsequently underwent intraportal ITx achieved similar stimulated C-peptide levels, glycemic outcomes, and graft function to those who underwent initial intraportal transplant. Overall, similarities in our primary outcome and most secondary outcomes support the notion that a failed extrahepatic ITx does not impact the success of subsequent intraportal ITx, and that improvement in techniques for extrahepatic ITx are necessary. However, an unplanned finding of this study demonstrated that patients receiving intraportal ITx who experience early graft failure or graft primary non-function may be at risk of subsequent graft failure and studies evaluating this patient cohort would be of interest. Additionally, although allosensitization was uncommon in patients receiving extrahepatic ITx, this risk remains a potential consideration in this patient population who are at risk of requiring future islet or solid organ transplants. The risk of allosensitization may have been mitigated in this series because maintenance immunosuppression was continued while on the wait list for a subsequent intraportal islet infusion.

Our findings contrast somewhat with the promising preliminary results with omental islet transplantation from Baidal et al, with outcomes that are similar to subject 2 in their study.[14]

Our omental transplants did demonstrate sustained but low C-peptide production over time but remained inferior to intraportal infusion in our hands. Our experience is limited to only four omental ITxs and variance in technique or islet quality could explain this difference. Alternatively, it is likely that human-to-human variation in vascularity and fatty infiltration in the omentum between subjects, or age and species-specific differences in omental anatomy or immunological response could explain the discrepancy between preclinical experience and clinical translation of this approach in murine[13] and macaque[12] models. While omental cell composition remains similar between species,[33] fat density and vascular distribution is highly variable with animal models often having substantially less adiposity, potentially increasing diffusion capacity;[34] the latter remains crucial to islet engraftment and may further contribute to our findings.[35] Two recent studies evaluating omental ITx in humans have found similar results to ours, with >50% of patients having early graft failure, and others achieving marginal clinical benefit.[36, 37] Of note, the Van Hulle et al. group evaluated graft biopsies and demonstrated that substantial foreign body reaction may have led to their outcomes.[36]

Similarly, our results contrast with promising findings of allotransplantation of porcine islets within the gastric submucosa in immunosuppressed pigs reported by Echeverri et al.[10] Importantly, the gross and microscopic gastric anatomy differs between humans and porcine models, with the porcine stomach being 2-3 times larger and having much more cardiac mucosa than humans.[38] These cardiac cells produce primarily mucus, while the human gastric submucosa contains parietal and chief cells that produce hydrolytic enzymes and acid.[38] Again, these small inter-species differences may account for the findings in our study.

Finally, our results also diverge from those shown with ITx into the prevascularized subcutaneous space in mice.[15-17] However, a recent oral presentation by Dr. Witkowski's group

suggests that the prevascularized subcutaneous space (using similar techniques to the one employed in this study) can achieve engraftment and sustained C-peptide production and insulin dose reduction in humans when a lower islet tissue concentration is transplanted in the subcutaneous device within the rectus muscle fascia.[39] Our experience here only includes 3 patients and it remains possible that our observed failure with subcutaneous ITx is due to device capacity overload from the high islet masses that we implanted.[23] Alternatively, improved vascularization within intramuscular sites may offer potential improvements to current techniques.[40] Small iterative modifications may enable success of subcutaneous and other extrahepatic sites, and encourages ongoing refinements to further optimize these techniques.

Of note, for the gastric submucosal, subcutaneous and omental extrahepatic islet transplants we were more discriminatory in the selection of higher quality, higher purity preparations which should have lent favorably to improved islet survival and engraftment in these sites. Our aim was to reduce the amount of exocrine contamination in grafts placed in sites with more limited physical restraints. This may or may not have been wise in retrospect. We also selected young healthier recipients to optimize the conditions, which again should have lent favorably for extrahepatic sites.

A major limitation to the current study is the very small cohorts included in each of the extrahepatic sites, and the potential variability in our adoption of these new techniques. While this study presents only 9 subjects receiving extrahepatic grafts, it still represents the largest compilation of extrahepatic ITx to date. Additionally, the lack of substantial C-peptide production in every included patient suggests that even if additional patients were included, differences compared to intraportal infusion would still remain. Similarly, although we only present early outcomes following extrahepatic ITx, with the limited and relatively poor function

observed in the extrahepatic sites, it is unlikely that these grafts would spontaneously gain further function beyond the 1-3 month timeframe evaluated in this study; the fact that these patients were promptly given intraportal islet infusions also precluded a longer term analysis. We cannot however completely rule out that late onset graft function would have occurred. Comparison of extrahepatic ITx that occurred since 2012 to intraportal from 1999-2018 also introduces the potential of era related effects. In order to limit that risk, we performed a secondary analysis comparing era matched cohorts and showed similar results. It should also be noted that this is a single center experience and subject bias cannot be ruled out. It remains to be determined whether technical aspects have led to our negative results with extrahepatic ITx. Specialists assisted and trained our group with their omental transplant technique to minimize the likelihood that our findings are due to technical variability.[11, 14] All endoscopic procedures were performed by a single experienced interventional gastroenterologist following previously described techniques that are easily reproducible.[10] As previously described, surgeons who were successful with animal model participated in the human procedures to ensure technical consistency.[15-17, 23] Therefore, while technical differences could explain our findings, substantial efforts were made to reduce that likelihood. As discussed above, an additional variable that could contribute to the differences in our study compared to others is variance in islet cell preparations and transplanted islet tissue concentration. Herein, we report the IEQ/kg and purity of the extrahepatic and intraportal transplants but no data is available to compare our islet preparations to other centers. Mechanistic evaluation of the reasons for extrahepatic graft outcomes shown here are limited and may be beneficial to further improve these techniques. Nevertheless, the successful outcomes with intraportal transplantation in both groups is consistent with the high-quality islet preparations consistently provided by our islet isolation

team. Ongoing optimization of the islet preparation and transplant techniques may enable future success of extrahepatic sites and ongoing work is encouraged.

We present a comparative cohort study evaluating patients receiving extrahepatic and intraportal ITx at a single islet transplant centre. Patients who received extrahepatic ITx failed to achieve substantial C-peptide production when compared to intraportal transplantation. However, these patients did subsequently achieve similar graft function with a secondary intraportal ITx, suggesting that inadequate engraftment after extrahepatic transplant rather than graft or patient factors are implicated in graft failure after extrahepatic ITx. As we consider alternative sites for engraftment of islets or stem cell-islets, it is clear from our preliminary experience that more refinements will be needed to substantially improve cellular engraftment and survival if these sites are to match the current efficiency of the intraportal approach. Intraportal ITx, despite all of its limitations, prevails as a current gold-standard as the only implantation site to have consistently demonstrated the capacity to support long-term islet engraftment, glucose-responsive C-peptide production, glycemic outcome improvements, and sustained insulin independence. While the concept of achieving clinical success with extrahepatic ITx remains attractive, substantial work is required to transform this concept into a reality.

3.1.6 - References

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CHAPTER 4

PART 1 - PROGRESS IN TRANSLATIONAL REGULATORY T CELL THERAPIES FOR TYPE 1 DIABETES AND ISLET TRANSPLANTATION

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Review

Review

Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation

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Abstract

Regulatory T cells (Tregs) have become highly relevant in the pathophysiology and treatment of autoimmune diseases, such as type 1 diabetes (T1D). As these cells are known to be defective in T1D, recent efforts have explored ex vivo and in vivo Treg expansion and enhancement as a means for restoring self-tolerance in this disease. Given their capacity to also modulate alloimmune responses, studies using Treg-based therapies have recently been undertaken in transplantation. Islet transplantation provides a unique opportunity to study the critical immunological crossroads between auto- and alloimmunity. This procedure has advanced greatly in recent years, and reports of complete abrogation of severe hypoglycemia and long-term insulin independence have become increasingly reported. It is clear that cellular transplantation has the potential to be a true cure in T1D, provided the remaining barriers of cell supply and abrogated need for immune suppression can be overcome. However, the role that Tregs play in islet transplantation remains to be defined. Herein, we synthesize the progress and current state of Treg-based therapies in T1D and islet transplantation. We provide an extensive, but concise, background to understand the physiology and function of these cells and discuss the clinical evidence supporting potency and potential Treg-based therapies in the context of T1D and islet transplantation. Finally, we discuss some areas of opportunity and potential research avenues to guide effective future clinical application. This review provides a basic framework of knowledge for clinicians and researchers involved in the care of patients with T1D and islet transplantation.

Key Words: type 1 diabetes, islet, transplantation, regulatory T cells, tolerance

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4.1.1 - Abstract

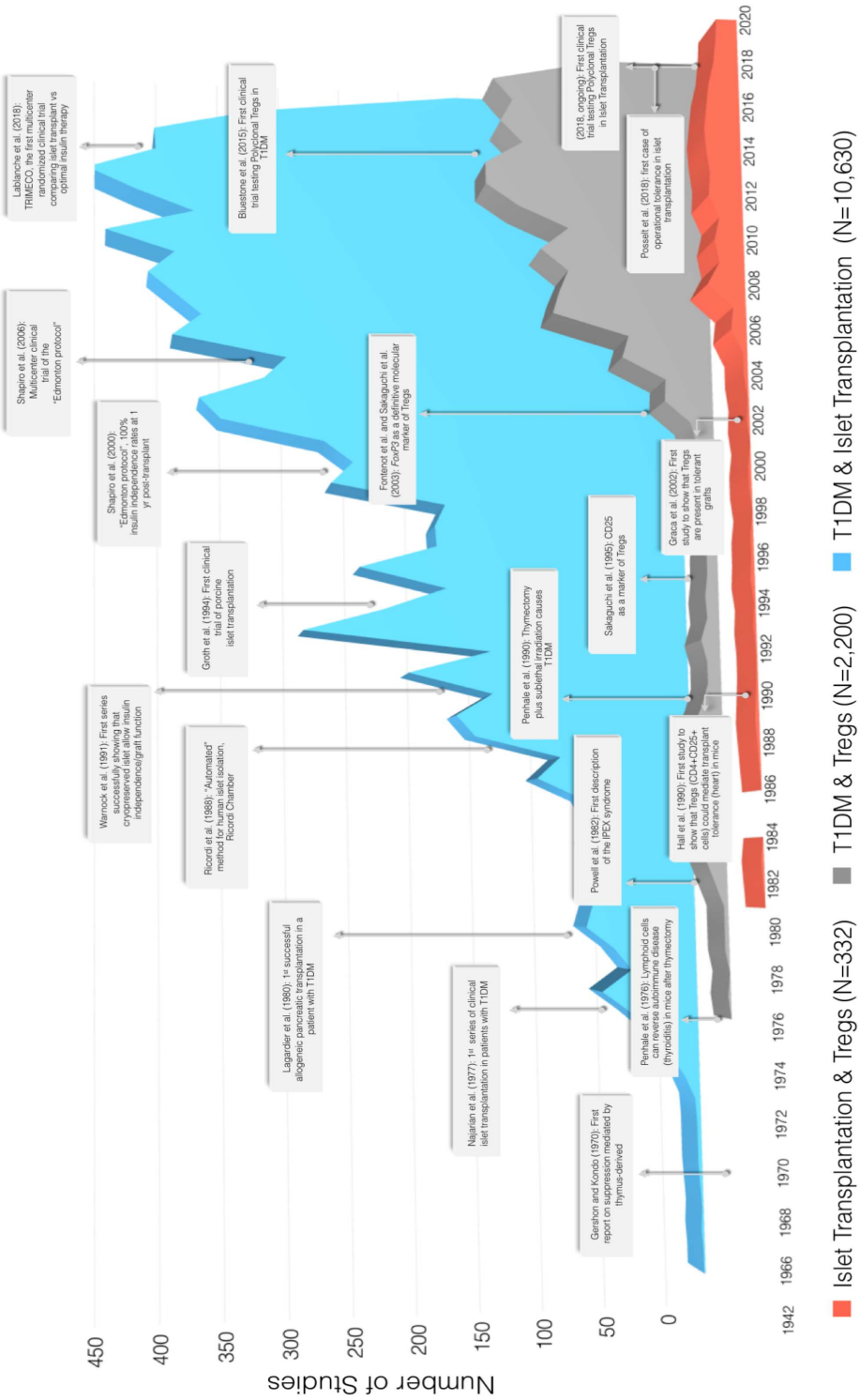
Regulatory T cells (Tregs) have become highly relevant in the pathophysiology and treatment of autoimmune diseases, such as type 1 diabetes (T1D). As these cells are known to be defective in T1D, recent efforts have explored *ex vivo* and *in vivo* Treg expansion and enhancement as a means for restoring self-tolerance in this disease. Given their capacity to also modulate alloimmune responses, studies using Treg-based therapies have recently been undertaken in transplantation. Islet transplantation provides a unique opportunity to study the critical immunological crossroads between auto and alloimmunity. This procedure has advanced greatly in recent years, and reports of complete abrogation of severe hypoglycemia and long-term insulin independence have become increasingly reported. It is clear that cellular transplantation has the potential to be a true cure in T1D, provided the remaining barriers of cell supply and abrogated need for immune suppression can be overcome. However, the role that Tregs play in islet transplantation remains to be defined. Herein, we synthesize the progress and current state of Treg-based therapies in T1D and islet transplantation. We provide an extensive, but concise, background to understand the physiology and function of these cells and discuss the clinical evidence supporting potency and potential Treg-based therapies in the context of T1D and islet transplantation. Finally, we discuss some areas of opportunity and potential research avenues to guide effective future clinical application. This review provides a basic framework of knowledge for clinicians and researchers involved in the care of patients with T1D and islet transplantation.

4.1.2 - Introduction

Type 1 diabetes (T1D) is a chronic, prevalent autoimmune disease treated by exogenous insulin, and associated with sequelae of end-organ complications and risk of inadvertent insulin overdose (i.e., hypoglycemia). While it can neither be prevented, nor cured presently, efforts are underway to reverse autoimmunity at the onset of diabetes using powerful immunomodulatory therapies, and to treat established disease with cellular transplantation of insulin-secreting cells within the pancreatic islets of Langerhans. A major challenge with the current practice of transplanting allogeneic adult human islets into patients with T1D (islet transplantation (ITx)), is the need for life-long immunosuppressive medications. ITx has been shown to be especially pertinent for patients complicated by recurrent severe hypoglycemia, where the risk-benefit ratio of metabolic stability more than offsets the potential risks of life-threatening infection, malignancy and other side effects from immunosuppression.[1] In this review, we highlight the potential beneficial role of regulatory T cell (Treg) therapies as a means to abrogate autoimmunity in new onset T1D, and to potentially mitigate the need for chronic immunosuppression after ITx.

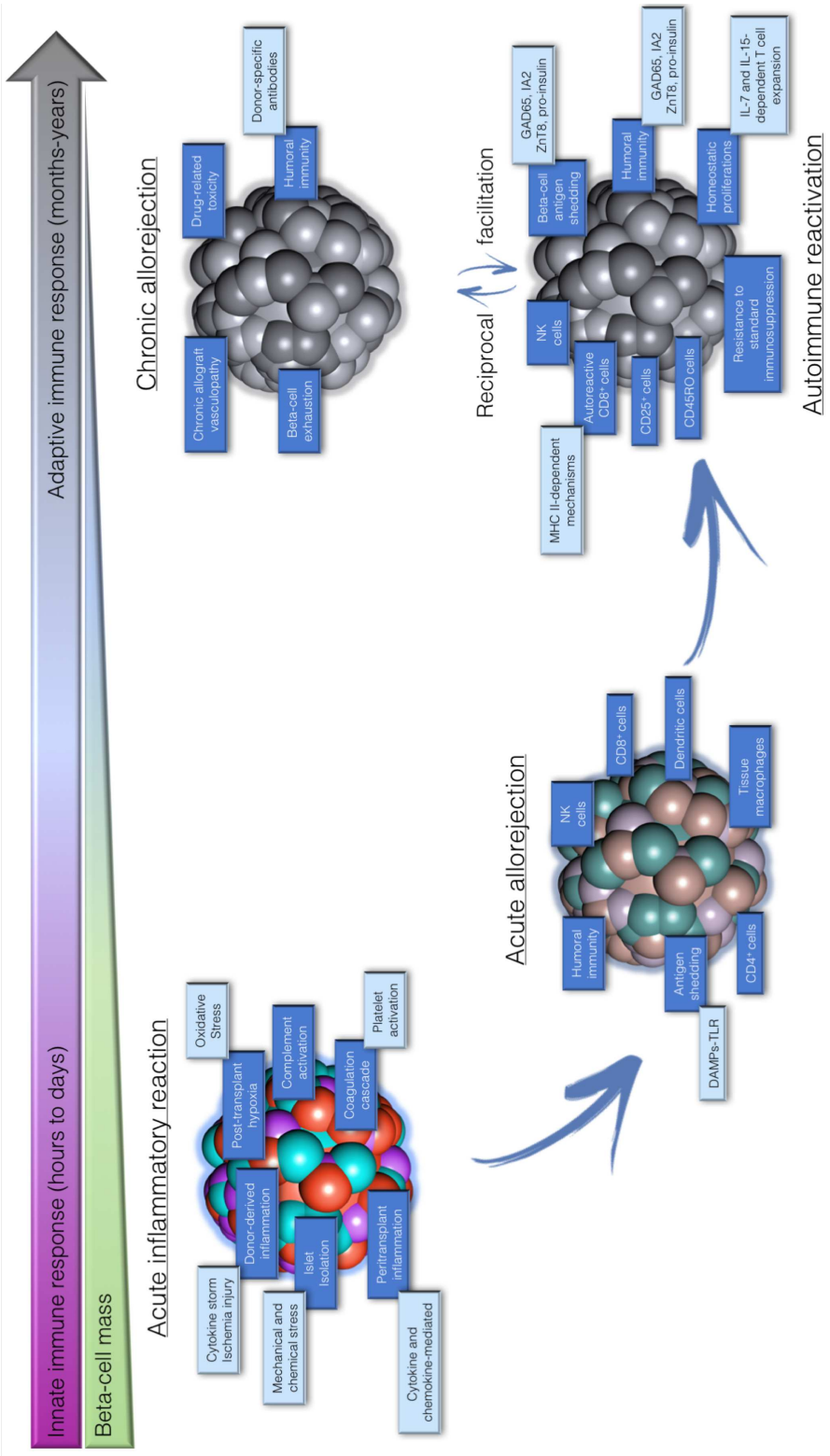
Refined protocols[2, 3] have substantially improved outcomes of ITx,[4] leading to long-term abrogation of severe hypoglycemic episodes in >90%, optimal glycemic control (HbA1c <7%) in ~60% and insulin independence in ~30% of patients.[5] State-of-the-art insulin pumps and glucose monitoring systems, even when tested in a randomized head-to-head fashion, cannot achieve this level of glycemic control.[6, 7] More importantly, ITx has shown to reduce progression of microvascular complications compared to intensive insulin therapy.[8] Despite this considerable progress (**Figure 4.1.1**), there are remaining challenges to convert ITx as a true cure for T1D, mainly, the need for chronic immunosuppression (**Figure 4.1.2**).[9, 10]

Figure 4.1.1. Studies published in PubMed/MEDLINE from 1957-June 11, 2020 related to "islet transplantation" and major landmarks in the field.



Note: This graph is purely illustrative and does not represent the results of a systematic search of the literature. Medical subject heading (MeSH) terms: "type 1 diabetes mellitus/diabet*" AND "islet transplantation", combined with several variations of "regulatory T cell". Used with permission from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev*. 2021;42(2):198-218.

Figure 4.1.2. Innate and adaptive immune phenomena during islet transplantation.



Note: Transplanted islets are exposed to diverse harmful responses that span from hours to days. During the first hours, immune reactions are of an innate nature and consist of cytokine- and chemokine-mediated damage, activation of the coagulation cascade, complement activation and damage mediated by oxidative stress. Concomitantly, the process of acute rejection ensues, and both humoral and cellular immunity have a prominent role in this process, with key players such as dendritic cells, CD8+ T cells, NK cells and tissue macrophages, among others. Chronic rejection and autoimmune rejection occur later during the life of the graft and both are potentiated through reciprocal facilitation, in which chronic allo-rejection promotes and perpetuates autoimmune reactivation, and *viceversa*. If these processes are not modulated and/or prevented, progressive loss of β-cell mass will inevitably occur, which will ultimately lead to graft failure. Used with permission from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev*. 2021;42(2):198-218.

Given the multiple side-effects and risks associated with these agents, alternatives that minimize or eliminate their need would expand the applicability of β-cell replacement therapies in T1D. Beyond the need for lifelong immunosuppression, other barriers need to be overcome to make ITx accessible and feasible for every patient with diabetes (**Table 4.1.1**).

Table 4.1.1. Challenges in clinical islet transplantation and potential solutions

Challenges	Potential Solutions
Pre-transplant	
• **Islet source (lack of sufficient islets)**	• Optimizing donor selection • Optimizing enzymatic digestion • Rescue gradient purification • Xenotransplantation (e.g., porcine islets) • Stem-cell-derived islets (e.g., hESC and hiPSC)
• **Cell death during culture** Stress induced Mechanical Chemical Hypoxia Contamination	• Anti-apoptotic/-necroptosis agents • Optimizing culture media (supplementation) • Lower temperature culture conditions • Improved perfusion (e.g., microfluidic perfusion) • Strict adherence to GMP practices
Peri-transplant	
• **IBMIR**	• Anti-coagulation • Anti-inflammatory agents • Complement inhibitors • Alternative implantation sites
• **Procedural complications (e.g., portal vein thrombosis, bleeding, etc.)**	• Thrombostatic agents • Low-packed cell volume → increased islet purity • Alternative implantation sites
• **Hypoxia/ischemia-reperfusion**	• Islet preconditioning • Pre-vascularization strategies • Pro-angiogenic agents • *In situ* oxygen delivery/generation
Post-transplant	
• **Immune responses** Autoimmunity Alloimmunity Chronic inflammation Toxicity with chronic immunosuppression	• Peptide/mimetope immunotherapy • Cell-based therapies (e.g., regulatory T cells) • Cellular encapsulation • Gene-editing strategies • Personalized stem cell therapies (e.g., hESCs and hiPSCs) • Immunosuppression minimization strategies

• **Graft attrition/metabolic exhaustion**	• Optimal glycemic control (e.g., early reintroduction of insulin) • Oral hyperglycemic agents (e.g., GLP-1 agonists) • Manage metabolic comorbidities (e.g., dyslipidemia) • Islet-friendly immunosuppression
Clinical translation	
• **Regulatory issues** • **Safety** • **Cost-effectiveness**	• Ensuring integrity of human islets during islet culture • Safer alternative implantation sites • Assessment of clinically relevant variables beyond insulin independence (e.g., abrogation of hypoglycemia, reduction in long-term diabetes-related complications, quality of life, etc.)

hESC: human embryonic stem cells, hiPSC: human induced pluripotent stem cells, GMP: good manufacturing practices, IBMIR: instant blood-mediated inflammatory reaction, GLP-1: glucagon-like peptide. Adapted from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev*. 2021;42(2):198-218.

Cell-based immunoregulatory therapies are strong candidates to enable ITx without immunosuppression. Among the many products,[11] regulatory T cells (Tregs) are one of the most promising. Given their capacity to regulate auto and alloimmune responses, Treg-based therapies are also highly relevant to control autoimmunity in T1D. We herein address and discuss current evidence for Treg-based therapies in T1D and ITx.

4.1.3 - Regulatory T Cells – A Brief Introduction

Tregs represent a specialized subset of CD4+ T cells (1-10%).[12] They suppress inappropriate immune responses to self-antigens, such as those occurring in T1D.[13] They are categorized by expression of the *Forkhead box protein 3* transcription factor (FOXP3)[14] (**Figure 4.1.3**). Tregs constitutively expressing FOXP3 (tTregs, previously known as naturally occurring Tregs) originate from the thymus during central selection, appear under physiological conditions, and are pre-committed to self-antigen recognition.[15] Conversely, Tregs expressing FOXP3 following tolerogenic stimuli are generated peripherally (e.g., intestinal mucosa) from naïve $CD4^+$ T cells (pTregs, previously known as adaptive Tregs), following cytokine-specific stimulation (e.g., IL-2, TGF-β).[16]

Figure 4.1.3. Regulatory T cells subpopulations

Note: In the thymus, T cells are exposed to multiple stimuli to select them for future functions. Cells reacting to self-antigens are negatively selected and undergo apoptosis. Thymus-derived Tregs (tTregs) have intermediate avidity for self-antigens and express several activation and functional markers that promote their survival. These cells are "primed" for immune regulatory functions and particularly relevant in the pathogenesis of autoimmune diseases. Naïve T cells migrate to the peripheral circulation and, subsequently, undergo differentiation into peripheral Tregs (pTregs) following exposure to specific cytokines (e.g., IL-2 and TGF-β) and activation by antigen-presenting cells (APC). The presence of additional cytokines, such as IL-27, further drives naïve T cell differentiation into peripheral Treg subtypes, such as Tr1 cells. Both types of pTregs have different mechanisms leading to T cell suppression: 1) cytokine production (e.g., IL-10, IL-35, TGF-β, etc.), 2) disruption of several metabolic pathways through metabolism of ATP to AMP by ectoenzymes CD39 and CD73, as well as production of adenosine and cAMP that directly modulate effector T cells, 3) modulation of APC maturation by CTLA4 costimulation blockade, upregulation of indoleamine 2,3-dioxygenase (IDO) and 4) induction of apoptosis through the production of granzymes A/B, perforins, but also through the Fas/Fas-ligand pathway, the galectin-TIM3. The same conditions for Treg differentiation can be replicated *in vitro* to generate and expand these cells, known as inducible Tregs (iTregs). Used with permission from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev.* 2021;42(2):198-218.

This cytokine-specific stimulation can be replicated to produce Tregs *in vitro* (inducible or iTregs).[14, 17] pTregs and iTregs are biased toward non-self-antigen recognition, making them relevant to transplantation. Additional markers allow further Treg characterization, such as CD45RA which classifies Treg functionality (i.e., naïve or activated/effector),[18] the methylation status of the Treg-specific demethylated region(s) of *FOXP3*, which identifies tTregs,[19] the sialyl Lewis (CD15s) marker, which identifies highly suppressor Tregs,[20] or their cytokine secretion/receptor profiles.[21] Tregs also express high levels of the IL-2 receptor α-chain (CD25) and low levels of the IL-7 receptor α-chain (CD127), which further determine their function, expansion/survival capacities and precise identification.[22]

Tregs suppress activation and proliferation of effector T cells (Teffs), NK cells, B cells, and dendritic cells.[23] Four mechanisms mediate these effects: 1) suppression through inhibitory cytokines, 2) suppression through cytolysis , 3) suppression through metabolic disruption and, 4) suppression through targeting of antigen-presenting cells (APC).[24] These mechanisms act in concert with T-cell receptor affinity to collectively determine the fate of Teffs (i.e., apoptosis, anergy or a dormant state).[25] Importantly, Treg-mediated suppression relies on stability/preservation of their phenotype, migration to relevant tissues (i.e., homing) and expansion of their T-cell receptor specificities (i.e., infectious tolerance).[26]

4.1.4 - Regulatory T Cells in Type 1 Diabetes

In 1995, Sakaguchi et al. showed that Treg-depleted mice developed severe autoimmunity (including diabetes) and that Treg reconstitution effectively prevented autoimmunity in a dose-dependent manner.[27] In humans, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) is a genetic disorder characterized by

dysfunctional Tregs,[28] that is associated with several autoimmune diseases, highlighting their importance in self-tolerance. Since T1D is a core manifestation of IPEX, profound defects of Tregs are suggested to play a vital role in the pathophysiology of T1D.[29]

The immunological cascade of events in T1D follows a two-checkpoint process. The first involves infiltration of autoreactive T cells within the islets of Langerhans, which is normally controlled by immunoregulatory mechanisms.[30] Indeed, autoreactive T cells to islet antigens are present in healthy individuals.[31] However, the second checkpoint, the loss of immunoregulatory mechanisms, is what eventually leads to tissue damage.[30] Tregs are central in these immunoregulatory mechanisms, and quantitative and qualitative defects have been proposed in T1D.[14] Unfortunately, a scarcity of studies (**Figure 4.1.1),** coupled with the relatively underdeveloped technology to study Tregs, have produced conflicting evidence.

Correcting quantitative defects has been attempted using *ex vivo* and *in vivo* Treg expansion. Preventing, delaying, and even reversing T1D with Treg expansion is frequently reported in preclinical studies,[14, 32] however, early clinical success has been more limited.[33-35] Most clinical studies have used autologous *ex vivo*-expanded Tregs, which suggests that increasing Treg numbers alone may be insufficient, given their potentially dysfunctional nature. Indeed, some studies have found that Treg numbers in patients with T1D are similar to healthy controls.[14, 36] However, other studies report that the degree of Treg expansion correlates with C-peptide levels and insulin use post-infusion, implicating suboptimal expansion as a potential reason for clinical failure.[35] Using functional markers (e.g., CD45RA) reveals decreased activated Tregs in patients with T1D, which correlates with decreased residual C-peptide secretion.[37]Thus, identifying more specific markers could allow for even better characterization of functional Treg defects in T1D.[37-39]

Genome-wide association studies (GWAS) have found susceptibility loci and SNPs in genes associated with Treg function in patients with T1D (e.g., *CTLA4, IL10, IL2, IL2RA).*[40] Studies comparing sorted Tregs of children and adults with new-onset T1D, type 2 diabetes, and BMI-matched controls have shown specific Treg gene expression "signatures",[41, 42] which predict future rapid vs slow C-peptide decline.[42] Functional defects of Tregs in T1D, mainly altered IL-2-related signaling pathways, but also unstable *FOXP3* expression, increased apoptosis, increased proinflammatory cytokine secretion and altered transcriptomes have been reported.[14, 43-45] Importantly, current evidence suggests that Treg dysfunction may be a cause and not a consequence of T1D.[46] Studies with high-risk subjects (e.g., high-risk *HLA* haplotypes) show defects in Treg survival prior to clinical disease.[47] However, subclinical islet inflammation cannot be excluded, and significant heterogeneity between populations prevents definite conclusions. Adequately-powered, long-term, multicenter, longitudinal studies are needed to define the role of both genetic and functional Treg profiling in T1D.

Studies evaluating Treg numbers or function in T1D are confounded by the fact that most assess peripheral Tregs and not those at the site of inflammation (i.e., pancreas or pancreatic lymph nodes [PLN]). In this regard, while studies assessing tissues obtained from deceased donors in the Network for Pancreatic Organ Donor with Diabetes (nPOD) suggest similar clonal diversity and frequency of Tregs in PLN vs peripheral blood,[48] a clearer difference in Treg function has been demonstrated by other studies. Indeed, Sebastiani et al. compared microRNA expression of sorted Tregs from peripheral blood or PLN in patients with T1D undergoing ITx and showed impaired *in vitro* suppressive capacities of PLN-isolated Treg vs those from peripheral blood.[49] The authors found that miR-125a-5p was hyper-expressed in PLN-isolated Tregs from patients with T1D and that this was inversely correlated with

expression of CCR2 (C-C chemokine receptor type 2), which is required for Treg migration to inflammatory (i.e., pancreas-specific migration of Treg cells). This is also supported by studies in deceased donors with early-onset T1D showing minimal Treg infiltration in the pancreas,[50] and suggesting that PLN may be the primary site of Treg dysfunction in T1D.[51] Finally, knowledge from T1D may not be extrapolated to ITx, given the occurrence of both auto and alloimmune responses occurring in this context. Still, autoimmunity remains a major challenge in ITx[52] and ongoing efforts in patients with T1D (NCT02691247, NCT02932826) will undoubtedly help guiding future interventions in ITx.

4.1.5 - Regulatory T Cells in Islet Transplantation

Operational tolerance (OT) remains the critically sought-after holy grail of transplantation. First described in 1975,[53] OT refers to the immune system becoming unresponsive to foreign tissues/organs without immunosuppression.[54] Within the complex mechanisms leading to OT, Tregs are of particular importance.[55] Treg-related biomarkers, including FOXP3 transcripts,[56, 57] $FOXP3^+$ cells and/or DNA methylation of the FOXP3 gene locus,[58] have been proposed as "tolerance signatures/footprints" to identify patients in which immunosuppression minimization or withdrawal could be attempted. However, "tolerance signatures" are limited by their heterogeneity due to small sample sizes, lack of standardization in sampling methods, and patient- and organ-specific variability in OT.[58, 59]

Another relevant concept in transplantation is infectious tolerance, also known as linked/bystander suppression. This refers to a state where tolerance to specific antigens is transferred to novel antigens if presented concomitantly with previously-tolerated antigens.[60] For example, after an allograft from donor A is accepted (primary acceptance), a graft from

donor B can also be accepted if antigens from donor A are presented concomitantly (secondary acceptance). After graft AB is accepted, subsequent grafts having only "B" antigens will be accepted (tertiary acceptance). These processes are facilitated in a Treg-rich environment.[60] In this process, Tregs regulate Teffs, but also B cells, thus modulating antibody-mediated rejection[61] and ensuring immunomodulation beyond the Tregs' specificities and half-lives. Promoting linked suppression is highly relevant in ITx since most patients require several infusions from different donors to achieve insulin independence.[62]

4.1.5.1 - Role of Tregs in Autoimmunity after Islet Transplantation

Autoimmunity has been shown to determine clinical success (e.g., insulin independence, graft survival) post-ITx.[52, 63-66] While the incidence of recurrent autoimmunity has not been thoroughly studied (or even properly defined), published case series comparing pre- and post-transplant cellular autoreactivity and/or antibody titers report *de novo*, recurrent and/or exacerbated autoimmunity in 18.7% to 62.5% of ITx patients.[52, 63, 64, 66] This contrasts with a low incidence of autoimmune recurrence following whole pancreas transplantation (6.6%); in this patient population, a decrease in antibody levels occurs in 17% of the cases.[67] A higher antigen load due to increased cell death immediately post-ITx as compared to pancreas transplant patients could explain these findings, however, this remains to be confirmed. The onset of recurrence and antibody-modification free time has also been shown to be positively correlated with graft survival and insulin-free time.[64] Importantly, no patient characteristics seem to be correlated with autoimmunity recurrence,[64, 66] albeit published studies are limited by small samples. Assessment of specific T cell populations (including Tregs) in clinical ITx and their relationship with autoimmunity recurrence has not been undertaken, nevertheless, two

important factors should be considered to contextualize the role of Tregs in autoimmunity after ITx. First, memory autoreactive T cells (e.g., $CD45RO^{+}$), which are central in T1D, are more resistant to drug- and Treg-mediated suppression compared to alloreactive T cells.[68] Second, drug-induced lymphopenia is compensated by homeostatic proliferation of the remaining lymphocytes.[69] This process, mainly mediated by IL-7 and IL-15, favors memory T cells and these cytokines decrease Tregs' suppressive capacities.[69-71] Indeed, IL-7Rα blockade has been shown to increase Treg:Teff ratios post T cell-depletion and led to indefinite islet graft survival in 80-100% of recipients in preclinical studies.[72] In clinical ITx, increased serum IL-7 and IL-15 correlate with post-transplant proliferation of autoreactive memory T cells and this response can be blocked *in vitro* with mycophenolate mofetil (MMF) and MMF plus sirolimus,[73] suggesting that homeostatic proliferation may be modulated with specific immunosuppressants. Harnessing specific aspects of homeostatic proliferation to promote tolerance in T1D and ITx, however, merits more exploration.

4.1.5.2 - Role of Tregs in Alloimmunity – Lessons from Solid Organ Transplantation

Several studies suggest that Treg-related biomarkers correlate with lower rates of rejection and improved patient and graft survival in solid organ transplantation (SOT).[74-77] A recent study found Treg values 1-year post-transplant to be particularly associated with death-censored graft survival.[78] However, studies are heterogeneous in their populations, clinical scenarios, methods of Treg assessment, tissues evaluated, and outcomes. Additionally, OT varies by organ; liver transplant recipients achieve it in 22 - 62.5%,[79-89] while kidney transplant recipients in 0.03% of the cases,[90] which may be related to each organ's immune microenvironment. For example, the liver is intrinsically tolerant to foreign antigens through

both hepatic stellate and Kuppfer cells[91] and has a Treg-nurturing environment involving a rich milieu of anti-inflammatory cytokines (e.g., IL-10, TGF-β);[92] less is known about the immune microenvironment in other solid organs. In pancreas transplantation, there is evidence suggesting that lower proportions of Tregs post-transplantation are associated with the appearance of *de novo* donor specific antibodies.[93] In this study, the use of alemtuzumab in simultaneous pancreas-kidney transplant patients was associated with lower Treg:Teff ratios as compared to basiliximab-treated kidney transplant patients. This study was limited by a small sample, highlighting the need for more research in this patient population. Treg-based therapies have been used to actively promote OT in solid organ transplantation, and published reports evaluating adoptive Treg transfer therapies (ATT) have consistently demonstrated safety **(Table 4.1.2).**[94-98] However, only one study using *ex vivo*-generated donor alloantigen reactive Tregs (darTregs) has achieved immunosuppression withdrawal, albeit in 7 of 10 liver transplant patients.[98] Thus, larger confirmatory studies are needed. The recent multicentric ONE Study, in which diverse cell products (including polyclonal Tregs and darTregs) were evaluated in kidney transplant recipients, showed promising results in terms of safety and immunosuppression withdrawal.[94] Specific reports of the "Treg-treated" arms of this trial could answer relevant questions regarding immunosuppression withdrawal, optimal dosing strategies and whether repeated infusions during follow-up are beneficial.

Table 4.1.2. Clinical trials evaluating Treg-based therapies in solid organ transplantation

Completed				
Author, year, country	**Organ**	**N**	**Intervention**	**Outcomes**
Sawitzki *et al.*, 2020 USA, England, France, Germany	Kidney	38 treated 66 controls	- Autologous *ex vivo*-expanded cell products, including 4 Treg products	- Demonstrated safety - Fewer infectious complications - No difference in acute rejection - 40% weaned from IS in treated group
Matthew *et al.*, 2018 USA	Kidney	9	- Autologous *ex vivo*-expanded PolyTregs - One dose of 0.5, 1.0, 5 x 10^9 cells/recipient	- No adverse events up to 2 years - IS withdrawal not attempted
Chandran *et al.*, 2017 USA	Kidney	3	- Autologous *ex vivo*-expanded PolyTregs - One dose of 31.9-36.9 x 10^7 cells/recipient	- No adverse events at 6 months - IS withdrawal not attempted
Sanchez-Fueyo *et al.*, 2020 England	Liver	9	- Autologous *ex vivo*-expanded PolyTregs - One dose of 0.5-1x10^6 or 3.5-4x10^6 cells/kg	- Demonstrated safety
Todo *et al.*, 2016 Japan	Liver (living donor)	10	- Autologous *ex vivo*-expanded darTregs (dose not specified)	- 7 IS-free for 16-33 months - 3 failed IS withdrawal
Ongoing				
Trial ID, Country	**Organ**	**N**	**Intervention**	**Estimated Completion**
NCT02739412 USA	Liver	7 (single arm)	*In vivo* Treg expansion using low dose interleukin-2	November 2020
NCT03284242 USA	Kidney	12 (single arm)	Autologous *ex vivo*-expanded PolyTregs in patients using everolimus	March 2021
NCT02711826 USA	Kidney	15 (per cohort)	Autologous *ex vivo*-expanded PolyTregs to reduce subclinical graft inflammation	October 2021
NCT04066114 USA	Kidney	10 (single arm)	*In vivo* Treg modulation with antagonists of CD28 and IL-6	October 2022
NCT03867617 Austria	Kidney	6 (per cohort)	Autologous *ex vivo*-expanded Tregs + donor bone marrow +	April 2023

			tocilizumab in HLA-mismatched recipients	
NCT03577431 USA	Liver	9 (single arm)	*Ex vivo* expansion of direct alloantigen reactive Tregs, targeting >2.5x10^6 cells given in single dose	March 2023
NCT03943238 USA	Kidney	22 (single arm)	Total lymphoid irradiation + ATG, followed by donor hematopoietic stem cells and autologous *ex vivo*-expanded PolyTregs in HLA-mismatched recipients	October 2024

IS: immunosuppression, ATG: anti-thymocyte globulin. Adapted from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev*. 2021;42(2):198-218.

4.1.5.3 - Role of Tregs in Alloimmunity – Efforts in Islet Transplantation

Despite reports of long-term outcomes in clinical ITx,[99-108] only a handful have evaluated its relationship with Tregs. The largest study assessing Tregs in clinical ITx included 42 patients with up to 2 years of follow-up after ITx and evaluated the effect of different induction immunosuppressants on Tregs. In this study, alemtuzumab transiently increased the proportion of $FOXP3^+$ cells (~70% of $CD4^+$ T cells at 1 month post-transplant), which was not observed with daclizumab or thymoglobulin (ATG). Unfortunately, this increase did not correlate with insulin independence.[109] Another report includes a female patient receiving a single ITx following two kidney transplants.[100] Induction consisted of ATG plus methylprednisolone, followed by maintenance with azathioprine, which was later switched to MMF. Insulin independence for this patient lasted > 10 years. At year 11, $CD4^+CD25^+FOXP3^+$ cells were significantly higher as compared to 5 healthy controls.[100] A recent case series[103] describes 10 patients receiving ATG and prednisone as induction, followed by maintenance with either efalizumab (EFA), which was later transitioned to combined sirolimus/MMF or MMF monotherapy, or Belatacept/sirolimus/MMF. Both groups (5 patients per group) showed

a 10-year insulin independence rate of 40%, but the EFA-treated group showed significant enrichment of Tregs compared to the Belatacept group. In the EFA-treated group, 1 patient achieved OT 5 years after transplantation, and remained insulin independent for up to 10 years; this patient had the highest proportion of Tregs from the whole cohort (67% of all $CD4^+$ T cells one-month post-transplant). To our knowledge, this is the only reported case of OT in ITx. EFA was also shown to improve insulin independence rates after single donor islet transplantation,[110] however, it was withdrawn from the market due to the potential risk of progressive multifocal leukoencephalopathy.

4.1.6 - Approaches for Regulatory T Cell Expansion

Higher Treg:Teff ratios promote operational tolerance.[16] Unfortunately, current immunosuppressants affect both Tregs and Teffs indiscriminately, maintaining the Treg:Teff ratio. An alternative is to increase the numerator of the Treg:Teff ratio through Treg expansion. This expansion can be done *ex vivo* or *in vivo*; the former can be antigen- or non-antigen-specific, and the latter can be systemic or localized. While seemingly straightforward, strategies for Treg expansion in T1D and ITx have yielded conflicting evidence.

4.1.6.1 - Ex vivo Treg expansion

Ex vivo Treg expansion entails Treg isolation, followed by cell- and/or cytokine-mediated stimulation to promote Treg-specific proliferation. Afterwards, expanded Tregs are reinfused to the patient (adoptive Treg transfer therapies, ATT). Although this process has been refined over the years, it is still limited by: 1) a lack of reproducibility,[111] 2) the time required

for expansion, 3) costs, and 4) the potential loss of the Tregs' regulatory/suppressive capacities.[111]

A central consideration with ATT is antigen-specificity. While non-antigen-specific or polyclonal Tregs (PolyTregs) are easier to expand, they induce a generalized, but not necessarily more effective, immunomodulatory response. Conversely, generating antigen-specific Tregs[16] limits their numbers, but increases their suppressive potency (up to 100-fold).[112, 113] Antigen-specific Tregs allow localized (at the site of antigen expression), more efficient and safer immunomodulation.[114] Recently, bioengineered Tregs expressing specific T/B cell receptors or chimeric antigen receptors (CARs) are surfacing as novel and more potent candidates to promote antigen-specific OT.[115]

4.1.6.1.1 - Ex vivo Treg Expansion in Type 1 Diabetes

Ex vivo Treg expansion and ATT induces long-lasting remission of T1D in preclinical models.[116, 117] Unfortunately, clinical experience has been more limited. Dr. Trzonkowski *et al.* first evaluated ATT in T1D.[34] In this trial, 10 children with newly-onset (2 months) T1D were treated with *ex vivo*-expanded autologous PolyTregs with no adverse effects. One year after infusion, two patients remained off insulin, compared to none of the controls, and higher C-peptide levels and lower insulin dosages were observed.[34, 35] Interestingly, a moderate correlation with Treg percentages and C-peptide levels was documented.[35] Dr. Bluestone and Dr. Tang's group, building on solid preclinical evidence,[118, 119] further confirmed safety of autologous PolyTregs in patients with newly-onset (3-24 months) T1D. Importantly, these researchers demonstrated preserved function, phenotype stability and up to 1-year half-lives of infused Tregs.[33] Finally, a short report evaluating autologous PolyTregs (two doses three

months apart) in 36 children with newly diagnosed T1D showed stable C-peptide levels and better glycemic control for up to 12 months, as compared to controls.[120] These outcomes further improved with rituximab,[120] suggesting that low-degree immunosuppression may be synergistic with ATT.

Ongoing trials assessing ATT in T1D (NCT02691247, NCT02932826, NCT02772679, NCT03011021) will strengthen the evidence and direct future research to define aspects such as the optimal timing of infusion, the benefit of "Treg-friendly", low-degree, immunosuppression and/or Treg-supporting cytokines, the need for antigen-specificity, and the role of recipient conditioning to enable optimal Treg engraftment (e.g., myeloablative and non-myeloablative immunodulation).[121, 122]

4.1.6.1.2 - Ex vivo Treg Expansion in Islet Transplantation

Despite promising data in HSCT[123] and SOT (**Table 2**), and the wealth of preclinical evidence in ITx showing benefit with Treg-based therapies,[32, 124-126] there is only one ongoing clinical trial evaluating ATT in ITx (NCT03444064). In this study, patients will receive one dose of 4-6 x 10^6 autologous *ex vivo* expanded PolyTregs 6 weeks after infusion. Induction immunosuppression with alemtuzumab, etanercept and anakinra, and maintenance immunosuppression with low dose tacrolimus + sirolimus will be indicated. This study could provide vital information about how Tregs modulate both auto and alloimmunity in the same patient. Results from this study are awaited and are expected to further contribute to our understanding of Treg-based therapies in ITx.

4.1.6.2 - In-vivo Treg expansion

Many interventions in preclinical models have allowed pronounced *in vivo* Treg expansion and, subsequently, prevention, delay and even reversal of T1D.[32] Similarly, in ITx preclinical evidence shows that *in vivo* Treg expansion enables prolonged, and sometimes indefinite, graft survival.[127] However, clinical translation has been hampered by suboptimal Treg expansion, cumbersome dosing strategies, adverse effects, and undesired consequences from concomitant immunosuppression strategies. Promising strategies and future research avenues will be discussed in this section.

4.1.6.2.1 - In vivo Treg Expansion in T1D

Clinical efforts to prevent, delay or reverse T1D have included approaches such as conventional immunosuppression (i.e., induction and maintenance immunosuppressants),[128, 129] HSCT,[130] islet antigen-based immune modulation,[131] BCG vaccination,[132-134] vitamin D supplementation,[135-139] anti-CD3 antibodies,[140-145] rituximab,[146, 147] imatinib (NCT01781975), etanercept,[148] anakinra,[149] abatacept,[150] alefacept,[151, 152] IL-7 blockade,[153] and IL-2 administration.[154-157] A comprehensive review on immunotherapies in T1D is beyond the scope of this article, however, aspects regarding *in vivo* absolute or relative Treg expansion will be discussed.

Most immunotherapies are not Treg-focused, thus, Treg assessment and reporting is frequently not undertaken. However, those including this information reveal the Tregs' potential relationship with improved clinical outcomes. For example, HSCT in patients with T1D has allowed sustained insulin independence for up to 9 years after treatment.[158] Importantly, Treg numbers are higher in patients achieving prolonged T1D remission after HSCT, compared to

those with short remission.[158] This has also been observed following HSCT for other autoimmune diseases,[159] suggesting that Treg numbers correlate with the degree of restoration of self-tolerance. Studies exploiting islet antigen-based immune modulation have shown that administration of islet autoantigens or peptides leads to more Treg-predominant immune responses in patients with T1D.[160] These efforts contribute to other clinical trials showing that administration of proinsulin peptide epitopes in patients with long-standing T1D can induce $CD4^+$ cells with a regulatory phenotype (i.e., increased IL-10 production).[161] In patients with new-onset T1D, administration of these peptide epitopes has been shown to increase FOXP3 expression levels in Tregs obtained from responders (C-peptide levels during follow-up equal or higher than baseline) as compared to non-responders.[162] Importantly, these therapies enabled significantly slower disease progression as compared to non-responders and no adverse effects, which are highly encouraging findings that support future efforts into these research avenues. Additionally, recent preclinical evidence in humanized mouse models has shown that stable and specific human $FOXP3^+$ cells can be induced *in vivo* using engineered insulin mimetopes, strengthening the possibility for vaccination to prevent T1D.[163] Equally promising results have been recently reported by the Type 1 Diabetes TrialNet ATG-GCSF Study group.[128] In this two-year follow-up report, patients receiving low-dose ATG showed higher stimulated C-peptide responses and lower HbA1c levels compared to placebo;[128] these results were positively correlated with higher Treg:Teff ratios and other Treg-related parameters. Since ATG is currently used as induction immunosuppression in patients undergoing ITx, these results are highly relevant to this patient population and will be addressed in sections below. Alefacept, a fusion protein that binds to CD2, was shown to increase Treg:Teff ratios,[151, 152] preserve C-peptide secretion, decrease insulin use and lower hypoglycemic events in patients with T1D for

up to 24 months of follow-up. Finally, a monoclonal antibody blocking the IL-7R receptor α (RN168) increased Treg:Teff ratios in patients with T1D, however, this study was not powered to evaluate metabolic control.[153] Of note, alefacept and RN168 increased Treg:Teff ratios mainly by decreasing Teffs, which suggests a Treg-sparing effect with both interventions.

IL-2-based immunotherapies are one of the few focusing specifically on *in vivo* Treg expansion.[154-156] Low-dose IL-2 therapy has been well-tolerated and dose-dependent Treg expansion and suppression of Teff responses have been observed.[154-156] In these studies, metabolic control remained stable, however, only slight improvements in C-peptide and HbA1c levels were reported.[156] Adequately powered and longer follow-up studies are ongoing (NCT02411253, NCT03782636, NCT02411253). Additionally, IL-2-based immunotherapies may prove synergistic when combined with other therapies and trials exploring this possibility are also ongoing (NCT04279613, NCT02772679). Of note, IL-2-based therapies should consider appropriate dosing, since high-dose IL-2 has been shown to transiently impair β-cell function in patients with T1D.[164]

4.1.6.2.2 - In vivo Treg expansion in Islet Transplantation – The Role of Conventional Immunosuppressants

While cellular therapies could allow immunosuppression-free transplantation in the future, a shift towards a more "Treg-centric" view of immunosuppression may help preventing graft rejection or even achieving operational tolerance in the present. This paradigm entails exploiting specific immunosuppressants with Treg-sparing/expanding properties.[165] This section synthesizes the available evidence with induction immunosuppression commonly used in clinical ITx, including anti-CD25 antibodies (daclizumab and basiliximab), anti-CD52

antibodies (alemtuzumab), thymoglobulin, and anti-CD3 antibodies. Maintenance immunosuppression will also be discussed, including studies with calcineurin inhibitors (CNIs), mTOR inhibitors, MMF, and costimulatory blockers.

Anti-CD25 antibodies are believed to negatively impact Treg numbers and function given their high expression of CD25. However, a study in patients undergoing ITx has challenged this notion by showing that induction with daclizumab (in combination with ATG, methylprednisolone and etanercept) in fact increased $FOXP3^+$ cells, which was sustained for up to a year of follow-up.[166] Whether other immunosuppressants compensated IL-2 blockade and/or whether Tregs can circumvent IL-2 blockade by other mechanisms (e.g., overexpressing the IL-7Rα),[69] was not assessed in this study. Alemtuzumab has significantly improved outcomes in clinical ITx.[167, 168] It depletes Teffs over Tregs, promotes Teffs conversion to Tregs, and increases anti-inflammatory cytokines associated with Treg survival and function.[109, 169] However, its Treg-favoring properties have not correlated with improved clinical outcomes, suggesting that most of its efficacy may be explained by its potent T-cell depleting effect. ATG (both rabbit- and equine-derived) is a potent and long-lasting "pan-lymphocyte-depleting" agent.[170] ATG favors Tregs during homeostatic proliferation by promoting faster Treg recovery and inducing other regulatory cells (e.g., Tr1 cells).[165, 171] However, dosing considerations are important with ATG, as low doses have shown to increase Treg:Teff ratios, maintain C-peptide secretion and decrease HbA1c levels after a two-year follow-up in patients with T1D,[128] while high-doses have opposite effects.[128] Interestingly, in the previously mentioned ATG TrialNet study, low-dose ATG was associated with increased Treg:Teff ratios for up to 24 weeks post-treatment, a persistent increase in Tregs with memory phenotypes ($CD45RO^+$) and increased percentage of Tregs expressing TIGIT as compared to placebo. These changes were correlated

with 2-year stimulated C-peptide responses.[128] Anti-CD3 antibodies have shown success in delaying onset of T1D in high-risk subjects[144, 145] and higher rates of insulin independence in clinical ITx.[167] Despite preclinical studies suggesting a Treg-sparing effect,[172, 173] this has been recently challenged.[144, 145] However, experience with anti-CD3 antibodies remains limited[167, 174], which should motivate future clinical trials using this agent that are accompanied with a comprehensive Treg assessment.

CNIs directly inhibit Treg activation and FOXP3 expression and indirectly inhibit Treg proliferation through decreased IL-2 production.[171] These effect on Tregs may be compensated by other processes enabling efficient and relatively safe immunosuppression, which explains its frequent use and favorable profile in clinical ITx.[4] Conversely, MMF and sirolimus represent "Treg-friendly" alternatives to CNIs.[171] Sirolimus was a key addition to the Edmonton protocol,[3] which was partly motivated by its minimal nephrotoxicity and lack of diabetogenicity as compared to CNIs. Indeed, many reports on long-term insulin independence rates using sirolimus as maintenance immunosuppression have shown no detrimental effect on metabolic outcomes.[99-108, 167, 175] However, adverse effects[101, 176] and/or local practices, have led to a decrease in the use of sirolimus in clinical ITx.[177] Importantly, in patients with T1D, sirolimus increases Tregs' suppressive capacities[178] and, in combination with IL-2 therapy, it leads to transient Treg expansion.[164, 179] *In vivo* Treg expansion with sirolimus has been corroborated in kidney transplantation, however, whether this correlates with improved clinical outcomes remains controversial.[180]

MMF, either as monotherapy or in combination with Daclizumab, has shown no effect in halting disease progression in patients with T1D,[181] albeit its effect on Treg numbers or function has not been assessed. *In vitro* studies using human cells suggest that MMF favors

Treg homeostasis and function and promotes Treg-predominant responses through inhibition of Th1 and Th17 effector cells[182] and, despite the lack of clinical evidence corroborating these phenomena in ITx, preclinical models show that MMF can expand Tregs *in vivo* and promote islet allograft tolerance.[183] These notions are also supported by clinical studies in liver and kidney transplantation.[184-186] A more comprehensive picture on the immunoregulatory properties of MMF, including Treg-related parameters, is expected to emerge as its use in clinical ITx increases.[62]

Costimulatory blockade with abatacept has been shown to prevent β-cell function decay, improved HbA1c levels, and slightly lowered insulin doses in patients with T1D.[150] Unfortunately, only a 5-patient case series using belatacept as maintenance immunosuppression after ITx has been published.[103, 187, 188] In these reports, belatacept (coupled with sirolimus and MMF) allowed long-term insulin independence after a single infusion in 5/5 patients, with 2/5 remaining insulin independent after 10 years.[103, 187] However, no changes in the percentage of peripheral Tregs were observed.[187] This coincides with evidence suggesting that Tregs are more susceptible to costimulatory blockade than Teffs, given their constitutive expression of CTLA4 (and CD28),[26] and that proper costimulatory signaling maintains stable peripheral Treg reservoirs and stable FOXP3 expression.[189, 190] Alternatively, some studies show that costimulation blockade, while affecting peripheral Treg numbers, increases Tregs at the tissue level.[191, 192] These and other conflicting aspects around costimulatory blockade, as well as investigation of other pathways (e.g., CD40/CD40L [anti-CD154], LFA-1, etc.) and their effects on Treg physiology should be further explored.

4.1.7 - Are Regulatory T Cells Safe?

Treg-based therapies have shown periprocedural safety and low morbidity in clinical trials.[122] The ONE Study even found fewer infectious complications with Treg-based therapies as compared to controls.[94] However, Treg-mediated tolerance relies on phenotype stability, and concerns regarding their potential for conversion to pathogenic Teffs in strong inflammatory, cytokine-rich contexts have arisen.[193] These pathogenic Teffs ("ex-Tregs") could exacerbate immune responses.[194, 195] Thus, optimal timing for interventions should always account for the inflammatory context. Indeed, preclinical[196] and clinical[98] studies show that operational tolerance can be achieved when ATT is performed after surgery-related inflammation has abated. Similarly, in preclinical models of ITx, *in vivo* Treg expansion before transplantation leads to indefinite graft survival, while synchronous *in vivo* expansion does not.[127]

Another concern is that, due to their immunosuppressive capacities, Tregs could also impair antitumor immunity. Tregs can be recruited to tumors through cytokine-mediated mechanisms (e.g., TGF-β, VEGF, GCSF),[197] and these cells favor tumor development, growth, and also protection from the immune system.[198] Thus, Treg-based therapies could be synergistic with drug-induced immunosuppression and further predispose to neoplasms.[199] As these and other cell-based therapies move forward, larger studies with longer follow-up will define their oncogenic potential.

4.1.8 - The Future of Regulatory T Cells in Type 1 Diabetes and Islet Transplantation

Many questions regarding the use of Tregs in T1D and ITx remain unanswered and there are several research avenues that remain to be fully explored. First, Treg-related tolerance signatures need to be refined and optimized to allow individualized patient follow-up. Cytokine-

based immune profiling and monitoring offers an alternative for this.[200] Periodic measurement of IL-2 (with or without concomitant IL-2 administration) and other cytokines involved in Treg modulation (e.g., IL-7, IL-10) to maintain "trough-levels" could be a potential approach.[201] Second, genetic editing/engineering could help overcome intrinsic or acquired defects impairing Treg survival, phenotype stability and regulatory capacities.[202, 203] CRISPR/Cas9 opens the possibility to modify genes involved in Treg physiology to enhance their suppressive capacity[204-207] and/or promote their expansion through *FOXP3* stabilization and upregulation.[205, 207-210] Alternatively, epigenetic regulation of *FOXP3* expression may also allow stability of the Treg phenotype.[208, 211] In terms of antigen-specificity, gene therapy in combination with epitope design algorithms[212] could facilitate generation of antigen-specific Tregs.[202] Finally, clinical trials in T1D and ITx should further explore localized Treg expansion or directed migration. Given that Teffs infiltrate grafts earlier than Tregs,[213] Treg-Islet coaggregation with/without localized T-cell depletion could reduce the number of Tregs needed for operational tolerance.[214-216] Conversely, directed Treg migration may be achieved by incorporating chemotactic factors, such as CCL22 or CXCL12, into islets or within encapsulation biomaterials. This could allow indefinite graft survival,[217-219] even without immunosuppression.[220, 221]

4.1.9 - Conclusions

In the past decades, a comprehensive framework around many aspects of Treg function in health and disease has been evolving. Preclinical studies in T1D and ITx show promising results with Treg-based therapies and reports of long-term diabetes reversal and operational tolerance are exceedingly frequent. In the clinic, Treg-based therapies have proven to be safe,

however, adequately powered, multicentric, long-term clinical trials are still needed to further characterize their efficacy in terms of clinically-relevant and patient-oriented outcomes. ITx remains an experimental procedure in many countries, thus, evidence supporting a role for Tregs in this scenario has been mainly extrapolated from other contexts (e.g., HSCT and SOT). Moving forward, we urge researchers to systematically evaluate and report Treg-related parameters and biomarkers as a part of detailed clinical trial follow-up. While complex and novel techniques, such as single-cell RNA-sequencing, cell-free DNA testing, or exosome composition analyses, may provide valuable information on various aspects of the immune response after transplantation, these technologies are not widely available yet. Thus, we suggest a framework focusing on four basic aspects during clinical follow-up (**Table 4.1.3**) including 1) comprehensive and standardized flow cytometry studies for whole blood immunophenotyping [222], 2) functional *in vitro* suppression assays [223], 3) detailed and accurate information regarding pharmacological immunosuppression to deepen knowledge regarding the specific effect of immunosuppressants in immune cells, including Tregs, and 4) comprehensive clinical information on relevant clinical outcomes. Naturally, multidisciplinary teams that truly embody the principles of translational research are needed to orchestrate these ambitious, but much-needed efforts**.** Islet transplantation provides a unique opportunity to study how Tregs could modulate both auto and alloimmunity within the same patient. This knowledge will enable discovery of precise aspects of Treg physiology that could allow personalized interventions that enable immune modulation in many contexts. This opens the possibility of a cure for many autoimmune diseases, such as T1D, and at the same time, to transplantation without immunosuppression.

Table 4.1.3. Proposed framework for follow-up of patients undergoing islet transplantation

Immunophenotyping	• Standardized flow cytometry protocols and reporting[222] ○ Baseline and follow-up assessments • Comprehensive analysis of Tregs and other relevant immune cells (e.g., dendritic cells, NK cells, B cells, etc.)
Suppression assays	• *In vitro* suppression assays to establish a link with immunophenotyping and clinical data[223]
Pharmacological immunosuppression	• Information on induction and maintenance regimes • Trough levels and changes in immunosuppression regimes during follow-up • Degree of adherence to immunosuppression regimes

Clinical outcomes	• Baseline and follow-up assessments of:
	  ○ Clinically relevant variables
	  ▪ Glycemic control (e.g., fasting glycemia, HbA1c levels, glucose tolerance tests)
	  ▪ Hypoglycemic events
	  ▪ Preservation/loss of C-peptide and rate of decline, including stimulated C-peptide measurements
	  ▪ Insulin usage
	  ▪ Diabetes-related complications
	  ○ Immunological status
	  ▪ Autoantibodies and donor-specific antibodies

Adapted from Marfil-Garza BA, Hefler J, Bermudez De Leon M, et al. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. *Endocr Rev*. 2021;42(2):198-218.

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CHAPTER 4

PART 2 - TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 25 (TNFRSF25) AGONISTS IN ISLET TRANSPLANTATION: ENDOGENOUS *IN VIVO* REGULATORY T CELL EXPANSION PROMOTES PROLONGED ALLOGRAFT SURVIVAL

CHAPTER 4, PART 2 - TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 25 (TNFRSF25) AGONISTS IN ISLET TRANSPLANTATION: ENDOGENOUS *IN VIVO* REGULATORY T CELL EXPANSION PROMOTES PROLONGED ALLOGRAFT SURVIVAL

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ORIGINAL ARTICLE

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Tumor necrosis factor receptor superfamily member 25 (TNFRSF25) agonists in islet transplantation: Endogenous in vivo regulatory T cell expansion promotes prolonged allograft survival

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Regulatory T cells (Tregs) modulate alloimmune responses and may facilitate minimization or withdrawal of immunosuppression posttransplant. Current approaches, however, rely on complex ex vivo Treg expansion protocols. Herein, we explore endogenous in vivo Treg expansion through antibody-mediated agonistic stimulation of the tumor necrosis factor receptor superfamily member 25 (TNFRSF25) pathway and its potential to prolong graft survival in a mouse model of islet allotransplantation. C57BL/6 male mice were treated with a single dose of TNFRSF25 agonistic antibodies (4C12 or mPTX-35) or IgG control. Diabetes was induced using streptozotocin. Four days later, flow cytometry was completed to corroborate Treg expansion, and 500 islets (CBA/J male mice) were transplanted. Glycemia was assessed thrice weekly until rejection/endpoint. Early intra-graft Treg infiltration was assessed 36 h post-transplant. TNFRSF25 antibodies enabled pronounced Treg expansion and treated mice had significantly prolonged graft survival compared with controls ($p < .001$). Additionally, the degree of Treg expansion significantly correlated with graft survival ($p < .001$). Immunohistochemistry demonstrated marked Treg infiltration in long-term surviving grafts; intra-graft Treg infiltration occurred early posttransplant. In conclusion, a single dose of TNFRSF25 antibodies enabled in vivo Treg expansion, which promotes prolonged graft survival. TNFRSF25-mediated in vivo Treg expansion could contribute to achieving lasting immunological tolerance in organ transplantation.

KEYWORDS
basic (laboratory) research / science, translational research / science, endocrinology / diabetology, immunosuppression / immune modulation, islet transplantation, immune regulation, immunosuppressant - fusion proteins and monoclonal antibodies, immunosuppressant - fusion proteins and monoclonal antibodies, costimulation molecule specific, tolerance, experimental, rejection, T cell mediated (TCMR)

Abbreviations: ACT, adoptive cell transfer; DR3, death receptor 3; MHC, major histocompatibility complex; STZ, streptozotocin; T1D, Type 1 diabetes mellitus; TCR, T cell receptor; TLA1, tumor necrosis factor-like cytokine 1A; TNFR1, tumor necrosis factor receptor 1; TNFRSF25, tumor necrosis factor receptor superfamily member 25.

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Title: Tumor Necrosis Factor Receptor Superfamily Member 25 (TNFRSF25) Agonists in Islet Transplantation: Endogenous *In vivo* Regulatory T Cell Expansion Promotes Prolonged Allograft Survival

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4.2.1 – Abstract

Regulatory T cells (Tregs) modulate alloimmune responses and may facilitate minimization or withdrawal of immunosuppression post-transplant. Current approaches, however, rely on complex *ex vivo* Treg expansion protocols. Herein, we explore endogenous *in vivo* Treg expansion through antibody-mediated agonistic stimulation of the tumor necrosis factor receptor superfamily member 25 (TNFRSF25) pathway and its potential to prolong graft survival in a mouse model of islet allotransplantation. C57BL/6 male mice were treated with a single dose of TNFRSF25 agonistic antibodies (4C12 or mPTX-35) or IgG control. Diabetes was induced using streptozotocin. Four-days later, flow cytometry was completed to corroborate Treg expansion and 500 islets (CBA/J male mice) were transplanted. Glycemia was assessed thrice weekly until rejection/endpoint. Early intra-graft Treg infiltration was assessed 36-hours post-transplant. TNFRSF25 antibodies enabled pronounced Treg expansion and treated mice had significantly prolonged graft survival compared to controls ($p<0.001$). Additionally, the degree of Treg expansion significantly correlated with graft survival ($p<0.001$). Immunohistochemistry demonstrated marked Treg infiltration in long-term surviving grafts; intra-graft Treg infiltration occurred early post-transplant. In conclusion, a single dose of TNFRSF25 antibodies enabled *in vivo* Treg expansion, which promotes prolonged graft survival. TNFRSF25-mediated *in vivo* Treg expansion could contribute to achieving lasting immunological tolerance in organ transplantation.

4.2.2 - Introduction

Pancreatic islet transplantation offers promise as an effective and potentially curative therapy for type 1 diabetes (T1D). In T1D, pancreatic β-cells in the islets of Langerhans are destroyed by the patient's own immune system, leading to insulin deficiency.[1] While insulin therapies are life-saving, they increase the risk of hypoglycemia.[2] Patients suffering from intractable severe hypoglycemia benefit greatly from islet transplantation. This therapy has advanced substantially since the first successful transplant over 40 years ago, and current evidence shows safety and efficacy in abrogating severe hypoglycemia, but also in providing optimal longer-term glycemic control.[3] However, recipients require lifelong immunosuppression to prevent rejection, which is a major limitation for the widespread use of this therapy.

Immunomodulatory cell therapies could diminish or abrogate the need for chronic immunosuppression post-transplant. Regulatory T cells (Tregs) are the most studied. Importantly, Treg-related biomarkers (e.g., FOXP3 transcripts, $FoxP3^+$ cells, DNA methylation of the FOXP3 gene locus) have been proposed as tolerance signatures to identify patients in which immunosuppression minimization or withdrawal could be attempted.[4] This has promoted the use of cell-based products, including Tregs, in clinical transplantation.[5-7] Clinical trials evaluating adoptive cell transfer (ACT) of *ex vivo*-expanded Tregs in T1D show safety and potential to delay progression of the disease.[8-12] However, these approaches rely on complex and costly *ex vivo* Treg expansion protocols to obtain sufficient cell numbers. While *ex vivo* expansion protocols continue to be refined and improved, these remain prohibitively expensive and logistically cumbersome. Thus, novel strategies to achieve endogenous *in vivo* Treg expansion without a need for a laboratory phase are actively being researched.

In this study, we explored *in vivo* Treg expansion through agonistic stimulation of the tumor necrosis factor receptor superfamily member 25 (TNFRSF25, *a.k.a.* death-receptor 3 (DR3)) pathway. TNFRSF25 is constitutively expressed on Tregs, and studies have previously reported *in vivo* Treg expansion using agonistic monoclonal TNFRSF25 antibodies.[13] Our primary objective was to evaluate whether *in vivo* Treg expansion using these antibodies could delay or prevent graft rejection in a preclinical mouse model of allogeneic pancreatic islet transplantation. Our secondary objective was to assess the dynamics of Treg expansion and whether systemic Treg expansion correlated with local intra-graft Treg infiltration.

4.2.3 - Material and Methods

4.2.3.1 - Diabetes Induction and TNFRSF25 antibodies administration

Diabetes was induced four days prior to transplantation by intraperitoneal (IP) injection of 175 mg/kg of streptozotocin (STZ; Sigma-Aldrich, ON, Canada) in acetate buffer, pH 4.5 (Sigma-Aldrich, ON, Canada). C57BL/6 (H-2k^{b}) male (10 to 16-week-old) mice were considered diabetic following a non-fasting blood glucose measurement ≥15.0 mmol/L on two consecutive days. Following STZ injection, mice were injected IP in a randomized order with a single dose (0.9 mg/kg) of either 4C12 (Biolegend®, clone: 4C12, Cat#:144412), mPTX-35 (provided by Pelican Therapeutics, Inc.), or Hamster IgG control (BioXcell®, Cat#:603-298-8564). mPTX-35 was produced as a surrogate mouse antibody to harbor mouse IgG1 heavy chains fused to the variable regions of PTX-35, a humanized clinical-grade TNFRSF25 agonistic antibody. The mouse surrogate antibody was required to test human PTX-35 activity *in vivo* as cross-linking is required for target trimerization (TNFRSF25). PTX-35 is the humanized, affinity matured version of the hamster TNFRSF25 antibody, 4C12. For Treg depletion, an anti-CD25 depleting antibody (BioXcell®, clone: PC-61.5.3, Cat#:BE0012) was administered IP at day 4 and 2 pre-transplant, at 400 μg/dose (200 μL/dose in phosphate-buffered saline [PBS]).

Donor and recipient mice were housed under conventional conditions with food and water access *ad libitum* and their care was in accordance with guidelines approved by the Canadian Council on Animal Care. This study was approved by the University of Alberta's Animal Care and Use Committee. Reporting of this study was done following the ARRIVE guidelines.[14]

4.2.3.2 - Islet Isolation and Transplantation

Pancreatic islets were isolated from 10- to 16-week-old CBA/J male mice (H-2k^k), as previously described[15]. Exocrine tissue and lymph nodes were removed from the preparation to ensure optimal purity (>90% $\pm$ 5%). Islet counting was done as previously described[16]. The islet preparation was separated into tubes with ~500 islets $\pm$ 10% and transplanted under the kidney capsule of C57BL/6 diabetic mice, as previously described.[15, 17] Institutional guidelines for perioperative care, anesthesia and pain management were followed.

To evaluate early local Treg infiltration mice were euthanized at 36-hours post-transplant and grafts collected. Tissues were processed as detailed below.

4.2.3.3 - Glycemic Control, Diabetes Reversal and Rejection

Glycemic control was assessed using non-fasting blood glucose measurements (mmol/L) thrice weekly after transplantation using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada). Diabetes reversal was defined as two consecutive readings < 11.1 mmol/L. Rejection was defined as two consecutive-day glucose measurements $\geq$ 15 mmol/L. Islet grafts were retrieved by total nephrectomy $\leq$ 24 hours following rejection.[15]

4.2.3.4 - Flow cytometry

Approximately 80 µL of blood were obtained from the tail vein. Approximately 20 µL of heparin and flow cytometry buffer (FACS buffer: phosphate-buffered saline (PBS), 2 mM EDTA, 1% bovine serum albumin) was added to the sample and placed on ice. Approximately 35 µL were added to each flow cytometry tube. Cell suspensions were stained using the following cell-surface antibodies targeting mouse epitopes: TCR-β*eFluor®450

(Invitrogen/eBioscience™, clone: H57-597, Cat#:48-5961-82, concentration: 0.2 µg/sample), CD4*FITC (Invitrogen/eBioscience™, clone: RM4-5, Cat#:11-0042-85, concentration: 0.5 µg/sample), CD8*NovaFluor Yellow 730 (Invitrogen/eBioscience™, clone:53-6.7, Cat#:M003T02Y07, concentration: 0.1 µg/sample), CD25*PE (Invitrogen/eBioscience™, clone: PC61.5, Cat#:12-0251-83, concentration: 0.2 µg/sample), NK1.1*APC (Invitrogen/eBioscience™, clone:PK136, Cat#: 17-5941-82, concentration: 0.2 µg/sample). After cell-surface incubation, RBC lysis buffer was added (8.02 g/L NH^4Cl, 0.1 g/L $KHCO_3$, 0.04 g/L Na_2EDT, pH 7.2-7.4 in L of MiliQ water), followed by a PBS wash, and permeabilization using FoxP3 staining buffer sets from eBioscience (Cat#:00-5523-00) following the manufacturer's instructions. Intracellular FoxP3 staining was done using FoxP3*AF647 (Biolegend®, clone: MF-14, Cat#:126408, concentration: 0.5 µg/sample). Isotype controls were used to run fluorescence-minus-one samples and set the gating strategy. Compensation (Invitrogen UltraComp Beads™, Cat#:01-2222-42, concentration: 35:100) and counting beads (123count™ eBeads counting beads, Invitrogen, Cat#:01-1234-42, concentration: 50 µL/300 µL of final volume) were also used. Islet grafts were processes as follows: 1) Graft was excised from the kidney using microscissors and placed on flow cytometry tubes containing cold RPMI 1640 media (Gibco Life Technologies Limited, Paisley, UK), 2) The graft was gently pipetted up and down 10 times using a 1000p pipette to dislodge cells from the kidney capsule, 3) The tube was gently vortexed and centrifuged at 360g for 5 min, 4) Supernatant was decanted and 10mM EDTA in PBS was added to the tube, 5) The graft was gently pipetted up and down 10 times using a 1000p pipette, 6) The tube was gently vortexed and centrifuged at 360G for 5 min, 7) Supernatant was removed and warm (37ºC) TrypLE™ express (Gibco Life Technologies Corporation, Grand Island, NY, USA) diluted in PBS

(concentration 1:3, 2mL/tube) was added, 8) Cells were gently pipetted up and down using a 1000p pipette ~10 times per minute for 10 minutes to further dislodge cells from the kidney capsule, 9) The tube was topped with 2% fetal bovine serum (FBS) in PBS to quench the enzyme, 10) The solution was put through a 70 μm cell strainer into a new flow cytometry tube, 11) The tube was centrifuged at 360G for 5 min, the supernatant was decanted and cells resuspended in FACS buffer. Flow cytometry staining continued, as described above. Samples were analyzed on a BD LSRFortessa X20 flow cytometer. Data were analyzed using FlowJo™ v10.0 (Ashland, OR: Becton, Dickinson and Company).

mPTX-35 dosing studies were conducted by Pelican Therapeutics, Inc. C57BL/6 mice were injected IP with 0.001, 0.01, 0.1, 1, or 10 mg/kg mPTX-35. On day 4, 7, 9, 11, and 14 post-injection single cell suspensions were generated from the thymus, spleen, and pancreatic lymph nodes (PLN) by placing organs over a 100 μm cell strainer then homogenizing with the rubber stopper of a syringe. Cells were pelleted at 340 rcf for 5 minutes. Red blood cells (RBC) were lysed in the spleen samples using RBC lysis buffer (Sigma-Aldrich, Cat#:11814389001). After 1 minute, 8.5 mL of PBS was added and cells pelleted. Cell surface antibody staining was completed using the following reagents targeting mouse epitopes (purchased from Biolegend®): anti-TCRβ*PE (clone: H57-597, Cat#:109207, concentration: 0.27 μg/sample), CD4*BV650 (clone: RM4-5, Cat#:100545, concentration: 0.1 μg/sample), CD25*PE-Cy7 (clone: PC61, Cat#:102015, concentration: 0.07 μg/sample). Intracellular protein staining was completed as done for blood samples, probing for Foxp3*AF488 (clone: FJK-16s, Cat#:53-5773-82, concentration: 1 μg/sample) and KI67*APC (clone:16A8, Cat#:652406, concentration: 0.4 μg/sample). Samples were acquired on a Beckman Coulter CytoFlexS instrument and analyzed using FlowJo™ v10.0.

4.2.3.5 - Fluorescence Immunohistochemistry

Kidneys were transected such that each half contained part of the islet graft, fixed in 10% formalin and then embedded in paraffin. The tissue was cut in 4 μm sections that were rehydrated and heat-treated for antigen retrieval for 20 min in citrate buffer. Then, they were blocked with 20% goat serum for 1 hour (Sigma-Aldrich, St. Louis, MI, USA). After blocking, sections were treated with FoxP3 primary antibodies (Novus Biologicals, Cat#:NB100-39002, concentration: 1:400) diluted in 5% normal goat serum (NGS), and incubated overnight at 4ºC. The next day, sections were washed with PBS, and secondary goat anti-rabbit antibodies (Invitrogen AlexaFluor® 568, Cat#:A11036, concentration: 1:200) diluted in 5% NGS were added and incubated for 1-hour at room temperature. Sections were washed and primary guinea pig anti-insulin antibodies (Dako, Mississauga, ON, Canada, Cat#:A0564, concentration: 1:100) diluted in 5% NGS were added and incubated for 1-hour at room temperature. Secondary FITC-conjugated goat anti-guinea pig antibodies (FITC, Jackson ImmunoResearch, West Grove, PA, USA, Cat#: 106-095-003, concentration: 1:200) diluted in 5% NGS were added and incubated for 1-hour at room temperature. Afterwards, sections were washed with PBS and counterstained with DAPI (ProLong Gold DAPI, Invitrogen, Calrsbadm CA, USA).

4.2.3.6 - Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) or as medians and interquartile ranges (IQR) as determined by normality using the Shapiro-Wilks test. Between-groups differences were analyzed using paired and unpaired *t*-tests, Wilcoxon matched-pairs signed rank tests or Mann-Whitney test, and one-way ANOVAs or Kruskal-Wallis test, as

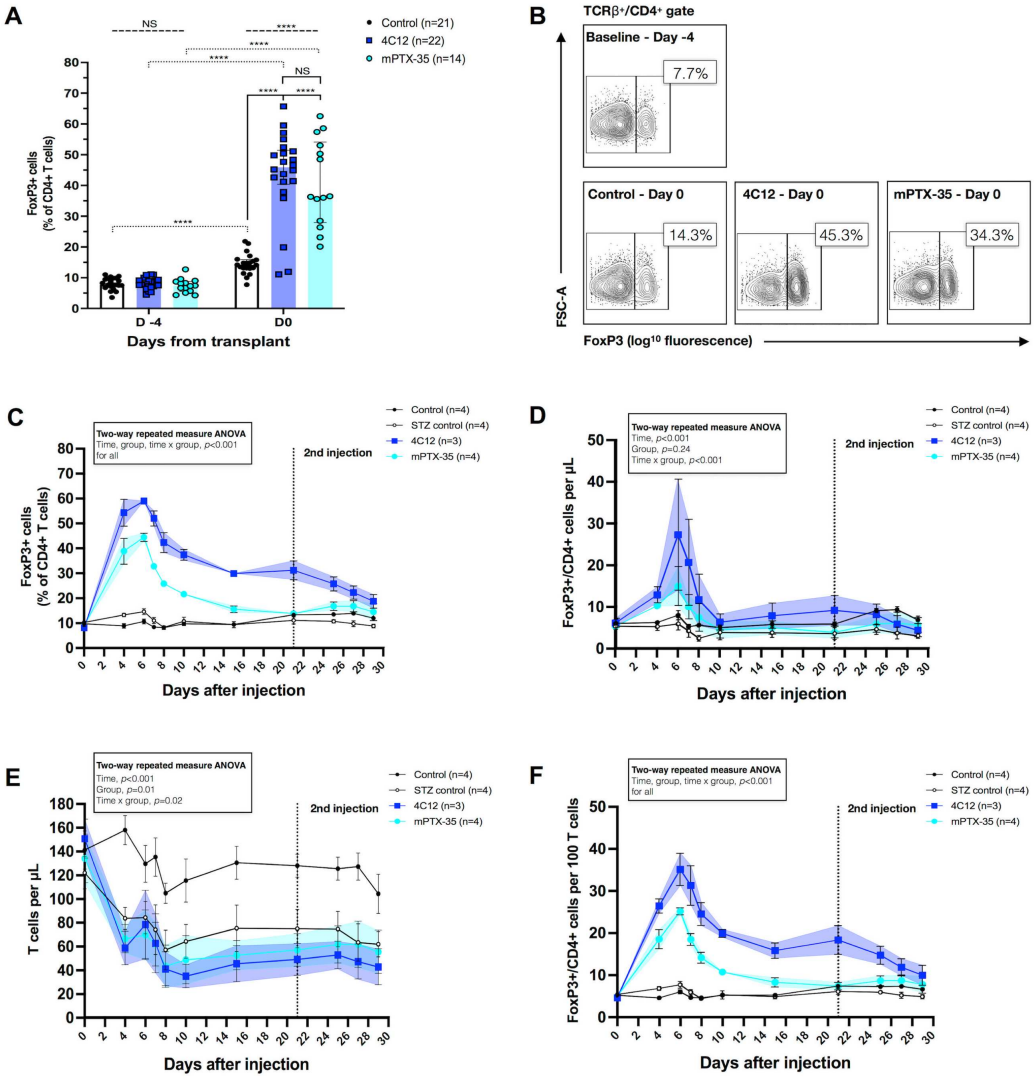
appropriate; Bartlett's test was used to assess for equal variances. Longitudinal flow cytometry data was evaluated using two-way ANOVA tests repeated measures tests. Kaplan-Meier survival function curves were compared using log-rank tests; graft survival is shown as median survival time and ranges. A p-value <0.05 was considered statistically significant. Correlation test were done using Pearson correlation coefficient. Analyses were non-blinded and performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

4.2.4 - Results

4.2.4.1 - Agonistic TNFRSF25 antibodies lead to Treg expansion

Pronounced Treg expansion was evident after administration of both TNFRSF25 antibodies (**Figures 4.2.1A and 4.2.1B).** In the 4C12 group, we observed an increase in Tregs from a median of 7.8% (IQR, 6.7 – 9.5) to 45.3% (IQR, 30.1 – 51.6).

Figure 4.2.1. Treatment with TNFRSF25 agonistic antibodies (4C12 or mPTX-35) leads to pronounced regulatory T cell expansion in peripheral blood.



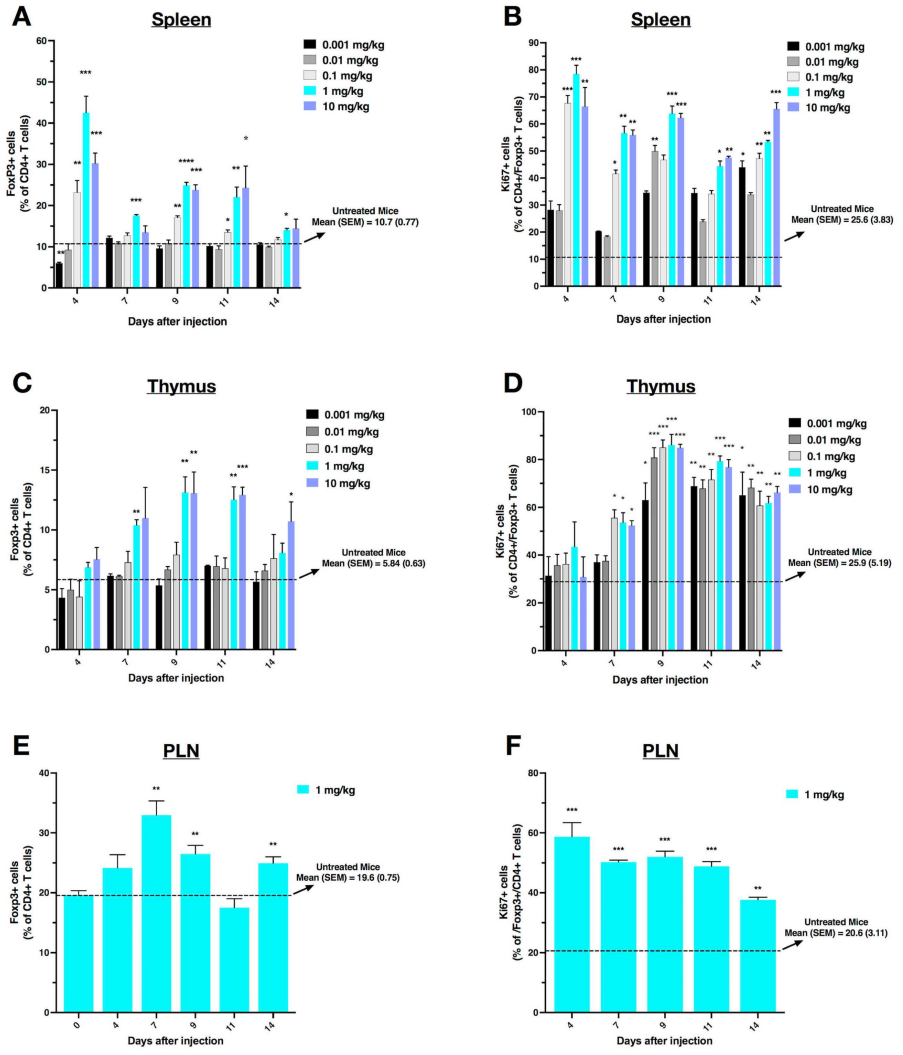
Note: Single cell suspensions were obtained from peripheral blood four days pre-transplant and at the day of transplant. **(A)** Pronounced endogenous *in vivo* expansion of $FoxP3^+$ cells (as a percentage of $CD4^+$ T cells) was corroborated at the day of transplant; (**B**) Representative raw data from flow cytometry analyses, and median percentage values for each group; **(C)** and **(D)** show the kinetics over time of $FoxP3^+$ cells as a percentage of $CD4^+$ T cells and absolute numbers (cells/μL). A second injection of each of the agents (4C12, mPTX-35 and IgG control) at 21 days after the first injection did not trigger a 2nd peak of Treg expansion; **(E)** kinetics over time of absolute numbers of T cells (TCR-β^+); **(F)** kinetics over time of the absolute numbers of $FoxP3^+$ $CD4^+$ cells per 100 T cells. Shown are medians and interquartile ranges (Shapiro-Wilks test showed non-normal distribution) for (**A**), and means and SEM for (**C**) and (**D**). In **(A)** statistics include Kruskal-Wallis tests (dashed line), Wilcoxon match-paired signed rank tests (dotted line) and Mann-Whitney tests (solid line): *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. In **(B)** and **(C)**, statistics include a two-way repeated measures ANOVA, results are shown in the figures.

In the mPTX-35 group, we observed an expansion of Tregs from 6.8% (IQR, 5.0 – 8.6) to 34.3% (IQR, 28.8 - 56). In the IgG isotype control groups, we observed an expansion of Tregs from 7.9% (IQR, 7.1 – 9.3) to 14.3% (IQR, 11.9 - 16). This effect of STZ on mild Treg expansion has been previously reported.[18, 19]

In parallel, we assessed the dynamics of systemic Treg expansion in peripheral blood over time and explored whether a second dose of these antibodies could induce further Treg expansion. Overall, we observed pronounced Treg expansion, with a peak at 6 days post-injection (**Figures 4.2.1C and 4.2.1D**). Both relative (%) and absolute (cells/μL) Treg expansion was observed. We found a significant decrease in T cell numbers (**Figure 4.2.1E**), likely related to STZ administration and uncorrected hyperglycemia, as this was detected early post-STZ administration and sustained until euthanasia. STZ's effects on leukocytes and T cells have been reported previously.[18, 19] Accordingly, we observed a pronounced increase $FoxP3^+/CD4^+$ cells per 100 T cells (**Figure 4.2.1F)**. A second dose of 4C12, mPTX-35 and hamster IgG at day 21 after the first injection did not induce a second peak of Treg expansion. Due to untreated diabetes and progressively deteriorating health status, mice were euthanized at day 30 after the first injection.

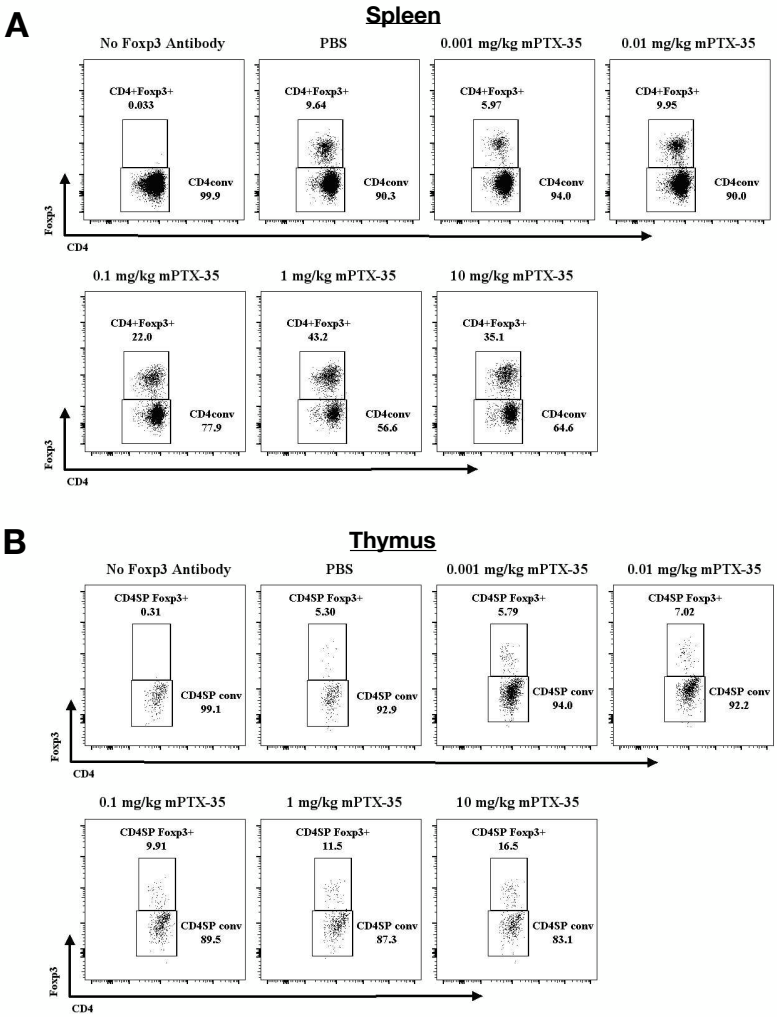
To further characterize the dynamics of mPTX-35, dose-response studies evaluating FoxP3^{+} cells in mouse spleens, thymus and PLN at different time points were done (**Figure 4.2.2**). These studies showed that the peak of mPTX-35-mediated Treg expansion occurred at different time points in the spleen (day 4), the thymus (day 9) and the PLN (day 7) (**Figure 4.2.2A, 4.2.2C and 4.2.2E**). Representative flow cytometry plots are shown in **Figure 4.2.3.**

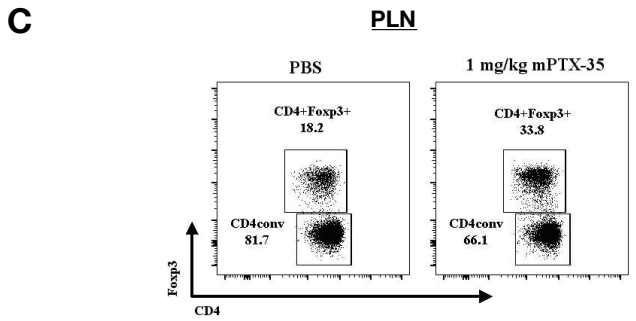
Figure 4.2.2. Treatment with a novel TNFRSF25 agonistic antibody (mPTX-35) leads to pronounced regulatory T cell expansion in spleen, thymus and pancreatic lymph nodes (PLN).



Note: Single cell suspensions were obtained from each tissue at different time points. Different doses were tested for these experiments. Live TCR-β-gated cells were analyzed. **(A)** Percent of FoxP3$^+$ cells (out of CD4$^+$/TCR-β$^+$ cells) within the spleen at different days post mPTX-35 injection (n=3 mice per cohort); **(B)** Percent of proliferating (Ki67$^+$) Tregs (CD4$^+$/FoxP3$^+$ T cells) within the spleen at different days post mPTX-35 injection (n=3 mice per cohort); **(C)** Percent of FoxP3$^+$ cells (out of total CD4SP/TCR-β$^+$ cells within the thymus at different days post mPTX-35 injection (n=3 mice per cohort); **(D)** Percent of proliferating (Ki67$^+$) Tregs (CD4SP/FoxP3$^+$ thymocytes) within the thymus at different days post mPTX-35 injection (n=3 mice per cohort); **(E)** Percent of FoxP3$^+$ cells (out of total CD4$^+$/TCR-β$^+$ cells) within PLN at different days post mPTX-35 injection (n=3 mice per cohort); **(F)** Percent of proliferating (Ki67$^+$) cells (out of CD4$^+$/FoxP3$^+$ T cells) within the PLN at different days post mPTX-35 injection (n=3 mice per cohort). Dashed lines represent the mean value within the untreated controls (n=4). Data are presented as means and SEM. Statistics include unpaired *t*-tests comparing values to untreated controls: *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$.

Figure 4.2.3. Representative flow cytometry data for (A) Spleen, (B) Thymus and (C) Pancreatic lymph nodes.

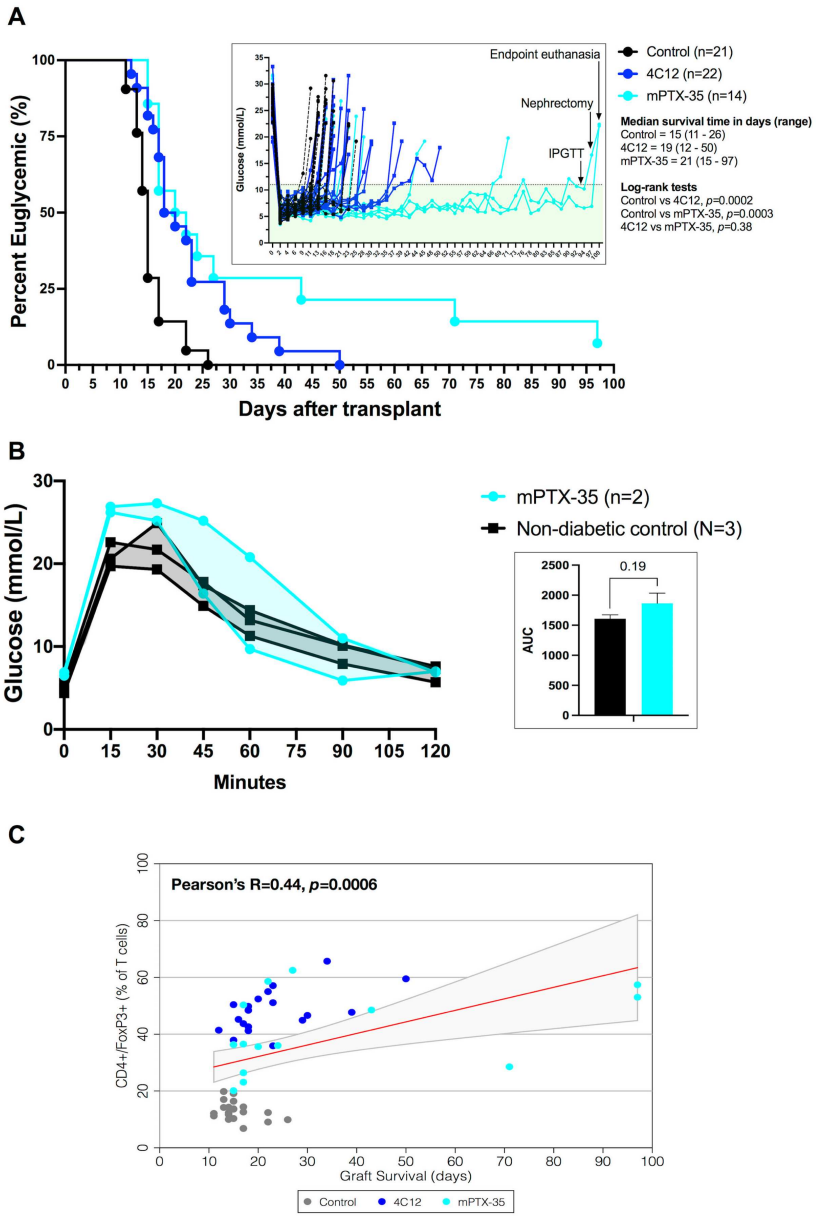




4.2.4.2 - TNFRSF25-treated mice show delayed islet allograft rejection

Islet transplant studies using a murine full-major histocompatibility complex (MHC) discordant allogeneic islet transplant model showed a median graft survival for the control, 4C12, and mPTX-35 transplant groups of 15 (range: 11-26), 19 (range: 12-50) and 21 (range:15-97) days, respectively (**Figure 4.2.4)**. In the mPTX-35 group, four mice (28.5%) had a graft survival >40 days. In the two mice with graft survival of 97 days, an IP glucose tolerance test showed that glucose responsiveness was slightly impaired compared to non-diabetic naïve mice (**Figure 4.2.4A** and **4.2.4B**). Grafts from the other groups could not be analyzed due to rejection. We observed a moderate, but significant correlation between the percentage of $FoxP3^+$ cells at transplantation and graft survival (**Figure 4.2.4C**).

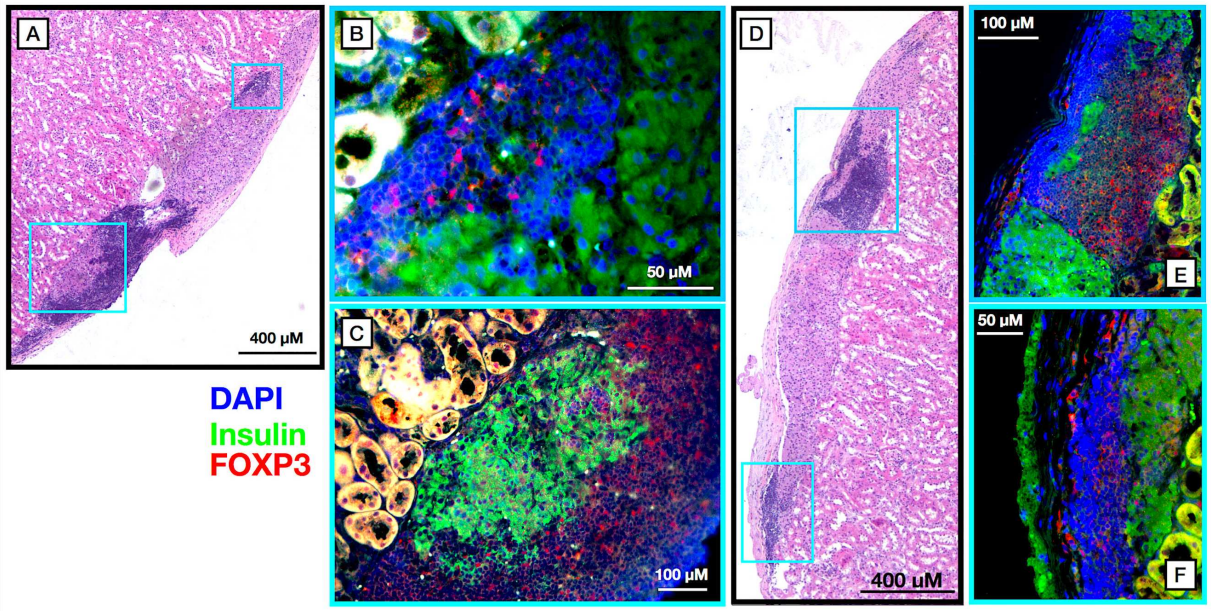
Figure 4.2.4. Treatment with TNFRSF25 agonistic antibodies (4C12 and mPTX-35) leads to delayed acute graft rejection and prolonged graft survival



Note: Graft survival was assessed by thrice weekly glucose monitoring. **(A)** Kaplan-Meier graft survival curves and glucose follow-up curves (insert) both showing prolonged graft survival. The green shaded area shows normoglycemia (glucose levels <11.0 mmol/L). Graft nephrectomies were done at day 95 post-transplant; **(B)** Results from an intraperitoneal glucose tolerance test (IPGTT) at day 95 post-transplant show no statistically significant differences (insert) between the areas under the curve (AUC) of non-diabetic controls and mPTX-35-treated mice; **(C)** A moderate correlation is observed between FoxP3$^+$ percentages (out of CD4$^+$/TCR-β$^+$ cells) and graft survival (days), the red line shows fitted values and the grey shaded area shows 95% confidence intervals. Statistics are shown in figure **(A)** and **(C)**. Unpaired *t*-test comparing area-under-curve is shown for figure **(B)**.

Immunohistochemistry of the two grafts surviving for 97 days from the mPTX-35 group showed dense, but localized cellular infiltrates, and substantial areas of tissue free of cellular infiltration (**Figure 4.2.5A and 4D**). The dense cellular pockets contained vast numbers of FoxP3^{+} cells (**Figure 4.2.5B-C and 4.2.5E-F**).

Figure 4.2.5. Localized FoxP3^{+} cell infiltrates in mice having long-term surviving graft following treatment with a novel TNFRSF25 agonistic antibody (mPTX-35)

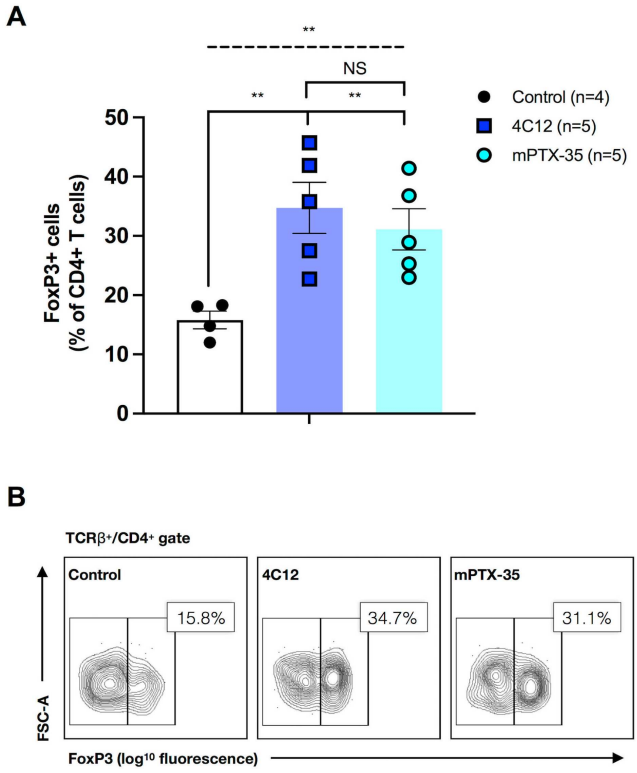


Note: (A) and **(D)** H & E staining showing two dense pockets of cellular infiltrates within the islet graft located in the kidney subcapsular space; **(B-C)** and **(E-F)** Immunohistochemistry staining shows abundance of FoxP3^{+} cells (red) within these infiltrates, as well as insulin^{+} cells (green).

4.2.4.3 - Administration of TNFRSF25 antibodies promotes local Treg infiltration

To assess whether the systemic Treg expansion correlated with early local Treg infiltration, we retrieved grafts at 36-hours post-transplant and conducted flow cytometric analysis**.** We observed a higher proportion of FoxP3^{+} cells out of CD4^{+} T cells within the graft in both the 4C12 and mPTX-35 groups, as compared to controls (**Figure 4.2.6**).

Figure 4.2.6. Treatment with TNFRSF25 agonistic antibodies (4C12 and mPTX-35) leads to increased intra-graft percentages of regulatory T cells



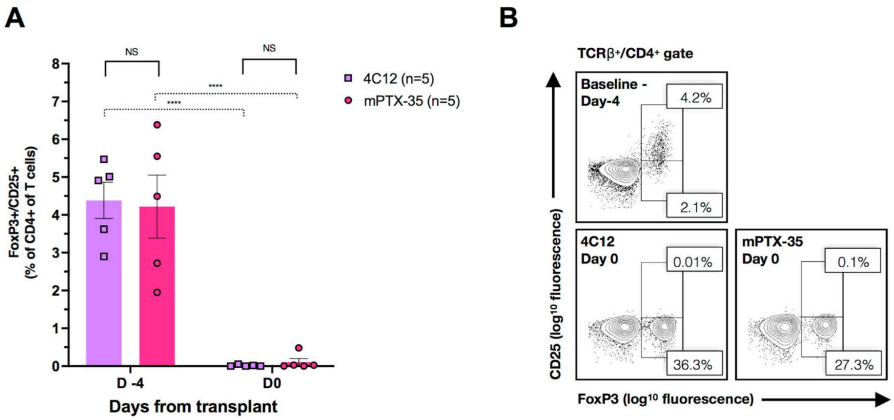
Note: Single cell suspensions were obtained from the islet grafts at 36-hours post-transplant. **(A)** Percentages of FoxP3$^+$ cells (out of CD4$^+$ T cells) are increased in both intervention groups as compared to controls. **(B)** Shown are the means and SEM. Statistics are presented in the figures. Statistics include one-way ANOVA tests (dashed lines) and unpaired *t*-tests (solid lines) comparing values to untreated controls: *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$.

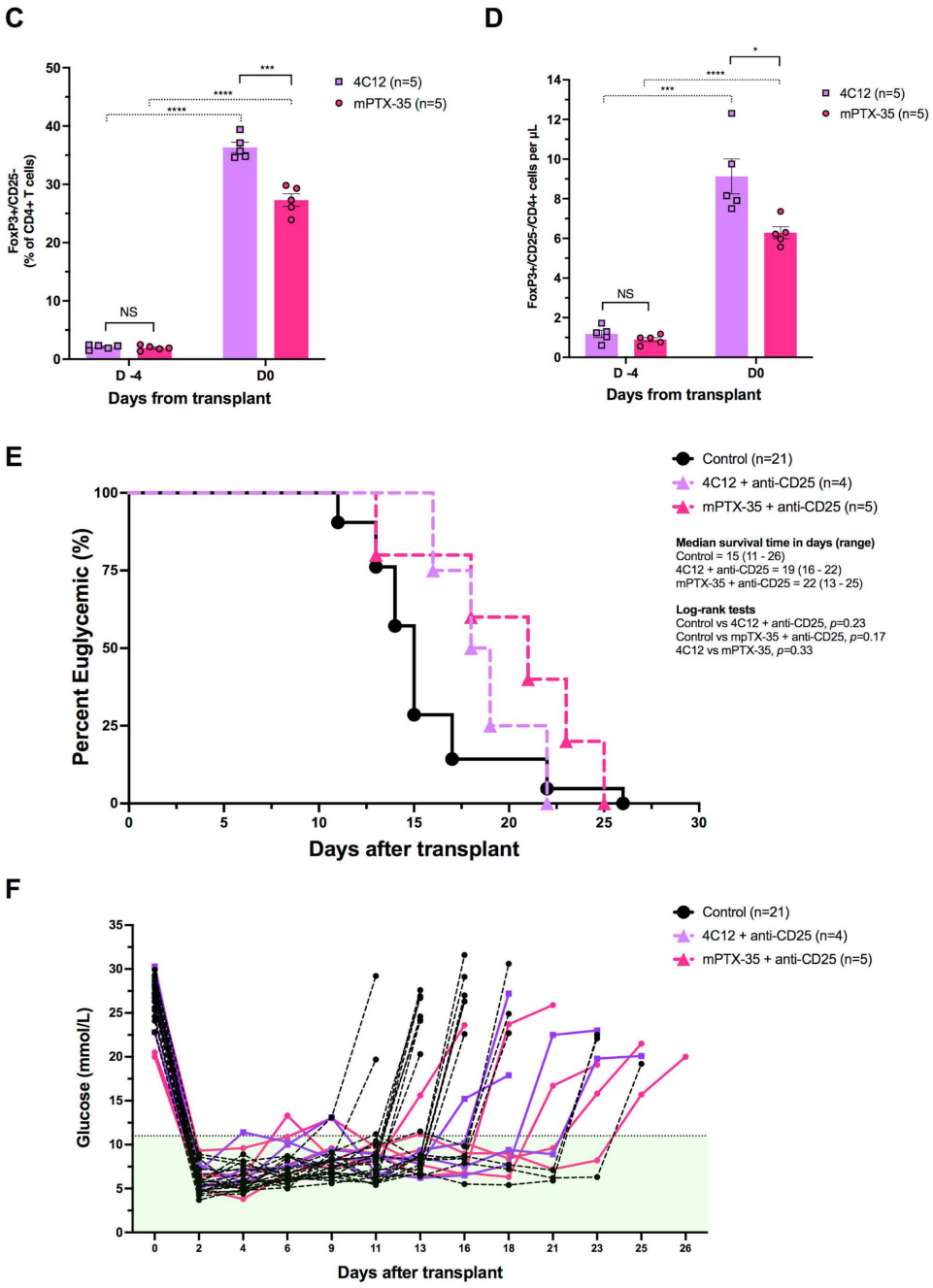
4.2.4.4 - Antibody-mediated CD25$^+$/FoxP3$^+$ CD4$^+$ T cell depletion partially abrogates the graft-preserving effect of TNFRSF25 antibodies

To determine causality between Treg expansion and prolonged graft survival, we performed islet transplants with TNFRSF25 administration in an antibody-mediated (anti-CD25 antibody) Treg depletion model. We corroborated depletion of CD25$^+$/FoxP3$^+$ CD4$^+$ T cells (**Figure 4.2.7A**), however, FoxP3$^+$ CD4$^+$ T cells were not depleted, and these were CD25$^-$ (**Figure 4.2.7C-D)**.

A delay in acute graft rejection as compared to controls was observed; however, this did not reach statistical significance (**Figure 4.2.7E**). While the lack of statistical significance could be explained by sample size, results suggest a remaining protective effect driven by TNFRSF25 antibodies despite $CD25^+$ Treg depletion. Notably, none of the anti-CD25-treated mice retained graft function beyond 30 days, as compared to 13.6% and 28.6% of mice in the non-depleted 4C12 and mPTX-35 groups, respectively, supporting that $CD25^+$ Tregs might be relevant to long-term graft survival. Other effector cell populations, such as $CD4^+$, $CD8^+$, and NK cells were similar compared to STZ-treated controls, which also supports a protective effect driven by $FoxP3^+$ $CD4^+$ regulatory T cells (**Figure 4.2.8**).

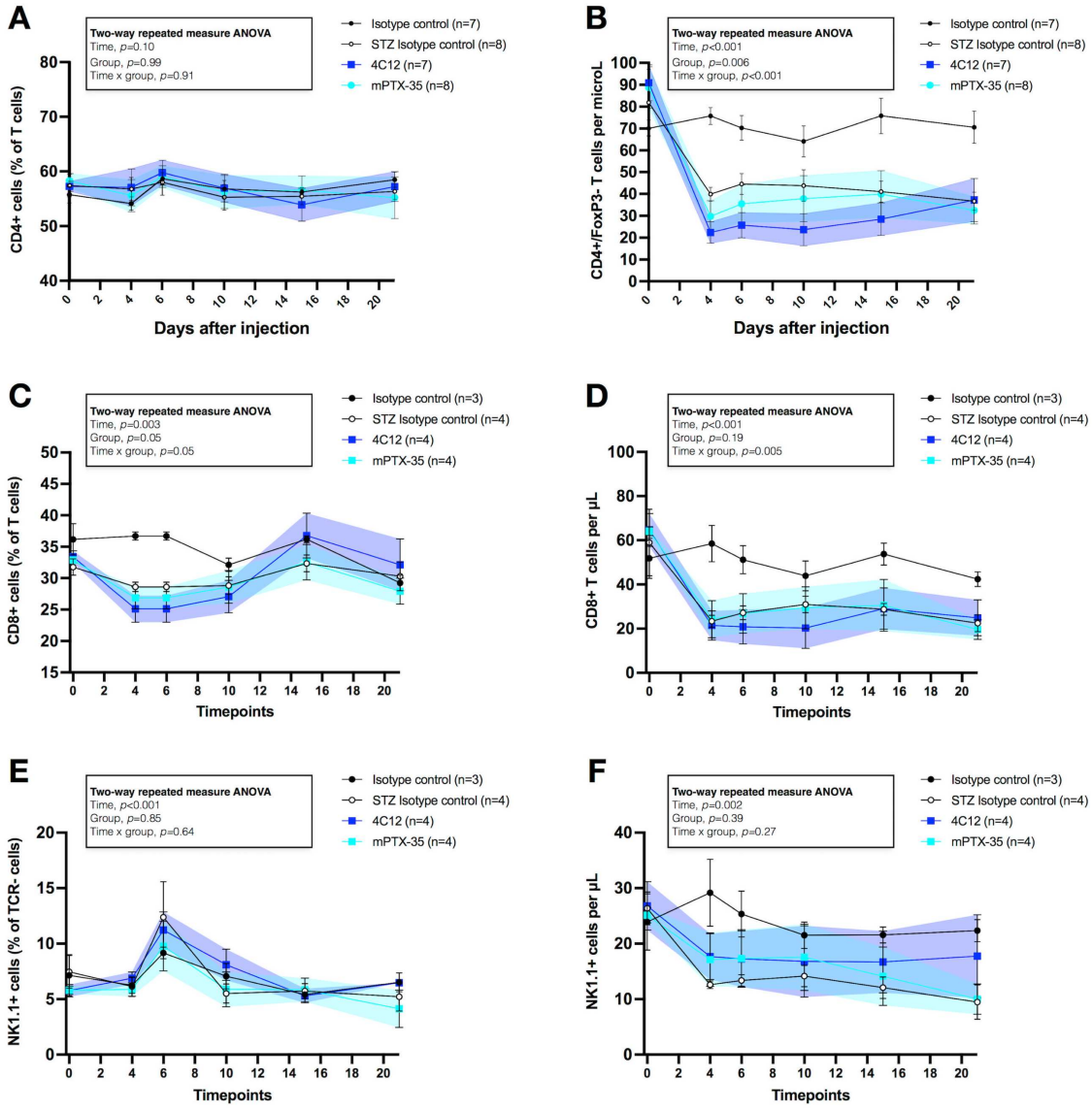
Figure 4.2.7. Treatment with TNFRSF25 agonistic antibodies (4C12 or mPTX-35) leads to $FoxP3^+CD25^-$ $CD4^+$ T cell expansion in peripheral blood, despite the administration of anti-CD25 *in vivo* depleting antibodies





Note: Single cell suspensions were obtained from peripheral blood four days pre-transplant and at the day of transplant. **(A)** Depletion of FoxP3$^+$/CD25$^+$ CD4$^+$ T cells was corroborated at the day of transplant; **(B)** Representative raw data from flow cytometry analyses, and median percentage values for each group; **(C)** Increases in relative numbers of FoxP3$^+$/CD25$^-$ cells (as a percentage of CD4$^+$ T cells); **(D)** Increases in absolute numbers of FoxP3$^+$/CD25$^-$ CD4$^+$ T cells (cells/µL); **(E)** Kaplan-Meier graft survival curves (one mouse died post-transplant due to surgical complications decreasing the sample size from 5 to 4 in the 4C12 group); **(F)** Glucose follow-up post-transplantation. The green shaded area shows normoglycemia (glucose levels <11.0 mmol/L). Shown are mean and SEM. In **(A), (C)** and **(D)** statistics include paired t-tests (dotted line) and unpaired *t*-tests (solid line): *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Statistics are shown in **(E).** No statistical comparisons were done in **(B)** and **(F)**.

Figure 4.2.8. The effect of TNFRSF25 antibody administration in different immune compartments over time



Note: (A) Percentage of $CD4^+$ T cells; **(B)** Absolute numbers (cells/µL) of $FoxP3^-CD4^+$ T cells (Tconvs); **(C)** Percentage of $CD8^+$ T cells; **(D)** Absolute numbers (cells/µL) of $CD8^+$ T cells; **(E)** Percentage of NK cells; **(F)** Absolute numbers (cells/µL) of NK cells.

Shown are mean and SEM. Statistics include two-way repeated measures ANOVA and results from these tests are presented in the figures. Individual multiple comparison test using Dunnett's correction for multiple comparisons showed no statistically significant differences between STZ isotype and TNFRSF25-treated groups at any time point either in terms of relative or absolute numbers.

4.2.5 - Discussion

Herein, we found that antibody-mediated agonistic stimulation of the TNFRSF25 signaling pathway led to pronounced endogenous *in vivo* Treg expansion. This correlated with a significant delay in acute graft rejection in a murine model of allogeneic islet transplantation. The degree of Treg expansion was moderately correlated with graft survival. Our results suggest that pre-transplant systemic *in vivo* Treg expansion may also favor early and persistent intra-graft Treg infiltration.

TNFRSF25 has the highest homology to TNFR1 amongst the TNF receptors (~ 63%).[20] Originally described in the 1990s, TNFRSF25 has recently attracted attention due to its capacity for *in vivo* Treg expansion. However, TNFRSF25 is also expressed by $CD4^+$, $CD8^+$ effector T cells, and natural killer T cells.[20] Activation of the TNFRSF25 signaling pathway by its cognate ligand, tumor necrosis factor-like cytokine 1A (TL1A), which is mainly expressed by antigen-presenting and endothelial cells,[21] promotes cytokine production.[22] These interactions serve as co-stimulatory signals and have been linked to exacerbations of inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, and skin psoriasis.[23-27] Additionally, agonistic stimulation of TNFRSF25 leads to antigen-specific T cell proliferation, a feature that has been explored in the areas of vaccinology,[28] infectious diseases,[29] and oncology.[30]

True to its co-stimulatory role, TNFRSF25 signaling, beyond the need for TL1A, requires T-cell receptor (TCR) engagement by cognate antigens and the presence of IL-2.[20] This becomes particularly relevant for Tregs, since they thrive on tonic, medium-affinity engagement of TCRs with available cognate self-antigens.[31] Exploiting this phenomenon, studies show pronounced Treg expansion following TNFRSF25 agonistic stimulation.[13, 32, 33] An explanation for TNFRSF25's apparently contradictory physiological functions (i.e., pro- vs. anti-

inflammatory) is that stimulation of TNFRSF25 may protect from bystander inflammation in the context of activated antigen-presenting cells displaying a combination of self and foreign antigens.[20]

In transplantation, Treg targeted therapies have become enticing candidates for induction of operational tolerance. Promising outcomes in liver[5] and kidney transplantation[6, 7] have been published recently. These strategies rely on *ex vivo* Treg expansion protocols, which are limited by prohibitive cost and logistics. Thus, *in vivo* Treg expansion represents an attractive alternative to harvest the benefits of co-adjuvant cellular therapies that could modulate allo- and autoimmune responses post-transplant, while avoiding challenges with *ex vivo*-expanded ACT therapies. Experience with TNFRSF25 antibodies (i.e., 4C12) and fusion proteins (i.e., TL1A-Ig) in preclinical models of hematopoietic stem-cell transplantation have consistently shown that pre-transplant Treg expansion ameliorates morbidity and mortality related to graft-versus-host disease.[32, 34-37] Importantly, TNFRSF25-expanded Tregs have a more profound suppressive effect compared to control isolated Tregs.[34] In solid organ transplantation, 4C12 enabled delayed acute allograft rejection in a murine model of fully allogeneic heterotopic heart transplant.[33] In this study, administration of a single dose of 4C12 four days before transplantation increased median graft survival from 8 to 17 days (p=0.0049), and ameliorated inflammatory cellular graft infiltration as compared to IgG-treated controls.[33] Additionally, 4C12 substantially increased intra-graft Tregs compared to IgG-treated controls. Our findings with 4C12 and the novel mPTX-35 agree with this previous study, suggesting a role for local Treg infiltration as the driving mechanism delaying acute graft rejection. It should be emphasized that these benefits were observed after a single dose of TNFRSF25 antibodies and without any additional immunosuppression. While we attempted to trigger another round

of Treg expansion with a second dose of TNFRSF25 antibodies, this was not achieved. This might be explained by the deteriorating health status of diabetic mice and sustained STZ- and hyperglycemia-induced T cell depletion; however, it remains possible that anti-idiotypic antibodies could have formed, or that TNFRSF25 expression decreased following engagement with agonistic antibodies. Future studies will address these issues to determine how to achieve recurrent Treg expansion with TNFRSF25 antibodies. Overall, these novel, specific immunotherapies are rapidly moving into clinical trials and results from these trials will be highly relevant as they harbor a robust potential for use in both cellular and solid organ transplantation. Agonistic stimulation of other TNFRSF receptors expressed in Tregs (i.e., GITR, CD27, OX40, and 4-1BB) has not shown to promote Treg expansion,[13] unless manipulation of the immunological milieu occurs concomitantly (i.e., cytokine depletion).[38] This introduces issues in terms of clinical translation. Evidence of Treg expansion with TLA1-Ig fusion proteins exists, however, more doses are required, concentrations are less sustained, and TLA1-Ig may be more prone to costimulation of effector T cells compared to TNFRSF25 antibodies (i.e., 4C12).[22] It remains to be seen how potent and durable TNFRSF25-mediated Treg expansion will be in humans, and how effective it may be in the setting of heterologous immunity.

There are several considerations and implications of *in vivo* TNFRSF25-mediated Treg expansion, particularly in the setting of T1D. While multiple approaches focusing on *in vivo* Treg expansion have reached clinical trials in patients with T1D (e.g., anti-thymocyte globulin, CD2-binding proteins, IL-7Rα blockers, low-dose IL-2-based immunotherapies),[4] scarce evidence exists in pancreatic islet transplantation. An example is the pre-clinical use of IL-2/IL2-mAb complexes proposed by Webster *et al.*[39] In this study, the authors found that IL-

2/IL-2-mAb complexes led to pronounced *in vivo* Treg expansion, and that injection of these cytokine-antibody complexes (daily doses for 3-days pre-transplant) prevented islet graft rejection in >80% of mice. Tolerance mechanisms remained elusive, however, intra-graft Treg infiltration in the form of cellular pockets was observed adjacent to the grafts.[39] These cellular pockets were also present in two of the mPTX-35-treated mice having long-term surviving grafts, and may be related to linked suppression, which involves graft acceptance when the tolerated and third party antigens coexist in close proximity; importantly, linked suppression is believed to be heavily mediated by Tregs.[40] Another approach for *in vivo* Treg expansion is the use of sirolimus/rapamycin with/without combined IL-10 administration. These strategies have shown to enable moderate (2-3 fold) *in vivo* Treg expansion and prolonged islet graft survival.[41, 42] However, sirolimus causes adverse effects in transplant recipients, including those undergoing islet transplantation, especially at high doses.[43] Recently, combination of sirolimus with low-dose IL-2[44, 45] or IL-2/IL-2-mAb complexes[46] has been proposed to expand Tregs, while avoiding adverse effects. Importantly, low dose IL-2 has been evaluated in combination with both 4C12 and TL1A-Ig in mouse models of graft-versus-host disease.[32] In these experiments, IL-2 supplementation increased Treg percentages in peripheral blood, but not in spleens or lymph nodes, with no observed improvement in recipient survival or graft-versus-host disease scores[32]. TNFRSF25 antibodies enable systemic and localized Treg expansion,[33] hence, TNFRSF25-based combinatorial strategies with sirolimus and/or IL-2/IL-2-mAb complexes could prove valuable and will be explored in future studies.

Contrary to sirolimus, IL-2, or IL-2/IL-2-mAb complexes, TNFRSF25 antibodies are more selective and potentially less prone to systemic adverse effects. However, a major limitation with TNFRSF25 antibodies and a vital consideration for future clinical trials pertains

to the timing of administration. As TNFRSF25 signaling depends on TCR engagement, researchers should consider that T cell activation upregulates TNFRSF25 (and most TNFR members). This co-stimulatory signaling could enhance T cell cytotoxic activity, or make effector T cells more resistant to Treg-mediated suppression.[47] Thus, a thoughtful way to proceed with clinical trials would be administering these compounds a few days before transplantation. This introduces logistical limitations, although it could prove feasible, especially in the setting of living donor transplantation. More importantly, in the context of an autoimmune disease such as T1D, consideration of possibly inducing recurrence of latent autoimmune responses poses additional challenges. Our study is limited in this regard, as our mouse model evaluated exclusively the alloimmune responses post-transplant. Studies using the non-obese diabetic (NOD) mouse, a commonly used model of T1D, are being planned to deepen our understanding regarding TNFRSF25-mediated *in vivo* Treg expansion in T1D and islet transplantation.

There are additional limitations that need delineation. First, given the modest effect on graft survival, it is likely that long-term immunosuppression is required concomitantly with TNFRSF25 antibodies to attain optimal effects. Immunosuppression should consider potential detrimental effects on Treg survival and function.[48, 49] Indeed, a Treg-centric view on immunosuppression should be promoted for future clinical trials involving TNFRSF25, considering that preclinical evidence suggests that calcineurin inhibitors preclude Treg expansion in this setting;[13, 22] but conversely, sirolimus does not.[13] Another limitation concerns Treg depletion model used herein, which did not conclusively show a causal role of Treg expansion in prolonged graft survival. This was possibly related to incomplete depletion of $FoxP3^+$ $CD4^+$ T cells. Notably, controversies exist around antibody-mediated Treg depletion,

including the claim that anti-CD25 antibodies shed the epitope rather than deplete the cells, as well as the notion that anti-CD25 antibody clone PC61 lingers in recipients and results in hindrance for fluorochrome-conjugated anti-CD25 antibodies used for flow cytometry.[50] Thus, it is possible that expanded FoxP3$^+$ CD4$^+$ T cells observed after anti-CD25 administration are conventional CD25$^+$ Tregs, although differences in the degree of absolute expansion in FoxP3$^+$ CD4$^+$ cells argue against this (**Figure 4.2.11D vs Figure 4.2.7D**). Additionally, it is possible that FoxP3$^+$ CD4$^+$ T cells observed following anti-CD25 administration represent CD25$^-$ /FoxP3$^+$ precursors, which mature into CD25$^+$ Tregs using distinct cytokine requirements (i.e., IL-15).[51, 52] Unfortunately, it is unknown whether these CD25$^-$/FoxP3$^+$ precursors express TNFRSF25 and respond to agonistic stimulation. Conversely, and favoring a Treg-driven effect, is the fact that other immune cell compartments were not significantly altered as compared to STZ-treated diabetic controls, albeit, immune cells not evaluated here could also contribute to graft preservation. Studies using other Treg depletion models (e.g., DEREG mice) could contribute to answering these questions.

As cellular therapies bring us closer to operational tolerance in transplantation, novel *in vivo* Treg expansion strategies that are safe, effective and clinically translatable are much needed. Herein, we show that *in vivo* Treg expansion using agonistic TNFRSF25 antibodies was associated with delayed acute graft rejection in a murine model of allogeneic islet transplantation. The degree of expansion was moderately correlated with graft survival and we observed that some long-term surviving grafts showed dense localized Treg infiltrates, which could be associated to linked suppression. While more research is required to elucidate the full potential of TNFRSF25, this study presents preclinical data showing its potential in islet transplantation.

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CHAPTER 5

PART 1 - CURRENT STATE AND EVIDENCE OF CELLULAR ENCAPSULATION STRATEGIES IN TYPE 1 DIABETES

CHAPTER 5, PART 1 - CURRENT STATE AND EVIDENCE OF CELLULAR ENCAPSULATION STRATEGIES IN TYPE 1 DIABETES

COMPREHENSIVE PHYSIOLOGY

Current State and Evidence of Cellular Encapsulation Strategies in Type 1 Diabetes

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ABSTRACT

Islet cell replacement therapies represent an effective way to restore physiologic glycemic control in patients with type 1 diabetes (T1DM) and severe hypoglycemia. Despite being able to provide long-term insulin independence, patients still require lifelong immunosuppression, which has myriad detrimental effects including an increased risk for opportunistic infections and some types of cancer. This vital issue precludes widespread application of these therapies as a true cure for T1DM. Encapsulation of islets into immunoisolating/immunoprotective devices provides the potential of abrogating the requisite for lifelong immunosuppression. The field of cellular encapsulation lies at a complex intersection between the areas of chemistry, physics, bioengineering, cell biology, immunology, and clinical medicine. In diabetes, cellular encapsulation has existed for nearly 50 years, nevertheless, a resurgence of interest in the field has been motivated by promising results in small- and large-animal models. Recent studies have demonstrated that long-term diabetes reversal without immunosuppression is indeed routinely achievable. Future researchers interested in exploring cellular encapsulation strategies will require a clear understanding of the basic theoretical and practical principles, guiding this rapidly expanding field. This article will provide essential considerations concerning the physicochemical properties of the most commonly used biomaterials, relevant aspects of the immune response to bioencapsulation, current encapsulation strategies, potential implantation sites for encapsulated cell therapies and, finally, a comprehensive review on the current state of clinical translation. © 2020 American Physiological Society. *Compr Physiol* 10:839-878, 2020.

Didactic Synopsis

Major teaching points

- Islet replacement therapies are effective therapies for patients with type 1 diabetes and severe hypoglycemia.
- Currently, the main issue limiting widespread use of islet replacement therapies for every patient with diabetes is the requisite for lifelong immunosuppression to avoid immune rejection.
- Cellular encapsulation represents a cost-effective, safe, and efficacious alternative to lifelong immunosuppression that could expand current indications for islet and β-cell replacement therapies.
- The main objective of cellular encapsulation is to provide maximal immune protection with minimal impact on cellular viability and function.
- The main driving force for oxygen and nutrient uptake, as well as hormone release for encapsulated islets, is passive molecule diffusion by electrochemical gradients.
- Immune responses such as the foreign-body response can be harnessed and tailored to foster a "friendlier" environment for encapsulated islet transplantation.
- Preclinical models have successfully and consistently achieved long-term diabetes reversal without immunosuppression following encapsulated islet transplantation.
- At present, only two reported patients have achieved insulin independence beyond 12 months after encapsulated islet transplantation.
- The field of cellular encapsulation lies at a complex intersection between chemistry, physics, bioengineering, cell biology and immunology, and clinical medicine. Thus, multidisciplinary efforts to allow well-designed and well-conducted research are strongly encouraged.

Introduction

As of 2014, an estimated 422 million people in the world live with some form of diabetes (166). Most recent studies report a steady increase in the cost associated with diabetes

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Running head: Cellular Encapsulation in Type 1 Diabetes

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5.1.1 - Abstract

Islet cell replacement therapies represent an effective way to restore physiologic glycemic control in patients with type 1 diabetes (T1D) and severe hypoglycemia. Despite being able to provide long-term insulin independence, patients still require lifelong immunosuppression, which has myriad detrimental effects including an increased risk for opportunistic infections and some types of cancer. This vital issue precludes widespread application of these therapies as a true cure for T1D. Encapsulation of islets into immunoisolating/immunoprotective devices provides the potential of abrogating the requisite for lifelong immunosuppression. The field of cellular encapsulation lies at a complex intersection between the areas of chemistry, physics, bioengineering, cell biology, immunology, and clinical medicine. In diabetes, cellular encapsulation has existed for nearly 50 years, nevertheless, a resurgence of interest in the field has been motivated by promising results in small- and large-animal models. Recent studies have demonstrated that long-term diabetes reversal without immunosuppression is indeed routinely achievable. Future researchers interested in exploring cellular encapsulation strategies will require a clear understanding of the basic theoretical and practical principles, guiding this rapidly expanding field. This review will provide essential considerations concerning the physicochemical properties of the most commonly used biomaterials, relevant aspects of the immune response to bioencapsulation, current encapsulation strategies, potential implantation sites for encapsulated cell therapies and, finally, a comprehensive review on the current state of clinical translation.

5.1.2 - Introduction

As of 2014, an estimated 422 million people in the world live with some form of diabetes [1]. Most recent studies report a steady increase in the cost associated with diabetes management, with an estimated cost of $327 billion in the US in 2017.[2] Although there is significant variability, studies in low- and middle-income countries support this notion and show that diabetes care is a substantial economic burden for governments, but particularly for patients, considering that out-of-pocket health expenditure is overwhelming in many countries.[3] Type 1 diabetes (T1D) accounts for approximately 10% of the total cases of diabetes.[4] T1D represents a multifactorial disease that arises from combined genetic predisposition and triggering factors from the external environment. Such a combination triggers a complex multi-stage autoimmune process that leads to destruction of insulin-producing β-cells within the pancreatic islets of Langerhans and, consequently, insulin deficiency and hyperglycemia.[5, 6] Clinical manifestations usually appear after destruction of approximately 80-90% of β-cells, although β-cell mass does not have a perfect correlation with clinical manifestations and/or β-cell function.[7] For nearly 100 years, the most common strategy to achieve glycemic control for these patients is subcutaneous injections of exogenous insulin; but, even with this treatment, glucose levels fluctuate considerably outside the normal physiological range, both into hyperglycemic and hypoglycemic values. As a result, continual hyperglycemia in patients with T1D can lead to chronic vascular complications such as retinopathy, neuropathy, and nephropathy. Conversely, recurrent hypoglycemia is no less threatening, since it can further lead to life threatening hypoglycemia unawareness, which refers to the presence of neurologic symptoms (e.g. loss of conscience) without the appearance of autonomic symptoms (e.g. tachycardia or sweating).[8] Recurrent hypoglycemia and hypoglycemia unawareness are often considered to be neglected

complications of diabetes, since most clinical guidelines focus on managing hyperglycemia. Still, hypoglycemia unawareness is common (20-40% of T1D patients)[8, 9] and it negatively impacts quality of life and increases both morbidity and mortality (4-10% of all T1D patients die of severe hypoglycemia), with its most feared complication being the "dead-in-bed" syndrome, a sudden unexpected death during nighttime hours.[10, 11] Limitations of current exogenous insulin therapies have led to the development of increasingly sophisticated automated insulin delivery systems, such as single or dual-hormone closed-loop "pumps", commonly referred to as "artificial pancreas", which have proven to abrogate extreme glucose excursions better than usual treatment.[12] In fact, one of the most recent clinical trials using a "closed-loop" insulin delivery system has reported achieving more than 70% of time within recommended glycemic target ranges (70-180 mg/dl), which translated into significant improvements in HbA1c (%) levels, while avoiding severe hypoglycemic episodes. Nevertheless, significantly more adverse events related to the trial device were also reported.[13] This trial attests to the complexity behind attaining optimal glycemic control with current insulin schemes that has motivated research into biological therapies such as whole pancreas transplantation, islet transplantation, and β-cell replacement therapies, which have quickly accumulated evidence supporting a more physiological glycemic control compared to exogenous insulin therapies.[14]

5.1.3 - Islet Transplantation

Islet transplantation is an effective therapy to restore physiologic glycemic control in patients with T1D. It is mainly indicated for patients suffering from severe hypoglycemia and hypoglycemia unawareness despite optimal and tailored exogenous insulin therapy.[15]

Specifically, islet transplantation is currently considered a last resource ("stage 4") in the current tiered algorithms to treat problematic hypoglycemia.[16] Research in the field of islet transplantation began with the seminal works by Minkowski and von Mering in 1892 which transplanted pancreas fragments into dogs, demonstrating transient improvement of glucosuria.[17] It took almost 100 years for the first patient to achieve insulin independence after an islet transplantation, albeit this only lasted for 9 months.[18] Ten years later, one of the ground-breaking studies in the field came with the "Edmonton Protocol", published in 2000.[19] By using a steroid-free immunosuppressive protocol, Shapiro et al. achieved 1-year insulin independence after islet transplantation in seven consecutive non-uremic T1D patients. Although patients required, on average, two infusions and >11,000 IEQ/kg, this study was the first to achieve such unprecedented results.[19] Nevertheless, despite such enthusiastic results, only 10% of patients maintained insulin independence for >5 years.[20] Subsequent efforts have significantly improved these numbers and the last report from the CITR (Collaborative Islet Transplant Registry) indeed reinforce this notion. With >1000 islet transplants to date and >2000 infusions, evidence supporting the use of this novel therapy has profoundly grown stronger.[21] Today, the 5-year insulin independence rate after last infusion is, on average, 30-50%. There are well-recognized factors associated with insulin independence such as induction immunosuppression with T-cell depleting agents and/or TNF-α inhibition, maintenance immunosuppression with mTOR and calcineurin inhibitors, an infused IEQ number >325,000, and recipient age >35 years, among others.[21] Beyond insulin independence (which should not be the sole determinant of clinical success following islet transplantation), other important outcomes include 5-year post-transplant HbA1c levels <7.0% in 60% of the patients and an absence of severe hypoglycemic episodes in >95% of the patients.[21] These outcomes vary depending on the experience of the

site, with centers reaching 5-year insulin independence rates equiparating those following whole pancreas transplant patients.[22, 23]

Currently, many challenges remain that prevent islet transplantation from replacing the gold standard, exogenous insulin therapy, for T1D management. These challenges are namely the shortage of matched organ donors and the requisite for lifelong immunosuppression. The former, although it inherently limits widespread use of β-cell replacement therapies, is currently not a major roadblock that precludes patients having an islet transplantation, if indicated. Nevertheless, this hindrance is actively being addressed by two attractive avenues: islet xenotransplantation and human pluripotent stem cells-derived β-cells (embryonic and induced).[24-28] Thus, once a limitless, efficient and safe β-cell source becomes clinically available, cellular replacement therapies will not be a viable option for all patients with T1D if the ideal immunological environment for cell survival is not attained. Currently, the risk-benefit ratio to indicate an islet transplantation favors the "risk" for most patients with T1D. This is mainly driven by the chronic effects of immunosuppression, since the current techniques of percutaneous infusion makes islet transplantation one of the safest transplants in the field. In fact, adverse events related to immunosuppression are the most commonly reported risk to islet transplant recipients, as indicated in the previously mentioned 10th CITR report. The most frequent adverse events related to immunosuppression include abnormalities of the granulocytes, lymphopenia, infection, diarrhea, abnormal liver function tests, and neoplasms, although their incidence has decreased over time (2003-2006: 44.6% to 2015-2018: 12.5%).[21] Importantly, patients in this report had a median follow-up of 4.2 $\pm$ 3.4 years, thus, some complications may be underrepresented. Additionally, commonly used anti-rejection drugs are inherently diabetogenic and they have deleterious effects on the transplanted islets themselves

which, in turn, can lead to graft attrition and failure.[29] Cellular encapsulation to provide effective and safe immunoisolation may be an attractive solution to circumvent these extremely relevant issues which currently limit the success and broad-spectrum application of islet transplantation and β-cell replacement therapies. This review will focus on the current notions and state of the field, the potential limitations for clinical translation and the future research avenues to promote widespread application of cellular encapsulation for patients with T1D and, perhaps, with other types of diabetes.

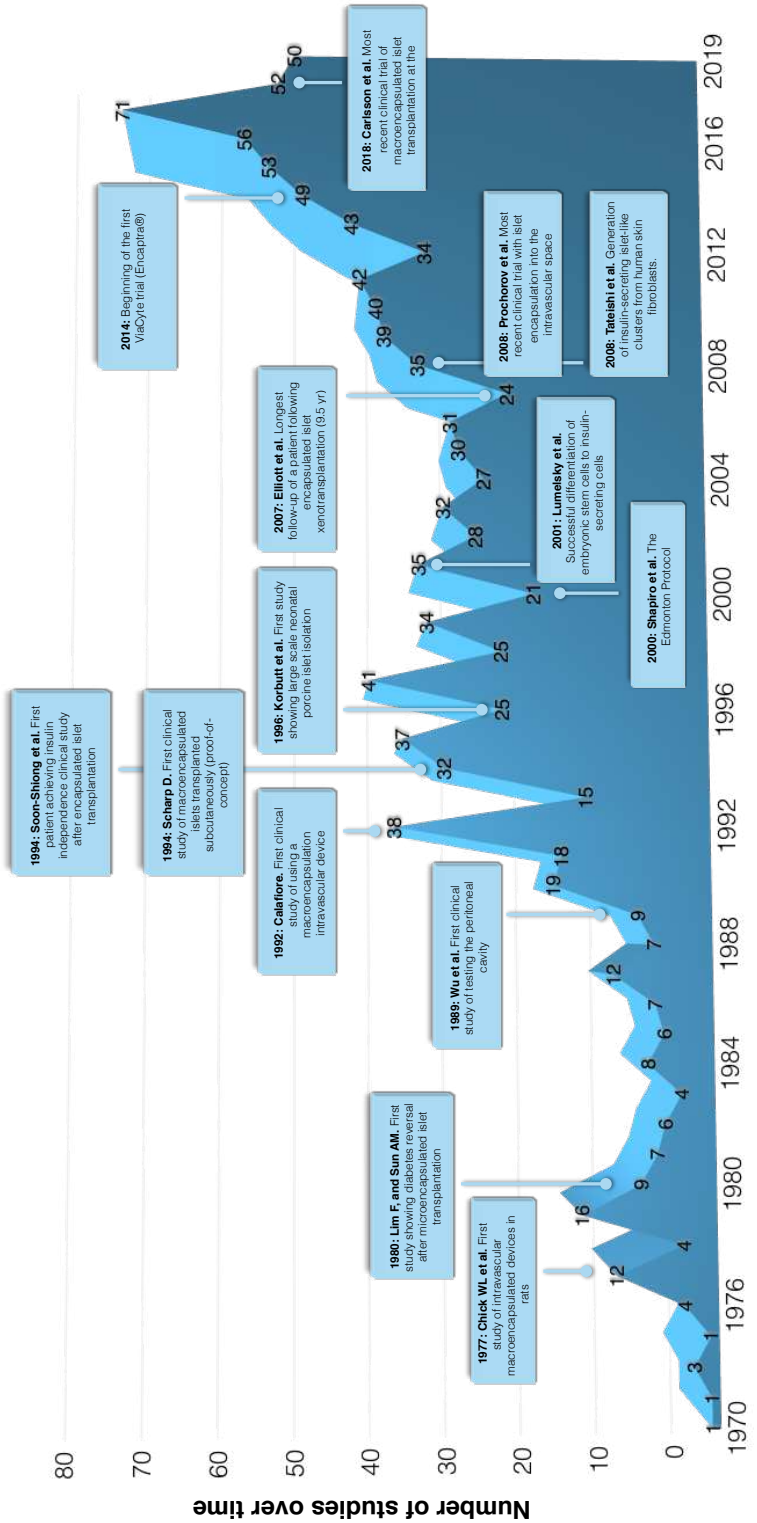
5.1.4 - Encapsulation of Pancreatic Islets

Cellular encapsulation has advanced greatly since early attempts at transplanting cells from a human insulinoma (insulin-producing tumor) contained within an immunoisolating device showed successful protection from the recipient's immune system in the early 1930s.[30] Addition of specialized biomaterials and the increasing complexity and refinement of encapsulation methods and technologies have motivated research endeavors utilizing a myriad of tissues and cell lines to treat diverse disease processes, such as renal failure, chronic anemia, myocardial infarction, bone and cartilage defects, neurological disorders and cancer, among others [31]. Interest in encapsulation strategies has also grown in parallel with the contemporary surge of efforts to move forward stem cell therapies in multiple research areas.[32]

Cellular encapsulation has always had a close and strong relationship with diabetes since one of the most straightforward models to test encapsulation efficacy has consistently been the pancreatic islet of Langerhans. In fact, islet encapsulation was attempted even before intraportal infusion of islets was successfully achieved by Dr. Lacy's group. In a short report published in 1970, Dr. Keith Reemtsma attempted xenotransplantation of fish islets inside a Milipore

macrochamber into the peritoneal cavity of rats; whether the chamber was made of nitrocellulose or polycarbonate was not specified in this report. With this approach, 63% of rats (85/135) showed >50% reduction in glycemic levels post-transplant, although only 8.2% (7/85) maintained these levels for >96 hours. Importantly, viable islets were observed as long as 20 days after transplant.[33] Ten years later, in one of the seminal papers in the field, Lim and Sun microencapsulated syngeneic islets and transplanted them into the peritoneal cavity of streptozotocin-induced diabetes rats, achieving diabetes reversal for a period of 3 weeks before graft attrition ensued.[34] Although ground-breaking, this report attests to contemporary limitations of the biomaterials, which was probably the culprit leading to graft attrition, given the syngeneic nature of the model. Ever since, encapsulation has remained at the frontlines of research in the field of islet transplantation and, subsequently, β-cell replacement therapies, mainly because of its translational potential. This is further driven by the absence of off-target effects due to both the use of innocuous materials and the possibility of eliminating the use of diabetogenic immunosuppressive drugs favored by current approaches. A non-systematic search up to September 15, 2019 in PubMed/MEDLINE using the terms "islet transplantation", "microencaps*", "macroencaps*", "coating", "compounding", "microsphere*" and "algin*" reveals an increasing number of publications related to these terms over the last decade (**Figure 1**)**,** although it seems that research in the field has followed a pattern of "peaks and valleys" which appears to be promoted by periodical landmark studies.

Figure 5.1.1. MEDLINE/PubMed search for studies involving diabetes and cellular encapsulation



Note: A non-systematic search up to September 15, 2019 in PubMed/MEDLINE using the terms "islet transplantation", "microencaps*", "macroencaps*", "coating", "compounding", "microsphere*" and "algin*" was conducted (N=1193). Major landmarks in the field are included in the figure.

The ideal encapsulation device would be safe, biocompatible, durable, allow for unhindered glucose-insulin metabolism, as well as optimal nutrient exchange and disposal of cellular waste products, preserve and protect the islet mass from inflammation and immune rejection and, be easily implantable and retrievable. Essentially, an ideal encapsulation device and/or strategy would yield what is sometimes referred to as a "bioartificial pancreas" (BAP). Achieving this is extremely relevant since, as previously emphasized, current insulin therapies do not recapitulate the complex interplay and physiological responses characteristic of an actual cell (or group of cells), including cell-to-cell interactions within the islet of Langerhans that provide the finesse required for optimal glycemic homeostasis.

5.1.5 - Physicochemical Considerations for the Use of Encapsulation in Cellular Therapies

A strong foundation on the physicochemical properties and characterization of different biomaterials and technologies is paramount to guide any research efforts attempting to achieve an "ideal" encapsulation device and/or strategy. Physical characterization of the type and mode of delivery of each encapsulation strategy should include information on the size, shape, surface and mechanical properties of the employed biomaterials, as well as porosity, among other parameters. Similarly, chemical characterization should involve a thorough assessment and reporting of the biomaterial properties such as chemical composition, molecular weight, ion binding capacities, interactions with other materials and purity.[35] The following section presents a short synthesis on these matters and summarizes vital concepts to consider when designing and implementing a novel encapsulation strategy which will also be valuable when interpreting reports in the field. The interested reader is referred to several recent reviews for a more comprehensive view on the matter.[35-37]

5.1.5.1 - Physiological and anatomical considerations

A comprehensive review of the physiology and anatomy of the islet is beyond the scope of this review, but basic aspects are relevant to understand the potential implications of encapsulation on the islets, mainly those concerned with vascularization, glucose sensing and hormone secretion. First, islet vascularity *in situ* has several distinct features. Islets have both afferent arterioles and efferent veins, which are subjected to autonomic and paracrine regulation, particularly at the precapillary level; this introduces a highly dynamic component to regulation of blood flow to the islet. Moreover, islets, as compared to acinar tissue, have more fenestrated, denser, and wider capillaries, which comprise 8-10% of the islet's volume; this translates into islets receiving around 5-20% of the blood flow to the pancreas, despite constituting 1-2% of the total pancreatic mass. The presence of nearly 10 times more fenestrae compared to acinar tissue coupled with significantly greater blood flow (5-6 ml/min x g vs 0.4-1.0 ml/min x g) and proximity of islets to arterioles jointly provide an ideal environment for effective oxygen and nutrient diffusion, as well as glucose sensing and hormone delivery.[38] In fact, blood flow is also regulated in response to glucose and insulinotropic stimuli, which provide effective feedback mechanisms. Finally, even though there is evidence that islets transplanted into tissues with continuous vasculature (e.g. kidney capsule) eventually develop fenestrae,[39] vascular density remains lower as compared to their native environment in the pancreas.[38] While encapsulation introduces a desirable physical barrier to protect islets from the cellular immune response, it hinders blood vessel ingrowth and, ultimately, blood flow to the islets. Furthermore, encapsulation intrinsically increases the average distance between capillaries and cells, which is inversely proportional to oxygen tension (PO_2) values. It is believed that a distance as low as

100 micrometers from the capillaries can promote cell hypoxia and lead to necrosis.[40] In this regard, the average diameter of current islet encapsulation capsules oscillates between 300-1000 micrometers, leading to estimated PO_2 levels of <10 Torr.[40] This affects oxygen and nutrient uptake leading to islet necrosis, but also to alterations in the kinetics of glucose sensing and hormone secretion. Together, changes in blood flow and molecule kinetics after encapsulation have an enormous impact on islet survival and function and, ultimately, become key determinants to establish normoglycemia after islet transplantation.

Following islet encapsulation, the sole mechanism enabling oxygen and nutrient exchange, as well as glucose sensing and insulin secretion is passive diffusion driven by electrochemical concentration gradients. In principle, this would support adequate glucose and insulin kinetics to achieve normal basal glucose levels. In fact, there is preclinical evidence that intravenous glucose tolerance tests (IVGTT) can be normalized after transplantation of encapsulated islets,[41, 42] which supports the notion that islet transplantation, even when encapsulated, can restore physiological glucose homeostasis. At this point, it is worth introducing the commonly-neglected fact that glucose influences its own disposal independent of changes in plasma insulin levels.[43] This phenomenon, termed "glucose effectiveness", has been demonstrated to play a role in improving insulin-independent mechanisms of glucose disposal. Thus, the main issue regarding glucose metabolism after islet transplantation and, more importantly, after transplantation of encapsulated islets, seems to be the impaired and far-from-physiological postprandial insulin response. In a healthy person, this response is highly dependent on the incretin effect, which refers to the potentiation of insulin secretion driven by neurogenic mechanisms (vagal-cephalic component) and gastrointestinal hormone (glucagon-like peptide 1, GLP-1) secretion following oral glucose intake. The incretin effect contributes

to approximately 60% of postprandial insulin secretion.[44] It has been shown that islet transplant patients, even those that have become insulin independent, have an impaired incretin effect after an oral glucose tolerance test, which resembles that of patients with type 2 diabetes.[45] Proposed mechanisms include islet denervation,[45] reduced β-cell mass[46] and GLP-1 resistance.[47] Additionally, the level of impairment would be affected by factors such as the site of implantation, which would determine the degree of physiological resemblance of insulin secretion. Particularly relevant is the fact that both neurogenic- and hormone-dependent mechanisms behind the incretin effect would be further disrupted (and not eventually recovered) if the islets are encapsulated. This, coupled with the fact that molecule diffusion (e.g. glucose, insulin, glucagon) depends on gradient concentration (which cannot be overemphasized), imposes a significant challenge for encapsulation strategies to recapitulate physiological glucose control. Whether improvements in device design and/or addition of medications to improve glucose sensing and insulin secretion (e.g. GLP-1 analogs) could overcome these hurdles remains to be tested.[43]

5.1.5.2 - Mechanical Considerations

Mechanical characterization of encapsulation methods has mainly focused on polymeric hydrogels in microsphere structures, although the same principles may be applied to other types of biomaterials and, perhaps, other structures. The most important mechanical aspects that should be described for every novel encapsulation approach include: mechanical resistance and stability, size and shape, spatial distribution, permeability, and leakiness.[35]

Mechanical resistance and stability of a biomaterial, in general, are determined by evaluating stiffness (resistance to deformation, including swelling) and toughness (resistance to

fracture). These two properties are essential to achieve durable and functional immunoprotection, since mechanical disintegration caused by deformation and/or fracture leads to increased permeability, epitope/cell exposure and, consequently, activation of the immune response. Mechanical resistance and stability can be optimized by modifying the physical and chemical interactions of and with the biomaterial. For example, mechanic stability of alginate, the prototypic biomaterial for encapsulation, is determined by the type of alginate, concentration and the size of applied gelling/crosslinking cation. It follows then that alginates with a high α-$_G$-guluronic acid content, highly concentrated, and that are crosslinked with larger cations (e.g. Ba^{++})[48] or at fixed-ratio combinations (Ca^{++} and Ba^{++}, 50:1)[49] have increased mechanical stability. Furthermore, polycation/polyamino acids layers around alginate beads can also improve mechanical stability (while decreasing pore size); commonly applied examples are poly-$_L$-Lysine, poly-$_D$-Lysine, poly-$_L$-Arginine and poly-$_L$-Ornithine. Other interesting approaches to increase mechanical resistance and stability have been carried forward by Dr. Minglin Ma laboratory at Cornell University. By combining alginate-based encapsulation with a central "backbone" made of a Ca^{++}-releasing nanoporous synthetic polymer (nylon), this group has achieved both uniform *in situ* cross-linking and improved handling capabilities, as well as effective short-term immunoprotection and function (100% diabetes reversal) in both an allotransplant and xenotransplant models using the intraperitoneal cavity as an implantation site.[50] Another approach by the same group included designing and building elastomer-reinforced interconnected toroidal structures covered with alginate-embedded islets. This novel encapsulation strategy improved stability and proved to be easily retrievable from the intraperitoneal cavity. It also showed adequate immune-protection in a xenotransplant model with 100% of the mice achieving long-term diabetes reversal until graft retrieval at 84 days

post-transplantation. Islet function and viability after retrieval remained intact.[51] These two reports attest to the importance of multidisciplinary bioengineering approaches to advance the field of cellular encapsulation. Mechanical resistance and stability can be assessed through different methods including: compression tests, micromanipulation, atomic force microscopy, swelling and explosion/osmotic stress assays, and agitation/shear force exposure assays.[52] Currently, the values for optimal mechanical resistance are still being defined, thus a recommendation or standard cannot be put forward, which precludes any attempt at homogenization of manufacturing practices.

Size and shape of the encapsulation structure have been shown to be relevant in terms of biocompatibility and biotolerability, particularly regarding the degree of foreign body response (FBR). Although there is no consensus, several conceptions have prevailed in the field; materials with smooth edges, less surface porosity, and smaller size are more biocompatible. These conceptions are not without controversy. First, although smooth edges are believed to be more biocompatible, the evidence is not recent and scarce,[53, 54] thus, the optimal geometry of encapsulation devices remains undefined. Second, although lower surface porosity would promote a lower degree of FBR, this is not always desirable, as the FBR can be harnessed to promote vascularization and habilitate inhospitable implantation sites such as the subcutaneous space.[55] Third, regarding size, a recent pre-clinical study by Veiseh et al. has elegantly shown that increasing sphere size (from 0.5 to 1.5 mm diameter) is associated with a significantly reduced immune response, both locally and peripherally. This phenomenon was found to be independent of the implanted biomaterials (alginate, glass and, stainless steel) and relevant to both small (mouse) and large (non-human primates) models. Finally, increasing the sphere size was found to improve outcomes after xenogeneic islet transplantation, such as diabetes reversal

and prolonged graft survival without immunosuppression.[56] Fortunately, size and shape are easily adjustable by current processing and manufacturing techniques, which allows specific tailoring of encapsulation structures to modulate the FBR and other aspects of the immune response, as well as optimization according to the specific conditions of the implantation site.

Spatial distribution refers to the disposition and organization of the biomaterials within the encapsulation structure. It is extremely relevant for encapsulation strategies involving hydrogel polymers. Spatial distribution is predominantly influenced by the gelling conditions (type and concentration of ions) and, to a lesser degree, by the type of biomaterials, which can lead, for example, to a wide range of different configurations spanning from a liquid, almost polymer-free membrane with a highly-concentrated core, to an almost homogeneous polymer distribution throughout the encapsulation structure.[35] Naturally, spatial distribution is a decisive factor for the long-term performance in relation to other factors such as of mechanical stability, permeability, and surface characteristics. Typically, spatial distribution is assessed using confocal laser scanning microscopy and post-processing 3D reconstruction.

Considerations regarding permeability are of utmost importance when designing and testing any encapsulation strategy. Permeability refers to the biomaterial's capacity to protect cells from destruction by the immune system while maintaining an adequate transport of oxygen and other molecules required for cell survival and function, as well as allowing for disposal of waste products outside the encapsulation structure. Despite being an essential aspect of cellular encapsulation, the specific characteristics and requirements for optimal performance concerning permeability are also not clearly defined.[57] Hence, there is significant controversy around the optimal effective molecular exclusion size, which is driven mainly by the perceived relevance of the components of the immune response to encapsulated cells: the cellular response or the

humoral/cytokine-mediated response. Although the debate is ongoing, most encapsulation strategies focus on preventing direct contact between encapsulated cells and immune effector cells. Furthermore, there is relative consensus around the fact that permeability should be adjusted depending on the specific cellular source for transplantation, with lower effective exclusion sizes recommended for xenotransplantation as compared to allotransplantation.[58] Assessment of permeability is mainly carried using size-based exclusion assays (molecular-weight cut-off, MWCO) as well as the rate of diffusion of macromolecules. The former involves obtaining a minimal molecular weight that will be excluded by the semi-permeable membrane, which is directly related to the membrane's pore size and inversely related to the size (and shape) of the molecules tested; the latter involves obtaining mass transfer, permeability and diffusion coefficients, which are usually determined by the chemistry of the membranes and solutes (e.g. membrane composition and thickness, chemical potentials/gradients).[37] Size exclusion and rate of diffusion are typically determined by methods tracing the ingress and/or egress of labeled molecules over time, the most common being proteins and polysaccharides (e.g. dextrans), although there are other methods described in the literature.[35] Characterization and adequate reporting of permeability assessments of the biomaterials (and type of solutes) used in encapsulation strategies should be a minimum requirement in the field of cellular encapsulation.

Finally, leakage of the capsular components, such as polymers (e.g. mannuronic acid from high-M alginate) or gelling ions (e.g. Ca^{++} or Ba^{++}), will influence the properties of the encapsulation structure over time. Consequently, leakage will stimulate immune responses by the host, but could also lead to toxicity secondary to accumulation of these components within the host. This is a particularly relevant issue with the commonly-used barium-containing

microspheres, since there is a latent possibility of leakage and accumulation of this cation that could lead to toxicity.[35] For example, studies have shown that high concentrations of barium in gelling solutions (20 mM $BaCl_2$) may reach limits that exceed recommendations to avoid toxicity, thus, it is recommended either low concentration of barium ions are used (e.g. 1 mM) or that barium leakage always be measured after implantation of high concentration barium-containing encapsulation devices.[59] Naturally, toxicity to islets would be also assumed to happen, although this could be inherently prevented because most studies testing new encapsulation strategies and/or biomaterials begin by assessing islet viability and function *in vitro* which functions as a "quality check" before proceeding with *in vivo* studies. Nevertheless, it should be mentioned that *in vitro* toxicity testing, although indispensable, will never recapitulate the post-transplant *in vivo* environment which could affect polymer degradation and, potentially, lead to islet and systemic toxicity. Leakage of capsular components has been quantified using mass spectrometry of diverse components in plasma, in the washing solutions or in different tissues.

5.1.5.3 - Chemical Considerations

Chemical characterization of biomaterials used for encapsulation involves analysis of their composition and its implications on biocompatibility. Most encapsulation strategies use polymers, thus, most evidence circles around this type of chemical structure. Two suggested starting points to characterize polymers would be 1) to describe the chemical composition of the monomeric units and their primary structure (amount and character of their functional groups), and 2) the molecular weight characteristics (weight number, average molecular weight and distribution).[37] The former is essential to predict interactions with other molecules

(including water), while the latter relates to the polymer's viscosity and rheological properties; both are also important for the process of sphere formation and gelation. Several techniques to conduct an appropriate chemical characterization of a biomaterial include: high-resolution nuclear magnetic resonance, X-ray photoelectron spectroscopy, time-of-flight (ToF) secondary ion mass spectrometry, and size-exclusion chromatography.[35, 37] Second, and particularly relevant to the medical field, is the documentation of the degree of purity of the biomaterial (e.g. endotoxin content, microbial contamination, and polyphenol and protein content).[37] Finally, additional chemical characterization includes hydrophilicity and charge, which are closely related to the surface properties of an encapsulation structure. The degree of hydrophilicity of a biomaterial is inversely proportional to the degree of protein adsorption and denaturation, which has a direct impact in epitope exposure and, consequently, the degree of the immune response; a positive charge has the same effect. Importantly, protein adsorption is the triggering factor for cellular overgrowth and the FBR (see below). Hydrophilicity is assessed by measuring the wettability and water contact angle (↑ wettability and ↓ water contact angle = ↑ hydrophilicity), while the electric charge is assessed by measuring the zeta potential (↓ zeta potential = ↓ charge). Consequently, an encapsulation structure with high hydrophilicity and negative charge would elicit a low-degree FBR and, thus, would have increased biocompatibility and biotolerability.

Biomaterials for cellular encapsulation can be divided in two large groups (**Table 5.1.1**): natural and synthetic.

Table 5.1.1. Advantages and disadvantages of natural and synthetic biomaterials used for cellular encapsulation

Biomaterial	Advantage	Disadvantage	References*
Natural: • **Alginate** • **Agarose** • **Hyaluronic acid** • **Chitosan** • **Collagen** • **Fibrin** • **Heparin**	• Extensive experience and evidence using multiple cell types and cargo (particularly for alginate) • Inexpensive to use and produce • Highly accessible in large quantities • Mass scale production for some • Lower immunogenicity, including a positive innate immune remodeling reaction • Some materials have bioactive properties such as binding sites for cells and adhesion molecules • Direct and indirect resemblance to the extracellular matrix	• Some reports (both successful and unsuccessful) used lower purity biomaterials • Significant lot-to-lot variability • Natural variability from *in vivo* sources • Added cost for sterilization • More limited mechanical properties • Variable and limited capacity to control and predict degradation rates • Unpredictable immune responses due to the variable content of impurities	54, 60, 91, 111
Synthetic: • **Poly-glycolide (PGA)** • **Poly-lactide (PLA)** • **Poly(lactic-co-glycolic acid)[PLGA]** • **Polycaprolactone (PCL)** • **Polyurethane (PU)** • **Polytetrafluoroethylene (PTFE)**	• Increased interest and more recent reports that include more thorough assessment of the physicochemical properties • Absent adaptive immune responses • High reproducibility and availability on demand • Mass scale production for some • High tunability of mechanical properties, shape and composition • More controlled and predictable degradation rates • Prolonged shelf life	• Stronger immune response to biomaterials, in general • Lower capacity to interact with cells • More expensive • Lower accessibility to some biomaterials	

*Adapted from Mariani et al. (54).

Natural biomaterials include polysaccharides such as alginate, agarose, hyaluronic acid and chitosan, proteins such as collagen, fibrin or heparin, and decellularized tissue matrices. They are extracted from living organisms by dissolving them in solvents or enzymes, they are relatively inexpensive to produce and biocompatible due to the already present binding sites for cells and adhesion molecules. Nevertheless, the "living" source of the materials inevitably leads to significant product variability which affects their chemical composition and structure and precludes predictability, replicability and, consequently, clinical translation.[54, 60-62] Synthetic biomaterials include mainly organic polymers such as poly-glycolide (PGA), poly-lactide (PLA), poly(lactic-co-glycolic acid)[PLGA], polycaprolactone (PCL), polyurethane (PU) and polytetrafluoroethylene (PTFE), among others.[61] Synthetic biomaterials are inert, can be easily tailored to different mechanical properties, shapes and chemical composition with high reproducibility and reduced costs, as well as increased durability. Nevertheless, they may have to be used in combination with other synthetic and/or natural materials to improve biocompatibility and biotolerability due to a stronger innate immune response and inflammatory reactions.

Although a surge of safe, biocompatible, durable and stable materials has become evident, most studies still use a limited number of biomaterials as the "backbone" of their encapsulation techniques. The following section presents information on the most common biomaterials used for islet encapsulation.

5.1.5.3.1 - Natural Materials

Alginate is the most common polymer used to produce microcapsules. Alginate is an anionic linear binary polysaccharide isolated from seaweed and comprised of blocks of (1→4)

linked β-$_D$-mannuronic (M blocks) and α-$_L$-guluronic (G blocks) acid residues that are joined together by glycosidic bonds.[63] Chemical characterization of alginate microcapsules mainly focus on variability of their G and M configuration and length of each bond, which is dependent on the source of algae extraction,[64] although alginates can also be extracted from several bacteria (e.g. *Azetobacter vinelandii)*. Alginates are usually characterized based on their G block and M block content into high-G/M alginates, intermediate-G/M alginates and low-G/M alginates. For example, high-G alginates (70% and more), and longer lengths of G-blocks, increase mechanically stability (more rigid) and permeability when compared to alginate microcapsules of high-M material.[63] Given that encapsulation of islets using alginate has some challenges, such as insufficient mechanical and chemical stability, and the lack of precise porosity control, these considerations become very important [65]. The polycation poly-$_L$-lysine (PLL),[34] and more recently poly-L-ornithine (PLO) and poly(methylene-co-guanide) [66] have been used as additional layers on alginate surfaces to improve mechanical stability and to provide better control of permeability.[65] Multilayered polyelectrolyte coatings also increase alginate's immunoprotection efficiency due to improved control over gel porosity.[67] Although commercially-available alginate is relatively pure, residual proteins that provoke inflammation and overgrowth of fibrotic tissue may be present thereby negatively affecting the long-term survival of the graft.[65] Overall, alginate provides a flexible platform to test encapsulation processes, structures, techniques and modes of delivery, since it is widely available and can be easily modified.

Other common *natural* biomaterials used for cellular encapsulation therapies include chitosan, cellulose and collagen. Chitosan is degraded by enzymatic hydrolysis from crustacean cells, mollusks, insects and fungi, it has a hydrophilic nature, thus, it has a low fibrotic and

inflammatory potential. It has been used in several disease models and with different cell types, but its widespread applicability is limited due to its low mechanical stability. Nevertheless, combination with other more mechanically stable materials, such as alginate, has been tested in pre-clinical models of xenogeneic and allogeneic islet transplantation, showing promising results.[59] Cellulose is highly abundant in nature as it is the predominant component of the cell wall of several plants. It cannot be degraded in humans, which, in theory, would be a highly desirable characteristic for an encapsulation material. Furthermore, it has been reported that cellulose has a MWCO (which would protect cells from humoral immune responses but could impair nutrient diffusion and glucose metabolism) as well as a low fibrotic potential. Survival of cellulose-encapsulated islets has been corroborated in *in vitro*[68] and *in vivo*[69] pre-clinical studies, although delayed insulin responses during glucose tolerance tests were also reported.[69] Further studies using this biomaterial will help elucidate its role in encapsulation strategies. Collagen is found abundantly in mammalian connective tissue. Several types exist, with type I being the most commonly used for cell encapsulation. Pre-clinical evidence supporting the relevance of collagen (type 1) encapsulation has already been reported for islet transplantation. In this report, researchers corroborated that type 1 oligomeric macroencapsulation does not have a deleterious role for islets *in vitro* and it may prove a valuable strategy for transplantation of encapsulated islets, even in the hostile subcutaneous site, where this strategy was found to promote "tissue integration" instead of a detrimental FBR.[70]

5.1.5.3.2 - Synthetic Materials

Synthetic biomaterials include: 1) Poly(ethylene glycol) (PEG), 2) Polytetrafluoroethylene (PTFE), 3) Polyesters, 4) Polyacrylates, 5) Polyamides, 6)

Polyepoxides, 7) Polyphosphazenes, 8) Poly(urethane) (PU), among others.[61] This section will discuss those most relevant to islet encapsulation. A detailed review and characterization of the chemical properties is beyond the scope of this review and the reader is referred to comprehensive reviews on the subject,[71-73] which will be used as a backbone for this section.

Poly(ethylene glycol) (PEG) is a versatile, water-soluble polyether compound and is probably the most commonly used synthetic biomaterial for islet encapsulation strategies. PEG polymers can be functionalized with the use of polyacrylates to form hydrogel networks with the added benefit that this can be done in the absence of toxic solvents. These PEG hydrogels are highly biocompatible and have biomechanical properties that can be fine-tuned for microencapsulation, conformal coating and layer-by-layer coating (LBL).[71] Microencapsulation studies using "acrylated" PEG have demonstrated no difference in viability and function in vitro compared to non-encapsulated islets. In vivo studies in xenotransplant rat models (pig to mouse) further showed immunoprotection when implanted in the intraperitoneal fat pads, albeit for a limited time (30 days). Conformal coating PEG-based techniques have also shown promising results in decreasing the instant blood-mediated inflammatory response (IBMIR).[74] New techniques for PEG-based encapsulation hold promise to improve outcomes in the field of islet transplantation, mainly membrane PEGylation, microfluidics-based encapsulation and immobilization of biologically-active particles (e.g. heparin) within PEG-based encapsulation membranes.[71, 73-76]

Polytetrafluoroethylene (PTFE), commercialized as Teflon, is a high molecular weight, non-biodegradable compound with high mechanical strength, high hydrophobicity and chemically inert. In clinical medicine, it is commonly used in its expanded porous form with interconnecting fibrils called ePTFE (Gore-Tex®). It induces none or little inflammation in the

body and is mainly applied as a leading component for vascular grafts, surgical meshes, stents, etc. [77] Thus, it is clinically safe. PTFE is mostly relevant to macroencapsulation and, in the field of islet transplantation several notable efforts have already been undertaken. Both Theracyte® and its successor, Encaptra® (PEC-Encap™ or VC-01™), which are devices being developed and tested by ViaCyte, Inc., have used a double PTFE membrane to immunoisolate islets and, most recently, β-cell precursors.[78, 79] Clinical efforts are ongoing (see below, NCT02239354), but preliminary reports have shown that "the Encaptra® device appears to be immune-protective with no evidence of allo- or auto-immune activation or sensitization in patients to date".[80] However, this information should be taken with caution given the very aggressive FBR that has been reported with the use of these devices which has hindered consistent and robust engraftment, leading to low cell viability and cell death[81] and thus, precluding any definite conclusions concerning specific aspects of the allo- and/or or auto-immune response to the encapsulated cells. These findings, coupled with PTFE's potential applications in various fields has motivated very active research efforts to refine this material. In this regard, W.L. Gore & Associates have partnered with ViaCyte, Inc., which will surely accelerate novel, more efficient and safer translation of these devices into the clinic, with particular benefits for the field of islet and β-cell replacement therapies. Further details on the structure and specific outcomes from these research efforts will be described in sections below.

Aliphatic polyesters include poly(glycolic acid) (PGA), poly(lactic-acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL), among others. All of them have been used for encapsulation of diverse cellular types. A common characteristic of the group is that they gradually degrade by hydrolysis in a bulk or surface erosion manner. Its degradation is dependent on several factors highly relevant to the field of cellular encapsulation:

structure, exposed surface area and size, level of hydrophobicity, applied stresses.[71] They are commonly copolymerized to adjust their degradation rate, which then becomes highly dependent on the ratio of glycolide to lactide. Besides the obvious concern with degradation of the encapsulation structure, degradation of particular aliphatic polyesters (e.g. PLGA) in itself promotes an immune response due to changes to the biomaterial that involve pH, instability and chemical modification. This is particularly relevant to controlled drug-delivery systems, but it can also affect proteins (e.g. hormones) released by cells. PLGA has been utilized in a murine islet xenotransplantation (pig donor to rat recipients) model, where islets implanted into the peritoneal cavity without immunosuppression showed capacity to ameliorate hyperglycemia (although not to normal levels). In vitro studies further showed impaired function of these retrieved encapsulated islets.[82] Other studies using combined strategies have shown more promising results. In a murine syngeneic islet transplantation model utilizing PLGA planar disks with a glycolyde:lactide ratio of 90%:10% (Vicryl®) coupled with Matrigel-embedded islets on the top of the disk and then implanted into the epididymal fat pad, all mice implanted with these composite scaffolds normalized glycemia. Mean glycemia was identical to control mice transplanted with islets into the kidney subcapsular space. Islets implanted into the epididymal fat without the PLGA scaffold did not achieve euglycemia, showing the relevance of the composite strategy.[83] Although this strategy did not provide immunoisolation, the technical simplicity and configuration of the scaffold opens the possibility for a macroencapsulation "sandwich" device in which Matrigel-embedded islets within "slices" of PLGA (Vicryl®).

Polyacrylates, in comparison to aliphatic polyesters, are non-biodegradable polymers. This category includes namely poly(methyl methacrylate)(polyMMA), poly(2-hydroxyethyl methacrylate)(polyHEMA), polyacrylonitrile (PAN) and polyacrylamide. Their composition

can vary from hard, crytal-like materials (polyMMA) to soft hydrogel-like materials (polyHEMA). Fine-tuning of the physical properties can be achieved by blending two or more polyacrylates in different ratios (e.g. HEMA-MMA). This last strategy is widely applied in encapsulation strategies for diverse cellular products.[71] HEMA-MMA has plenty of evidence supporting its benefits in terms of mechanical strength, permeability, cell viability and biocompatibility in different mammalian cells models. Unfortunately, not many studies have been conducted using islet encapsulation. The Sefton group, in 1990, showed that glucose kinetics of islets are relatively unaffected by encapsulation with HEMA-MMA,[84] nevertheless, due to its porosity (MWCO of 100 kDa), this biomaterial has not been shown to be fully immunoprotective.[73] Attempts using AN69 (PAN copolymerized with methallylsulfonic acid) have been also attempted in islet encapsulation. Despite initial studies in vitro showing optimal immunoisolation properties, in vivo murine studies demonstrated that a 1-day and 7-day intraperitoneal implantation period led to loss of permeability to glucose and insulin. The authors propose increased protein adsorption as a mechanism for these unfavorable results and supported this notion with in vitro studies where the alterations of glucose and insulin permeability seen in vivo were reproduced by coating AN69 capsules with fetal calf serum.[85]

Other synthetic biomaterials such as polyamides and polyepoxides have also been studied in the field of islet transplantation. Polyamides or poly(amino acids) are similar to proteins, but are composed of only one amino acid. There are natural polyamides (lysine, glutamate, aspartate, etc.) and synthetic (nylon [caprolactam]). Nylon was among the first materials used to encapsulate cells, although it was eventually abandoned due to its toxicity.[71] Nevertheless, recent efforts have tested a nylon external 3D-printed macrocapsule combined with an inner core of high-G-alginate-encapsulated insulin-producing cells (INS1E-β–cells) and

have shown nearly intact viability and function, although no in vivo studies were reported.[86] Polyepoxides, on the other hand, are formed by adding epoxy functional groups to polyamines to increase their crosslinking capacities. Originally developed by IBM (International Business Machines Corporation), SU-8, an epoxy-based polymer, has been used to manufacture single-cell "micro-boxes" with removable lids that can be used to encase/encapsulate a variety of cells, including islets.[71] Using this material, Gimi et al. first showed that islets within this "micro-boxes" retain their viability and function in vitro.[87] No *in vivo* studies using islets have been conducted with this synthetic biomaterial. Ongoing efforts using more refined and even newer synthetic and hybrid natural-synthetic biomaterials will significantly contribute to formulate more informed decisions on future research avenues to advance islet encapsulation.

5.1.6 - Biocompatibility Considerations for the Use of Encapsulation in Cellular Therapies

Besides physicochemical characterization of encapsulation devices and biomaterials, essential aspects concerning biocompatibility and biotolerability need to be consciously studied as the field moves forward into the clinical realm. Biocompatibility is defined by "the ability of a material to locally trigger and guide non-fibrotic wound healing, reconstruction and tissue integration", while biotolerability is defined by "the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reactions".[88] Traditional operational definitions and concepts of biocompatibility align more with the definition of biotolerability, while recent evidence now allows a shift in the definition of biocompatibility, which undoubtedly encompasses more complex processes than just the absence or attenuation of "inflammatory reactions". Encapsulation therapies, thus, should aim at designing inert biomaterials and devices that comply with these new definitions of biocompatibility and

biotolerability. Optimizing these properties will improve diffusion efficiency of oxygen, nutrients and relevant cargo, as well as safety and durability.

A pivotal process surrounding biocompatibility and biotolerability is the foreign body response (FBR). It can be characterized by acute and chronic inflammatory phases, followed by a long-lasting granulation tissue phase. The acute phase is typically of the innate kind and consists of responses to the biomaterial itself and to the procedure-related injury. This phase lasts from minutes to days and the central phenomenon is adsorption/desorption of proteins to the implant (Vroman effect), as well as activation of effector cascades (e.g. complement, coagulation), local exposure to cytokines and migration of cells (e.g. neutrophils and macrophages). The chronic phase lasts from days to weeks during a normal tissue response, but it can persist for the *in vivo* lifetime of the implant. It is mainly characterized by persistence of neutrophils and an increased presence of macrophages and lymphocytes which perpetuate a chronic state by secretion of diverse chemokines (CCL2, CCL4, CXCL8).[54] The granulation phase occurs if the insult persists, it may last up to years, and is characterized by a varying degree of fibrosis promoted by infiltrating fibroblasts and deposition of components of the extra-cellular matrix, as well as infiltration of foreign body giant cells (fusion of monocytes and macrophages), capillary formation and, finally, encapsulation. Capillary formation (neovascularization) has emerged as an extremely relevant aspect of the FBR. Several studies have been able to harness neovascularization by accelerating and increasing capillary formation with the use of pre-implantation strategies that elicit and modify the FBR to achieve nurturing environments for islet transplantation.[55, 89] Temporal considerations to maximize these approaches should be always considered as there is evidence suggesting that the timing of pre-

implantation before transplantation could be a decisive factor determining subsequent success of these strategies.[90]

The FBR, particularly during its chronic phase, also has an adaptive immunity component. Particles, ions and/or degradation products originating from the biomaterial, but also from the cells themselves, can be recognized by macrophages and dendritic cells and presented to T cells. In fact, a more pronounced adaptive immune response can be elicited by allogeneic or xenogeneic implanted cells. The cells in charge of perpetuating adaptive immune responses are helper T cells (Th) and, whether these cells display a pro-inflammatory phenotype (Th1) or an anti-inflammatory/regulatory phenotype (Th2) depends on many factors. Helper T cells have direct communication with tissue macrophages and, analogously, these cells can be driven into a pro-inflammatory phenotype (M1 macrophages) or an anti-inflammatory/regulatory phenotype (M2 macrophages). These profiles are dictated, partly, by secretion of pro-inflammatory (TNF-α, IL-1, IL-6) or anti-inflammatory cytokines (IL-10).[54] Thus, Th2 cells and M2 macrophages have an indispensable role in promoting an anti-inflammatory, constructive, immunoregulated response to biomaterials. The fate and success of every encapsulation strategy is highly dependent on the degree and direction of both innate and adaptive immune responses.[91]

There are several factors affecting the immune response to biomaterials and, consequently, their biocompatibility, biotolerability and long-term efficiency. These include, but are not limited to: the type of biomaterial (e.g. natural vs synthetic), the physical and chemical properties of the biomaterial, donor-host interactions (e.g. allogeneic vs xenogeneic), the implantation site, and the use of different immunosuppressive and non-immunosuppressive drugs. The FBR should be considered as any other physiological process (e.g., coagulation) for

which innovative ways can been successfully designed and developed to modify it to our advantage. Indeed, several strategies have emerged to modulate the FBR elicited by biomaterials and foster a "friendlier" environment for encapsulated cellular products. Following the conceptual framework recently put forward by Ernst et al., these strategies can be classified as: 1) Biomaterial selection and modification, 2) Local delivery of immunomodulating drugs, and 3) Co-delivery and immobilization of biological agents.[92]

Every biomaterial has the potential to elicit a FBR, but the degree and specific characteristics of this response vary between the different type and composition of the biomaterial. As previously mentioned, synthetic biomaterials are inexpensive, easily obtained, highly and reliably modifiable, however, they are considered to promote inflammatory reactions due to intrinsically foreign nature which prevents tissue integration and long-term tissue fibrosis. In contrast, natural biomaterials, despite considered to be less immunogenic, they are less amenable to modifications and less reliable in terms of manufacturing variability, which can lead to batch-to-batch differences in the immune response thus hampering clinical translation (**Table 5.1.1**).[63] There is evidence suggesting that natural biomaterials promote a more anti-inflammatory phenotype response (M2 macrophage) as compared to synthetic biomaterials.[91] These differences may be attributed more to their chemical composition than to their natural or synthetic status, since it has been shown that immunological responses can vary significantly, even for the same biomaterial. In fact, a recent large-scale chemical screening to find alginates with better immunological, biocompatibility and biotolerability properties has been recently undertaken which supports this notion and shows promising results. The Langer/Anderson group found three triazoles-containing alginate analogs (among >750 screened) that showed a favorable profile in terms of a low-degree FBR characterized by

decreased macrophage and neutrophil infiltration in both rodents and non-human primates.[93] Large efforts like these, which are analogous to high-throughput screening used for drug discovery, will prove to be extremely valuable for the field of cellular encapsulation.[94] Naturally, combinatorial strategies (hybrid materials) using both natural and synthetic biomaterials could prove more beneficial at establishing an equilibrium that allows obtaining the best properties of each type of biomaterial.[95]

It is currently believed, although with some degree of controversy, that tuning specific physical properties can also have an impact in the FBR, biocompatibility and biotolerability. Hydrophobicity, for example, has been positively correlated with the degree of FBR due to the notion that water on the surface can be more easily replaced by a layer of hydrophobic proteins and/or cells (e.g. monocytes/macrophages).[96] Similarly, surface charges are also considered to affect the degree of protein adsorption, with positive surface charges associated with a more potent cellular immune response and protein adsorption as compared to negative ones, following electrostatic forces of repulsion (cellular membranes and proteins are negatively charged).[54] Another interesting example is pore size, which has also been directly related to the degree of immune response, although this relationship is complex. The current canon is that small pore size, by decreasing the contact surface area, leads to less protein adsorption, macrophage infiltration and protein adsorption. It has also been shown that a small pore size leads to a higher percentage of M2 macrophages and increased vascularization in the fibrotic capsule. This was proposed from studies using poly-HEMA sphere-templated scaffolds.[97] Nevertheless, there is controversy in the field about this aspect of encapsulation, since other studies have shown that larger pore size (using a PTFE device) led to higher vascularization around the encapsulation device.[98] Whether these contradictory findings can be explained by

the different biomaterials used in these studies is unknown. Size has always been a relevant issue regarding the physical characteristics of encapsulation structures and the immune response. A recent study has shown that spheres of larger diameter (1.5 mm vs lower) induce a lower degree of FBR, which translated into less fibrosis, less macrophage infiltration and, consequently, better viability and function in a model that used islets as the encapsulated cellular product.[56] These positive findings were replicated in similar size spheres using different materials besides alginate (stainless steel, glass, etc.). Studies *in vivo* showed that this improved profile led to higher rates of diabetes reversal in an islet xenotransplant model (rat to mouse).[56] The same group later published a report describing three triazoles-containing alginates that were found to ameliorate the FBR (see *above*). Interestingly, a combination of these novel compounds with a 1.5 mm-diameter sphere configuration seemed to be synergistic in its positive effects on the FBR.[93] Thus, harnessing and modifying immune and inflammatory responses by tailoring physico-chemical characteristics of biomaterials is a complex task and current efforts, although promising, still have a long way before they can be used in the clinic. Currently, mechanistic studies emphasize protein adsorption as the central event triggering the FBR, however, ongoing studies exploring changes in protein conformation and macrophage polarization may provide alternative insights into this process that could provide additional strategies to harness the FBR to improve biocompatibility and biotolerability of biomaterials.[92]

Local delivery of immunomodulatory drugs by embedding them within biomaterials and/or encapsulation structures represents another strategy to modulate the immune response and the FBR to biomaterials (and to cells) while eliminating the toxic effects of systemic immunosuppression. Glucocorticoids, which are steroidal anti-inflammatory drugs, provide a perfect example of this later concept. While preventing islet toxicity through avoidance of

systemic glucocorticoid administration was believed to be responsible for the success of the Edmonton Protocol,[19] more recent pre-clinical evidence suggests that local administration of dexamethasone may not only be non-toxic to islets but it may also prove to be a valuable adjunct to modulate the FBR and decrease fibrosis towards biomaterials, even at the subcutaneous space.[99, 100] Further research efforts testing innovative ways of drug delivery are being actively explored which, coupled with evidence from large animal models, will be extremely valuable to delineate the role of local glucocorticoid administration in islet transplantation. Several other compounds to regulate the inflammatory and the foreign body response to biomaterials *in situ* include nonsteroidal anti-inflammatory drugs such as ketoprofen,[101] polyphenols such as tannic acid[102] and curcumin,[99] other anti-inflammatory drug such as pentoxifylline,[103] as well as direct macrophage inhibitors.[104] Alternatively, local delivery of immunosuppressants such as Tacrolimus, which is one of the cornerstones of immunosuppressive regimes in clinical islet transplantation, may provide extremely useful, as recent pre-clinical models of encapsulated islet transplantation suggest.[105] The complexity and redundancy in the pathways involved in these immune responses may require a combinatorial approach to achieve maximum efficacy towards clinical translation.

Additional strategies to modulate the immune response include the use of composite approaches involving co-encapsulation with supporting cells, chemokines, ligands or trophic factors that are co-embedded within a supporting structure made of polymers and/or extracellular matrix.[106] These are commonly referred to as bioscaffolds. In the case of cellular co-encapsulation, two main cell types have been used in the field of islet transplantation: mesenchymal stem cells (MSC) and regulatory T-cells (Tregs). Currently, there is substantial pre-clinical evidence supporting many beneficial effects following co-transplantation of MSC

with islets, including improved graft survival and function.[107] These effects may be explained by cell-to-cell interactions and their secretory profile. Islet co-encapsulation with MSC has been recently associated with diminished pericapsular fibrotic overgrowth following implantation. Additionally, co-encapsulation and transplantation of these composite structures into the peritoneal cavity showed sustained viability and 100% diabetes reversal up to 50 days post-transplant, even in immunocompetent mice. Importantly, these promising results were only observed when MSC were stimulated pre-encapsulation with a cytokine cocktail including IFN-γ and TNF-α. Still, co-encapsulation with unstimulated MSC showed 71.4% diabetes reversal at 50 days compared to 9.1% with encapsulated islets alone.[108] Treg co-encapsulation has also been shown to be successful in pre-clinical models. Using an NOD (non-obese diabetic) mouse model, Graham et al. showed that co-encapsulating islets and Tregs in PLGA scaffolds led to improved allograft survival and long-term diabetes reversal rates of 50% without immunosuppression, compared to control mice treated with systemic (IV) Tregs and without any treatment, which lost their grafts approximately 15 days after transplantation.[109] Interestingly, no systemic Treg expansion was shown in the islet-Treg PLGA scaffold recipients, which contributes to the safety of this strategy by preventing any potential off-target or adverse effects. Several bioactive molecules may also be locally delivered by co-encapsulation in order to modify the inflammatory and immune response after implantation. Examples include the C-X-C Motif Chemokine Ligand 12 (CXCL12) which has proven to ameliorate the immune response in islet allo- and xenotransplantation models by locally recruiting Tregs,[110] transforming growth-factor-β 1 (TGF-β1) which showed to decrease cytokine expression and leukocyte infiltration as well as delayed islet allograft rejection,[111] TNF-α sequestering mimicking peptides which have shown to ameliorate TNF-α-induced

toxicity to encapsulated islets *in vitro,*[112] IL-1 inhibitory peptides that have demonstrated protection to encapsulated islets after exposure to a cytokine cocktail (IL-1β, TNF-α, IFN-γ),[113] and a conjugated streptavidin-FasL compound that resulted in prolonged survival of transplanted allogeneic islets when combined with rapamycin.[75] Alternatively, co-encapsulation with pro-angiogenic factors (e.g. VEGF) has shown promising results to further enhance neovascularization after encapsulated islet transplantation, which improves islet survival, engraftment and function, even in hostile environments, such as the subcutaneous space.[114] Finally, co-encapsulation using components of the extracellular matrix has also shown promising results in terms of improved cell viability, survival and function.[115] The surge of new compounds and biomaterials will allow infinite potential combinations to be tested, however, large-scale, structured and collaborative approaches will be required to select the most promising strategies for successful clinical translation.

5.1.7 - Encapsulation Strategies

The current goal of cellular encapsulation is to protect cells from the host's immune response by preventing cell-to-cell interactions between foreign and immune effector cells. This cellular response is believed to be the main driver for immune rejection, cell death and, subsequently, graft loss. Nevertheless, complement-mediated and/or cytokine-mediated cell death are also relevant pathways leading to vigorous immune responses, particularly in the case of xenotransplantation.

Encapsulation of pancreatic islets has followed two main approaches: macroencapsulation and microencapsulation. **Figure 5.1.2** provides a schematic description of these conventional encapsulation approaches. **Figure 5.1.3** provides a schematic description of

alternative and composite encapsulation approaches. Microencapsulation includes strategies such as conformal coating and layer-by-layer (LBL) encapsulation, which has sometimes been classified as an entirely separate approach, nanoencapsulation. Overall, these strategies rely on different techniques to immobilize islets within a semi-permeable structure that simultaneously permits the exchange of small molecules, such as glucose, insulin, oxygen, and other metabolites (including waste products) allowing islet survival and preserving their function, but also acting as a physical barrier that protects them from immunological insults and abrogates the need of immunosuppressive medications, which have been ligated to myriad toxic and diabetogenic effects.[116] Both macro and microencapsulation have its advantages and disadvantages, which will be presented in this following section.

Figure 5.1.2. Schematic representation of classical approaches for cellular encapsulation

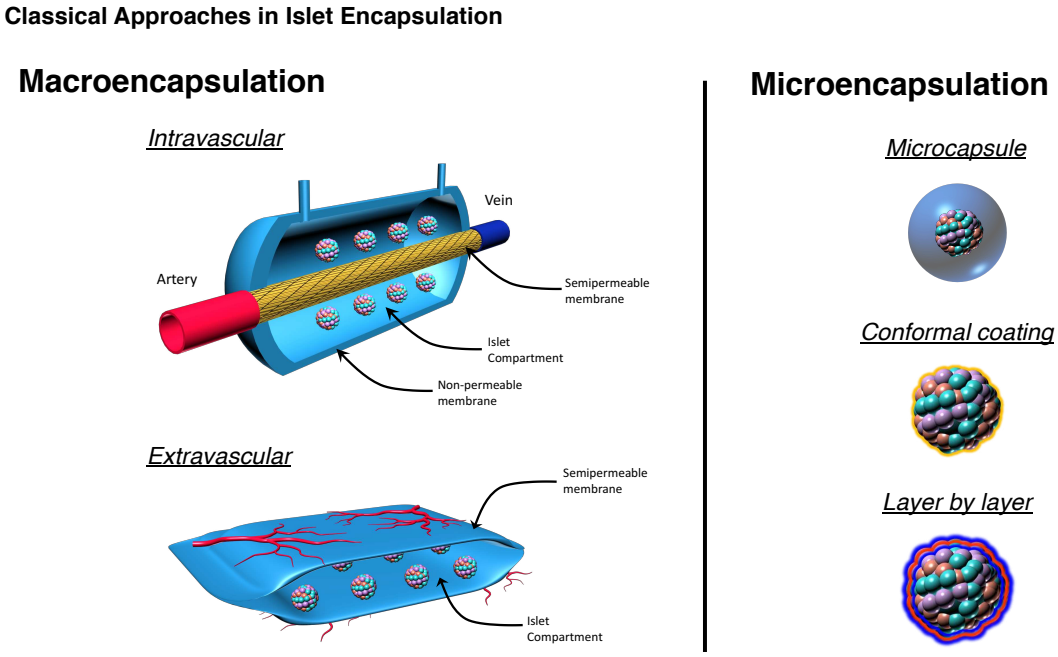
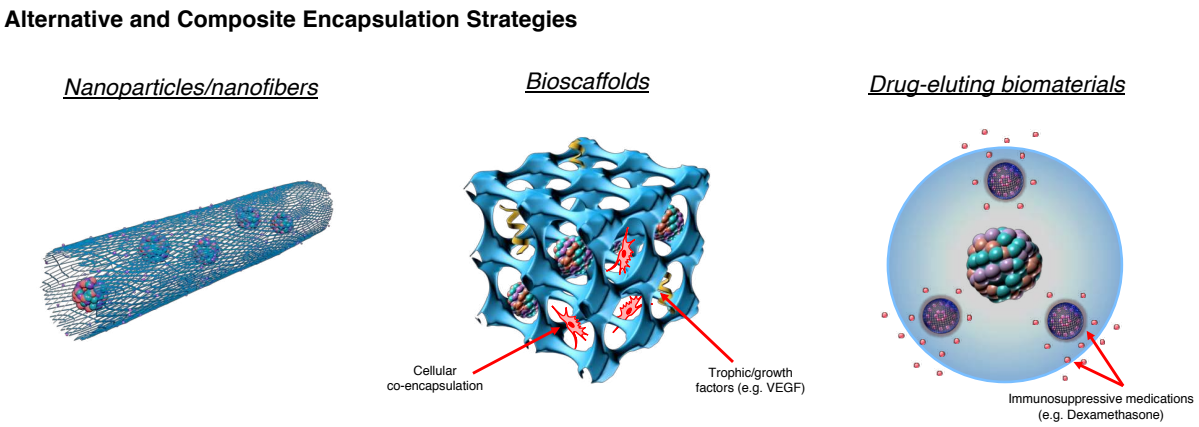


Figure 5.1.3. Schematic representation of alternative approaches for cellular encapsulation



5.1.7.1 - Macroencapsulation

Macroencapsulation involves housing multiple islets into a single encapsulation device or structure. Although there is no consensus, macroencapsulation structures are defined as those with >1 mm in diameter. The main advantages as compared to microencapsulation includes economization of space and volumes, prevention of cell clustering, protection from mechanical stress and, perhaps the most important, localization of the islet mass which allows full graft retrieval if needed for graft surveillances, in case of graft attrition or other adverse effects. The latter aspect has become extremely relevant with the use of novel stem-cell-derived β-cell replacement therapies. Macroencapsulation devices are divided into two groups: intravascular and extravascular.

5.1.7.1.1 - Intravascular Macroencapsulation

Intravascular devices mainly consist of a hollow device containing islets that are encapsulated within a permselective membrane (**Figure 5.1.2**). These devices are connected

directly to the circulatory system by surgical anastomoses thereby providing substantial oxygen and nutrient supply, as well as unhindered glucose sensing and, consequently, improved insulin secretion kinetics.[117] Conversely, the use of a permselective membrane promotes islet survival by sequestrating the graft from the immune system with the aid of a membrane barrier.[118] Given these two favorable aspects, intravascular macroencapsulation was among the first encapsulation strategies tested in the field, with several promising results obtained in pre-clinical studies.[117] The initial studies required *ex situ* placement of the device due to their size, thus, the devices were connected to the circulation but were maintained outside the animals. Naturally, these studies precluded long-term follow-up. In a study by Tze et al. in 1976, devices were fabricated from a polyvinyl chloride-acrylic copolymer fiber and loaded with allogeneic islets into streptozotocin-induced diabetic rats.[119] This study showed adequate insulin responsiveness with the device, nevertheless, this was a short-term study lasting only 4 hours due excessive bleeding secondary to heparinization to maintain patency of the device.[119] Using P-100 Amicon fibers to prevent thrombosis, Sun at al., in 1977, reported rapid diabetes reversal in rats (<1 hour) as well as near-normal glycemia in monkeys (<4 hours), that was sustained for 48 hrs; despite the use of the P-100 Amicon fibers, thrombosis of the device was reported as a common adverse event.[120] Two relevant devices were carried forward in the 1990s by Monaco's and Calafiore's groups. The first, referred to as the "hybrid artificial pancreas", consisted of a single-coiled, hollow fiber membrane contained within a disk-shape acrylic housing into which cellular cargo was compartmentalized. Within the acrylic housing, a tubular ultrafiltration membrane made from acrylic, with a wall thickness of 120-140 nM and a MWCO of 50 kDa, was introduced and connected to PTFE vascular grafts that provided the place for the vascular anastomosis. Two seeding ports were used to introduce the islets in the compartment between

acrylic housing and the tubular ultrafiltration membrane. This approach resulted in optimal long-term glycemic control in dogs.[121] However, efforts to translate this approach into the clinic were halted due to severe problems with thrombosis and bleeding. The second, an artificial vascular prosthesis, was comprised of two co-axial tubes, with the inner tube made of permeable Dacron® (poly(ethylene terephthalate)) and the outer tube made of PTFE. Islets were then encapsulated with alginate and placed in the space between the inner and outer tube. With this device, Calafiore's group reported long-term diabetes reversal, insulin independence and sustained C-peptide levels in a model of xenotransplantation (human islets into dogs).[122] Furthermore, a pilot clinical trial with two patients showed some promising results, although, robust control obtained in canines could not be recapitulated.[122] Further details on this and other pre-clinical and clinical studies will be presented in the following sections. Nevertheless, it should be emphasized that considering the advantages of intravascular encapsulation such as abundant oxygen supply, isolation of the graft from immune cells, and coupled with the tremendous progress in vascular surgery techniques and biomaterials, reassessment of this macroencapsualtion approach may be warranted.

5.1.7.1.2 - Extravascular Macroencapsulation Devices

Extravascular macroencapsulation devices can be grouped into either tubular or planar. These devices, as compared to microencapsulation devices have a lower surface:volume ratio which affects oxygenation and nutrient diffusion. Contrary to intravascular devices, oxygen and nutrient support for encapsulated islets in these devices depends solely on passive diffusion from the surrounding microcirculation, thus, the cellular cargo is prone to hypoxia and necrosis.[123] This is extremely relevant when choosing an implantation site, since partial oxygen

tension values are highly variable in different parts of the body.[124] Adding to this challenge are issues related to the biocompatibility and biotolerability of the biomaterial. Most biomaterials used for macroencapsulation lead to tissue overgrowth and fibrosis, which exacerbate oxygen and nutrient diffusion, as well as glucose and insulin diffusion kinetics. As previously mentioned, fine-tuning the physico-chemical characteristics of a biomaterial can direct the immune response and harness or accelerate intrinsic process such as neovascularization (which usually takes 3-4 weeks) to foster a less hostile environment during the early engraftment period.[125] These are known as prevascularization strategies and can be attained either by pre-implantation of a biomaterial that would elicit capillary formation before islet transplantation or by co-delivery of growth factors acting on vessel formation (e.g. vascular endothelial growth factor).[126] Other alternatives involve oxygenation to the islets by using direct oxygen administration,[127] oxygen-generating/releasing biomaterials,[128] or gene-therapy.[129]

Tubular chambers are typically made of polyacrylonitrile (PAN) and polyvinyl chloride (PVC), both of which are biocompatible materials.[118] Several research efforts have used these biomaterials as extravascular tubular chambers or sealed hollow fiber devices.[130-132] For example, Lanza et al., using a large animal model (canine) of allotransplantation, demonstrated that islets within tubular devices made from permselective acrylic membranes (similar to those used for the hybrid artificial pancreas previously described) implanted in the peritoneal cavity could lead to insulin independence in 50% of the recipients (51-82 days), as well as significant reductions in insulin doses without the use of immunosuppression.[132] Although promising, these studies showed shorter graft survival times than those with the intravascular devices, most likely because of insufficient oxygen and nutrient diffusion.

Planar devices are usually made from two circular or rectangular flat sheets that are sealed at the edges and that implement a loading port for cellular infusions. This planar configuration is believed to provide more mechanical resistance and stability to the device, a greater possibility of increasing islet seeding density and potentially improved oxygenation as compared to tubular devices.[118] Generally, devices of this type are implanted in the subcutaneous tissue or in the peritoneal cavity because of their configuration and size. Also, a prevascularization phase of weeks-months before transplantation is commonly pursued to further enhance the implantation environment. One of the most notable efforts using this encapsulation is the Islet Sheet, designed by Islet Sheet Medical and later supported by the Hanuman Medical Foundation. Its immunoprotection function is provided by acellular alginate layers while diffusion of nutrients, oxygen, glucose and insulin, as well as metabolite exchange occurs by passive diffusion from the surrounding microcirculation.[133] This sheet is comprised of alginate-embedded islets (optionally with a reinforced polymer) contained ("sandwiched") between two subsequent immunoprotective films of alginate-laden cellulose filter membranes. Optimal diffusion is facilitated by setting a thickness of the sheet to ~250 μm.[133] In an *in vivo* testing of the Islet Sheet transplanting 75,000 allogeneic IEQ (distributed in six Islet Sheets) to the omentum of a diabetic dog, normoglycemia was maintained for 84 days.[133] Importantly, at retrieval, Islet Sheets were intact and easily procured; graft-dependent normoglycemia was confirmed by showing hyperglycemia after retrieval of the Islet Sheet.[133] This attests to one of the major advantages of the extravascular macrodevices, the easy and complete retrievability.

More complex macroencapsulation devices consisting of inner and outer membranes with and without alginate-embedded islet cores, commonly referred to as chambers, have been also been attempted. One example is the TheraCyte™ device (Baxter Healthcare, Round Lake,

Ill., USA). This device is constructed with a bi-layered chamber of polytetrafluoroethylene (PTFE). The outer layer has a 5 μm thickness that encourages neovascularization[98] which then is glued to a second 0.45 μm inner layer to provide immunoisolation. At one end of the device, a polyethylene port permits loading of islets into the inner chamber. This chamber is encased in a woven polyester mesh to improve mechanical stability and durability. TheraCyte™ devices showed protection against rejection for an extended period of time in immunocompetent animal recipients.[78] Further modification of the TheraCyte device led to the Encaptra® Cell Delivery System, designed by ViaCyte, Inc. This system (also called PEC-Encap™ or VC-01) follows the same design principles and is currently undergoing Phase I/II clinical trials for safety testing (NCT02239354, NCT02939118); no definitive results have been published, although, as previously mentioned, preliminary reports show a need to optimize the biomaterials in order to decrease the FBR,[81] which is actively being addressed by this company, in association with *W.L. Gore & Associates***.** A derivation of this system, the PEC-Direct™ (also called VC-02), is currently also undergoing clinical testing (NCT03162926, NCT03163511). Importantly, iterations to the PEC-Direct™ include perforations to the PTFE membranes, which naturally eliminates the immunoisolation properties of these devices and, thus, immunosuppression would still be required. Of note, the cellular product used for these devices involves human stem-cell-derived pancreatic endocrine cells (PEC) and not mature islets, therefore, retrievability is an attractive attribute of these two devices.

An innovative and well-designed alternative to avoid intra-device hypoxia after implantation into the subcutaneous space is being carried forward by BetaO_2 Technologies Ltd. through its ßAir macrochamber. This device consists of two main components: 1) An oxygen module/tank that is connected to two subcutaneous polyurethane tubes that serve as oxygen-

refueling ports and that is separated by a gas-permeable membrane and 2) an islet module containing tightly-packed (>2,000 IEQ/cm^2) encapsulated islets within an ultrapure, high-G (62%) alginate, which is further immunoisolated by a layer of alginate-impregnated PTFE membrane (25 μm thick and 0.4 μm pore size).[41] Protocols for the optimal use of this device included daily oxygen (60% O_2, 5% CO_2) injections into the subcutaneous ports. This device, and several later iterations, showed promising results in pre-clinical models in terms of biocompatibility, as well as sustained islet viability and *in vitro* function after device retrieval.[41] Subsequent studies using immunocompetent rats were able to show diabetes reversal and recovery of metabolic capacity lasting throughout the duration of the study (3 months).[134] These results supported two clinical studies, but unfortunately, results were not so promising as none of these patients achieved insulin independence and only 1 (out of 5) attained a reduction in daily insulin doses. However, it must be noted that a sub-optimal islet doses were used for these studies.[127, 135] Further details of these studies will be discussed below.

A recent macroencapsulation device has been put forward by Dr. Desai's group at UCSF and now commercialized by Encellin. This device is composed of a polycaprolactone (PCL) thin-film macroencapsulation chamber arranged into a disk-shaped structure (1-5 cm in diameter) which is maintained using a peripheral nichrome wire. There are two versions of this device that vary in their pore size, from microporous (2 μm) to nanoporous (30-100 nm), both have a membrane thickness set at 10 μm.[136] These two characteristics optimize immunoisolation properties as well as oxygen and nutrient diffusion, providing a bridge between micro and macroencapsulation. Cells are loaded into the chamber through an opening of the two-layered structure which is then sealed using heat. A pre-clinical syngeneic mouse model using the "Encellin device" showed maintained viability and function of MIN6 cells and islets implanted

cells within these devices. Additionally, clear signs of increased vascularization starting at day 14 were observed, with a steady increase of 1.5% daily for 2 months.[136] Of note, testing of different biomaterials, including PLGA and polyvinylidene fluoride showed worse performance in terms of vascularization than PCL. Studies testing the Encellin device to sustain functional β-cell *in vitro* and *in vivo* differentiation from human embryonic stem cells have proven successful and have shown long-term *in vivo* function in terms of glucose-stimulated C-peptide secretion.[137] Further research investigating the immune response and overall performance of the "Encellin device" on allo- and xenograft models are ongoing. A very relevant characteristic of this device is that PCL degradation can be tuned to match the lifetime of the encapsulated cells, which eliminates the need for device removal. Nevertheless, this could have a direct impact on immunoisolation, and it is not clear if long-term immunosuppression would still be required.

5.1.7.2 - Microencapsulation

Microencapsulation involves encasing one or a small number of islets into immunoisolating structures which frequently have <1 mm in diameter, although this cut-off is not a standard in the field. Microspheres composed of alginate represent the prototypical microencapsulation structure, but other shapes and biomaterials have also been used. The sizes vary widely, from 350 μm to 1.5 mm, but current efforts are focusing on achieving increasingly smaller capsule sizes. This reduction in size has been facilitated with the use of modern fabrication techniques, such as electrostatic and/or air jet driven deposition methods.[138] Microencapsulation is advantageous due to the increased surface to area/volume ratio which is driven by the commonly used spherical configuration of the structures; this configuration also provides immunological and physico-mechanical advantages, as well as feasible large-scale,

highly precise and easily tunable manufacturing capabilities.[72] Consequently, this maximizes oxygen and nutrient diffusion dynamics which are indispensable for islet tissue survival and function.[34] Another advantage pushing microencapsulation forward is that implantation of these structures normally requires a minimally invasive procedure (e.g. percutaneous techniques) that can be tailored to the implantation site. There is substantial information, both from pre-clinical and clinical studies, endorsing microencapsulation as a safe, effective and feasible strategy for cellular encapsulation, including islet transplantation.[139] Furthermore, microencapsulation strategies have proven to support β-cell differentiation and maturation, which will further motivate future research endeavors in the field.[79]

Microencapsulation encompasses two main approaches: conformal (also conformational) coating and layer-by-layer (LBL) encapsulation (also called nanoencapsulation). As previously mentioned, nanoencapsulation is sometimes considered a separate category and commonly refers to those immunoisolating structures using membranes with pore sizes in the nanometer range. Nevertheless, we believe that the terms macroencapsulation and microencapsulation, in the field of islet transplantation, are more related to the number of islets being included within the encapsulation structure, with the former including tens to thousands, and the latter including one to a few islets. We acknowledge that there's no consensus in the field, and these terms and conceptual frameworks are likely to evolve as the field moves forward. A short description of conformal coating and LBL encapsulation follows.

Conformal coating refers to a strategy where very thin immunoprotective membranes and/or biomaterials cover individual cells or cell aggregates following their "landscape", thus, they "conform" to their irregular shapes and surfaces. These strategies can decrease the

molecular transit time between cells and their environment, thereby allowing more dynamic and sensitive cellular responses. Such a thin coating also significantly reduces graft volume by decreasing the biomaterial's contribution to the total transplanted volume, which could be relevant to clinically-proven implantation sites, such as the intraportal space.[140] Several biomaterials have been used to test this strategy, the most common being PEG and poly-HEMA-MMA.[140-142] Several methods to achieve cross-linking of the biomaterials used for conformal coating have been evaluated. One of the most common includes photo-cross-linking, that is, exposure to visible/UV light couple with photoinitiatior-mediated polymerization. Early attempts in islet encapsulation with these method achieved membranes with a thickness of 10-100 μm with a MWCO as low as 30 kDA. By "PEG-coating" adult porcine islets, Hill et al. achieved diabetes reversal in a rat model (xenotransplantation) without immunosuppresion.[142] A similar approach in non-human primates, albeit with a modified PEG component, was carried by Novocell (now ViaCyte) and demonstrated adequate allograft function with low-dose immunosuppression. Attempts to carry these forward in two patients with T1D were not as promising, as these patients did not achieve insulin independence, nevertheless, a significant decrease in hypoglycemic episodes was observed and C-peptide levels were detectable, although lower than expected.[141] Importantly, the authors report sub-therapeutic doses for these transplants, as patients received 45% and 58% of the calculated therapeutic islet dose. Other techniques for conformal coating involve microfluidic devices, which have shown to be able to provide adequate coating and preserved function *in vitro* and *in vivo*. A shortcoming of PEG is that it is less biocompatible than other biomaterials and susceptible to cytokine attack (particularly shown in large animal models), which demands more research efforts exploring other biomaterials for conformal coating.[137]

Layer-by-layer (LBL) encapsulation is one of the newest approaches in cellular encapsulation, including pancreatic islets. This approach allows significantly decrease in the coating thicknesses coating, even when compared to conformal coating. LBL encapsulation is as an alternative technique for islet surface modification. This type of coating uses electrostatic alternating layer deposition of polycations and polyanions, resulting in precise control over the thickness of the coating.[143] The choice of polymers, their concentration, and the number of layers can be modulated to control coating thickness in the nanometer range. Some examples of widely used polymers are alginate, polystyrene sulfonate (PSS), and poly(acrylic acid) (PAA), while examples of polycations are poly(allylamine hydrochloride) (PAH), poly (diallyldimethylammonium chloride) (polyDDA), and poly-$_L$-lysine (PLL).[65] LBL coating has the advantage that it can be performed at room temperature, requiring neither sophisticated instruments nor subsequent annealing of the deposited film, making it more suitable for widespread use.[143] Kozlovskaya et al. (2012) have proposed using hydrogen bonds to achieve LBL coating assembly instead of the widely used electrostatically-bonds due to cytotoxicity of the latter method.[144, 145] This group used poly(N-vinylpyrrolidone) (PVPON) and tannic acid (TA) to coat islets, which was proven to restore euglycemia in diabetic mice and protect the graft from autoreactive T-cell responses.[102, 146] Nevertheless, further studies on PVPON/TA coating using allo- and xenotransplants model are needed. Recently, Park et al. have used LBL coating with PEG and heparin to provide "nanoshielding" for islets being transplanted into the portal vein. These authors used induction and maintenance immunosuppression since their main objective was to protect islets from IBMIR. With this strategy, 60% (3/5) of subjects achieved long-term insulin independence as compared to none in the control and PEG alone (non-heparin) "nanoshielded" groups.[147] These are promising results, which attest to the immense

potential of nanoencapsulation and nanotechnologies as a future solution to many of the immunological obstacles for the clinical success of islet transplantation. LBL coating techniques have become extremely versatile and thus have extended to several other fields of study. Due to this technique's capability to incorporate molecules that recapitulate the properties of extracellular matrices of multiple tissues,[148] LBL coating has been also used for encapsulation of other cell types besides pancreatic islets.[149] Additionally, its favorable performance profile and its continuous technical refinement has allowed LBL coating to be used to encapsulate probiotics,[150] growth factors,[151] multiple drugs,[152] but also, to be used as a contributing technique for 3D tissue bioengineering[153] and even as a coating for biosensors.[154] The vast repertoire of possibilities and scenarios in which LBL coating can be used facilitates composite encapsulation approaches that could harness each technique's main advantages to provide maximum immunoisolation while allowing optimal cell survival and function.

5.1.8 - Implications of the Implantation Site

With the advent of more complex encapsulation strategies including newer biomaterials, composite bioscaffolds and drug-eluting biomaterials, evaluation of implantation sites beyond the clinically-proven intraportal circulation have become increasingly more feasible and attractive. This section will summarize current evidence testing implantation sites for encapsulated islet transplantation. **Table 5.1.2** shows major implantation sites relevant to islet encapsulation therapies, as well as their advantages and disadvantages.

It should be emphasized that implementation of encapsulation devices and/or strategies should always consider the implantation site. An intrinsic issue regarding islet and β-cell replacement therapies is the fact that these cells will be implanted outside their native

environment; the sole process of obtaining these cells puts them in a non-physiological and stressful situation. Several specific aspects to consider include: 1) Type of encapsulation approach (macro vs micro), 2) Route of insulin delivery (portal vs systemic), 3) Space to accommodate physiologically-relevant volumes, 4) Blood and/or oxygen supply, 5) Technical aspects of implantation, monitoring and, potentially, retrieval, 6) Inflammatory response and engraftment process and, 7) Potential for immune protection.[155] Ongoing and future efforts should always put these aspects into balance to select the most appropriate implantation site for each encapsulation device and/or strategy. The following section presents a synthesis of the most relevant aspects of current implantation sites for islet transplantation, with a focus on those having the most evidence for those used for islet encapsulation.

Table 5.1.2. Summary of different anatomical sites used for implantation of cellular encapsulation therapies

Transplant Site	Clinical Trials	Advantage	Disadvantage	Reference*

Intraportal	No	• Minimally invasive (in humans)
		• Clinically established as the gold-standard
		• Physiological recapitulation of insulin delivery (intraportal)
		• Potentially favorable long-term immunological environment
			• 60-70% graft loss due to instant blood-mediated inflammatory reaction (IBMIR) and ischemia/reperfusion injury
			• Exposure to high levels of immunosuppressive drugs
			• Procedural risks: hemorrhage, hematoma, thrombosis
			  o Limited graft mass/volume to avoid complications
			  o Potentially riskier with encapsulated islets
			• Graft retrieval nearly impossible
			  o Limitation for stem-cell therapies
			  o Limitation for histological analysis and follow-up (e.g. in case of rejection)
			• Difficult to test in small animal models
			• Low clinical translation potential for novel encapsulation methods
				19, 147, 156, 157

Kidney (subcapsular)	No	• Pre-clinically established (current “gold-standard"), particularly in murine models
		• Highly vascularized
		• Minimal IBMIR
			• Invasive
			• Lower oxygen tension (vs intraportal)
			• Non-physiological recapitulation of insulin delivery (systemic)
			• Procedural risks: hemorrhage, hematoma
			• Graft retrieval nearly impossible
			  o Limitation for stem-cell therapies
			• Low clinical translation potential for novel encapsulation methods
			  o Tight capsule in humans → limited graft mass/volume
				7, 161, 162

Peritoneal cavity	Yes	• Minimally invasive • Highly vascularized • Increasing evidence generated from pre-clinical studies	• Medium clinical translation potential for novel encapsulation methods • Graft retrieval nearly impossible o Limitation for stem-cell therapies	178, 183, 184, 185, 186, 187,

		• Most studied encapsulate clinical method to date • No limitation on graft mass/volume • Physiological recapitulation of insulin delivery (intraportal)	• Limitation for histological analysis and follow-up (e.g. in case of rejection) • Inflammatory response o Risk of adhesions	188, 189, 190, 191,
Subcutaneous	Yes	• Non-invasive • Increasing evidence generated from pre-clinical studies • Safest • Easy monitoring, including biopsy o Ideal for stem cell therapies • Easy retrieval • No limitation on graft mass/volume • High clinical translation potential for novel encapsulation methods	• Lower oxygen tension (vs intraportal) • Requirement for preconditioning and/or priming (e.g. prevascularization) • Non-physiological recapitulation of insulin delivery (systemic) • Delayed engraftment period • Exposure to physical or thermal injury	41, 55, 125, 127, 135, 211, 213, 214
Intravascular	Yes	• Highly-vascularized • Low hipoxia • High exposure to nutrients	• Non-physiological recapitulation of insulin delivery (systemic) • Low clinical translation potential for novel encapsulation methods • High potential for intra-device thrombosis o Need for anticoagulation • Potential for severe bleeding (rare)	119, 122, 207, 210, 216,

Adapted from Pepper et al., 2015 (55). * A detailed description of clinical studies is shown in Table 5.1.3.

5.1.8.1 - Liver/Intraportal Space

Pioneered by Paul Lacy in 1973,[156] intraportal infusion of islets is the preferred route of administration in the clinic and the first site to show that insulin independence can be achieved after islet transplantation in patients with T1D.[18] Until recently, it was the only site shown to be able to foster long-term insulin independence.

Intraportal delivery of encapsulated islet is limited by many factors (**Table 5.1.2**), but mainly, by the increased size of the islet graft after encapsulation. Previous studies have shown that one of the main factors to avoid complications such as portal vein thrombosis is the packed cell volume and, in fact, there is evidence supporting that a packed cell volume <5 mL has been shown to prevent this complication.[157] Volumes of up to 100 times greater are expected with current encapsulation strategies, which would probably increase the risk of vascular complications. Ongoing efforts to improve encapsulation strategies, such as conformal coating and layer-by-layer encapsulation methods may limit packed cell volume of encapsulated islets. Furthermore, the use of bioscaffolds and/or drug-eluting materials could confer immunomodulatory characteristics to shied islet from the hostile immunological environment,[147] particularly, from IBMIR, which has been shown to be responsible for up to 60% islet loss in the immediate post-transplant period and, consequently, the main cause for primary non-function after islet transplantation.[158] Similar to the kidney, the liver has a capsule and, therefore, a subcapsular space. This implantation site has been recently tested in an encapsulated (alginate magnetocapsules) xenotransplant pig model (human islets to nondiabetic pigs) and has shown to be a better implantation site in terms of a decreased FBR and fibrosis when directly compared to the muscle and subcutaneous site.[159] Importantly, this last report did not compare the liver surface vs intraportal infusion, which is the clinical standard, thus it

requires future efforts to define its role in clinical islet transplantation.[159] Similar methods using islet cell sheets and adhering them to the liver surface using immunocompromised murine models have also shown a favorable profile for this site. The liver subcapsular space may be a new home for islet transplantation that would not be too far from the current. Although subcapsular implantation is achievable using minimally invasive methods such as ultrasound-guided implantation,[159] which can also be used for the kidney subcapsular site, whether the liver subcapsular site would preserve physiological recapitulation of insulin responses (which is a major advantage for the intraportal site) is currently unknown. Unfortunately, there are currently no published studies in humans evaluating transplantation of encapsulated islets into the portal vein which precludes any comparison to other clinically-tested sites such as the peritoneal cavity, the subcutaneous space and the intravascular space.

5.1.8.2 - Subcapsular Kidney Space

Islet transplantation into the subcapsular space of the kidney (also referred to as kidney capsule, KC) is the gold standard for pre-clinical rodent models, which is by far the most common model used in research. Direct comparison with other sites such as the liver (intraportal), muscle and omentum has shown that the KC has the advantage of having lower mortality, lower mean operative times, requires a lower marginal mass to achieve diabetes reversal, and achieves faster time to euglycemia.[160] Despite this favorable profile in rodents and the fact the it shares some disadvantages with intraportal transplantation (e.g. relative hypoxic environment), islet transplantation into the KC has not adequately permeated to large animal models due to partial or negative results, and only a few clinical attempts have been conducted over the years, mostly with negative results.[161] For example, islet autotransplantation in non-

human-primate models has shown that the KC was inferior to intraportal administration in terms of diabetes reversal, albeit only a 10-day follow-up after transplantation was conducted.[162] Similar results have been observed in human clinical trials of autotransplantation in terms of insulin independence (0% in the KC vs 42.8% in the portal circulation),[163] albeit only two patients were treated using the KC as an implantation site in this study. In a clinical study of islet allotransplantation comparing KC vs intraportal infusion, only 2/3 patients in the KC group had detectable, but transient, C-peptide levels as compared to 6/6 patients in the intraportal group which all had detectable and persistent C-peptide levels. Importantly, no patient in any group achieved insulin independence or sustained insulin reductions.[164] This evidence, coupled with the relatively higher invasiveness of the procedure in humans compared to intraportal infusion (percutaneous vs open surgery approach) and other anatomical considerations such as the relatively more rigid capsule in humans that precludes infusion of large volumes, add to the hurdles with clinical translation. Of note, clinical evidence supporting a less favorable profile for the KC was generated before the Edmonton protocol and before more advanced islet isolation methods yielding purer preparations were established, which could decrease packed cell volume. Furthermore, the introduction of islet xenotransplantation, where islet preparations of high purity (>90%) could further decrease packed cell volume, urges a reconsideration of the notion of space limitation in the KC. Thus, a current reappraisal of the KC as a potential transplantation site should be considered. Evidence that IBMIR could be avoided and that toxicity from immunosuppressants may be ameliorated at this location further supports this line of thought. Recent studies using a fully allogeneic non-human-primate model where autologous islets (from 50-70% pancreatectomies) were transplanted into the KC to create composite islet-kidney grafts before subsequently transplanting this composite islet-kidney grafts to allogeneic

recipients have shown better outcomes in terms of glucose homeostasis and insulin independence when compared to both free islets into the KC and the intraportal circulation.[165] This study suggests that the main disadvantage of transplantation into the KC could be a delayed neovascularization leading to hypoxia and early necrosis. Additional combination of this pre-vascularized islet-kidney composite graft with expansion of hematopoietic stem cells and subsequent allotransplantation of both the islet-kidney composite graft and hematopoietic stem cells confirmed the same promising findings and allowed for tolerance to the graft without immunosuppression.[166] Clinical translation for this approach is complicated, but this proof-of-concept studies shows that the KC is effective for islet transplantation but adequate vascularization may require more time as compared to the intraportal site.

Regarding encapsulated islet transplantation, several pre-clinical reports have shown that implantation at the KC is associated with a more favorable immunological profile (less macrophage recruitment), higher capsule retrieval rate, weaker capsular overgrowth and optimal viability as well as function,[155, 167, 168] even when directly compared to the SC space and the intramuscular space.[159] As previously stated, islet encapsulation, depending on the approach and method, could increase the packed cell volume more than 100-fold, which hampers the potential applicability of the KC as an implantation site due to current paradigms on space and technical limitations associated with this site.[169] Whether improving human islet preparations purity by refining current isolation methods or using pig islets which are usually purer would allow for clinically-feasible volumes after encapsulation should be explored in the future.

5.1.8.3 - Peritoneal cavity/Omentum

The peritoneal cavity is rapidly advancing as the preferred site for transplantation of encapsulated islets and is currently the most commonly used implantation site to test novel encapsulation strategies or devices. Such phenomenon is mainly driven by pre-clinical evidence showing very promising results in small and large animal (non-primate) models.[50] This site is favored, at least in part, because it is not technically challenging and it allows large transplant volumes (suited for both micro and macroencapsulation). Furthermore, it is believed to allow physiological insulin secretion due to its communication with the portal circulation. It does have several drawbacks. A particular situation with intraperitoneal implantation of encapsulated islet was noticed when the first studies in non-human primates (NHP) were conducted. When encapsulated islets were implanted in the peritoneal cavity, the capsules migrated to the pelvis due to the upright position of NHP, which promoted clumping, triggered hypoxia and, consequently, necrosis. Naturally, this is directly relevant to humans. Laparoscopic approaches (vs laparotomy) and implantation into naturally-defined compartments, such as the *bursa omentalis* partly solved this issue.[170, 171] Furthermore, implantation of encapsulated islets into the peritoneal cavity still has some physiological caveats, some of these are common to every implantation site, but some are unique. First, the peritoneal cavity is relatively hypoxic as compared to the portal circulation. The peritoneum (visceral) receives around 3% of the splanchnic blood flow and an absolute blood flow of around 9.7 ± 1.9 ml/min x 100 g of tissue (~60 times less than the pancreatic islets, ~40 times less than the kidneys, ~6-8 times less than the liver, similar to the greater omentum and skin, and ~4 times higher than the striated muscle).[172, 173] This hypoxic environment is believed to worsen after encapsulated islet transplantation due to the increased metabolic demand at this, already precarious location.[155]

Second, although the peritoneal cavity is believed to recapitulate physiological insulin responses due to its venous drainage to the portal vein, this is controversial, since studies have shown impaired and delayed insulin secretion kinetics with intraperitoneal administration of insulin (as would happen with intraperitoneally-implanted islets) when compared to intraportal and systemic (IV) administration.[174, 175] In contrast, islet transplantation (non-encapsulated) into the peritoneal cavity in a murine model has shown to be associated with lower areas under the curve in glucose tolerance tests as compared to the KC, intraportal, and muscle implantation sites, which translates into better glucose clearance and metabolic capacity of the graft.[160] Of note, only intraperitoneal glucose tolerance tests were done in this study and no comparative IV or oral glucose tolerance tests were done, which demands caution when interpreting these results since glucose kinetics may be affected by the route of administration and islets in the peritoneal cavity would be rapidly and directly exposed to glucose, as compared to those in the kidney capsule, liver or muscle, a scenario that would only be exacerbated with encapsulation of the islets (see above).[176] Third, it is believed that the peritoneal cavity has a suboptimal capacity for revascularization. Although this may be true, there is significant data showing that diseased (e.g. inflamed) peritoneum does have a high potential for VEGF-dependent and -independent neoangiogenesis.[173] This may be relevant for transplantation of both free and encapsulated islets. Fourth, immunological responses at the peritoneal cavity seem to favor fibrosis and inflammation, since one of the natural roles of the peritoneal cavity is to contain any inflammatory process and avoid their dissemination. Thus, one of the major factors limiting optimal function of intraperitoneally-implanted encapsulated islets would be the FBR and fibrotic response, which has been shown to be significantly higher at sites such as the subcutaneous and KC. The fibrotic response (assessed by $CD68^+$ staining) has also been

correlated with lower capsular integrity, islet viability, insulin content and secretion.[177] Modified biomaterials, such as alginates, have also shown to hamper this response and sustain islet viability for more prolonged periods.[171] but complete correction of this immunologically hostile environment remains to be completely achieved.

Within the peritoneal cavity, a special mention should be done for the greater omentum and surgically engineered omental pouches. The greater omentum consists of a fatty, highly vascularized structure that originates from the greater curvature of the stomach and covers the intestines in an apron-like fashion. It is configured into micro mesh-like disposition containing blood vessels, nerves, lymphatics, adipose, mesenchymal and immune cells, as well as a supportive extracellular matrix. It originates from the visceral peritoneum and it is the structure that divides the greater sac (peritoneal cavity proper) and the lesser sac (*omental bursa*). It is believed to function as the "abdominal policeman" since it helps clear bacterial and foreign material and to contain inflammatory processes within the peritoneal cavity.[178] The greater omentum is commonly used in protective (e.g. post-perforation) and reconstructive abdominal surgery. The greater omentum has recently attracted attention as a potential implantation site for islet transplantation. Recognizing potential limitations with intraportal infusion of islets, the greater omentum as an alternative transplant site was introduced in 1977 by Ferguson and Scothorne. This early proof-of-concept study showed that, by "folding" the omentum over the implanted islets, islet rejection could be spared in this implantation site; the authors suggested that the greater omentum had an immunoprivileged nature.[179] This "folding" approach is still carried by most studies evaluating this potential implantation site for islets. A few variations utilizing sequential pre-conditioning strategies to create a more vascularized environment for

implantation,[180] as well as composite bioscaffolds using growth factors (e.g. VEGF), autologous plasma and thrombin have also been attempted.[178]

Implantation in the greater omentum demands a more invasive and technically-challenging approach as compared to other sites such as the intraportal circulation and the subcutaneous space. Nevertheless, this site conserves some of the advantages of the peritoneal cavity, such as the capacity for large volumes and physiological recapitulation of insulin responses.[160] It is also associated with improved vascularization and immunological conditions that can foster prolonged islet survival and function as well as a nurturing environment for stem cell-derived β-cell replacement therapies as compared to other sites.[181, 182]

Despite all these caveats, which are shared by other sites such as the subcutaneous space and the muscle, the peritoneal cavity represents a readily accessible, safe, and clinically feasible implantation site for both free and encapsulated islet transplantation. Whether the greater omentum will be better than the peritoneal cavity remains to be defined. Certainly, some of the advantages and disadvantages of this implantation site have been already delineated with the support of several published clinical attempts which will be reviewed in the following section and are summarized in **Table 5.1.3.**[183-191] Ongoing clinical trials utilizing novel cellular co-encapsulation strategies, and/or drug-eluting/coated biomaterials will undoubtedly contribute to delineate further potential avenues to optimize this site.

Table 5.1.3. Published clinical reports testing encapsulated islet transplantation

Author, year	Source (dose)	N	Encapsulation Strategy	IS	Comments
Intraperitoneal					
Wu et al., 1989 (188)	Human fetal islets (5, 3, 2 "transplantations")	3	• Microencapsulation • Alginate-poly-$_L$-lysine • 7-10-days culture period pre- and post-encapsulation	NS	▪ Follow-up: 6 months ▪ Procedural complications: None reported ▪ Adverse events: Persistent hypoglycemic episodes 1-3 months after transplant ▪ **Outcomes:** o Insulin independence: No o Insulin reduction: Yes, 2/3 patients, 20-30% reduction o HbA1c (%): not reported o C-peptide: 1 patient had an increase on days 10-12 (<0.08 → 0.37 pmol/ml). Returned to baseline at 6 months ▪ Device/capsules were not retrieved
Soon-Shiong et al., 1994 (189)	Human islets, pooled from 8 donors (1st dose: 9,957 IEQ/kg, 2nd dose: 5000 IEQ/kg 6 months after Total: 14,957 IEQ/kg)	1	• Microencapsulation • High-M alginate-polylysine	Yes I: none M: cyclosporine and azathioprine (continued due to previous kidney transplant)	▪ Follow-up: 9 months ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o Insulin independence: Yes ▪ Onset: 9 months post-transplant o Insulin reduction: Yes. From 0.7 U/kg/d ± 0.01 → 0.2 U/kg/d (1st dose) → None (2nd dose) o HbA1c (%): From 9.3 → 7.8 o C-peptide: From 0.1 ng/mL → 0.6 ng/mL (1st dose) → 1.0 ng/mL (2nd dose) o Improvement in neuropathy and stable renal function ▪ Device/capsules was not retrieved
Calafiore et al., 2006 (212)	Human islets (400,000 and 600,000 IEQ)	2	• Microencapsulation • Highly-purified 1.6% alginate	No	▪ Follow-up: 1 year ▪ Procedural complications: None reported ▪ Adverse events: None reported

			• Double-coat of poly-$_L$-ornithine (0.12 and 0.06%), and another layer of 0.04% alginate		▪ **Outcomes:** o Insulin independence: No o Insulin reduction: Yes. From 32 U/d (patient 1) → 20 U/d (48 wk, patient 1). From 37 U/d (patient 2) → 27 U/d (24 wk, patient 2)1 o HbA1c (%): From 9.75 → 6.7 (16 weeks) → 7.3 (24 weeks, patient 2) → 7.7 (48 weeks, patient 1) o C-peptide: From undetectable → 0.225 ng/mL (pre-meal and 0.6 ng/mL (post-meal) at 6 months → 0.25 (pre-meal) at 1 year (patient 1) o Abrogation of hypoglycemic episodes ▪ Capsules were not retrieved
Elliott et al., 2007 (190)	Neonatal pig islets (15,000 IEQ/kg)	1	• Microencapsulation • High-M alginate-poly-$_L$-lysine	No	▪ Follow-up: 9.5 years ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o Insulin independence: No o Insulin reduction: Yes, 30% reduction within 14 months post-tx. Return to baseline afterwards. o HbA1c (%): From 9.3 (pre-tx) → 7.1-8.4 (9.5 years follow-up) o Porcine C-peptide (urinary): From 0 ng/mL → 9.4 ng/mL (4 months) → 0 ng/mL (14 months onward) o Porcine insulin detected after glucose challenge (OGTT) at 9.5-years follow-up o No evidence of PERV ▪ Biopsy of capsules: viable cells (glucose-responsive *in vitro*)
Tuch et al., 2009 (187)	Human Islets Median of 178,200 IEQ (98,200-227,900)	4	• Microencapsulation • Barium-alginate	No	▪ Follow-up: 2.5 years ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o Insulin independence: No o Insulin reduction: No.

					○ HbA1c (%): No change ○ C-peptide (urine): undetectable, except for one patient (4 infusions): 0.06-0.34 nmol/L. ○ Positive anti-GAD antibodies developed and remained positive 2.5 years after initial infusion. ○ No detectable levels of anti-HLA I or II antibodies ▪ Capsule retrieval revealed necrotic islets with pericapsular fibrosis
Basta et al., 2011 (184)	Human islets (540,000-1,600,000 IEQ/patient	4	• Microencapsulation • Ultra-purified in-house prepared alginate/polyaminoacidic	No	▪ Follow-up: 7 years ▪ Procedural complications: 1 patient inadvertently had intramuscular implantation ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: Yes. From 34.25 ± 2.6 U/d → 24.7 U/d ± 5.0 (24 months) → 27 U/d ± 1.7 (36 months). Back at baseline doses by 7 years. ○ HbA1c (%): From 8.7 ± 0.5 → 7.6 ± 0.2 (24 months) ○ C-peptide: From undetectable → 0.23 ± 0.12 (pre-meal) and 0.60 ± 0.13 (post-meal) at 36 months ○ No detectable changes in levels of anti-GAD and anti-islet antibodies ○ No detectable levels of anti-HLA I or II antibodies ▪ No device retrieval analysis
Jacobs-Tulleneers-Thevissen et al., 2013 (191)	Human islets (300,000 IEQ)	1	• Microencapsulation • Alginate	Yes (failed intraportal islet transplant) I: basiliximab M: MMF + Tac	▪ Follow-up: 3 months ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: No ○ HbA1c (%): No change ○ C-peptide: increased to functional levels only after the first week post-tx (0.17 nmol/L)

					○ No anti-islet, anti-GAD antibodies, anti-HLA antibodies were induced. ▪ Device/capsules were retrieved and appear intact, free of fibrosis, with viable and functional, albeit impaired, islets.
Matsumoto et al., 2014 (186)	Neonatal pig islets (Group 1 [n=4]: 5,087 ± 84 IEQ/kg, Group 2 [n=4]: 10,416 ± 613 IEQ/kg, Group 3 [n=4]: 14,456 ± 334 IEQ/kg, Group 4 [n=2]: 19,822 ± 716 IEQ/kg)	14	• Alginate-poly-$_L$-ornithine-alginate	No	▪ Follow-up: 3 months ▪ Procedural complications: hypersensitivity, post-procedural discomfort and anxiety. Resolved without residual effects. ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: No ○ HbA1c (%): No change. No relationship between dose and HbA1c levels (Group 1 had the lowest HbA1c, although not statistically significant). ○ C-peptide: not reported ○ Group 1 and 2 (lower doses) had decreased hypoglycemic episodes. ○ No PERV detected ▪ Device/capsules were not retrieved and analyzed
Matsumoto et al., 2016 (185)	Neonatal pig islets (Group 1 [n=4]: 10,273 ±278 IEQ/kg Group 2 [n=4]: 19,099 ± 491 IEQ/kg [4 pts])	8	• Microencapsulation • Alginate-poly-$_L$-ornithine-alginate	No	▪ Follow-up: >600 days (1.6 y) ▪ Procedural complications: 1 episode of paralytic ileus, full recovery with conservative treatment. ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: No. ▪ Group 1: 58 ± 18.2 U/d → 51.0 ± 18.3 U/d ▪ Group 2: 59.1 ± 25.2 U/d → 43.4 ± 14.5 U/d ○ HbA1c (%): ▪ Group 1: 9.3 ± 1.4 → 7.7 ± 0.9 ▪ Group 2: 8.4 ± 0.4 → 6.6 ± 0.5 ○ Serious hypoglycemic events ▪ Group 1: no reduction in hypoglycemic events

					▪ Group 2: 43.5 ± 16.7/month → 16.4 ± 3.2/month o No evidence of PERV ▪ Retrieved encapsulated islet were viable and capsules intact, mild fibrosis. No *ex vivo* functional tests reported.
Baidal et al., 2017 (183)	Human (602,395 IEQ)	1	• Macroencapsulation (not immunoprotective) • Islets + Autologous plasma (1:2 ration) • 2 layers: thrombin (inner) + autologous plasma (outer)	Yes I: ATG + ETA M: MMF + Tac → MMF + Sir	▪ Placed in the greater omentum. Part of NCT02213003 ▪ Follow-up: 1 year (ongoing) ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o Insulin independence: Yes ▪ Onset: 17 days-post-transplant ▪ Duration: through follow-up (1 year) o HbA1c (%): 6.8 (pre-tx) → 6.0 (post-tx) o Beta score: 7 (75 d), 8 (6 mo), 7 (1 y) o Beta-2 score: 16 (75 d), 15 (6 mo), 10 (1 y) ▪ Device/capsules were not retrieved
Subcutaneous					
Scharp et al., 1994 (131)	Human islets (150-200 IEQ)	9	• Macroencapsulation • Acrylic-copolymer hollow fibers + 1% alginate • Permselective outer and inner membrane	No	▪ Three patients with T1D, three with T2D and three controls ▪ Follow-up: 2 weeks ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o No changes in glucose metabolism outcomes (proof-of-concept study) ▪ Device/capsules were retrieved o Encapsulated islets from patients with T1D and T2D did not respond to *in vitro* glucose challenge. Only responded after stimulation with theophylline. o Viability remained 90-95% in all patients except for one who had a blunt trauma at the site of implantation (70% viability) o Minimal pericapsular fibrosis was observed

Valdes-Gonzalez et al., 2005 (214)	Neonatal pig islets + Sertoli cells (1:30-100) Infusions: 1 (12/12), 2 at six months (11/12), 3 at 36 months (4/12) Total dose: 13,927 ± 1819.99 IEQ/kg	12	• Macroencapsulation • Pre-implanted collagen-generating empty devices: surgical-grade steel mesh tube + interior PTFE rod • Devices implanted 2 months prior to transplant	No	▪ Follow-up: 4 year (ongoing) ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** o Insulin independence: Yes, transient ▪ 2/12 patients, after 2nd infusion, <3 months duration. o Insulin reduction: Yes/No → two groups defined ▪ Group A (6/12 pts): 72.46% reduction at 1 year post-tx, 68.5% reduction at 2 years ▪ Group B: (6/12 pts): 15.7 increase at 1 year post-tx, 22.7% increase at 2 years o HbA1c (%): ▪ Group A (6/12 pts): From 11.02 ± 0.71 → 9.13 ± 1.14 (36 months follow-up) ▪ Group B: (6/12 pts): From 10.80 ± 1.13 → 7.83 ± 0.29 (36 months follow-up) o No detectable porcine C-peptide after 12 months in Group B and after 36 months in group A. o Low levels of anti-porcine C peptide antibodies detected. Increased titers of IgG and IgM anti-Gal ▪ Device retrieval (4/6 in Group A at 36 months) showed insulin-positive cells. No *ex vivo* functional tests reported
Scharp et al., 2006 (141)**	Human islets Patient 1: 17,500 IEQ/kg Patient 2: 18,800 IEQ/kg	2	• Microencapsulation • PEG conformal coating	Yes. Low dose CsA from day -5 to 30 post-transplant	▪ Follow-up: 6 months ▪ Procedural complications: None. The authors report significant fibrosis at the injection sites due to repeated and chronic insulin injections. ▪ Adverse events: not reported ▪ **Outcomes:** o Insulin independence: No o Insulin reduction: not reported o HbA1c (%): not reported o Decrease in percentage of days in hypoglycemia ▪ Patient 1: from 43.2% to 17.6% ▪ Patient 2: from 48.5% to 23.3

					○ Detectable C-peptide levels (previously non-detectable) ○ Increased titers of anti-insulin, anti-GAD, and anti-IA2 ○ Histological analysis: not reported.
Valdes-Gonzalez et al., 2007 (211)	Neonatal pig islets + Sertoli cells (1:30-100) Dose: 250,000 IEQ per infusion Total: 19,320 IEQ/kg Number of infusions: 3	1	• Macroencapsulation • Pre-implanted collagen-generating empty devices: surgical-grade steel mesh tube + interior PTFE rod • Devices implanted 2 months prior to transplant	No	▪ Follow-up: 35 months ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: Yes ▪ Onset: 30 weeks post-first-transplant ▪ Duration: 18 months ○ Insulin reduction: Yes. From 0.72 U/kg → 0.2 U/kg/d ○ HbA1c (%): From 8.2 (post-device-implantation period) → 7.8 (last follow-up) ○ Increase in porcine C-peptide after glucose stimulation ○ Increased titers of IgG anti-Gal after infusions. Unprovoked increased titer of IgM and IgG anti-Gal related to loss of insulin independence ○ "Reversal" in microalbuminuria ▪ Insulin, glucagon and Sertoli cells present in graft after 20 months. ▪ No PERV detection
Valdes-Gonzalez et al., 2010 (213)	Neonatal pig islets + Sertoli cells (1:30-100) Dose: 250,000 IEQ per infusion Median infusions: 3	23 (2 withdraw)	• Macroencapsulation • Pre-implanted collagen-generating empty devices: surgical-grade steel mesh tube + interior PTFE rod • Devices implanted 2 months prior to transplant	No	▪ Follow-up: 5.7 years (2.6-7.7) ▪ Procedural complications: None reported ▪ Adverse events: None reported ▪ **Outcomes:** ○ Insulin independence: Not reported. ○ Insulin reduction: Yes. From 1.11 $\pm$ 0.43 → 0.86 $\pm$ U/kg/d ○ HbA1c (%): From 7.8 $\pm$2.0 (post-device-implantation period) → 6.9 $\pm$ 0.9 (post-1st infusion) → 8.2 $\pm$ 1.5 (post-2nd infusion) → 8.2 $\pm$ 1.7 (post-3rd infusion) → 9.26 $\pm$ 2.4 (last follow-up) ○ Increase in porcine C-peptide after arginine stimulation

					○ Low levels of anti-porcine C peptide antibodies detected. Increased titers of IgG and IgM anti-Gal ○ "Decrease" in microvascular complications ▪ No device retrieval analysis
Ludwig et al., 2013 (127)	Human (2,100 IEQ/kg)	1	• Macroencapsulation • βair device	No	▪ Follow-up: 10 months ▪ Procedural complications: none ▪ Adverse events: None ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: Yes, 52 UI/day (pre-tx) → 43 UI/day (post-tx) ○ HbA1c (%): From 7.4% → ~6.5% (no specific values reported for follow-up) ○ C-peptide: detectable but below physiological ranges ○ Beta and Beta-2 scores: not reported ○ *In vitro* test of the chamber following retrieval showed C-peptide response to glucose challenge ▪ No *in vitro* test for encapsulated islets outside chamber
Carlsson et al., 2018 (135)	Human (155-180,000 IEQ) One patient received 360,000 IEQ	4	• Macroencapsulation • βair device: chamber with macroencapsulation (PTFE + alginate membrane) fueled with oxygen through ports	No	▪ Follow-up: 180 days ▪ Procedural complications: local, minor ▪ Adverse events: None ▪ **Outcomes:** ○ Insulin independence: No ○ Insulin reduction: No ○ HbA1c (%): No ○ C-peptide: undetectable ○ Beta and Beta-2 scores: not reported ○ No *in vitro* function after device retrieval ▪ Encapsulated islet outside the device showed normal function

Intravascular					

Calafiore, 1992 (122)	Human Islets (150,000 and 250,000 islets)	2	• Micro/Macroencapsulation
			  • Alginate microencapsulation
			• Macroencapsulation with tubular intravascular device with inner membrane of Dacron and outer membrane of PTFE
				No	▪ Follow-up: Patient 1: 240 days, Patient 2: 60 days
					▪ Procedural complications: None reported
					▪ Adverse events: None reported
					▪ **Outcomes:**
					  o Insulin independence: Yes (1 patient)
					  ▪ Onset: 18 days after implantation
					  ▪ Duration: 7 months
					  o Insulin reduction: Yes. Patient 1: ~75%, Patient 2: ~40%.
					  o HbA1c (%): Not reported.
					  o C-peptide: Increased in both patients. Specific values not reported.
					▪ No device retrieval for analysis

Prochorov et al., 2008 (216)	Rabbit fetal islets (>6,000 IEQ/kg)	19	• Macroencapsulation
			• Nylon microporous (1-2 μm pore) capsule
			• Arteria profunda femoris or forearm cubital vein
			• Antithromboti therapy with repoliglucin, pentoxyphilin and fraxiparin
				No	▪ Follow-up: 2 years
					▪ Procedural complications: lymphorrhea at the femoral site in one patient, pseudoaneurysm in one patient's *arteria profunda femoris* (required capsule removal).
					▪ Adverse events: None reported
					▪ **Outcomes:**
					  o Insulin independence: Yes.
					  ▪ Onset: 24 hours after surgery
					  ▪ Duration: days (not specified)
					  o Insulin reduction: Yes. Overall, 60-65% reduction. Three patients with non-T1D had 85% insulin reduction.
					  ▪ Implants in the forearm cubital vein had better performance
					  o HbA1c (%): Not reported. Serum fructosamine decreased significantly from 340.0 ± 24.0 mcM (baseline) → 250.0 ± 24 mcM (2 years post-surgery)
					  o Beta and Beta 2 scores: Not reported
					  o C-peptide: From 2.9 ± 1.4 nM → 89.0 ± 5.2 (14 days post-surgery) → 29.3 ± 2.7 nM (2 years post-surgery)

		○ No serious hypoglycemic events during follow-up ▪ No device retrieval for analysis
Total patients	**112**	

IS: immunosuppression, NS: not specified, IEQ: islet equivalent, I: induction immunosuppression, ATG: antithymocyte globulin, ETA: Etanercept, M: maintenance immunosuppression, MMF: mycophenolate sodium, Tac: tacrolimus, Sir: sirolimus, DSA: donor-specific antibodies, PERV: porcine endogenous retrovirus, OGTT: oral glucose tolerance test. *Device was implanted in the pre-peritoneal space. ** Details from this trial were obtained from Scharp DW, and Marchetti P. Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution. Adv Drug Deliv Rev 67-68: 35-73, 2014 (141).

5.1.8.4 - Subcutaneous Space and Intramuscular

The subcutaneous (SC) space is currently the most clinically-desirable implantation site. Multiple efforts, both pre-clinical and clinical are ongoing to optimize this location for islet transplantation, both for free and encapsulated islets, but also for stem-cell derived β-cell replacement therapies.[155] Combining a limitless cell source, complete elimination of immunosuppression and a safe, and probably ambulatory procedure, would immediately position cellular therapies as a potential true cure for diabetes. Even if the challenge of graft attrition due to non-immune causes (e.g. β-cell exhaustion and/or death) could be partially solved, thus allowing only limited periods of insulin independence, being able to completely retrieve and replenish encapsulation devices (or products) without major hurdles and complications could still be considered a highly desirable therapeutic choice due to the optimal glucose control compared to most exogenous insulin formulations, dosing schemes or delivery systems.

The SC space has several advantages and disadvantages (**Table 5.1.2**). The main issue, as with other implantation sites except for, perhaps, the intravascular space, is hypoxia. In this regard, it is worth emphasizing that measurements of tissue oxygenation are highly dependent on the instrument and/or technique used. For the SC space, one of the most commonly-used methods to measure tissue oxygenation is the transcutaneous oximeter ($TcPO_2$). With this method, values have ranged between <1 mmHg to 81 mmHg, which are highly dependent on two conditions: skin temperature and anatomical location of the electrode.[192] Most recent reports have acknowledged that $TcPO_2$ electrodes heat the skin to about 42-45º C, which dilates blood vessels and facilitates blood flow and, consequently, tissue oxygenation. In this regard, a temperature increase (37ºC to 44ºC) has been corroborated to significantly improve blood flow

and tissue oxygenation as much as 43.6 $\pm$ 20.7 mmHg.[193] One of the most recent reports has validated a novel method to measure interstitial tissue oxygen in human healthy subjects by using a phosphorescence-based porous poly-HEMA hydrogel containing a covalently bound palladium-benzoporphyrin which reacts to oxygen levels. Results from this study show that subcutaneous values of tissue oxygenation assessed by $TcPO_2$ center around a median of 57.0 mmHg, as compared to those assessed with this hydrogel-based method, which center around 5.5 mmHg.[194] Unfortunately, whether heating of the electrode led to these findings was not assessed by the authors. Given the significant surge in pre-vascularization strategies to enhance the SC space prior and/or during implantation,[155] tissue oxygenation measurements before and after pre-vascularization would be a valuable addition to future reports in the field. Additionally, the capacity to increase tissue oxygenation at the SC space by increasing local temperature, if confirmed, could be a useful and safe adjunct to future clinical trials testing encapsulated (or free) islet transplantation into the SC space by using heating pads for a short period of time after implantation. Despite the significant limitation conferred by hypoxia, there is abundant pre-clinical evidence showing that prevascularization strategies of the SC site are effective in "priming" this highly-desirable site for nearly optimal islet engraftment and survival, even with encapsulated islets. This has been thoroughly reviewed recently by Zhu et al.[155]

Additional potential roadblocks to advance the SC space as a new home for islets include the altered non-physiological insulin kinetics and the potentially increase in immunogenicity at this site. Non-physiological insulin kinetics are an obvious concern, even with transplantation at the intraportal site, which is believed to provide the closest physiological resemblance compared to healthy patients. At the SC, insulin response has been shown to be delayed as compared to healthy controls;[43] this is, obviously, a shared problem with even the most

advanced and automated exogenous insulin therapies, which even require a pre-meal bolus administered by the patient to adequately regulate post-prandial glucose levels. Additionally, changes in the SC space architecture secondary to chronic insulin administration, such as fibrosis and scarring, could further impair insulin secretion kinetics. These factors precluding adequate glucose sensing and insulin secretion would be further aggravated by islet encapsulation, and directly related to the encapsulation structure/biomaterial characteristics.[176] Regarding immunogenicity, a recent study has shown that the SC space shows a greater immunoresponsiveness to alginate capsules as compared to the intraportal and subcapsular kidney space. Whether this could be related to decreased viability and apoptosis, with the subsequent release of relevant immunogenic cargo, was not directly assessed[159] and, whether this would be the case in humans remains to be tested. Thus, the potential benefits in terms of safety and applicability of the SC for islet transplantation warrant vigorous efforts which should be continued and extended in a generalized fashion in the field.

The intramuscular (IM) site has also surfaced as a favorable implantation site for islet transplantation in the last couple of years. This site provides limited direct contact with blood (minimizing IBMIR), adequate vascularization and oxygenation,[195] easy accessibility for implantation and monitorization and, if required, easy retrievability.[195, 196] Although introduced in pre-clinical models since 1978 by Weber et al.,[197] the first successful clinical report came in 2008 and involved a 7-year old girl with severe hereditary pancreatitis that underwent a total pancreatectomy and autotransplantation of 160,000 IEQ into the brachioradialis muscle. This intervention maintained long-term C-peptide secretion and supported normal HbA1c levels with low insulin doses, and no recurrent hypoglycemic episodes up to 2-years after the procedure.[198] Conversely, results from a small case series describing islet allotransplantation into the IM site

in four patients were not as promising. Despite patients achieving slightly higher C-peptide levels, only one patient had a decrease in insulin requirements (50% decrease at 1 year); however, suboptimal numbers of IEQs were transplanted in these patients.[199] The IM site appeared safe and no severe complications were reported, although the authors emphasize that strategies to support improved vascularization and oxygenation are needed to promote this site as a feasible alternative for islet transplantation.[199] In this regard, the IM site has shown to be particularly advantageous to support the high metabolic demands of endocrine tissues, as has been thoroughly demonstrated in patients having parathyroid gland autotransplants.[200] In fact, an ongoing clinical trial, the Pancreatic Islets and Parathyroid Gland Co-transplantation for Treatment of Diabetes in the Intra-Muscular Site (PARADIGM, NCT03977662) seeks to build on the success seen with parathyroid gland transplantation into the IM site and harness its pro-angiogenic and pro-survival effects to support engraftment and function of islets into the IM site. These effects have been recently reported to be driven by a unique subset of $CD34^+$ cells residing in the parathyroid glandular tissue and proven to improve engraftment of mouse and human islets, as well as stem cell-derived insulin-producing cells.[201] Results of the PARADIGM trial will be extremely relevant to advance further efforts to transplant encapsulated β-cell replacement therapies into more hypoxic environments (e.g. the SC and IM sites) that, nevertheless, are more desirable and clinically translatable. To date, there are no clinical studies evaluating encapsulated islet transplantation into the IM site, nevertheless, there is evidence from pre-clinical studies that could guide future research endeavors exploring this implantation site. An early study tested implantation of agarose embedded rat islet allografts into the intermuscular site which was prevascularized using a polyethylene terephthalate (PET) mesh bag filled with a collagen sponge seeded with gelatin microspheres containing basic fibroblast

growth factor. After two weeks of prevascularization, agarose-embedded islets were introduced into the PET mesh bag. With this approach, the authors report reversal of hyperglycemia without immunosuppression that lasted >35 days, which did not occur in the other groups testing no prevascularization or different iterations of the previously described procedure.[202] Another example comes from a PhD thesis chapter of a graduate student from Dr. Oberholzer's group at the University of Illinois at Chicago. This work shows that the extent of fibrotic overgrowth and the FBR elicited by alginate-microencapsulated islet transplantation into the IM site in rats is extremely dependent on the source of the islets. The author, Dr. Bochenek, reports that syngeneic encapsulated islets showed no tissue overgrowth and intact islet viability, while encapsulated allogeneic islets showed loss of islet morphology which was associated with 100% tissue overgrowth and a multilayered cellular infiltrate composed of α–smooth muscle actin-positive myofibroblasts, $CD68^{+}$ and $CD3^{+}$ cells. Both types of encapsulated islet were implanted for a period of 8 weeks.[203] The author cautions on the possible lack of protection from allorejection with the biomaterial used for encapsulation (ultrapure, low viscosity, high glucuronic acid, alginate with Ca^{++}/Ba^{++} as gelling ions), but also, mentions that a rat model may not be optimal for IM transplantation studies, given the small size of the animal and the difficulty targeting specific muscle groups that would provide ideal conditions for islet function after implantation (e.g., type 1 muscle fibers), which should be considered for future studies.[203] A more recent study assessing alginate capsules into different anatomical sites in pigs reported that capsules implanted at both the SC and IM sites provoke a more pronounced FBR, more inflammation and a thicker immune response, but a lower degree of stromal fibrosis, as compared to those implanted at the kidney and liver subcapsular space.[159] Although these findings are informative to predict an immune response at both the SC and IM site in humans,

they were only obtained from a single pig, and no islets were encapsulated and implanted at these sites. This is relevant given that alginate capsules containing human islets at both the kidney and liver subcapsular spaces promoted a higher degree of FBR, inflammation and a significantly thicker immune response as compared to empty alginate capsules.[159] Thus, definite conclusions regarding the specific differences in immune responses to encapsulated islets implanted at the SC and/or IM sites compared to other sites cannot be made, and further studies are needed.

Overall, clinical evidence obtained from both non-encapsulated islet transplantation studies and pre-clinical studies evaluating encapsulated islet transplantation corroborate that the SC and IM site are safe and technically convenient, however, strategies to improve engraftment and immune responses early after transplant are essential to advance these implantation sites forward as feasible alternatives for islet and β-cell replacement therapies.

5.1.8.5 - Intravascular

The intravascular space has several advantages and disadvantages, both centered around the fact that this location provides "direct" contact with the blood. As previously stated, adequate diffusion of oxygen and nutrients is vital to promote optimal viability and adequate function of encapsulated islets. Islets are accustomed to living and functioning in higher oxygen concentrations than other cells. As compared to other implantation sites, having a direct contact with arterial blood would expose islet to a partial pressure of oxygen of 75-100 mmHg,[124] but also it would positively-impact nutrient and glucose kinetics, as well as waste clearance from the encapsulation structure. Of these factors, post-transplant hypoxia is believed to be the main contributor to decreased islet viability (apoptosis) and function. Intravascular implantation of

encapsulated islets could solve this issue, which has been tackled using innovative and safe, albeit cumbersome engineering approaches, such as the βAir macrochamber,[127, 135] but also with approaches involving islet preconditioning, increased local oxygen solubility, soluble factor and gene delivery, or *in situ* oxygen generation.[92] Unfortunately, given the scarcity of studies, no definite conclusions on the physiology of islets in intravascular encapsulated structures, which are indispensable to motivate any clinical studies. On the other hand, having direct contact with the circulation could be detrimental for islets in terms of the immune response, particularly IBMIR. Two of the most important processes occurring during IBMIR is activation of the coagulation (including platelet activation and adhesion) and complement cascades.[204] Whether pore size could be adjusted to efficiently block interaction with the effectors of these processes (e.g. platelets, complement), and prevent IBMIR entirely, remains to be tested.

The first attempt of transplanting encapsulated islets in the intravascular space was done three years after Kemp et al. described their initial pre-clinical success with intraportal infusion of islets. Dr. Tze's group tested intravascular implantation of what they then called an "artificial endocrine pancreas" connected directly to the aorta and successfully showed diabetes reversal shortly after implantation in Lewis rats.[205] Nevertheless, animals where only kept alive for a few hours due to excessive bleeding secondary to heparinization to keep the device permeable. After Dr. Tze's initial success, many other device modifications and improvements were carried forward by many groups over the following years,[141] leading to one of the most promising efforts in the history of encapsulated islet transplantation, the "Hybrid Artificial Pancreas",[206] designed and tested by Drs. Monaco's and Chick's group. Two back-to-back reports showed that the Hybrid Artificial Pancreas, a chamber containing islets within a copolymer membrane that connects to standard vascular grafts at the time (PTFE), showed long-term patency, even

with aspirin therapy alone.[121, 206] This was associated with insulin reduction and improved glycemic control and, in one case (1/13, 7.6%), with long-term, insulin independence without immunosuppression.[206] Further studies replicated these initially promising findings, showing insulin independence without immunosuppression in 50% of the treated dogs (6/12),[121] although in some instances, two devices were needed to achieve insulin independence.[207] A more compact intravascular tube-shaped device composed of an outer membrane of PTFE with a semipermeable inner membrane of Dacron (polyethylene teraphtalate), both of which are commonly-used biomaterials in the clinic, was tested in parallel to the "Hybrid Artificial Pancreas" by Dr. Calafiore's group at the University of Perugia.[208] These authors infused alginate-encapsulated islets (porcine and human) into a tubular-shaped device and implanted it via anastomosis to the iliac artery and vein. This approach proved to maintain islet viability and, although it did not lead to insulin independence, it was associated with improved glycemic control, including correction of post-prandial hyperglycemia, with an insulin dose reduction of 50-60%. This device was quickly moved forward to a pilot clinical trial in two patients, in which one patient achieved insulin independence for 7 months and the other had a 40% reduction in daily insulin doses.[122] Despite these promising results, no further clinical trials were attempted until 2008, where Prochorov et al., in Belarus, tested intravascular macroencapsulation of rabbit fetal islets in 19 T1D patients and achieved a 65% reduction in insulin doses. Further details of these efforts are exposed in following sections and are summarized in **Table 5.1.3**. From this account of the evidence, the intravascular site would seem a feasible and attractive alternative, nevertheless, these previously-described pre-clinical reports, although promising in terms of glycemic-related outcomes, were plagued with procedural complications and serious adverse events during follow-up, including systemic and local infections, thrombosis of the devices,

lack of clinical recovery following implantation (even despite insulin independence) and, even death. Nevertheless, thirty-years of advances in surgical techniques, engineering processes and improved biomaterial quality and physicochemical properties, may lead to a decreased or lack of both procedural complications and serious adverse events. In fact, recent pre-clinical studies have used agarose-embedded islets encased in a PTFE macrochamber and immunoisolated by a silicon nanopore membrane (similar to the original "Hybrid Artificial Pancreas") and shown promising *in vivo* results in pigs characterized by sustained viability and C-peptide release, although no glycemic-related outcomes were assessed.[209, 210] Unfortunately, the surgical approach used by these authors (PTFE vascular grafts anastomosed to the carotid artery) recapitulates from previous studies, which would naturally raise the same concerns in terms of safety, risk-benefit ratio and, overall, clinical translation.

5.1.9 - Advances in the Clinical Realm and Persistent Obstacles for Widespread Clinical Translation

A total of 112 patients included in 19 reports (some patients may have been included in two or more subsequent reports) have been transplanted with encapsulated islets using several encapsulation strategies and into three implantation sites: peritoneal cavity/omental pouch, subcutaneous space and intravascular. Overall, safety and viability aspects of these therapies have been encouraging since most of these studies have reported islet viability after variable follow-up periods even without immunosuppression (15/19, 78.9%). Furthermore, in contrast to pre-clinical studies only scarce and minor procedural complications and/or adverse events have been reported in clinical studies (**Table 5.1.3**). Nevertheless, clinically-relevant results have not been favorable, consistent and, in some reports, they have been disappointing, although

some of these reports were proof-of-concept studies and suboptimal doses of islet may have been administered. The fact is that insulin independence has been achieved only transiently in 5/112 patients (4.5%), and only 2 patients have achieved insulin independence for >12 months.[183, 211] Only one of these patients was free of immunosuppression; interestingly, neonatal pig islets were used in this patient,[211] which by itself would constitute a strong argument to continue pursuing efforts using this cell source. In contrast to insulin independence, results regarding reduction of insulin doses and HbA1c levels have been more favorable, where 9/19 studies (47.3%) reported some degree of reduction on this two parameters. In some cases, these positive outcomes were observed even with minimal graft function measured by C-peptide (human or porcine). This suggests that, although insulin independence may be "off the table" for these therapies, encapsulated islet transplantation without immunosuppression may be a potentially cost-effective, powerful and safe adjunct to current therapies to improve glycemic control in selected patients. This has yet to be proven, but given the proven safety profile with these therapies, clinical trials to prove this concept should be considered. Details on published and ongoing clinical efforts in the field of encapsulated islet transplantation will be summarized in the following paragraphs. A systematic review of the literature is beyond the objectives of this review, but **Table 5.1.3** contains a detailed profile of every published study to date.

5.1.9.1 - Intraperitoneal/Omental Pouch

The intraperitoneal cavity is currently the most commonly tested implantation site for encapsulated islet transplantation. In fact, the first clinical effort to transplant encapsulated islets was carried forward using the intraperitoneal cavity by Wu et al. in 1989 and led by Anthony M. Sun, which we have mentioned before as one of the key and first researchers advancing the

field of islet encapsulation. In this study, three patients were treated with human fetal islets encapsulated in alginate microspheres. No dose was specified, only that patients received "2-5 transplantation within 1-2 months"; also, whether immunosuppression was used is not specified in this report. Human C-peptide was detected, which confirmed islet function, nevertheless, patients suffered severe hypoglycemic episodes 1-3 months after transplanted. None achieved insulin independence, although 20-30% insulin reduction was achieved in 2/3 patients.[188] Five years later, the first clinical report of insulin independence after encapsulated islet transplantation was authored by Soon-Shiong et al. In this report, a 38-year-old man with T1D and a kidney transplant was implanted with encapsulated islet within high-M alginate poly-$_{L}$-Lysine microspheres for a total dose of 14,957 IEQ/kg from 8 pooled human donors. Although the patient did not receive induction immunosuppression, he continued his usual maintenance immunosuppression with cyclosporine. In addition to achieving insulin independence, the patient reported significant improvement in his quality of life and return to full-employment. Unfortunately, the report only includes 9-months of follow-up, thus, the possibility of a longer period of insulin independence cannot be discarded.[189] Surprisingly, there were not any studies continuing these efforts, and it took 12 years for the next report to be published. Thus, in 2006, Calafiore et al., from The University of Perugia in Italy, reported the first two cases of a pilot clinical trial transplanting human islets (400,000 and 600,000 IEQ) encapsulated in high-purity alginate. No insulin independence was achieved, but both a reduction in HbA1c and daily insulin requirements, as well as abrogation of hypoglycemic episodes were achieved.[212] In 1996, Robert B. Elliot, founder of Living Cell Technologies™, and colleagues transplanted 1.3 million (15,000 IEQ/kg) encapsulated neonatal pig islets into the peritoneal cavity of a 41-year-old male patient in Australia. No initial report or early follow-up of this attempt was published

and it was until 2007 when they published a 9.5 yr follow-up of this patient; importantly, this was the first long-term follow-up report to date. Encapsulated islets were analyzed and confirmed to be viable and *in vitro* studies showed only slightly impaired function. Porcine insulin was detectable in serum. Clinically, the patient did not achieve insulin independence but referred near total abrogation of hypoglycemic episodes and, although insulin reduction was not maintained past 14-months post-transplant, HbA1c levels had a sustained decrease. Of note, this is the longest follow-up reported to date (in any implantation site) regarding islet encapsulation *in vivo* and it confirms conserved islet viability and function. Whether new materials (compared to those used 23 years ago) could achieve similar or better results should be a research priority. Two years later, in 2009, Tuch et al. transplanted 4 patients with a median of 178,200 Ba^{++}-alginate-encapsulated human IEQ. Very disappointing results were reported for this trial, although no major safety issues were documented. Whether this had to do with the encapsulation strategy (Ba^{++} gelation) remains unknown, but other factors such as the low viability of pre-implanted islets (73%) and low purity (68%) could have negatively impacted these results.[187] Another report from the University of Perugia's group was published in 2011. Using a highly-purified prepared-in-house alginate, Basta et al. transplanted four long-standing T1D patients with a median of 650,000 human IEQ (range 500-1,600,000). Besides none of them achieving insulin independence, insulin reduction was transient and increased to baseline doses by 7 years of follow-up. HbA1c reduction was sustained for 3 years, although the same 7-year follow-up for this parameter was not reported.[184] Next, a disappointing report in 2013 by Jacobs-Tulleneers-Thevissen et al. in which a 61-year-old female patient was transplanted with 300,000 IEQ showed no benefit from this transplant. Interestingly, this happened despite induction immunosuppression with basiliximab and maintenance immunosuppression with

mycophenolate sodium and tacrolimus.[191] Still, this study may have used a suboptimal dose. Two of the largest studies using the peritoneal cavity as an implantation site for encapsulated islets have been carried forward by Matsumoto et al., in partnership with Robert B. Elliot. Together, they transplanted a total of 22 patients with different doses of encapsulated neonatal pig islets ranging from 5,000 to 20,000 IEQ/kg. While their first report only showed a decrease in hypoglycemic events as a clinical benefit, their second report did show a decrease in HbA1c (despite no daily insulin reduction). Still, thorough evaluation of porcine endogenous retrovirus (PERV) showed no transmission, which confirmed safety for using these islets as a potentially limitless source for transplantation.[185, 186] The latest report comes from the Miami group, led by Drs. Alejandro and Ricordi. By transplanting human islets (602,395 IEQ) combined with autologous plasma (1:2 ratio) and embedded in a two-layered structure composed of recombinant thrombin and a second layer of autologous plasma into the omentum, the authors achieved insulin independence by 17 days post-transplantation in a 43-year-old woman with a 25-year history of T1D. One-year follow-up showed sustained insulin independence. Importantly, these results were achieved with standard immunosuppression protocols, since the multi-layered bioscaffold may not be entirely immunoprotective. Further reports will delineate this issue and present additional results of this ongoing study (NCT02213003).

5.1.9.2 - Subcutaneous Space

The SC space, as we've emphasized before, is a highly desirable implantation site, even for transplantation of non-encapsulated islets. Unfortunately, little progress has been made to make this "the new home" for islet transplantation. The first group to attempt transplantation of encapsulated islets into the SC space was the Lacy group in 1994. This was a proof-of-concept

and not a curative attempt in which they implanted 150-200 previously-cryopreserved, alginate-embedded IEQ macroencapsulated in a hollow-fiber made of acrylic-copolymer (MWCO of 65 kDa) into three groups of patients (n=3 in each group): healthy, type 2 diabetes (T2D) and T1D. A two-week period of implantation showed that viability was similar in all three groups, but in both T1D and T2D the degree of "cellular process development" was higher, which was assessed by fluorescein diacetate (FDA) staining.[131] No *ex vivo* functional studies were performed which precludes any conclusions about functional aspects of the retrieved encapsulated islets. The following efforts, although controversial, contributed with most of the clinical evidence regarding islet encapsulation into the SC space in humans. Three reports by Dr. Valdes-Gonzalez' group in Mexico amounting to a total of 36 patients (probably patients were included in more than one report) consistently showed procedural safety and a lack of serious adverse events with transplantation of encapsulated neonatal porcine islet into the SC space.[211, 213, 214] These reports were supported by Robert B. Elliot and Diatranz™, a partnership venture between Living Cell Technologies™ and Otzuka Pharmaceutical Factory, Inc. This company provided islets from neonatal pigs bred at a pathogen-free facility in Australia, then, the islets were combined in a 1:30-100 ratio with Sertoli cells and transplanted. Both cells were isolated in Australia and then flown to Mexico. The authors recruited adolescents with T1D and implanted proprietary devices composed of surgical-grade steel mesh tubes with a PTFE rod in its interior. Two months of intensified diabetes treatment were conducted while the FBR allowed "maturation" of the device by promoting formation of a vascularized collagen layer around the steel mesh. Afterwards, surgical exposure of one end of device and removal of the PTFE rod permitted islet infusion; a PTFE cap was then used to seal the newly generated pocket. By implanting multiple devices in a single patient, this group performed >70 infusions with an

average of 250,000 islets (with their corresponding Sertoli cells) per infusion (more than 17 million islets in total).[213] Overall, only 3 patients from these attempts achieved insulin independence, albeit transiently. Of note, one of them achieved insulin independence for 18 months, which has been the longest period of insulin independence documented for encapsulated islet transplantation.[211] Regarding insulin reduction doses and HbA1c levels, the first report of this series documented a significant decrease in insulin requirements and HbA1c levels, nevertheless, subsequent reports with pooled data and longer follow-up showed that daily insulin doses and HbA1c levels returned to baseline eventually (median follow-up of 5.7 years, range: 2.6-7.7).[213] These reports, although ground-breaking, have not been replicated and results are not free of questioning. Moreover, there was significant controversy in the field due to ethical and technical concerns with these studies; analysis of these considerations is beyond the scope of this review and the reader is referred to relevant literature.[215] It is worth noting that the authors thoroughly emphasized the fact that this clinical trial was approved by National Regulatory Agencies and Bioethics Committees in Mexico. The fact is that Dr. Valdes-Gonzalez device and approach was further tested by the Miami group in a model of syngeneic rat islet transplantation and showed comparable results to intraportal transplantation in terms of reversal of diabetes and metabolic capacity.[125] Unfortunately, no further large animal models were tested and further research efforts from Dr. Valdes-Gonzalez have not been documented since 2010. The other two clinical reports using the subcutaneous space as an implantation site for islet have used the βAir device (BetaO2 Technologies Ltd, Israel), which has been already described in previous sections. The first report by Ludwig et al., showed safety and technical feasibility of this device and successful graft function for a period of 10 months. Although a suboptimal mass to achieve insulin independence was implanted (2,100 IEQ/kg), this

intervention proved to reduce daily insulin doses despite below-physiological C-peptide levels.[127] Nevertheless, technically, this device was implanted at the pre-peritoneal space and not strictly into the SC space. Although initially promising, four additional patients didn't have the same response and the device had to be removed at 4-5 months post-transplant according to protocol due to a lack of function in terms of C-peptide detection and glycemic-related outcomes. The authors suggest that the lack of positive results could have been related to insufficient vascularization, an inadequate waste disposal mechanisms due to macroencapsulation which led to toxic product accumulation (e.g. Ca^{++} or islet amyloid polypeptide) and/or a subcutaneous location vs a pre-peritoneal in comparison to their first report.[135] Importantly, the βAir chamber required daily oxygen refilling by the patients, which was reported to generate some stress related to remembering and performing this procedure. This would constitute a significant drawback for this approach and somewhat resemble the cumbersome practice of daily insulin injections and glucose monitoring already experienced by T1D patients.

Overall, the clinical experience using the SC space has been only attempted using two approaches, the "Valdes chamber" and the βAir chamber. Both have reported initial promising reports that have not been able to be extended to larger samples and longer follow-up periods. Ongoing efforts using ViaCyte's VC-01™ (PEC-Encap, NCT02239354) and VC-02™ (PEC-Direct, NCT03163511) devices that will be coupled with pancreatic endoderm cells that could be more resistant to hypoxic environments, will provide extremely valuable information to guide the field forward.

5.1.9.3 - Intravascular Space

The intravascular space as an implantation site for clinical encapsulated islet transplantation was heavily pushed forward in the 1990s. In fact, regulatory approval by the FDA was already in motion for intravascular devices, but they were abruptly suspended due to a sudden death by rupture of the vascular-device anastomosis in one of the long-term surviving animals treated with this approach which led to death by exsanguination. This dog was part of an experimental group that had achieved long-term insulin independence with a xenograft and without any immunosuppression.[141] Before this, a pilot clinical trial was already attempted by R. Calafiore from the University of Perugia. Two patients, one with T2D and one with T1D were implanted with an artificial vascular prosthesis consisting of two coaxial tubes, the inner tube made of a permeable Dacron® membrane and filled with alginate-encapsulated human islets and an outer tube made of PTFE. The patient with T2D (patient 1) was transplanted with 150,000 islets and achieved insulin independence 18 days after device implantation, while the patient with T1D (patient 2) didn't achieve this despite a higher dose of 250,000 islets. Nevertheless, both had a significant reduction on their insulin requirements of ~40-75 %. Sustained C-peptide levels were documented after 240 days in patient 1 and 44 days in patient 2. Importantly, no procedural complications and/or adverse events during follow-up were reported.[122] Almost 30 years later, only one clinical study using the intravascular space has been published. In 2008, Prochorov et al. treated 19 T1D patients with macroencapsulated fetal rabbit islets (>6,000 IEQ/kg) into either the *arteria profunda femoris* or into the venous fragment of an arteriovenous anastomosis on the forearm.[216] A nylon microporous macrocapsule was used for this approach but, unfortunately, no further details on the macroencapsulation design and/or the implantation procedure are provided. The authors report no cases of insulin independence,

but a 60-65% insulin reduction as well as sustained (2-year follow-up) insulin and C-peptide levels was documented. No HbA1c is reported, but the authors refer that serum fructosamine levels decreased substantially. No procedural complications occurred, and only one serious event, the formation of a pseudoaneurysm of the *arteria profunda femoris* that required capsule extraction, was reported. Overall, this report does not provide many details to allow replication of the results, but there are also many concerns about the study design and rationale in terms of islet source, surgical procedures and follow-up methods. A reappraisal of the intravascular implantation site should be considered in light of these findings. The carotid artery may have been a suitable place in the 1990s due to the size of the devices at that moment in time, but current biomaterials, vascular grafts, engineering and surgical techniques may allow "less vital" vessels to be used as niches for implantation of encapsulation intravascular devices.

5.1.10 - Future Directions and Research Avenues in the Field

The field of cellular encapsulation is intensely expanding as it attempts to challenge treatments that are conventionally accepted to transform the field of islet transplantation.[217] Although successful efforts in the field have been recently populating the literature, several key challenges remain.[218] Moving forward, we propose focusing on the following potential research avenues: 1) Standardizing current reporting practices for novel encapsulation methods and structures, 2) Promoting large-scale efforts analogous to high-throughput drug screening to find optimal biomaterials for cellular encapsulation, 3) Exploring novel techniques and strategies to optimize immunoprotection as well as molecule diffusion properties of future encapsulation structures, and 4) Characterizing the specific conditions for optimal encapsulation of potentially

limitless cell sources such as pig islets and/or stem cell-derived islets as well as aspects related to their long-term safety.

One of the most limiting aspects for clinical translation and replicability of novel encapsulation strategies remains the lack of standardized reporting on the physico-mechanical and chemical properties of the used biomaterials and/or compounds, as well as a lack of detailed manufacturing processes to allow replication and/or migration to different models or, even to the clinic. While some efforts to provide a framework for cellular encapsulation have been put forward,[37] currently there is not a consensus on the matter. Additionally, these recommendations have not permeated everywhere and there is still a generalized lack of adequate reporting in the field. We believe that an international consensus as well as mandatory requirements for publication should be contemplated in the near future. This will ultimately lead to safer, faster, and more efficient and effective clinical translation endeavors.

Another potential research avenue involves migrating the valuable lessons obtained from high-throughput drug screening to the field of biomaterials. This approach for drug discovery has advanced greatly in the few years and has played a role in the discovery of multiple new drugs. Using high-throughput approaches for biomaterial development and discovery is not a novel concept,[94, 219] but it was not until recently that it has proven to yield relevant results with the potential for clinical translation.[93] Notably, integration of artificial intelligence and machine learning, analogous to what is currently occurring in the field of drug discovery, would allow finding not only new and better biomaterials, but also potentially beneficial combinations of current and future materials.

Encapsulation techniques and strategies are rapidly evolving, most of them involve nanoencapsulation and are being currently developed by several groups. In this regard,

nanoencapsulation technologies moving away from the conventional encapsulation methods such as electrostatic spraying or pump-based encapsulation, such as microfluidics or electrospinning technology, have started to show promising results,[118] and future efforts would help elucidate their role in the field. Overall, nanoencapsulation allows a more tailored approach to accommodate the physiological needs of different cells types in terms of nutrient and oxygen diffusion (e.g. human or pig islets, immature of mature β-cells) and, concurrently, introduces the capacity to fine-tune immunoprotection and vascularization properties by allowing incorporation of immunomodulating and/or pro-angiogenic biomaterials or drugs, commonly referred to as bio-functionalization.[106] Alternatively, strategies to improve macroencapsulation are also continuously improving which, coupled with exploration of previously "forgotten" implantation sites, such as the intravascular space, could prove equally promising given the current biomaterials and surgical techniques. Future studies in the field will still have to compare microencapsulation and macroencapsulation head-to-head to provide optimal evidence to guide future clinical trials.

A highly-relevant issue guiding research efforts in cellular encapsulation concerns the encapsulated product. A cell's metabolic requirements and specific performance vary from cell to cell and even from species to species. For example, it has been reported that neonatal pig islets are more resistant to hypoxia. As such, they may be more suitable inhospitable sites such as the SC space or, alternatively, they could thrive despite a more aggressive immunoisolation approach, both of which have been suggested by recent studies,[220] as well as previous clinical studies.[211, 213, 214] Similarly, stem cell-derived β-cells are becoming an increasingly feasible alternative cell source and promising studies have recently shown that alginate encapsulation can foster cell survival and allow long-term diabetes reversal in allogeneic mouse models.[221]

Although immature stem cells are also believed to be resistant to hypoxia, contrary to neonatal pig islets, encapsulation of these cells may counteract this favorable profile, as have been shown in studies reporting that alginate-encapsulated stem cells have suboptimal differentiation in the SC space as compared to the intraperitoneal space.[182] Importantly, no pre-vascularization strategy was performed in this recently published study. These, and other previously mentioned research efforts (e.g. ViaCyte's Encaptra ®), represent the first wave with many yet to come and demonstrate that a one-size-fits-all encapsulation approach may not be optimal. This paradigm has direct implications for clinical translation.

5.1.11 - Conclusions

Cellular replacement therapies for type 1 diabetes have been consistently advancing over the last three decades. While the issue of limited cell sources is being actively and successfully tackled by several research groups, the need for chronic immunosuppression remains a major challenge to allow widespread application of these therapies. Cellular encapsulation strategies represent a safe, cheap and definitive alternative to chronic immunosuppression. Current technologies have allowed precise tailoring of biomaterials to optimize immunoprotection and, at the same time, maximize long-term cell survival and function. Abundant pre-clinical evidence in small- and large-animal models has demonstrated that diabetes reversal can be routinely attained and maintained in the absence of immunosuppression. Nevertheless, the path to clinical translation has not been straightforward, as only a few patients have shown a benefit from these therapies. This delay in clinical success may be attributed, at least in part, to a current lack of reporting standardization of encapsulation strategies that precludes replicability and scalability,

a significant heterogeneity in encapsulation techniques and methods, as well as an absence of clinical guidelines to conduct these efforts.

As future scientists embark in increasingly more complex research endeavors involving cellular encapsulation, multidisciplinary collaborations should be the norm, as this field requires deep understanding of many intricate aspects of chemistry, physics, engineering, cell biology and immunology. Working in this manner will undoubtedly increase the probabilities of successful clinical translation. Currently, the unfavorable risk-benefit ratio supports islet transplantation only for those patients with type 1 diabetes and problematic hypoglycemia that have failed conventional treatment algorithms. Achieving safe and efficient immunoisolation for cellular therapies without immunosuppression could extend the spectrum of indications to every patient with type 1 diabetes and, perhaps, to selected patients with type 2 diabetes.

5.1.12 - Acknowledgements

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5.1.13 - References

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CHAPTER 5

PART 2 - IMMUNOSUPPRESSION-FREE ISLET TRANSPLANTATION WITH A REPLACEABLE AND SCALABLE ENCAPSULATION DEVICE INTO A VASCULARIZED SUBCUTANEOUS SITE

CHAPTER 5, PART 2 - IMMUNOSUPPRESSION-FREE ISLET TRANSPLANTATION WITH A REPLACEABLE AND SCALABLE ENCAPSULATION DEVICE INTO A VASCULARIZED SUBCUTANEOUS SITE

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Title: Immunosuppression-free islet transplantation with a replaceable and scalable encapsulation device into a vascularized subcutaneous site

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5.2.1 – Abstract

Cell encapsulation represents an attractive strategy to realize immunosuppression-free cellular therapies for type 1 diabetes. However, long-term cell survival in encapsulation systems remains a challenge after transplantation, especially in the poorly vascularized subcutaneous site. Herein, we present a sequential approach that harnesses temporary, controlled inflammation-induced neovascularization to create a modified subcutaneous cavity that supports transplantation and optimal function of a customized, thread-like islet encapsulation device that geometrically matches the modified subcutaneous cavity. Increased oxygenation is corroborated at the vascularized site, and computational modelling predicts physiological glucose responsiveness and improved islet survival with our approach. Sustained diabetes reversal is validated in immunocompetent syngeneic, allogeneic, and xenogeneic murine models. Additionally, long-term human islet survival is observed in an immunocompetent discordant human-to-mouse xenogeneic model. We further show that impaired devices can be readily replaced *in situ* into the existing vascularized cavity, with prompt return to normoglycemia. Finally, we demonstrate the scalability of our approach using a minipig model, supporting future clinical translation endeavors. This work presents a promising platform to realize subcutaneous immunosuppression-free islet transplantation.

5.2.2 - Introduction

Type 1 diabetes (T1D) is a devastating disease characterized by the loss of endogenous insulin production due to autoimmune destruction of the pancreatic β cells and affects millions of people worldwide.[1] While advancements in blood glucose (BG) monitoring and insulin therapies have alleviated the burden of T1D, suboptimal glycemic control, potentially complicated by disruptive and occasionally fatal hypoglycemia, remains a key limitation for optimal disease management.[2] Islet transplantation into the liver intraportal circulation is an effective therapy for a selected population of people with T1D suffering from intractable severe hypoglycemia.[3, 4] Recent reports show patient survival rates > 90% at 20-years[5, 6] and graft survival rates ~80% at 10-years,[7, 8] coupled with marked and sustained improvements in glycemic control and near complete abrogation of severe hypoglycemia for most patients.[6-8] Despite these achievements, the need for lifelong immunosuppression and cell supply remain major impediments to realize islet transplantation as a curative therapy for T1D and other forms of diabetes. To overcome this issue, encapsulating islets within immune-isolating devices, which permit bidirectional transport of nutrients and therapeutics, has been proposed as a solution.

Great progress in encapsulation approaches has been achieved thanks to notable accomplishments in the field of biomaterials and bioengineering,[9-11] though no efforts to date have rendered patients free of insulin using cell encapsulation systems in a clinical setting. Approximately 120 people with T1D have undergone implantation of cell encapsulation devices in Phase I/II clinical trials.[12-14] Over half of these, received implants in the subcutaneous space, which is a highly desirable implantation site given its technical feasibility, accessibility for monitoring, capacity to accommodate large tissue volumes, and minimally invasive nature.

Unfortunately, the subcutaneous space is inhospitable to implanted cells due to limited vascularization and aggressive local fibrotic deposition, which together hamper optimal engraftment and induce extensive islet cell necrosis.[15] Given the practical advantages of the subcutaneous space, it is of interest to explore ways to overcome these limitations.

Accordingly, much attention has been devoted to enhancing vascularization of the subcutaneous site through myriad approaches, such as those harnessing materials-mediated inflammatory responses[16-20] and releasing pro-angiogenic factors locally[21-24]. In addition, the use of bioscaffolds containing extracellular matrix components (*e.g.*, fibrin[25], collagen[26, 27] and incorporation of cellular products (*e.g.*, mesenchymal stem cells[28] or endothelial cells[29]) have shown improvement with subcutaneous islet transplantation in preclinical studies. However, limitations remain, including fibrotic scar overgrowth with the use of device-facilitated neovascularization, the complexities of advancing multiple biological products into clinical trials, a lack of clinical translation with most pharmacological strategies, and the need for immunosuppression.

Oxygen (O_2) tensions in the subcutaneous site are lower compared to other implantation sites, which further impacts cell survival and engraftment.[30] Many strategies have been developed to provide exogenous O_2 supply in cell encapsulation devices and sustain early engraftment and long-term cell survival, including O_2-generating[31, 32] and O_2-filling[33-36] systems. For example, the βAir device (Beta-O2 Technologies, Israel) demonstrated the benefits of supplemental O_2 provision by frequent O_2 injections in early preclinical studies and a preliminary human trial.[34-36] However, a more recent clinical trial reported minimal circulating C-peptide levels with no impact on glycemic control despite supporting allogeneic islet survival; this was likely a consequence of the unfavorable mass transfer between the device and the host

resulting from inadequate vascularization and fibrotic capsule formation around the devices.[33] Therefore, even in systems with enhanced oxygenation of encapsulated cells by exogenous means, adequate vascularization and minimal fibrotic deposition are essential for optimal function of encapsulated islets, particularly at the subcutaneous site which usually features a delayed glyco-insular response with marked hysteresis.[37]

Herein, we present a sequential system involving the creation of a vascularized subcutaneous cavity and the subsequent implantation of a cell encapsulation device that geometrically matches the vascularized subcutaneous cavity. First, a medical nylon catheter is implanted in the subcutaneous space, which induces a controlled host inflammatory response[38] creating a vascularized pocket a few weeks after catheter implantation. The catheter is then withdrawn and a perfectly-fitted, thread-like alginate-based cell encapsulation device[39] is transplanted into the modified subcutaneous pocket. We name the system **SHEATH** (**s**ubcutaneous **h**ost-**e**nabled **a**lginate **th**read) for the sheath/sword-like analogy to the modified pocket and the implanted encapsulation device. In this body of work, we characterize the improvement of local O_2 tension in the modified subcutaneous site following vascularization and evaluate mass transfer properties *in silico* and *in vivo*. Additionally, SHEATH's potential for effective immunoisolation and long-term diabetes reversal in allogeneic and xenogeneic transplant models are assessed. Finally, we describe proof-of-concept studies evaluating the possibility of *in situ* device replacement to restore normoglycemia in the event of graft failure and explore the potential for scalability using a large animal (minipig) model. Overall, we demonstrate that the SHEATH system provides a potentially feasible platform to achieve immunosuppression-free subcutaneous islet transplantation.

5.2.3 – Materials and Methods

5.2.3.1 – Study design

The objective of this study was to develop an approach to enable immunosuppression-free islet transplantation into a modified vascularized subcutaneous space using a cell encapsulation device. We characterize the modified subcutaneous cavity using histological analysis, as well as semiquantitative and quantitative local measures of O_2 tensions. The function of the cell encapsulation device implanted into the modified site was validated in immunocompetent syngeneic, allogeneic, and xenogeneic murine models. We also developed surgical procedures in a large-animal minipig model to explore the potential for scalability and clinical translation of the system. All sample sizes in each study are indicated in the figure legends, and the sample sizes were sufficient to conduct reasonable statistical analyses, where applicable. No data was excluded from analysis. All experiments were performed at least twice, but more often more than three times. Details about the replication of experiments are indicated in the figure legends where applicable. For *in vivo* mouse studies, mice were randomly assigned to different experimental groups. No blinding was used for the experiments in this study. However, the blood glucose monitoring was performed by different individuals and the therapeutic function of the device was validated by different researchers in both laboratories at Cornell University and University of Alberta.

5.2.3.2 – Materials

Sodium chloride (ACS reagent, ≥ 99%), calcium chloride dihydrate (ACS reagent, ≥ 99%), barium chloride dihydrate (ACS reagent, ≥ 99%), *D*-glucose (BioXtra, ≥ 99.5%), poly(methyl methacrylate) (Mw ~350,000 Da by GPC), and *N*,*N*-dimethylformamide (HPLC

grade, ≥ 99.9%) were purchased from Sigma-Aldrich. Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning. Sterile sodium alginate (Pronova SLG100) was purchased from NovaMatrix. 7-French, medical-grade nylon angiographic catheters were purchased from Cook Medical. Ultrapure water was obtained using a Type 1 Synergy UV Water Purification System.

5.2.3.3 – Animals

Male BALB/c (10-16 weeks old) mice and male C57BL/6 mice (10-16 weeks old) were purchased from the Jackson Laboratory. The mice were maintained at a temperature of 70–72 °F with 30–70% humidity under a 14 h light/10 h dark cycle. Male Sprague-Dawley rats (weight of ~300 g) were purchased from Charles River Laboratories. Female Göttingen minipigs (6 months old) were purchased from Marshall Bioresources (North Rose, NY). The care for all animals within this study were approved by the Canadian Council on Animal Care or Cornell Institutional Animal Care and Use Committee and complied with relevant ethical regulations.

5.2.3.4 – Characterizations

Stereo microscope images were taken using a stereo microscope (Olympus SZ61). Optical and fluorescent microscope images were taken using a digital microscope (EVOS FL) or an Aperio Scanscope (CS2). Immunofluorescence images were taken using a confocal microscope (ZEISS LSM 710). GraphPad Prism 8 software was used for data plotting.

5.2.3.5 – Creation of the vascularized subcutaneous site

Prevascularized site for subcutaneous encapsulated islet transplantation was created using medical-grade nylon angiographic catheters.[38] Briefly, the mice were anesthetized with 3% isoflurane in O_2 and their abdomens were shaved and sterilized using betadine and 70% ethanol. A small (~4 mm) transverse incision was made on the side using scissors. A subcutaneous pocket was created using a saline-wet tip or blunt surgery tool and a catheter (7-French, Cook Medical) was inserted into the subcutaneous pocket (one or two catheters on each mouse). The incision was closed using a 5-0 nylon suture. The catheter was kept in place for 4-6 weeks before islet transplantation.

5.2.3.6 – Assessment of inflammatory responses promoting neovascularization

The localized inflammatory state was assessed 1 week post-implantation in a subset of mice (C57BL/6, Jackson Laboratories, Canada). Briefly, 1 week after catheter implantation, the peri-catheter tissue was retrieved, placed in microcentrifuge tubes, flash frozen with liquid nitrogen, and stored at -80°C until further use. A similar procedure was performed to obtain subcutaneous tissue (*i.e.*, untreated) from the contralateral side of each mouse to enable paired comparisons. The day of assay, 1 mL of lysis buffer (150 mM NaCl, 20 mM Tris Ultrapure, 1 mM EDTA, 1% Triton X 100, 1% BSA, 0.05 SDS, diluted in deionized water) per 200 μg of tissue was added to the microcentrifuge tube. Homogenization (PowerGen, Fisher Scientific, Ontario, Canada) of the sample was then performed while the samples were maintained on ice (30 s × 2 replications), followed by sonication (VirSonic, VirTis, NY, USA) with 10 short pulses, also while maintaining the samples on ice. The tubes were centrifuged at 1,500 RCF for 10 minutes at 4°C, followed by immediate collection of the supernatant. These tubes were then

centrifuged again at 18,000 RCF for 10 minutes at 4°C and the resulting supernatant was collected. Finally, 10 µL of protease inhibitor cocktail (Sigma-Aldrich Canada Co., Oakville, ON, Canada) per 1 mL of supernatant was added. Cytokine and chemokine analysis was conducted using the Multi-spot Mouse Proinflammatory 7-plex Ultra-sensitive kit (Meso Scale Discovery, Gaithersburg, MD, USA), requiring 25 µL of lysate per replicate, read on a Meso QuickPlex SQ120 and analyzed using the MSD Discovery Workbench software v4.0.

5.2.3.7 – O_2 measurement using the Lumee™ Oxygen Platform

The Lumee™ Oxygen Platform (Profusa, Emeryville, California) was used for the comparison of O_2 tension at unmodified and vascularized subcutaneous sites. The mouse abdomen was shaved and dehaired using hair removal cream. Then, a PdBMAP-containing O_2 sensor was injected into the prevascularized site created by the catheter or a control subcutaneous site without any pretreatment. The O_2 measurements were performed one day after placement of the O_2 sensors. The mice were anesthetized with 3% isoflurane in air and placed on a heat pad. A wireless patch reader containing a light-emitting diode (emitting at 630 nm), a photodetector (detecting at 800 nm), and a temperature sensor was placed on the skin over the location of the injected O_2 sensor for O_2 measurement.

5.2.3.8 – Electron paramagnetic resonance (EPR) for O_2 mapping

O_2 mapping was performed on a 25 mT EPR imager (JIVA-25, O2M Technologies, LLC). The JIVA-25 operates at 720 MHz using electron paramagnetic resonance O_2 imaging (EPROI) principles and utilizes O_2 sensitive electron spin-lattice relaxation rates (T_1) of Lithium phthalocyanine (LiPc) for reporting pO_2.

First, a cylindrical LiPc/PDMS solid probe (6 mm length, 1.5 mm diameter; each containing 20 mg LiPc) was made by mixing LiPc in fresh prepared PDMS resin (silicone elastomer base and curing agent in a 10:1 weight ration) and curing in a 3D-printed mold. Then, a LiPc/PDMS solid probe was placed into the prevascularized site created by the catheter or unmodified subcutaneous site without any pretreatment.

EPR imaging was performed one day after the placement of the EPR probes. Briefly, the mice were anesthetized with 3% isoflurane in air and placed in the temperature-controlled (37 ^{o}C) resonator of the EPR imager. Surface plots of the pO_2 distribution of the EPR probes were collected to obtain the average O_2 tension at the unmodified and vascularized sites.

5.2.3.9 – Mass transport modeling

Mass transfer models were developed for studying insulin release kinetics in a hypothetical perifusion test and pO_2 distributions at steady state in devices in the unmodified and vascularized sites. The mathematical representation of O_2, glucose, and insulin consumption, as well as glucose-stimulated insulin release, followed the methodology developed for a similar problem.[40-42] Certain adjustments to this model were made regarding the geometry and dimensionality of the system (we considered the model in 3 dimensions), O_2 consumption rate of human islets, and implementation in finite element software (COMSOL Multiphysics with MATLAB). For determining O_2 profiles in rat and human-islet containing devices, the problem was considered at steady state and in 3 dimensions. Monte Carlo simulations were performed, wherein each time the positions and selected sizes of the islets, and in one case the boundary O_2 tension, were randomized according to quantified distributions.

Model development and implementation in finite element solver software is described in detail in Appendix A.

5.2.3.10 – Islet isolation and purification

Mouse islets were harvested from BALB/c mice for syngeneic transplants to BALB/c and allogeneic transplants to C57BL/6 mice. Mice were euthanized by sodium pentobarbital injection. Before pancreatectomy, the common bile duct was cannulated using a 27 G needle, followed by infusion of 2.5 mL (0.125 mg/mL) of cold Liberase TL research-grade collagenase (Roche Diagnostics, Laval QC, Canada). The pancreata was mechanically digested in a 50-mL tube at 37°C water bath for 14 min with gentle shaking. Following digestion, cold Hank's Balanced Salt solution (HBSS) was added to the tubes, and the tissue was filtered using a 400 μm sieve and then purified using histopaque-density gradient centrifugation (1.108, 1.083, 1.069 g/mL, Sigma-Aldrich Canada Co., Oakville, ON, Canada) at 800 RCF for 11 min with 0 break and 0 acceleration. The islets were collected from the gradient and washed twice using HBSS media. Islet equivalent (IEQ) number of purified islets was counted by reported IEQ conversion factors.[43] The islets were then cultured overnight (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, and 1% penicillin/streptomycin) at 37°C and 5% CO_2 overnight before further use.

Rat islets were isolated from Sprague-Dawley rats for xenogeneic transplants to C57BL/6 mice. The rats were anesthetized using 3% isoflurane in O_2 throughout the whole surgery. Briefly, the pancreas was distended with 10 mL 0.15% Liberase (Roche) in M199 media (Gibco) through the bile duct and then digested at 37°C in a circulating water bath for ~28 mins (digestion time varied slightly for different batches of Liberase). The digestion was

stopped by adding cold M199 media with 10% FBS (Gibco). After vigorously shaking, the digested pancreases were washed twice with media (M199 + 10% FBS), filtered through a 450 μm sieve, and then suspended in a Histopaque 1077 (Sigma)/M199 media gradient and centrifuged at 1700 RCF with 0 break and 0 acceleration for 17 min at 4°C. This gradient centrifugation step was repeated for higher purity. Finally, the islets were collected from the gradient and further isolated by a series of gravity sedimentations, in which each top supernatant was discarded after 4 min of settling. Islet equivalent (IEQ) number of purified islets was counted by reported IEQ conversion factors.[43] Islets were then washed once with islet culture media (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, and 1% penicillin/streptomycin) and cultured in this medium overnight at 37°C and 5% CO_2 overnight before further use.

Human islets were purchased from the Alberta Diabetes Institute IsletCore at the University of Alberta[44] for xenogeneic transplants to C57BL/6 mice. The donor record ID for this batch of human islets is R334 with a purity of 95% with 10% trapped in acinar (more information is available at www.isletcore.ca). The obtained human islets (shipped overnight) were cultured in CMRL medium supplemented with 0.5% BSA, 2 mM glutamine, 0.0067 mg/L of selenium, 10 mg/L of insulin, and 5.5 mg/L of transferring, in an incubator (37°C, 5% CO_2) for 1 day before transplantation.

5.2.3.11 – Fabrication of the islet encapsulation devices

Thread-like islet encapsulation devices were fabricated using nylon sutures.[39] Briefly, a 5–0 suture (Ethilon nylon, monofilament) was twisted, folded, and knotted at the ends to obtain a quadruple-helix structure. To modify its surface, the twisted structure was then embedded into

poly(methyl methacrylate)/*N*, *N*-dimethylformamide (7% w/v) solution containing $CaCl_2$ (2.5% w/v) for a few seconds for the surface modification and air dried for 24 h in a chemical hood. All threads above were sterilized by UV exposure or a hydrogen peroxide plasma sterilizer before use. The modified thread was used as a "backbone" for an alginate hydrogel islet-loaded fiber which was created by filling a tubular mold (polyethylene tube, 1.5 mm inner diameter) with alginate solution (2%, wt/vol in saline) and inserting the modified thread into the mold, where the alginate was crosslinked by Ca^{2+} ions diffused from the thread surface. After 5-min of cross-linking in the mold, the device was further crosslinked in a buffer bath containing 95 mM $CaCl_2$ and 5 mM $BaCl_2$. Islets were premixed into the alginate solution for the fabrication of islet-containing devices.

To fabricate syngeneic or allogeneic transplants for BALB/c and C57BL/6 mice), 500 IEQ of BALB/c islets in ~35 μL alginate were incorporated onto two 1-cm long threads (250 IEQ for each thread). To fabricate concordant xenogeneic transplants for C57BL/6 mice, 500 IEQ of rat islets in ~35 μL alginate were incorporated onto two 1-cm long threads (250 IEQ for each thread). To fabricate discordant xenogeneic transplants for C57BL/6 mice, 2000 IEQ of human islets in ~70 μL alginate were incorporated onto two 2-cm long threads (1000 IEQ for each thread). To fabricate cell-free transplants for minipigs, 5-cm or 12-cm long threads were used.

5.2.3.12 – Transplantation and retrieval in mice

Prevascularized sites were created 4–6 weeks prior to islet transplantation. Diabetes was induced 1 week prior to transplantation by intraperitoneal injection of fresh prepared STZ solution (acetate buffer, pH 4.5) at a dosage of 150–175 mg STZ/kg mouse. The mice were

considered diabetic following a non-fasting BG measurement above 350 mg/dL on three consecutive days. All mice were anesthetized with 3% isoflurane in O_2 and the abdomen area was shaved and sterilized using betadine and 70% ethanol before surgery. Institutional guidelines for perioperative care, anesthesia and pain management were followed.

For device transplantation at the prevascularized site, a small incision close to one end of the implanted catheter was made to expose the catheter lumen. The catheter was then slightly exposed, and the device was gently introduced into the catheter lumen. Simultaneously, the catheter was slowly and completely pulled out. The skin incisions were closed using 5-0 nylon sutures. These procedures were repeated on each side of the abdomen. For free islet transplantation at the prevascularized site, an islet-loading polyethylene tube (PE-50) was inserted through the catheter lumen, and the islets were infused into the prevascularized site after withdrawing the catheter.

For device transplantation at the unmodified control site, a subcutaneous pocket was created right before the transplantation using a saline-wet tip or blunt surgery tool. Then, the device was inserted into the subcutaneous pocket.

For transplantation at the kidney capsule site, a left lateral paralumbar subcostal incision was made and the left kidney was exposed. The renal capsule was incised at its upper pole and islets were infused using a polyethylene (PE-50) tube and a microsyringe. The incision was closed with suture followed by skin closure using surgical staples (Autoclip, Becton Dickinson, Sparks, MD), and mice received 0.05 mg/kg subcutaneous bolus of buprenorphine. Mice were transplanted with an islet mass of 500 IEQ.

For device retrieval at the prevascularized site, the device was located, and a small incision was made at one end of the device. After dissecting the surrounding tissue, the device

was pulled out from the vascularized site using micro tweezers. In some cases, a long incision on skin was made along the device, and the device was harvested together with surrounding tissue for *in-situ* characterizations. For device retrieval at the control site, the device was harvested together with surrounding tissue in a similar fashion. Mice received 0.05 mg/kg subcutaneous bolus of buprenorphine. The incisions were sutured, and mice were monitored for blood glucose levels after device retrievals. A subset of mice were euthanized right after retrieval without following BG monitoring.

5.2.3.13 – In situ device replacement at the vascularized site

A small incision on the skin was made at one end of the device, and the surrounding tissue was dissected to expose the device end. The device was then gently pulled out, leaving the vascularized pocket site in place. A modified polyethylene tube (PE-50) with one angled end and the one expanded funnel-shaped end was made for the device replacement (see results). The angled end was designed to fit into the vascularized pocket and the expanded opening end was designed to facilitate device replacement.

5.2.3.14 – Implantation and retrieval in Göttingen minipigs

Göttingen minipigs (female, 6 months old) were premedicated with glycopyrrolate and butorphanol, induced with propofol, and anesthetized with isoflurane in O_2. The ventral skin of the minipig was shaved and sterilized before surgery.

For the catheter implantation, a small (~4 mm) incision was made on the side using a scalpel and a deep subcutaneous pocket was created by a blunt surgical tool. Then, the catheter (5 cm or 12 cm long) was attached through a metal rod and inserted into the subcutaneous

pocket. The rod was then withdrawn, leaving the catheter within the subcutaneous space. Finally, the subcutaneous tissue and the incisions on skin were closed using sutures. The catheter was kept in place for 4-6 weeks before device implantation.

For device implantation, a small incision close to one end of the implanted catheter was made to expose the catheter lumen. The device was placed in a silicone tube and loaded through the tunnel of the implanted catheter, and the catheter was withdrawn. Finally, the subcutaneous tissue and the skin incision was closed using sutures. The location of the transplanted device was recorded to guide subsequent device retrieval.

For the device retrieval, the device was located and a small incision on skin and surrounding subcutaneous tissue was made to expose the device end (ultrasound imaging can be used to locate the device, if necessary). The device was pulled out from the vascularized site using tweezers. For device replacement, a modified PE tube was inserted into the vascularized pocket as a guide.

5.2.3.15 – BG monitoring and intraperitoneal glucose tolerance test (IPGTT)

Mouse BG levels in syngeneic and allogeneic transplantation experiments were measured using a glucometer (OneTouch Ultra 2, LifeScan, Canada). Mouse BG readings in xenogeneic transplantation experiments were measured with another glucometer (Contour Next EZ, Bayer). Diabetes reversal was defined as two consecutive readings below 200 mg/dL. Rejection was defined as three consecutive readings over 250 mg/dL.

For the IPGTT, mice were fasted overnight for approximately 16 h, then intraperitoneally injected at a dosage of 2 g glucose/kg. BG was measured at 0, 15, 30, 60, 90, and 120 min following the glucose injection.

5.2.3.16 – Ex vivo static glucose-stimulated insulin secretion (GSIS) assay

Krebs Ringer Bicarbonate (KRB) buffer was prepared according to the follow formula: 98.5 mM NaCl, 4.9 mM KCl, 2.6 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 25.9 mM $NaHCO_3$, 0.1% BSA (all from Sigma-Aldrich), and 20 mM HEPES (Gibco). The retrieved devices were incubated in the KRB buffer for 2 h at 37 ˚C, 5% CO_2. Devices were transferred and incubated in KRB buffer supplemented with 2.8 mM glucose, then 16.7 mM glucose for 75 min each. The buffer was collected after each incubation step, and insulin concentration was measured using an ultrasensitive rat insulin ELISA kit (ALPCO).

5.2.3.17 – Morphology and immunohistochemistry of islets in retrieved devices

The retrieved devices and samples were fixed with 10% formalin and embedded in paraffin. 5 μm paraffin-embedded sections were collected on glass slices. H&E and Masson's trichrome staining was performed by Cornell's Histology Core Facility.

For immunofluorescent staining for blood vessels, slices were deparaffinized in xylene and sequentially rehydrated in 100% ethanol, 95% ethanol, 75% ethanol, and PBS. After antigen retrieval in boiled citric acid buffer (10 mM citric acid and 0.05% Tween 20, pH 6.0) for 20 min and following blocking with 2% BSA for 1 h at room temperature, primary antibody goat anti-mouse CD31 (R&D systems, AF3628, 1:200 dilution) was applied and incubated overnight at 4˚C. After washing with PBS, secondary antibody Alexa Fluor 488-conjugated donkey anti-goat (Invitrogen, A-11055, 1:400 dilution) was applied and incubated for 60 min at room temperature. Finally, slides were washed with PBS, applied with antifade/DAPI, and covered with glass coverslips.

For immunofluorescent insulin and glucagon staining, similar to the procedures described as above, after deparaffination, rehydration, antigen retrieval, and blocking, rabbit anti-rat insulin (Abcam, ab63820, 1:200 dilution) and mouse anti-rat glucagon (Abcam, ab10988, 1:200 dilution) were used as the primary antibodies. Alexa Fluor 594-conjugated goat anti-rabbit IgG (Thermofisher, A11037, 1:400 dilution) and Alexa Fluor 488-conjgated donkey anti-mouse IgG (Thermofisher, A21202, 1:400 dilution) were used as secondary antibodies.

5.2.3.18 – Statistics

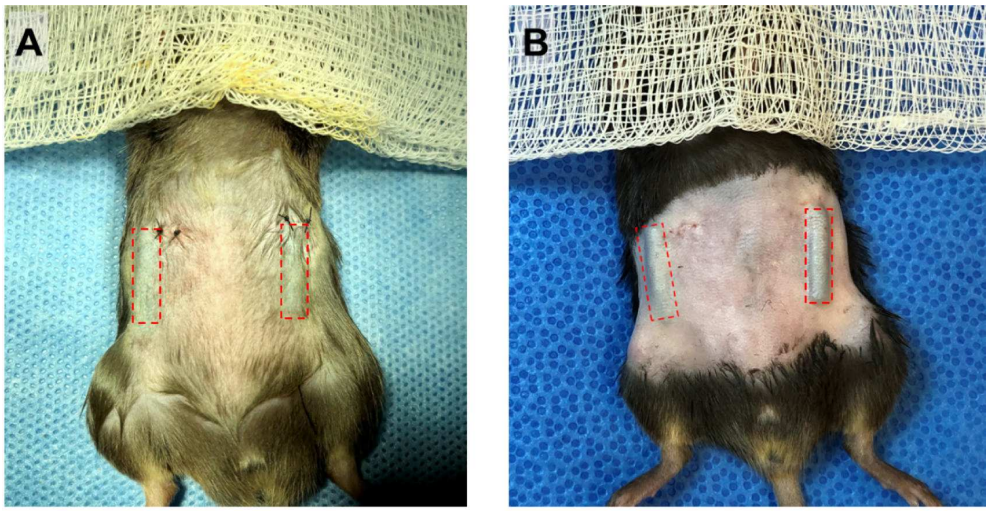
Data are expressed as raw values, mean ± SD, or mean ± SEM, as indicated in the relevant figure captions. To determine the selection of the appropriate statistical test, data were assessed for normality (Shapiro-Wilk) and equality of variances (F-test) before analysis. Cytokine concentrations were compared using a paired-samples Student's *t* test. Local O_2 measurement data were analyzed by an unpaired two-sided Welch's *t*-test and an unpaired two-sided student's *t*-test, as appropriate. Modeling results from the Monte Carlo simulations of variable external boundary oxygen were compared using a two-sided Mann Whitney U test. GSIS data were analyzed using an unpaired two-sided student's *t*-test. BG measurements during IPGTTs were analyzed via a two-way analysis of variance (ANOVA) with Greenhouse-Gessier's correction (for unequal variances) and with Tukey's *post hoc p*-value adjustment for multiple comparisons. Random BG measurements were analyzed by a one-way analysis of covariance (ANCOVA) where time was considered a continuous covariate; data at days 0 and 1 were excluded from the analysis. Survival curves were analyzed by a Mantel-Cox (log-rank) test. Statistical significance was concluded at $p < 0.05$.

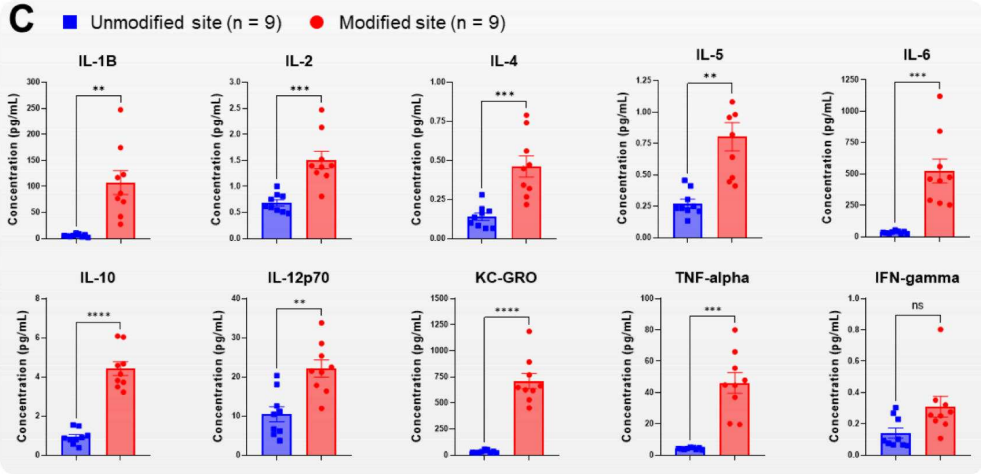
5.2.4 - Results

5.2.4.1 - Design and concept of the SHEATH

The SHEATH approach was designed to provide a vascularized site to promote the engraftment and survival of encapsulated cells without the need for immunosuppression. Subcutaneous implantation of a clinical-grade nylon catheter for 4-6 weeks prior to islet transplantation triggers a controlled neovascularization-promoting foreign-body response (**Figure 5.2.1A and B**). Indeed, an increase in tissue cytokine levels was observed post-implantation, with a generalized immune response involving both pro- (*e.g.*, IL-1β, IL-6, TNF-α) and anti-inflammatory (*e.g.*, IL-4, IL-10) cytokines, suggesting no specific pattern related to M1 or M2 macrophage responses[45] (**Figure 5.2.1C**). Catheter implantation ultimately created a sheath-like pocket surrounded by a dense network of blood vessels amenable for cell transplantation (**Figure 5.2.2A**). Subsequently, a thread-like cell encapsulation device matched in size and shape was implanted into the vascularized subcutaneous pocket formerly occupied by the catheter via a minimally invasive procedure (**Figure 5.2.2A**).

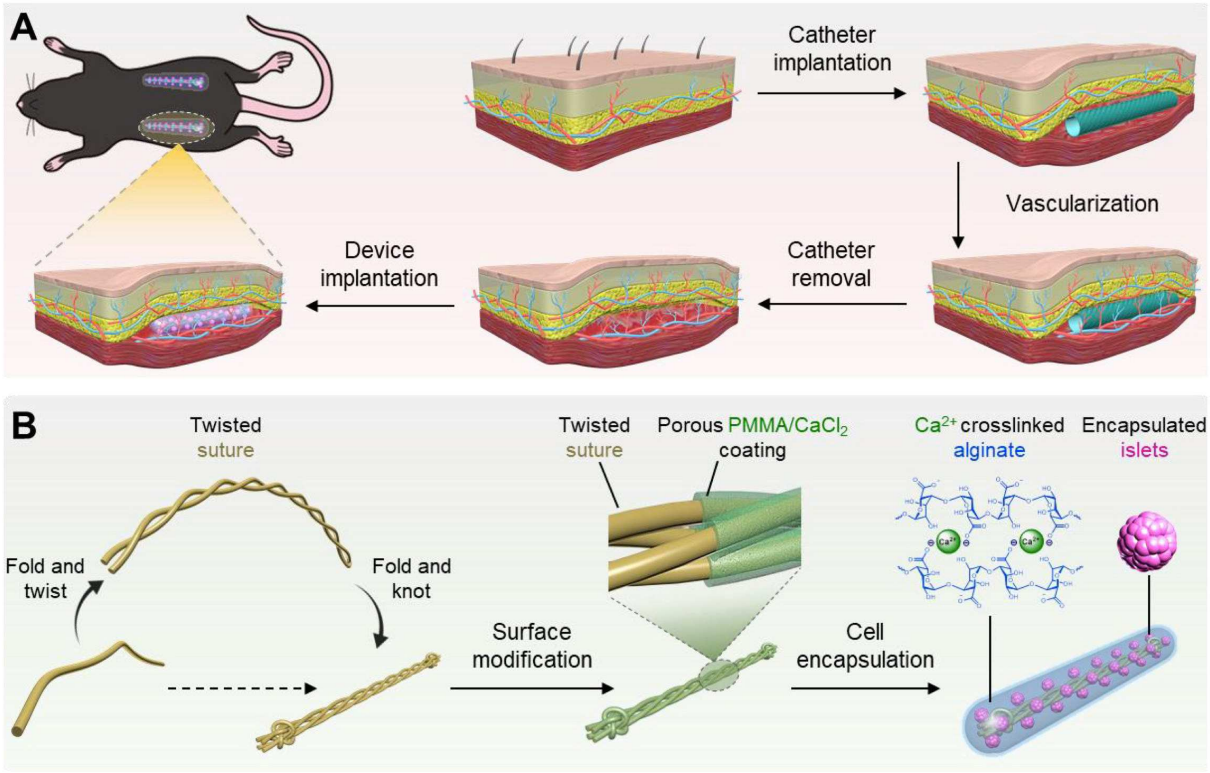
Figure 5.2.1. Characterization of the catheter-induced inflammatory response

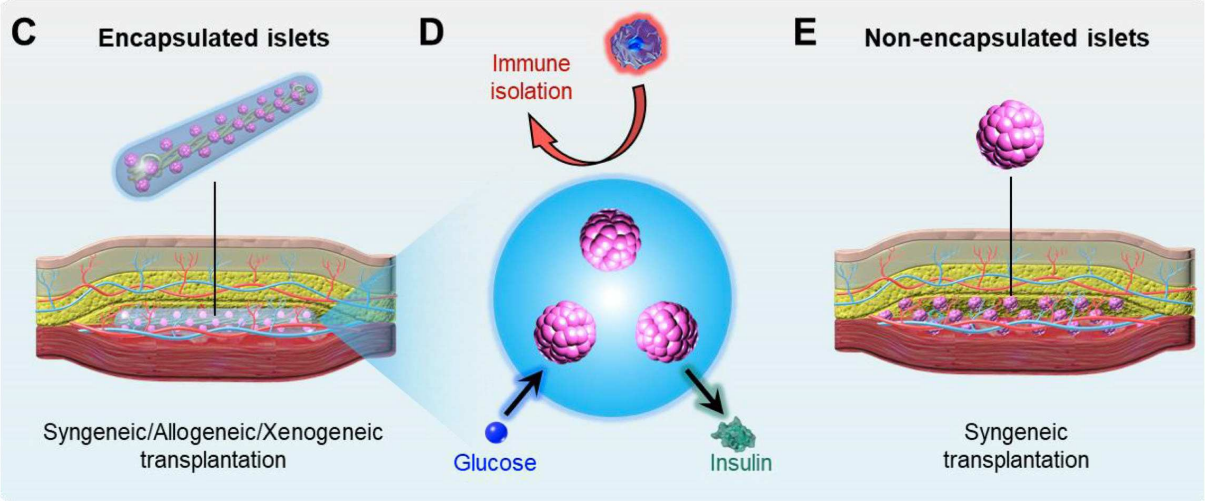




Note: Digital images showing a mouse implanted with 1-cm long catheters at both sides immediately after implantation (A) and six weeks post-surgery (B). (C) Inflammatory assay showing significantly higher tissue cytokine levels at the unmodified (control) vs modified subcutaneous site; shown are medians and interquartile ranges. Groups are compared using Mann-Whitney U tests; $*p < 0.05$, $**p < 0.01$.

Figure 5.2.2. Design of the SHEATH system.





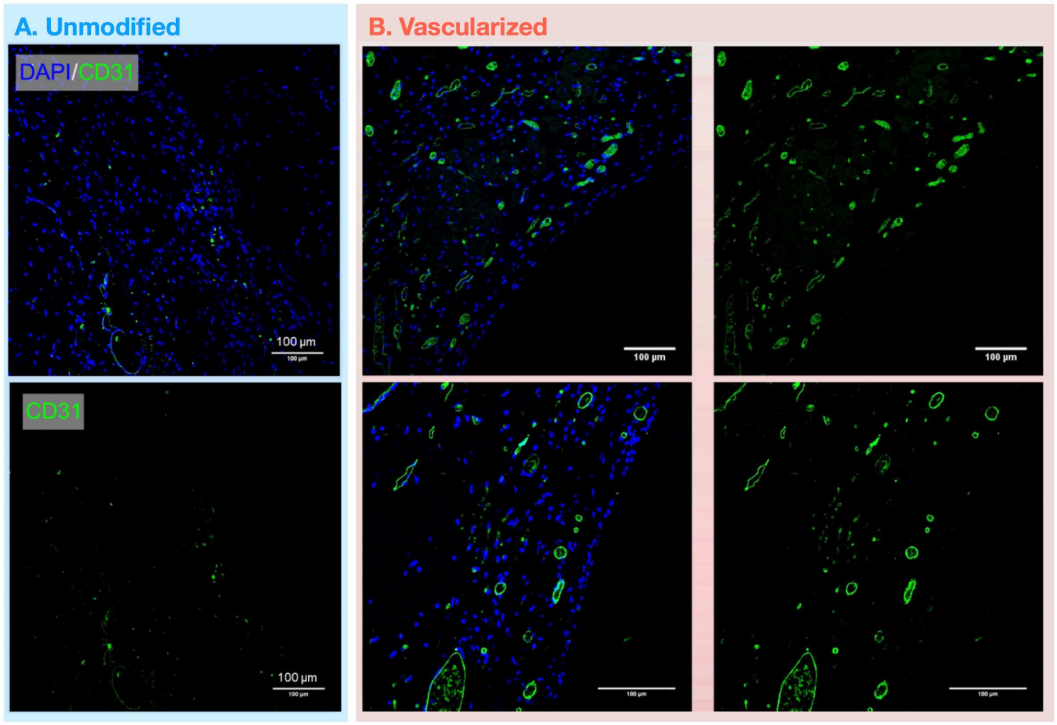
Note: (**A**) Schematic illustrating the creation of the vascularized subcutaneous site achieved by implantation of a catheter 4-6 weeks before transplant. Removal of the catheter created a vascularized pocket that can be used for implantation of the islet encapsulation devices. (**B**) Schematic illustrating the fabrication of the islet encapsulation device. (**C** and **D**) Schematic illustrating the concept of the immuno-isolating cell encapsulation system. The alginate matrix (blue) protects cells from immune interference while allowing the free passage of glucose and insulin required for therapeutic function. (**E**) Illustration of non-encapsulated islets within the vascularized site as an alternative delivery mechanism, suitable only for syngeneic transplants or allogeneic transplants with immunosuppression.

The cell encapsulation device was designed to be robust and scalable, consisting of a modified nylon suture thread with an islet-seeded alginate hydrogel coating (**Figure 5.2.2B**). Specifically, a $CaCl_2$-containing polymer coating was applied on the surface of a twisted and knotted nylon suture thread via a dip-coating process. A porous microarchitecture was achieved in the coated layer via a phase separation process. This coating improved attachment of the alginate hydrogel crosslinked by Ca^{2+} ions diffusing from the central thread. The alginate hydrogel, notable for its biocompatibility, allows bidirectional nutrient and hormone exchange, as well as immune protection for encapsulated cells (**Figure 5.2.2C and D**), which non-encapsulated (naked) islets lack (**Figure 5.2.2E**).

5.2.4.2 - Improvement of local O_2 environment at the modified vascularized subcutaneous site in SHEATH

Following creation of the subcutaneous pocket, we evaluated changes in vascular density and corresponding local oxygenation at the modified vascularized subcutaneous site using histological assessment, O_2-sensing luminescence probes, and Electron Paramagnetic Resonance (EPR) O_2 imaging. Since the native unmodified subcutaneous site typically has a low vascular density (**Figure 5.2.3A**), we hypothesized that the pro-vascularization effects of the pre-implanted catheter would significantly improve the oxygenation environment at the modified site.

Figure 5.2.3. Immunohistochemical to assess vascularization

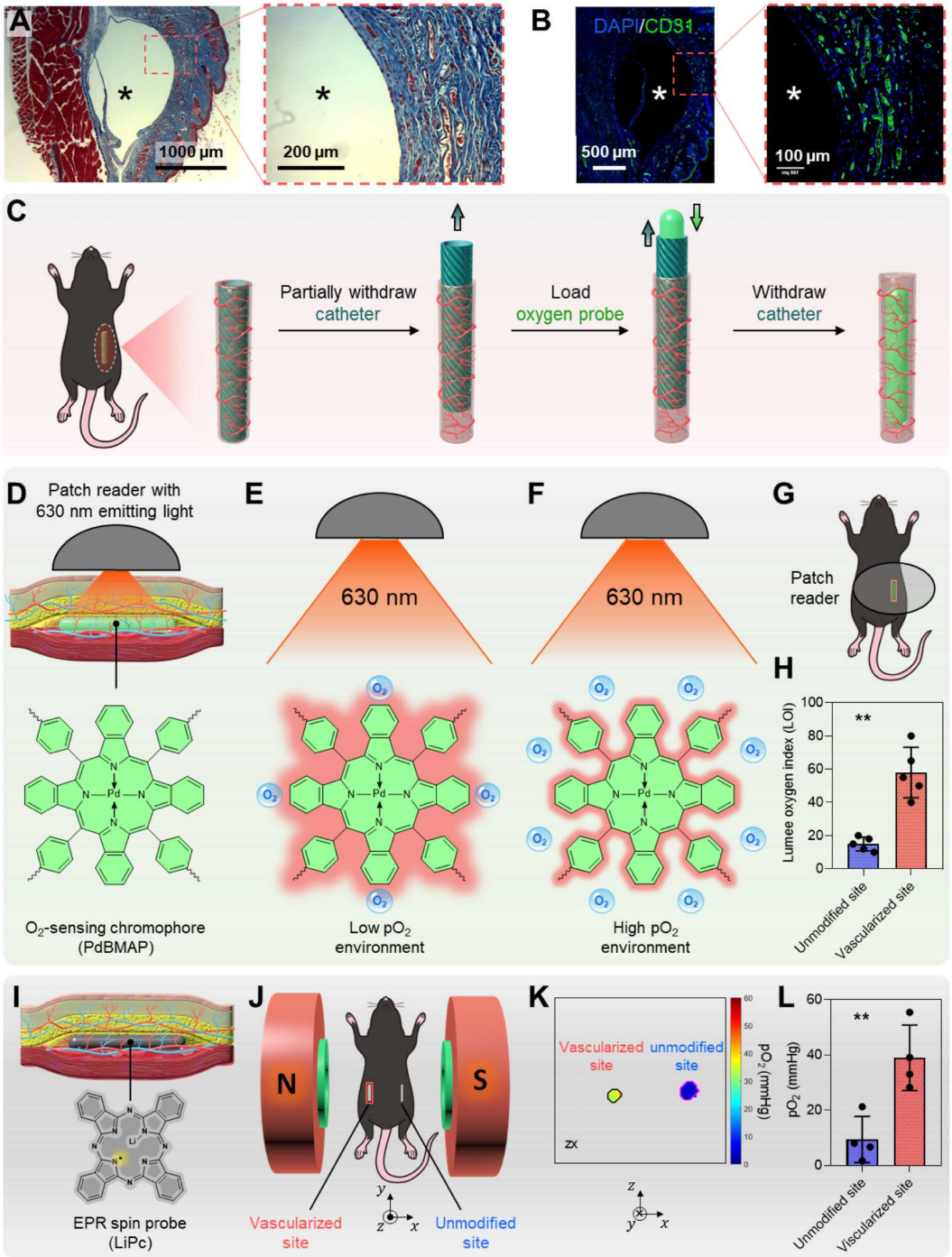


Note: Immunohistochemical staining of CD31 (green) to visualize blood vessels at the unmodified subcutaneous site (A) and vascularized subcutaneous site (B) in C57BL/6 mice, revealing that the catheter-modified site significantly increased local neovascularization in comparison to the native control site. Nuclei were stained using DAPI (blue). The asterisk indicates the luminal space created by the removed catheter.

Masson's trichrome staining revealed the presence of a dense network of blood vessels at the modified site (**Figure 5.2.4A**), which was also observed using fluorescence immunohistochemistry (**Figure 5.2.4B and Figure 5.2.3B**). We then examined whether increased blood vessel density enhanced local O_2 levels within the prevascularized subcutaneous pocket. First, we used a pseudo-quantitative method, consisting of an O_2-sensing chromophore Pd (II) tetramethacrylated benzoporphyrin (PdBMAP)-integrated hydrogel probe, which was injected into the modified and unmodified control sites to compare O_2 concentrations (**Figure 5.2.4C**). Interrogation of the phosphorescence lifetime decay of the PdBMAP-probes was achieved using a small wireless patch reader equipped with 630 nm emitting light, expressing Lumee Oxygen Index (LOI) readings which are positively correlated with O_2 concentrations (**Figure 5.2.4D-G)**. A significantly higher LOI was observed within the modified vascularized site compared to the unmodified site (**Figure 5.2.4H**), indicating increased oxygenation.

We confirmed these findings using EPR O_2 imaging which produces an absolute pO_2 reading (**Figure 5.2.4I and J)**. Analogous to what was performed with the chromophore probes, O_2-sensing lithium phtalocyanine (LiPc) spin probes were implanted into the vascularized site. An identical probe was inserted contralaterally into the unmodified site as a control. EPR interrogation showed significantly higher pO_2 levels within the vascularized subcutaneous pocket as compared to the control site, with a mean pO_2 of ~40 mmHg and ~10 mmHg, respectively (**Figure 5.2.4K and L)**. Collectively, these results demonstrate that catheter implantation increases local vasculature density and O_2 levels in the modified subcutaneous site.

Figure 5.2.4. Characterizations of O_2 environment improvements at the vascularized subcutaneous site



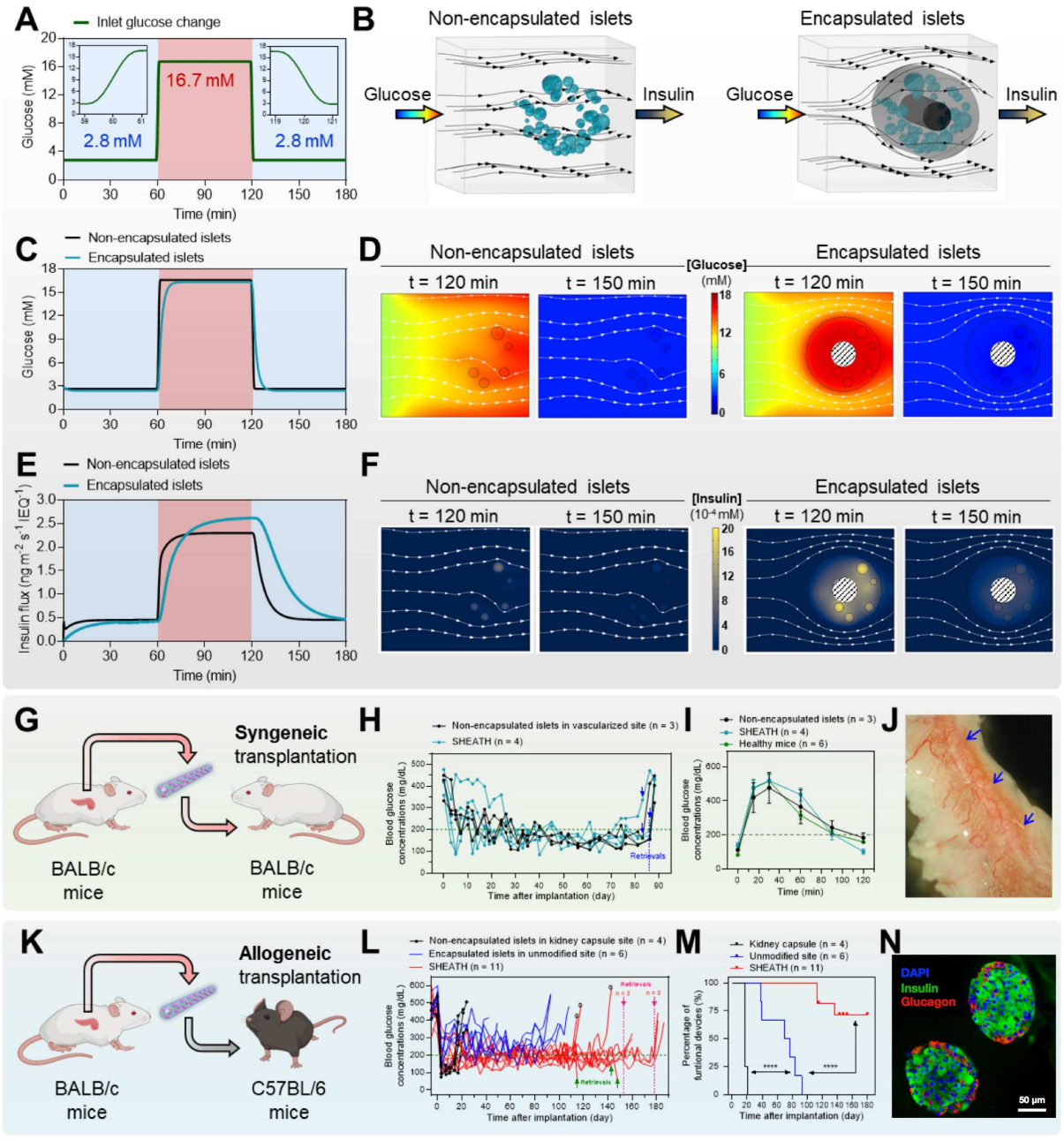
Note: (A) Masson's trichrome staining showing cross-sectional images of the vascularized subcutaneous site in C57BL/6 mice (asterisk indicates the space left following catheter removal). (**B)** Immunohistochemical staining of the vascularized site for CD31. (**C**) Schematic illustrating oxygen probe placement into the vascularized pocket. (**D** to **G)** Comparison of O_2 tensions at the unmodified and vascularized subcutaneous sites using a semiquantitative

O_2 measurement method based on the Lumee™ Oxygen Platform. An O_2-sensing porphyrin chromophore hydrogel probe was injected into the desired site, and a wireless patch reader was attached on the skin above the probe's location (**D**). The O_2-sensing probes respond to 630-nm emitting light produced by the patch reader (**E** and **F**) and the phosphorescence lifetime decay of their emission light was collected by the patch reader (**G**). Phosphorescence decay times are then processed to calculate the Lumee oxygen index, providing a semiquantitative evaluation of O_2 tension at the target site. (**H**) The vascularized site showed a significantly higher Lumee oxygen index; mean ± SEM, $^{**}p = 0.0023$ (unpaired two-tailed Welch's *t*-test). (**I** to **L**) O_2 measurement using EPR imaging. (**I)** Chemical structure of the EPR spin probe, which is embedded within an implanted rod-shaped solid EPR sensor. (**J**) Schematic representation of the EPR oxygen measurement for a mouse with two EPR sensors placed in the unmodified and vascularized sites. (**K)** Representative surface plot collected for O_2 tensions from one plane of the EPR measurement at the unmodified and vascularized sites. (**L**) Quantifications of the average O_2 tension at the unmodified and vascularized sites in mice collected by EPR imaging; mean ± SEM, $^{**}p = 0.0065$ (unpaired two-tailed student's *t*-test).

5.2.4.3 - The SHEATH system preserves acceptable mass transfer properties, achieves diabetes reversal and prevents allorejection in mice

A potential concern with encapsulated insulin-secreting cells is delayed insulin delivery due to additional mass transport limitations for insulin and glucose within the hydrogel matrix and non-vascularized surrounding tissue. We therefore studied the effect of islet encapsulation in the thread-like device on glucose-responsive insulin kinetics (**Figure 5.2.5)**. Briefly, a computational model was developed for a hypothetical *in silico* perifusion test, in which naked (non-encapsulated) or encapsulated islets were exposed to flowing media with variable concentrations of glucose (**Figure 5.2.5A and B,** Appendix A**)**. Specifically, the glucose regimen consisted of three 60-min phases (2.8–16.7–2.8 mM glucose) with 2-min transition periods. Measurements of the glucose concentration in islets over time showed that encapsulation did not significantly impair glucose diffusion in comparison to the non-encapsulated islets (**Figure 5.2.5C and D**). However, encapsulated islets exhibited a slight delay in insulin flux out of the system in comparison to non-encapsulated islets (**Figure 5.2.5E and F)**.

Figure 5.2.5. Characterization of mass transfer properties of the islet encapsulation device and diabetes reversal potential in mice

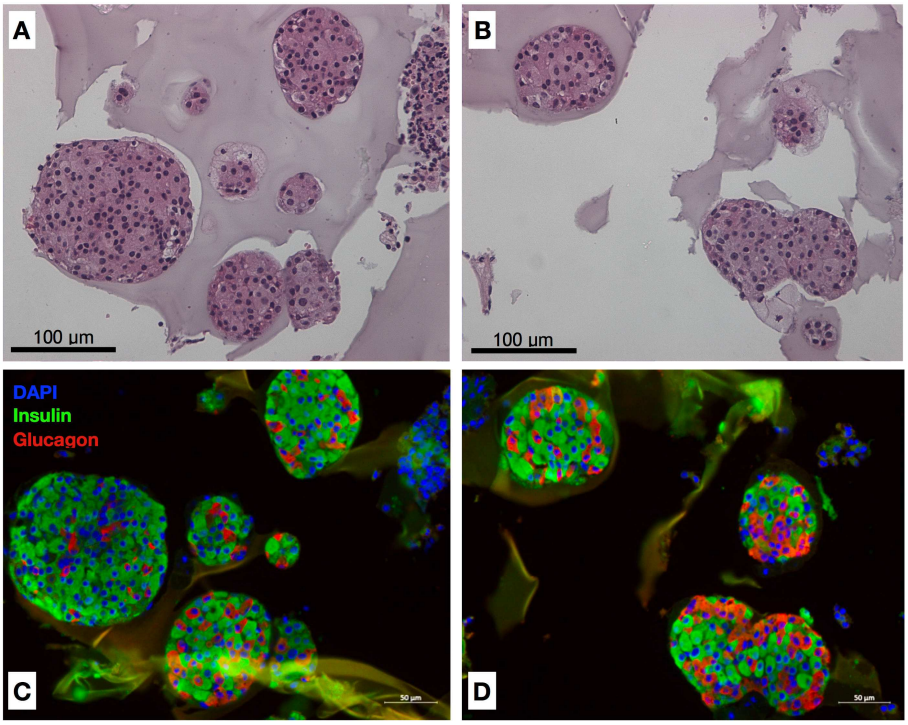


Note: (A to **F)** Settings and results of the *in silico* dynamic perifusion simulation to compare glucose-stimulated insulin secretion kinetics in non-encapsulated and encapsulated islets. (**A**) Inlet glucose concentration settings of the perifusion simulation, featuring three 60 min glucose regimens: an initial low-concentration (2.8 mM) phase, followed by a high-concentration (16.7 mM) phase, and finally a return to the low concentration (2.8 mM) regime; inset plots show the continuous transition of glucose concentration between regimens occurring over 2 min. (**B**) Schematic showing the *in silico* representation of the perifusion test. Non-encapsulated (left) and encapsulated (right) islets were positioned in flowing media and exposed to the variable glucose regime, producing a simulated

insulin outflux. (**C**) Glucose concentration (as a volume-average) in islets over time during the *in silico* perfusion test. (**D**) Surface plots of the glucose concentration in the perifusion system at 120 and 150 min with non-encapsulated (left) and encapsulated (right) islets. (**E**) Outlet flux (normalized by IEQ) of insulin over time during the *in silico* perifusion test. (**F**) Surface plots of insulin concentration in the perifusion system at 120 and 150 min with non-encapsulated (left) and encapsulated (right) islets. (**G** to **J**) Diabetes reversal with the islet encapsulation device in a BALB/c syngeneic transplantation mouse model (G). (**H)** BG measurements of diabetic BALB/c after transplantation of non-encapsulated islets and encapsulated islets in the vascularized subcutaneous site. Blue arrows indicate the retrieval time points; n.s., $p = 0.3754$ (one-way ANCOVA). (**I**) IPGTT on day 80 post-transplantation (healthy mice were used as control); mean ± SEM; n.s., $p = 0.9646$ (non-encapsulated islet-treated mice versus encapsulated islet-treated mice), n.s., $p = 0.9889$ (non-encapsulated islet-treated mice versus healthy mice), n.s., $p = 0.8985$ (encapsulated islet-treated mice versus healthy mice); two-way ANOVA with Greenhouse-Gessier's correction for unequal variance of differences and a post-hoc Tukey's *p*-value adjustment for multiple comparisons. (**J**) Stereo microscope image of a retrieved BALB/c islet encapsulated device with surrounding tissue on day 86 showing the neovascularization surrounding the device (arrows indicate the location of the device). (**K** to **N**) Diabetes reversal of the islet encapsulation device in a BALB/c-to-C57BL/6 allogeneic mouse model (K). (**L**) BG measurements in diabetic C57BL/6 mice after transplantation of naked islets to the kidney capsule site or encapsulated islets to the unmodified and vascularized subcutaneous sites; green arrows indicate retrievals without following BG monitoring; ****$p < 0.0001$ (unmodified site versus vascularized site, one-way ANCOVA). (**M**) Kaplan-Meier graft survival estimates, ****$p < 0.0001$, (all comparisons, Mantel-Cox test). (**N**) Immunohistochemical staining of one retrieved device from the vascularized subcutaneous site on day 178 showing insulin/glucagon-positive islets.

To validate *in silico* models, we assessed the SHEATH's performance *in vivo*. First, we performed syngeneic islet transplants in streptozotocin (STZ)-induced diabetic BALB/c mice (**Figure 5.2.5G)**. For comparison, we included mice transplanted with non-encapsulated syngeneic islets into the vascularized subcutaneous site. We observed that mice with encapsulated islets maintained similar non-fasting BG levels compared to those with non-encapsulated islets (**Figure 5.2.5H)**. Moreover, intraperitoneal glucose tolerance tests (IPGTTs) not only demonstrated similar metabolic responses between groups, but also to those of healthy non-diabetic mice (**Figure 5.2.5I**). Following device extraction, dense vascular networks were observed surrounding the devices (**Figure 5.2.5J**). Moreover, integrity of the islet architecture was fully preserved (**Figure 5.2.6**).

Figure 5.2.6. Immunofluorescence of retrieved graft in a syngeneic transplant mouse model



Note: (A and B) Hematoxylin and eosin staining of retrieved grafts in a syngeneic transplant mouse model. (**C and D**) Immunofluorescence staining for insulin (green), glucagon (red) and nuclear staining using DAPI (blue) of retrieved grafts in a syngeneic transplant mouse model.

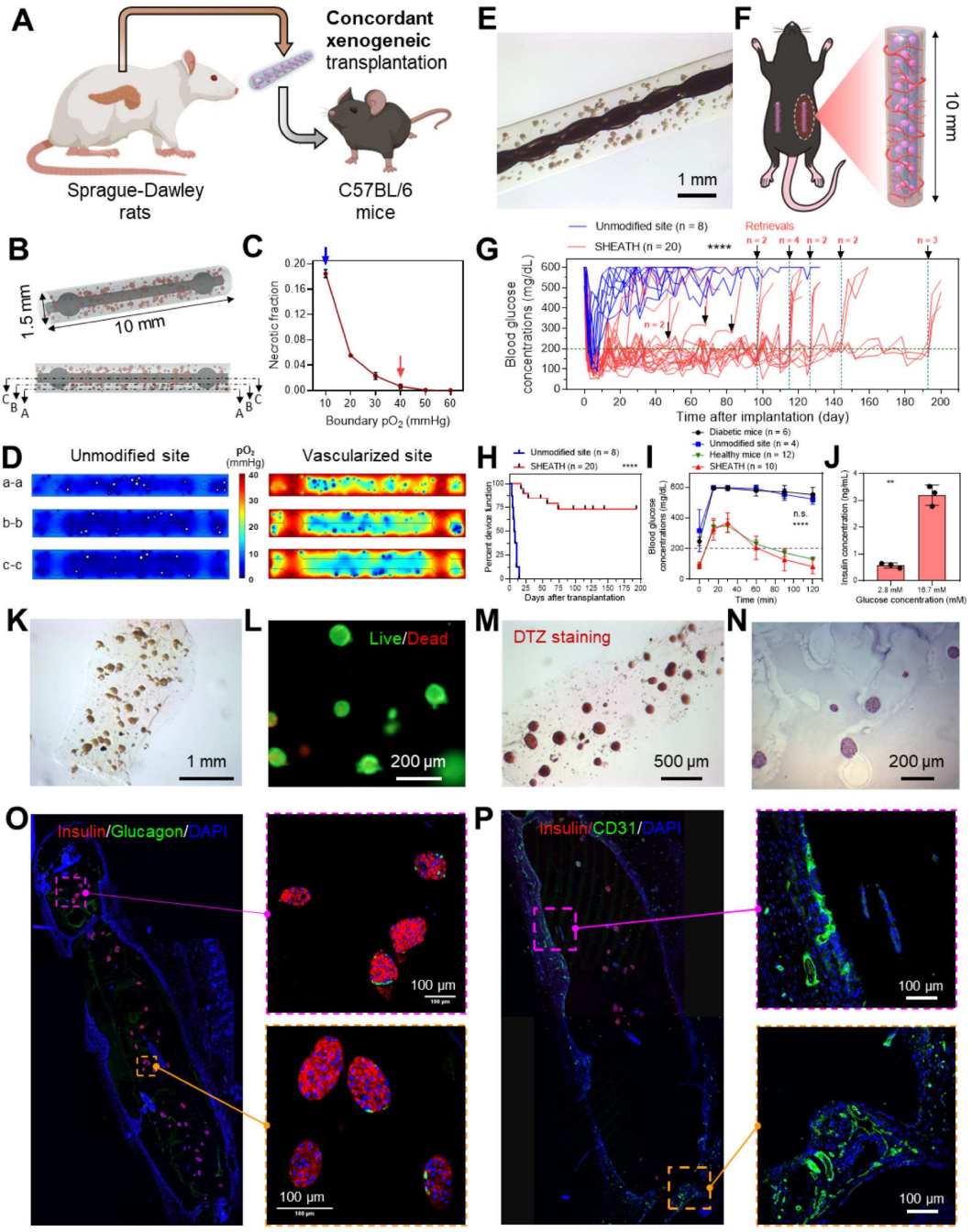
We also tested the potential of the SHEATH system in enabling diabetes reversal in a fully major histocompatibility complex-mismatched mouse model, in which BALB/c islets were transplanted into immunocompetent C57BL/6 mice (**Figure 5.2.5K**). For these experiments, we compared the SHEATH system to non-encapsulated islet transplantation into the subcapsular kidney space (*i.e.*, to corroborate rejection) and encapsulated islet transplantation into a control (*i.e.*, unmodified) subcutaneous site. We observed that the SHEATH system supported significantly better diabetes reversal than controls (**Figure 5.2.5L and M**). Similar to what was observed in the syngeneic model, interrogation of the grafts at device retrieval demonstrated fully preserved islet architecture (**Figure 5.2.5N)**. Altogether,

these results suggest that the facile mass transfer properties of the thread-like islet encapsulation device and its surrounding vascular network jointly achieved with the SHEATH system sustain diabetes reversal whilst preventing allogeneic rejection without immunosuppression.

5.2.4.4 - SHEATH enables long-term diabetes reversal in a concordant xenogeneic rat-to-mouse islet transplant model

Building on the favorable results with syngeneic and allogeneic models, we evaluated the immune-isolating capacity and therapeutic function of the SHEATH system in a more stringent immunological context, a concordant xenogeneic model in which rat islets were transplanted into fully immunocompetent C57BL/6 mice (**Figure 5.2.7A**). First, a complementary computational model was developed to predict pO_2 distributions in devices transplanted in the control and vascularized sites (**Figure 5.2.7B**). Site placement was implemented in the model by the boundary oxygen concentration, which was 10 and 40 mmHg in the unmodified and vascularized sites, respectively, based on the average values obtained from EPR measurements (**Figure 2.2.8A-E**). Stochastic simulation results indicated that the expected necrotic volume fraction of islets was strongly dependent on the boundary oxygen value (**Figure 5.2.8F and G**), with predicted values of 18.5 ± 0.3% versus 0.6 ± 0.2% of the total islet volume in the unmodified and vascularized sites, respectively. Surface plots of the simulated pO_2 distributions also suggested a marked improvement in device and cellular oxygenation (**Fig. 5.2.7D**).

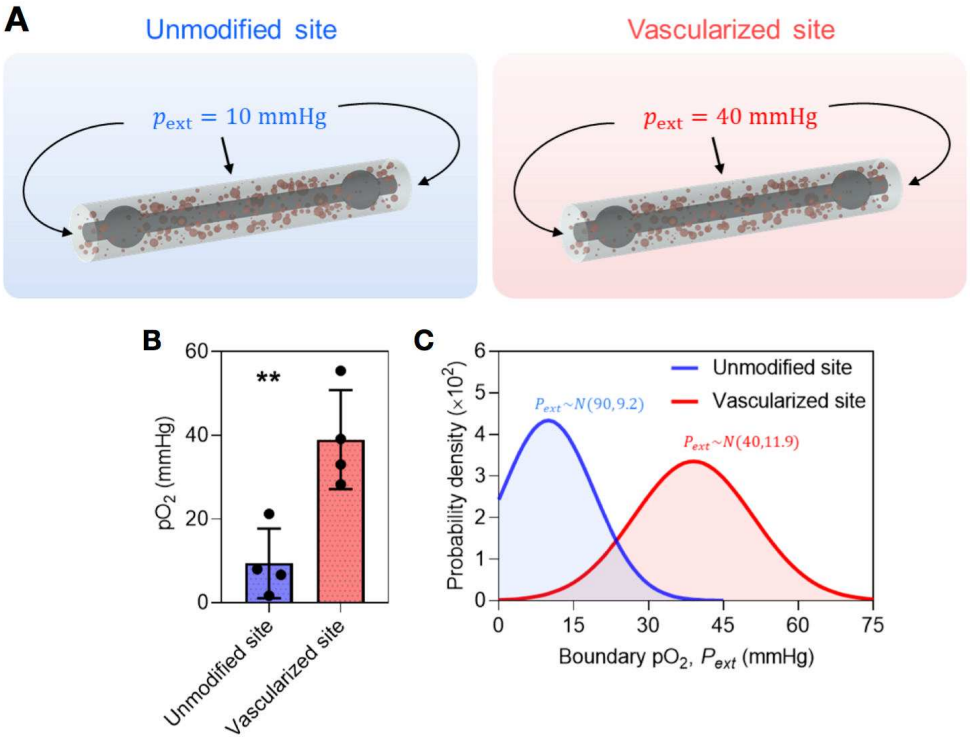
Figure 5.2.7. A vascularized subcutaneous site enables long-term diabetes reversal in a concordant xenogeneic rat-to-mouse encapsulated islet transplant model

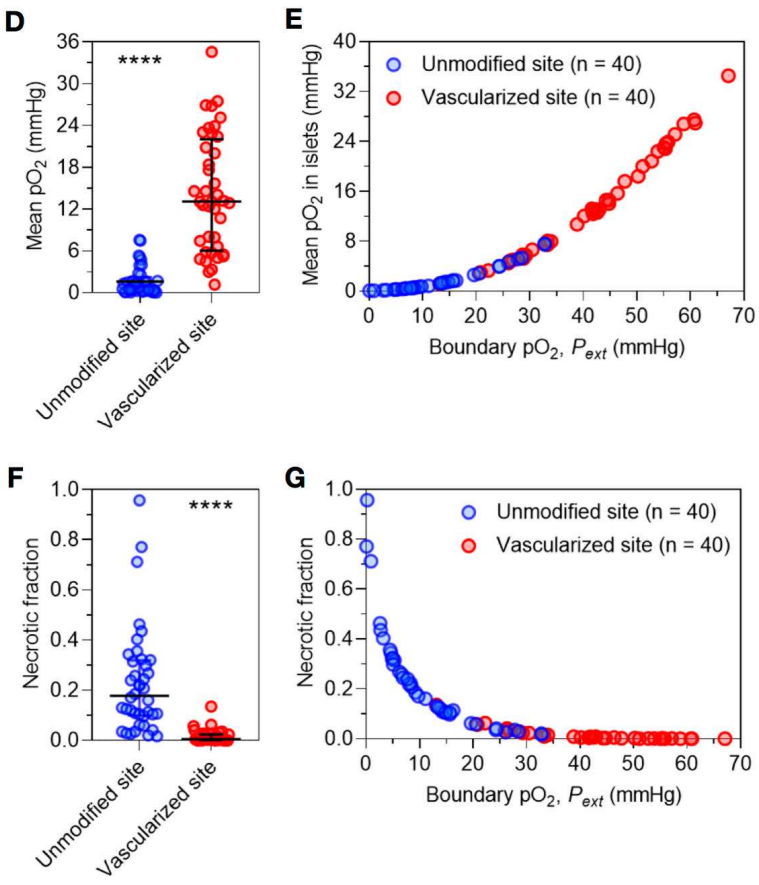


Note: (A) Illustration of the xenotransplantation model. (**B**) Schematic representing the simulated geometry of the encapsulated device containing rat islets. (**C**) Simulation-predicted volume-fraction of necrosis in the encapsulated islets at variable boundary O_2 tensions. Arrows indicate the expected boundary pO_2 of the unmodified site (blue arrow, 10 mmHg) and vascularized site (red arrow, 40 mmHg), as determined by the information obtained from EPR measurements. (**D**) Simulation-predicted pO_2 distributions within the islet encapsulation device at three

tangential cross sections as labeled with a-a, b-b, and b-c with boundary O_2 tensions of 10 mmHg (unmodified site) and 40 mmHg (vascularized site). White regions in the islets represent necrosis. (**E**) Stereo microscope image of a rat islet encapsulated device. (**F**) Illustration of the vascularized subcutaneous pocket for device transplantation. (**G**) BG measurements of diabetic C57BL/6 mice after transplantation of encapsulated rat islets in the unmodified and vascularized subcutaneous sites, ****p < 0.0001 (unmodified site versus vascularized site, one-way ANCOVA). (**H**) Kaplan-Meier graft survival estimates; ****p < 0.0001, (all comparisons, Mantel-Cox test). (**I**) IPGTTs before device retrievals, mean ± SEM; n.s., p = 0.9952 (mice with implants at unmodified site versus diabetic control mice), n.s., p = 0.8372 (mice with implants at vascularized site versus healthy mice), ****p < 0.0001, (all other comparisons); two-way ANOVA with Greenhouse-Gessier's correction for unequal variance of differences and a post hoc Tukey's p-value adjustment for multiple comparisons. (**J**) Static GSIS test of devices retrieved on day 193; mean ± SD, **p = 0.0071 (unpaired Student's t-test). (**K**) Stereo microscope image of collected hydrogel from a retrieved device on day 97. (**L** and **M**) Live/dead staining (L) and DTZ staining (M) of islets from retrieved devices on day 115. (**N**) H&E staining of a retrieved device on day 144 showing healthy islets. (**O**) Immunohistochemical staining showing intact morphology and insulin/glucagon-positive islets. (**P**) Immunohistochemistry of retrieved devices on day 127 showing peri-device vascularization as shown by positive staining for CD31.

Figure 5.2.8. *In silico* modelling of local oxygen levels and its impact on islet necrosis





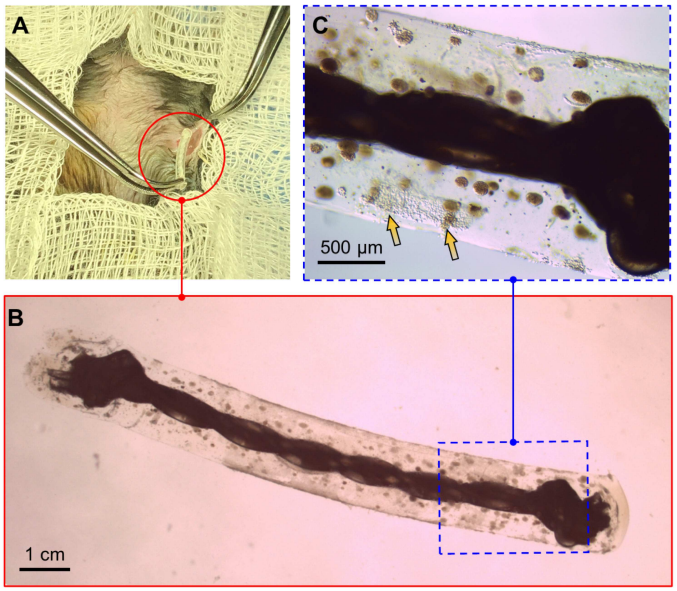
Note: (**A**) Values of the external boundary oxygen tension applied in simulations (based on the average value from EPR oxygen measurements) for devices at the unmodified and vascularized site, respectively. (**B**) Distribution of EPR oxygen measurements in the unmodified and vascularized sites. (**C**) Probability density functions of the best-fit normal distributions to the absolute pO_2 measurements at the unmodified and vascularized sites shown in **B**. (**D to G**) Results from Monte Carlo simulations of the rat islet device where the external boundary pO_2, p_{ext}, was treated as a random variable described by the normal distributions shown in **C**. Mean pO_2 (**D and E**) and net necrotic fraction within rat islets (**F and G**) in devices in the unmodified and vascularized sites. Data was compared in bar graphs (**D and F**) and in relation to the simulated value of p_{ext} (**E and G**). c and e: ****$p < 0.0001$ (two-sided Mann-Whitney U test).

We then proceeded with transplantation studies to validate these results using identical protocols for device implantation as previously described for syngeneic and allogeneic models (**Figure 5.2.7E and F**). These experiments showed a robust capacity for long-term diabetes reversal wherein SHEATH enabled correction of hyperglycemia for over 190 days while the devices implanted into the unmodified control site failed within 2 weeks (**Figure 5.2.7G and**

H). Additionally, in an IPGTT, mice treated with the SHEATH system displayed near-physiological glycemic profiles, whereas glucose responsiveness in control mice resembled those of diabetic non-transplanted mice (**Figure 5.2.7I**). In addition, some devices were retrieved to evaluate *ex vivo* glucose-stimulated insulin secretion (GSIS), which also revealed adequate glucose-responsiveness of the encapsulated islets (**Figure 5.2.7J**).

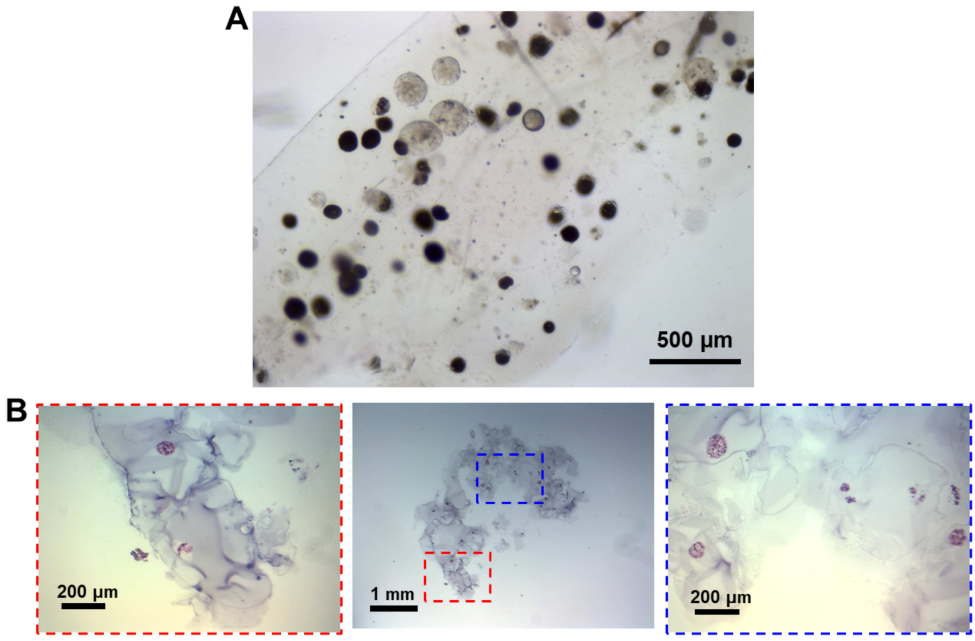
Graft retrieval to assess islet integrity and function was performed by either directly pulling the device out from the modified site or by harvesting it with the surrounding tissue. We observed that the device could be smoothly retrieved from the modified subcutaneous pocket, and that minimal fibrotic deposition accrued on the device surface (**Figure 5.2.9A-C**). Following retrieval, examination of the devices revealed round islets with a yellow hue and smooth surfaces, which indicated preserved islet morphology (**Figure 5.2.7K**). Preserved cell viability (**Figure 5.2.7L**), and positive dithizone identification staining (**Figure 5.2.7M**) were also corroborated. Hematoxylin and eosin (H&E) staining (**Figure 5.2.7N**) and insulin/glucagon immunostaining (**Fig. 5.2.7O**) further confirmed the integrity and sustained function of the islets in the retrieved SHEATH devices. Moreover, *en bloc* removal of the SHEATH graft demonstrated adequately preserved peri-device vascularization without significant additional fibrotic layers (**Figure 5.2.7P**). Comparatively, encapsulated islets retrieved from the unmodified control site appeared as dark clusters (**Figure 5.2.10**) under stereo microscope imaging and largely fragmented or necrotic in H&E staining. Overall, the SHEATH system enabled effective immune protection and supported long-term diabetes reversal in a concordant xenogeneic transplant model.

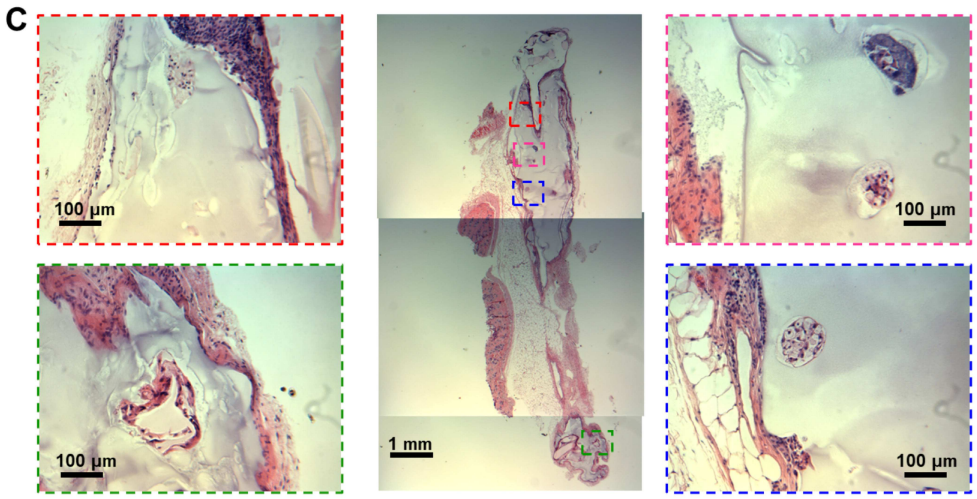
Figure 5.2.9. Macroscopic assessment of fibrotic tissue deposition in a retrieved device



Note: (**A**) A digital image of a retrieved device smoothly withdrawn from the vascularized site on day 97 after transplantation. (**B and C**) Stereo microscope images of the retrieved device showing minimal fibrotic deposition (yellow arrows) surface.

Figure 5.2.10. Histological assessment of retrieved devices implanted in the unmodified subcutaneous site.



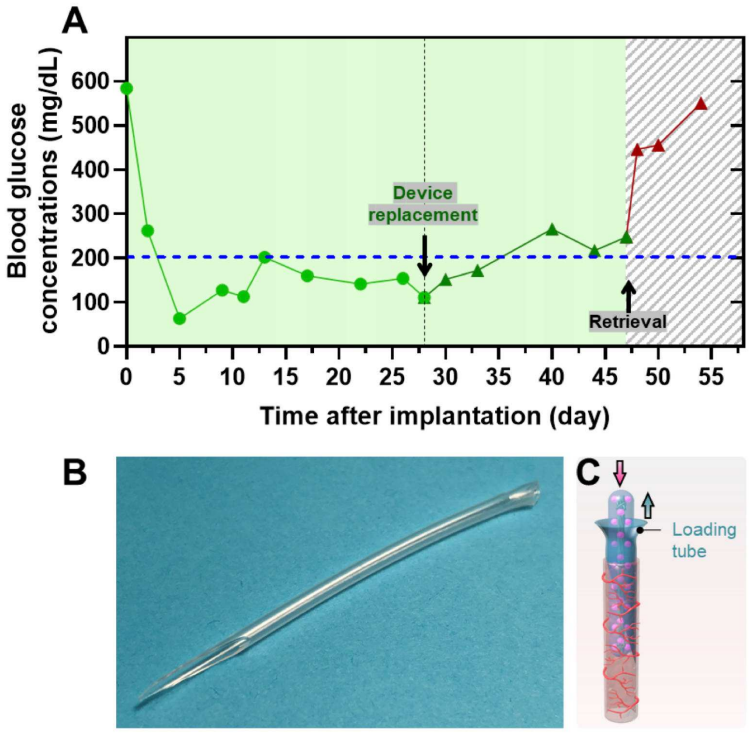


Note: (A) Stereo microscope images of encapsulated islets collected from a control device retrieved from the unmodified subcutaneous site on day 58. Most islets appeared as dark clusters, indicating cell death. (B and C) H&E staining of encapsulated islets in control devices showing severe islet damage with pyknosis (shrunken and dark nuclei)/karyorrhexis (fragmented nuclei) or complete loss of nuclei.

5.2.4.5 - SHEATH allows in situ replacement of a failing graft

Despite the clear success observed with the SHEATH system in syngeneic, allogeneic, and concordant xenogeneic models, we observed that some mice showed impaired graft function before device retrieval (**Figure 5.2.5L and Figure 5.2.7G**). We corroborated that the vascularized pocket in the SHEATH system remains intact after device retrieval in a preliminary test, which demonstrated continued glycemic control when a functional device was replaced *in situ* with a new one (**Figure 5.2.11**). Taking advantage of this fact, we explored the possibility of *in situ* replacement of failing devices in mice.

Figure 5.2.11. Preliminary assessment of *in situ* device retrieval and its impact on a functioning device

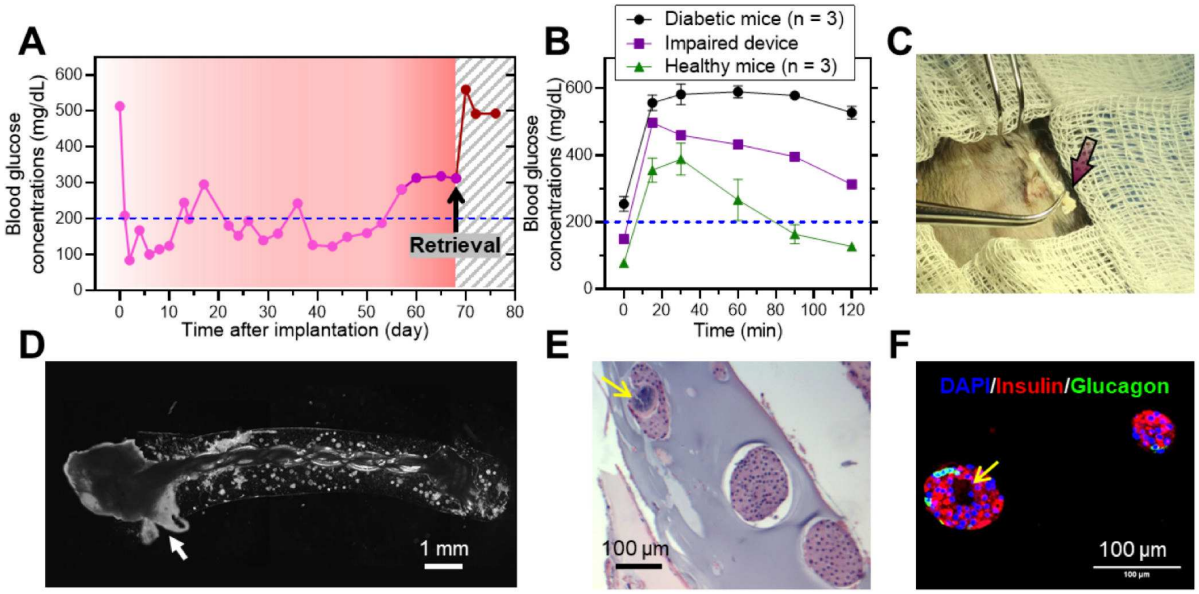


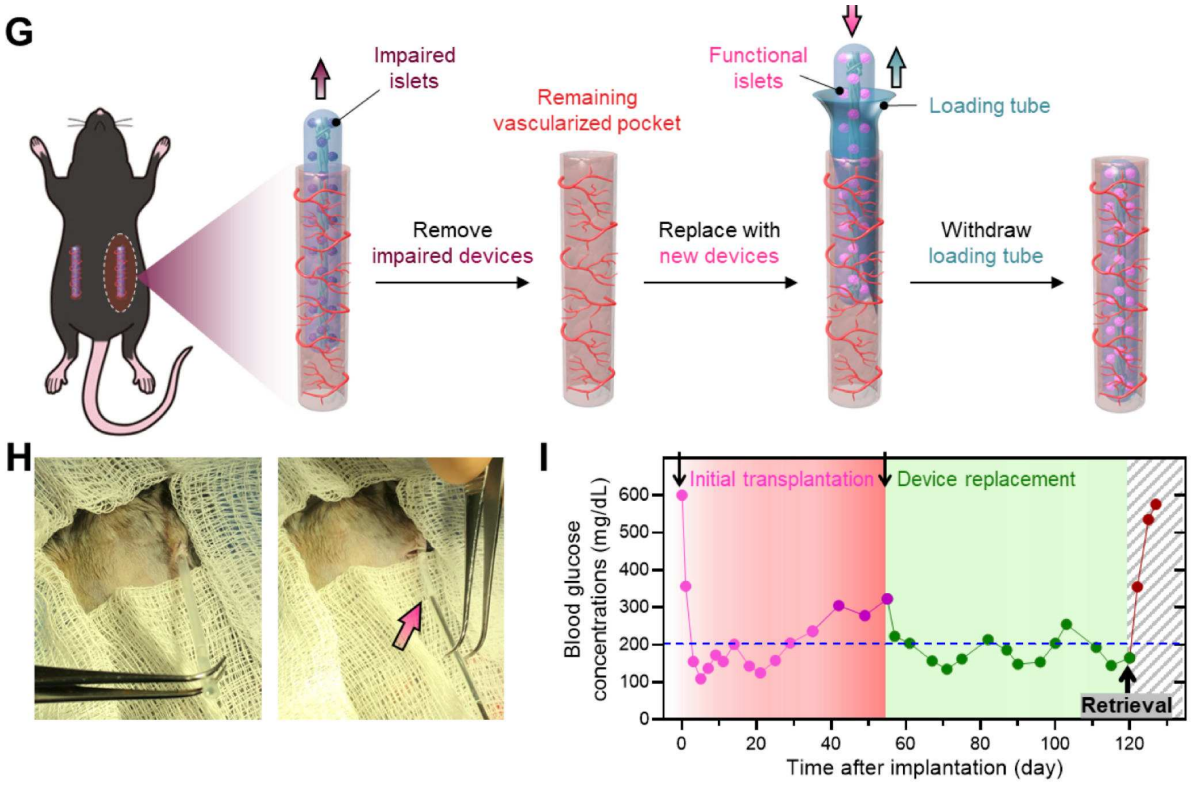
Note: (**A**) BG measurements of one mouse with a functional device replaced by a new one in a preliminary replacement test. (**B**) Customized PE tube that was manufactured to enable in situ device replacement. (**C**) Schematic showing the process of device replacement. Following device retrieval, the sharp end of the customized PE tube is inserted to maintain patency of the vascularized subcutaneous pocket. Once the PE tube is in place, a new device is inserted through the expanded funnel-shaped side of the customized PE tube. Following device implantation, the PE tube is completely removed.

A typical impaired device was characterized by elevated BG levels (**Figure 5.2.12A**) and showed partially impaired glucose responsiveness during a glucose tolerance test (**Figure 5.2.12B**). Upon retrieval, this device showed tissue adhesion at one exposed end of the thread (**Figure 5.2.12C and D**), while histological evaluation revealed islet damage characterized by cluster dispersion and necrotic core formation (**Figure 5.2.12E and F**). To test the prospect of *in situ* replacement, mice displaying impaired function following xenogeneic rat-to-mouse transplants using the SHEATH system were selected for device retrieval and replacement

(**Figure 5.2.12G**). Briefly, impaired devices were removed by performing an incision close to the device end, followed by microdissection of the surrounding tissue. After device retrieval, a customized polyethylene (PE) loading tube was inserted into the vascularized pockets to guide device replacement (**Figure 5.2.11B**). A freshly prepared device was then loaded into the tube and gently pushed into the established vascularized pocket (**Figure 5.2.12H**). This strategy allowed the existing vascularized subcutaneous site to support optimal engraftment and graft survival of a supplementary device. Strikingly, normoglycemia was successfully restored, which was sustained until device retrievals (**Figure 5.2.12I**). Together, these experiments provide proof-of-concept that the SHEATH system is amenable for future *in situ* device replacement, if required.

Figure 5.2.12. *In situ* device replacement at the modified vascularized site





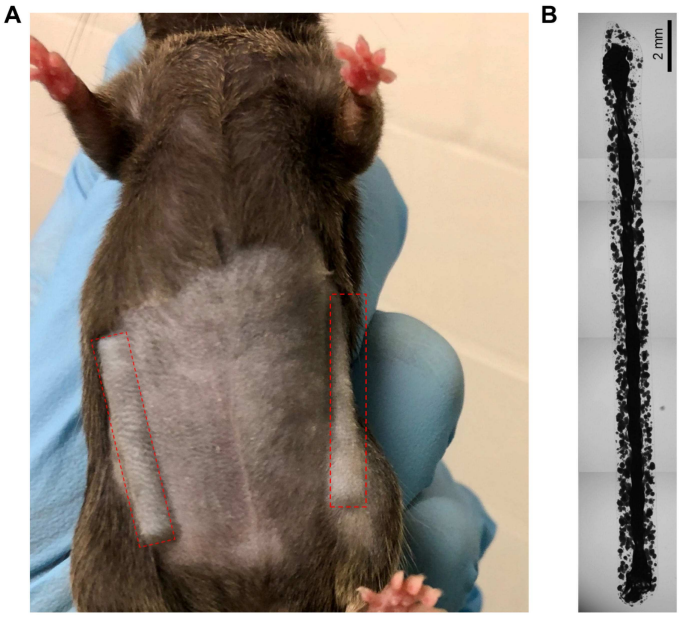
Note: (A) BG data from one subject (**Figure 5.2.7**) showing return to hyperglycemia at ~60 days indicative of partial graft attrition. BG dramatically increased following device retrieval. (**B**) IPGTT on day 60 showing impaired glucose responsiveness. (**C** and **D**) A digital image (**C**) and a dark-field microscopy image (**D**) of the retrieved device showing tissue adhesion (white arrow) at one end of device where the central thread was exposed. (**E** and **F**) H&E (E) and immunohistochemical staining (F) of retrieved impaired device showing some islets with necrotic core regions (yellow arrows). (**G**) Schematic illustrating the procedure for device replacement at the vascularized site. (**H**) Digital images demonstrating device replacement through a modified PE tube, which served as a portal for the delivery of a new device into the vascularized subcutaneous site (pink arrow identifies the device in the loading tube). (**I**) BG measurements of one mouse experiencing partial graft attrition and device replacement. Return to normoglycemia was observed shortly following device replacement.

5.2.4.6 - SHEATH supports long-term islet survival in a discordant human-to-mouse xenogeneic transplant model

Building on the elevated local O_2 environment and effective immunoisolating capacity of the SHEATH system observed in allogeneic and xenogeneic transplant models, we evaluated its therapeutic function in a discordant xenogeneic model of human-to-mouse islet transplantation. Considering that higher doses of human islets are required to achieve normoglycemia in mice due to their resistance to human insulin,[46] longer catheters were

implanted prior to transplantation (**Figure 5.2.13A**). Correspondingly, longer devices (*i.e.*, ~2 cm) were fabricated (**Figure 5.2.13B**).

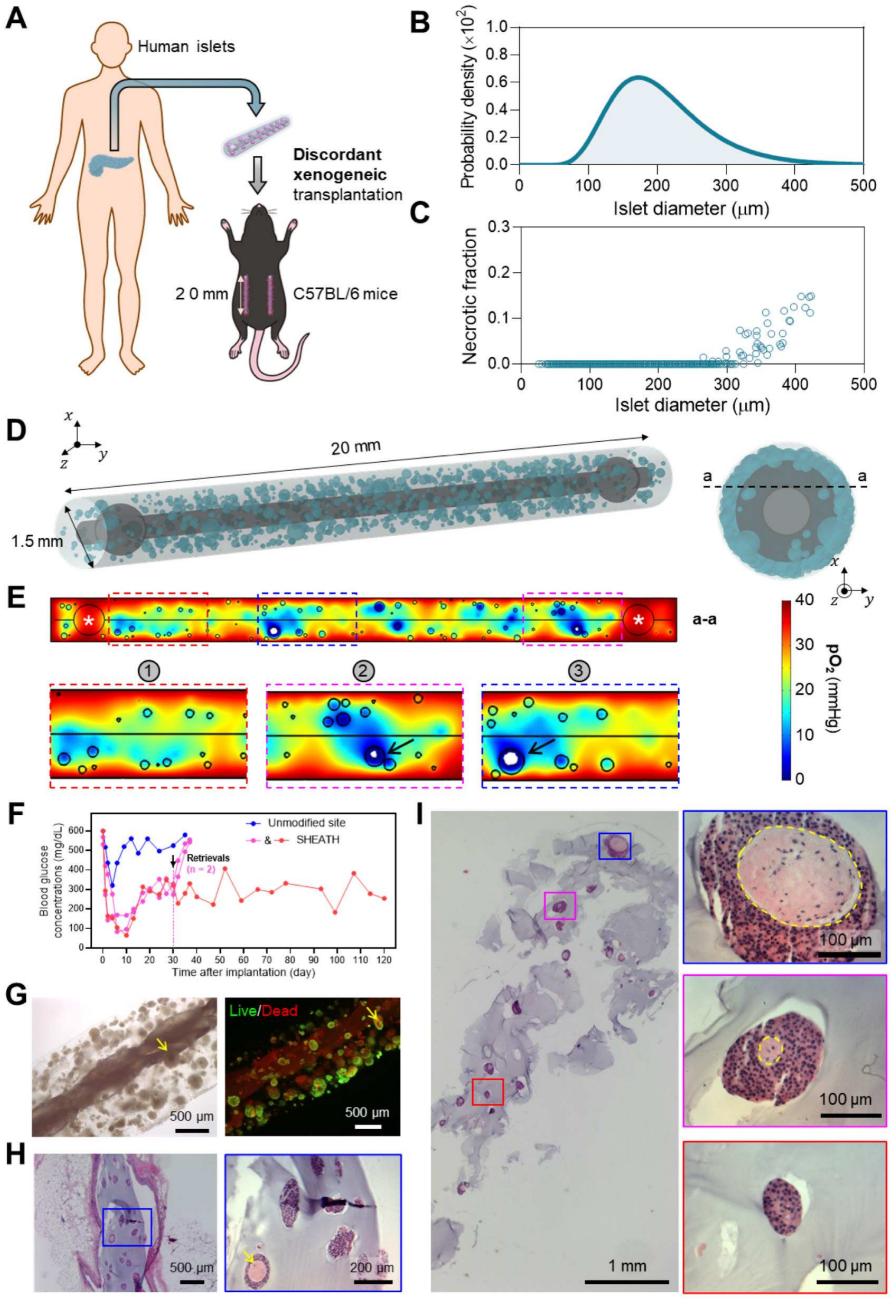
Figure 5.2.13. Catheter implantation and encapsulation device manufacture for discordant xenogeneic human-to-mouse transplant studies



Note: (A) A digital image showing a mouse implanted with ~2-cm long catheters (outlined with red dashed frame) on both sides. (B) A microscopy image of a 2-cm long device with 1000 IEQ human islets for implantation on one side.

Computational models were adapted to account for these updated device dimensions, as well as islet size distribution and seeding density (**Figure 5.2.14B-E** and Appendix A). *In silico*, it was anticipated that most large islets (*i.e.*, > 300 μm) would be more prone to necrosis even at the vascularized site (**Figure 5.2.14C and E**), though higher pO_2 levels throughout the device were predicted, as compared to the control site (**Figure 5.2.15**).

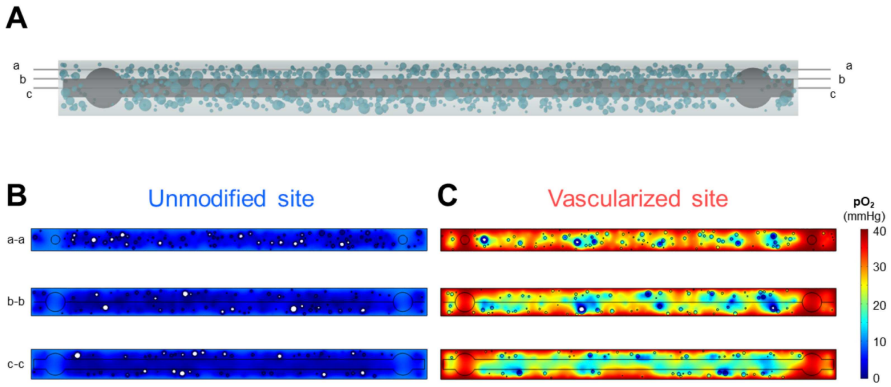
Figure 5.2.14. Performance of the SHEATH system in a discordant xenogeneic human-to-mouse islet transplant model



Note: (A) Illustration of the human-to-mouse xenogeneic model. (**B**) Size distribution of the batch of human islets used for transplantation. The probability density is shown on a volume basis. (**C**) Simulation predictions of the necrotic volume fraction of individual islets showing that necrosis is anticipated in the larger islets. (**D**) Schematic representing the simulated geometry and distribution of the human islets within the encapsulated device. (**E**) Simulation-predicted pO_2 distributions in the islet encapsulation device in the oxygen environment of the vascularized subcutaneous site (40 mmHg) at the cross section labeled a-a in (D). Black arrows indicate the necrotic

core (white area) in one simulated large islet. (**F**) BG measurements of diabetic C57BL/6 after transplantation of encapsulated human islets in unmodified and vascularized subcutaneous sites. (**G**) Stereo microscope image (left) and live/dead staining (right) of islets in a retrieved device on day 30. Yellow arrows indicate the necrotic core regions in large islets. (**H** and **I**) Histologic characterization of devices retrieved from a mouse at 120-days post-transplant**. (H)** H&E staining of a device retrieved with the surrounding vascularized subcutaneous pocket on day 120 showing that most small islets were healthy, while some large islets exhibited core necrosis (indicated by the yellow arrow). (**I**) H&E staining of the contralateral device retrieved on day 120 showing that small islets were healthy while some large islets exhibited core necrosis (dashed yellow outlines circumscribe the necrotic area in islets).

Figure 5.2.15. Predicted differences in pO_2 levels within encapsulation devices at different implantation sites

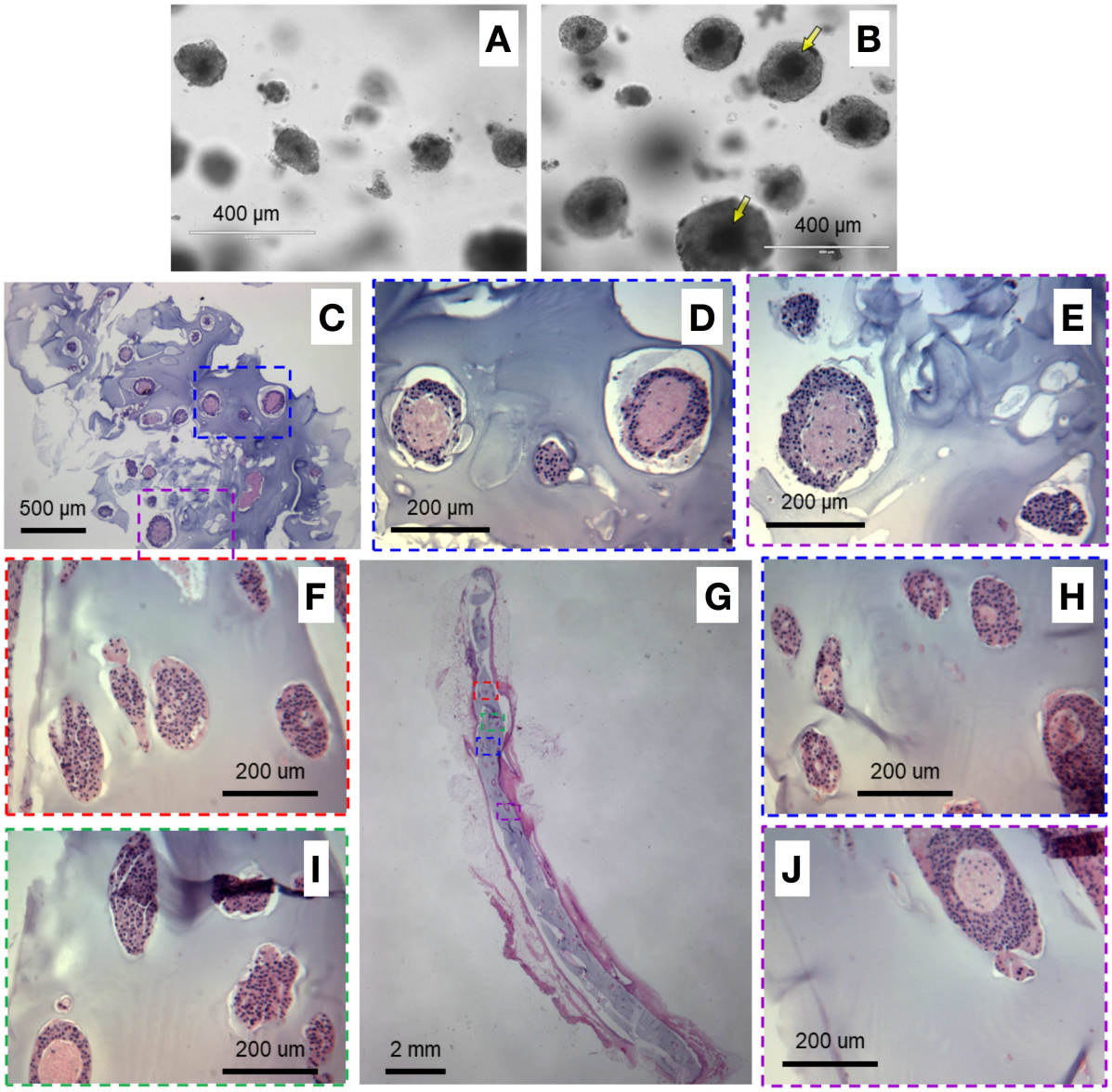


With these considerations in mind, we proceeded with *in vivo* testing of the SHEATH system in this discordant xenogeneic model. The implantation process was similar to that previously described in the rat-to-mouse model. A marked decrease in BG levels was observed shortly after device implantation and maintained over the following 2 weeks, indicating the transplanted human islets were able to correct hyperglycemia. Unfortunately, BG levels in all recipients gradually increased to approximately 300 mg/dL at about 20 days post-transplant (**Figure 5.2.14F)**. While long-term normoglycemia was not completely supported in this challenging transplant model, partial correction of hyperglycemia was evidenced by an increase in BG levels to over 500 mg/dL upon device retrieval, which suggests ongoing graft function.

Live/dead staining of retrieved devices confirmed cell viability in many islets, although necrotic core formation was also evident in the larger islets (**Figure 5.2.14G and H, and Figure 5.2.16**). Importantly, devices could be smoothly retrieved from the modified subcutaneous pocket and cell viability of human islets was preserved even in devices retrieved at day 120, though necrotic cores were observed in large islets (**Figure 5.2.14I and Figure 5.2.16**).

Overall, these results suggest that the SHEATH system can support cell survival and partial correction of hyperglycemia in a human-to-mouse transplant model. It should be emphasized that human islets, particularly large islets, are more susceptible to hypoxia-related cell death.[47, 48] Indeed, as predicted by computational models, necrotic core formation seemed to be directly related to islet size (**Figure 5.2.14I and Figure 5.2.16**). Incorporation of human islets with smaller size could significantly favor device function in the SHEATH system. Thus, further optimization of the SHEATH system might be necessary to improve engraftment, specifically, when considering the large variations in size distribution, functional heterogeneity, and purity of research-use human islets.

Figure 5.2.16. Imaging of human islets within retrieved encapsulation devices



Note: (**A** and **B**) Stereoscope image showing dark cores (yellow arrows) in large islets. (**C to J**) H&E staining showing islets within encapsulation devices. Some of these islets show preserved architectures (F), while necrotic core formation was observed predominantly in the larger islets (**I and J**).

5.2.4.7 - Surgical procedures to scale-up the SHEATH system in a minipig model

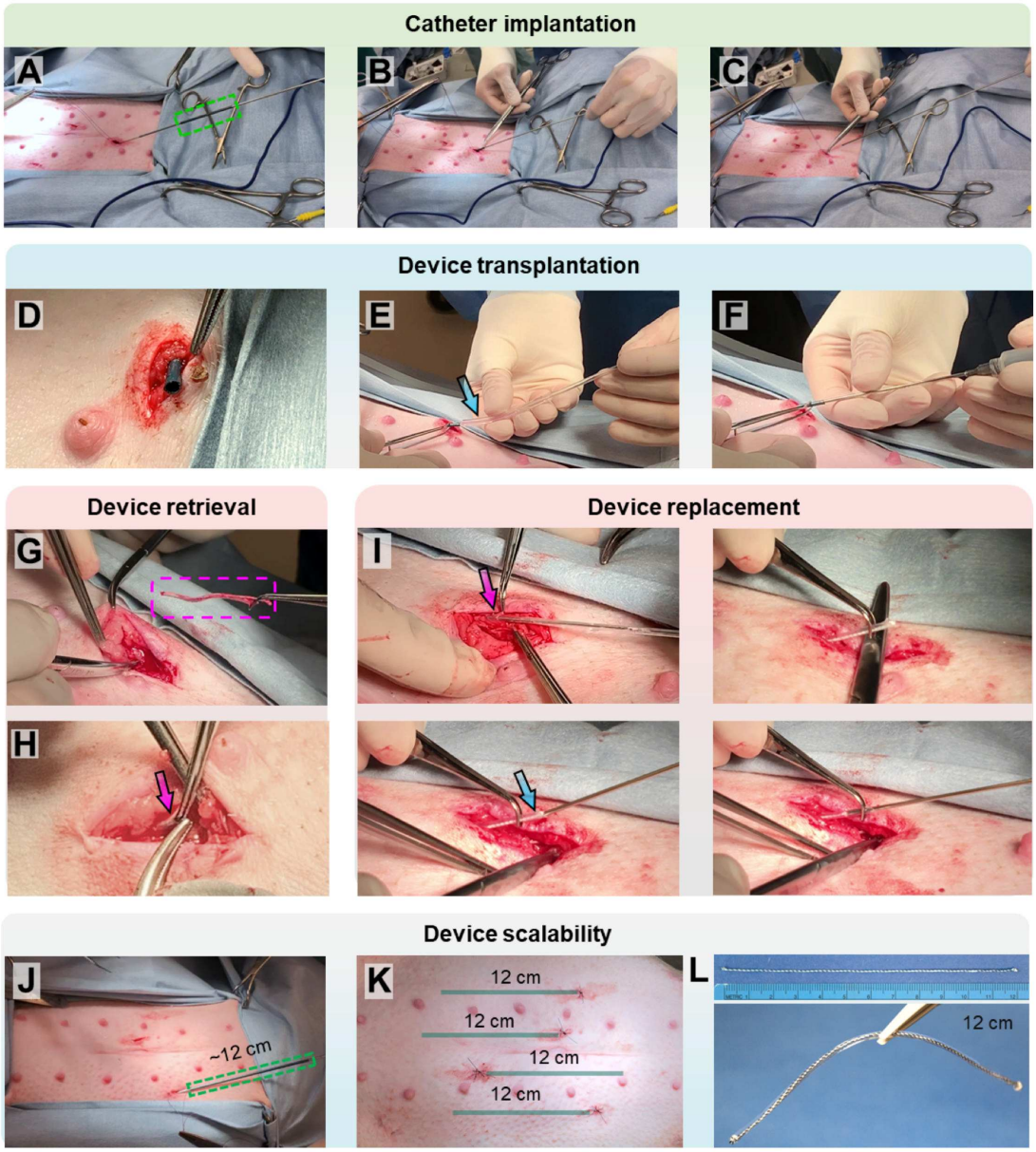
To explore the potential for scalability and clinical translation, we developed procedures to implement the SHEATH approach in Göttingen minipigs **(Figure 5.2.17)**. Briefly, a small incision was made on the ventral abdominal skin, followed by blunt dissection to create a deep subcutaneous pocket. A metal rod was inserted within this subcutaneous pocket, followed by introduction of a nylon catheter using the metal rod as a guidewire (analogous to the clinical Seldinger technique). Once the catheter was positioned inside the subcutaneous pocket, the metal rod was withdrawn (**Figure 5.2.17A-C).** Afterwards, an encapsulation device was implanted into the same site by first making a small incision close to one end of the catheter to expose the lumen, followed by loading of the device into the catheter lumen through a silicone tube (**Figure 5.2.17D-F**). The catheter was then slid out of the vascularized tunnel while the device was kept in place.

We also investigated the feasibility for *in situ* device retrieval and replacement in this large animal model. Devices were removed 4-6 weeks after implantation and replaced using a custom-made polyethylene loading tube (**Figure 5.2.17G-I**). Several iterations of these procedures using multiple 12-cm long catheters were done to evaluate the scalability of the SHEATH system (**Figure 5.2.17J-L**).

For clinical applications, we anticipate that subcutaneous implantation in the lower anterior abdominal wall, volar side of the forearm, lateral side of the thigh, medial aspect of the leg or indeed the entire length of the upper and lower limbs may be potential anatomical locations to explore the scalability of the SHEATH system (**Figure 5.2.18**). A recently initiated clinical trial (NCT05073302) of the prevascularization technique will prove extremely informative in guiding the further optimization and clinical translation of the current SHEATH

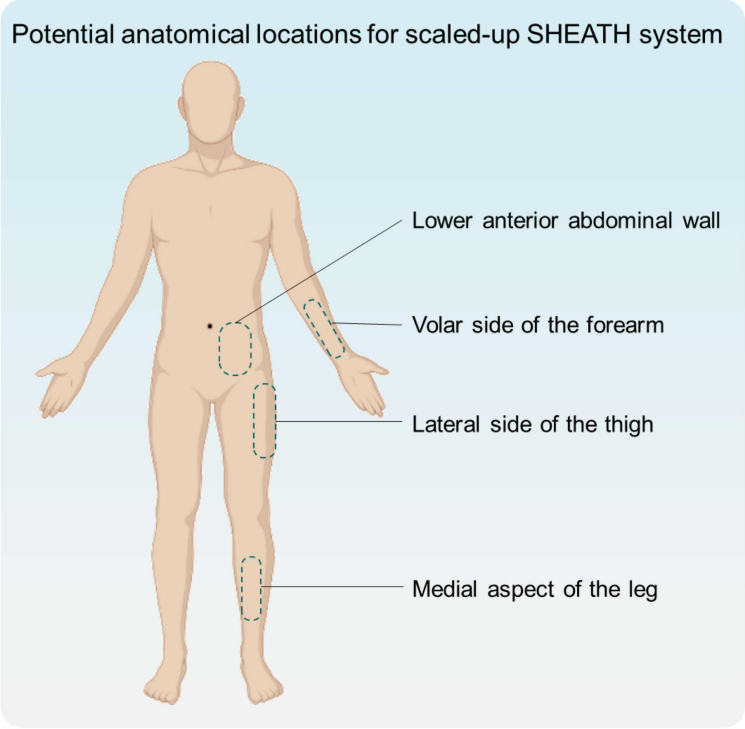
system. Overall, the procedures for device implantation, removal and replacement described herein are minimally invasive, technically feasible, and scalable, which together could facilitate successful clinical translation.

Figure 5.2.17. Demonstration of scalability potential of the SHEATH system in a minipig model



Note: (A to **C)** Digital images of the surgical procedure for catheter implantation in the ventral subcutaneous space in a Göttingen minipig: (**A**) a metal rod acting as a "guidewire" was placed through a catheter (5 cm in length); (**B)** the catheter and rod were inserted into a deep subcutaneous pocket created by a blunt surgical tool; (**C**) the rod was removed, and the catheter was left in the subcutaneous space. (**D** to **F**) Digital images (captured from Movie S5) of the surgical procedure for device implantation into the vascularized space 4-6 weeks after catheter implantation: (**D)** a small incision was made at one end of the implanted catheter to expose the catheter's end; (**E**) a cell-free device (indicated by a blue arrow) was placed into a silicone tube and loaded through the lumen of the implanted catheter; (**F**) the catheter was withdrawn, aided by a guide rod, leaving the device in the vascularized space created by the implanted catheter. (**G** to **I**) Device retrieval and replacement at the vascularized site. (**G)** Digital image of a 5-cm long device (outlined with pink dash line) removed from the vascularized site after 4-6 weeks. (**H**) Image of one vascularized pocket (pink arrow) after device retrieval. (**I**) Digital images showing the device (blue arrow) replacement into the vascularized pocket (pink arrow) through a customized PE tube. (**J**) Image captured from Movie 5 showing the implantation of a 12-cm long catheter. (**K**) Image of the ventral side of a Göttingen minipig after implantation of multiple long catheters. (**L**) Images of a 12-cm uncoated thread (top) and alginate coated (bottom) device.

Figure 5.2.18. Potential anatomical locations for clinical translation of the SHEATH system



5.2.5 - Discussion

In clinical trials, several vascularization strategies have been implemented to improve cell survival and device function of subcutaneously implanted insulin-producing cell encapsulation systems. ViaCyte Inc., one of the leading companies in the field, has developed multiple cell macroencapsulation devices in clinical trials (PEC-Encap, NCT02239354; PEC-Direct, NCT03162926) using pro-vascularization polytetrafluoroethylene/polyester membranes. Ongoing basic and clinical research efforts from this company are currently directed towards incorporating innovative materials to reduce local fibrotic deposition and maintain good vascularization surrounding the device.[49] However, even if these endeavors succeed, hypoxia-mediated necrosis of transplanted cells at early stages post-transplant would remain a pressing issue, since complete material-induced vascularization usually occurs weeks after device implantation. Sernova Corp., another device company, has proposed a prevascularization strategy to address this limitation. Early clinical trials evaluating a polymer-based Cell Pouch^{TM} implanted subcutaneously weeks prior to islet transplantation to provide a prevascularized device to host islets, however, demonstrated marginal engraftment and a limited impact in glycemic control in three patients.[50] A more recent clinical report evaluating an iteration of this Cell Pouch^{TM} device showed some clinical impact in terms of glycemic control[51], although this was observed only when supplementary intraportal islet infusions were administered. While potentially promising, a major limitation of the PEC-Direct and the Cell Pouch^{TM} systems is that both still require immunosuppression and result in substantial cell loss.

Herein, the sequential combinatorial SHEATH system overcomes these challenges by creating a vascularized subcutaneous site for a hydrogel-based cell encapsulation device which together enable immunosuppression-free islet transplantation and long-term diabetes reversal

in mice. The prevascularized subcutaneous site provides a well-oxygenated environment immediately after islet transplantation to prevent early hypoxia-induced cell death. This approach differs from other indefinite-dwelling device/material-based vascularization methods in several key aspects. SHEATH uses a medical-grade catheter with a smooth surface to induce neovascularization prior to islet transplantation by harnessing a temporary inflammatory response, which is abrogated upon catheter withdrawal. This process ultimately creates a well-oxygenated subcutaneous "device-less" cavity that supports implantation of a customized cell encapsulation device. Additionally, removal of the catheter prevents continued collagen deposition that would lead to degeneration of the neovascular network,[52] which usually occurs with material-induced inflammatory response-mediated vascularization systems. Furthermore, in addition to supporting effective immune exclusion, the use of a thread-reinforced alginate-based encapsulation device provided mechanical robustness, high biocompatibility with minimal peri-device cell overgrowth or fibrosis, and synergized with the perfectly-fitted vascularized subcutaneous pocket to enable smooth retrieval and even *in situ* replacement. While highly effective in these models, it remains to be further validated in first-in-human studies, especially in the setting of autoimmune T1D.

Several considerations were made in SHEATH's design to facilitate clinical translation. For example, the use of Food and Drug Administration (FDA)-approved biomaterials, such as alginate, was essential. To date, sterile ultrapure alginate has been commercially produced and widely applied in cell and other cargo delivery systems in preclinical and clinical studies because of its biocompatibility, non-immunogenicity, facile mass transfer properties, convenient gelation process, good mechanical stability, and favorable immunoisolating capacities. Furthermore, as a natural polymer, alginate provides versatility to introduce chemical

modifications onto its backbone, which facilitates the development of alginate derivatives highly tailored for specific biomedical applications. For example, Vegas et al. created a library of triazole group-modified alginates that substantially reduced foreign body reactions.[53] Similarly, our previous work reported that zwitterion-modified alginates reproducibly mitigated cellular overgrowth for cell encapsulation systems.[54] These low-fowling modified alginate hydrogels can be easily incorporated into the current SHEATH system without changing any of the device's fabrication protocols.

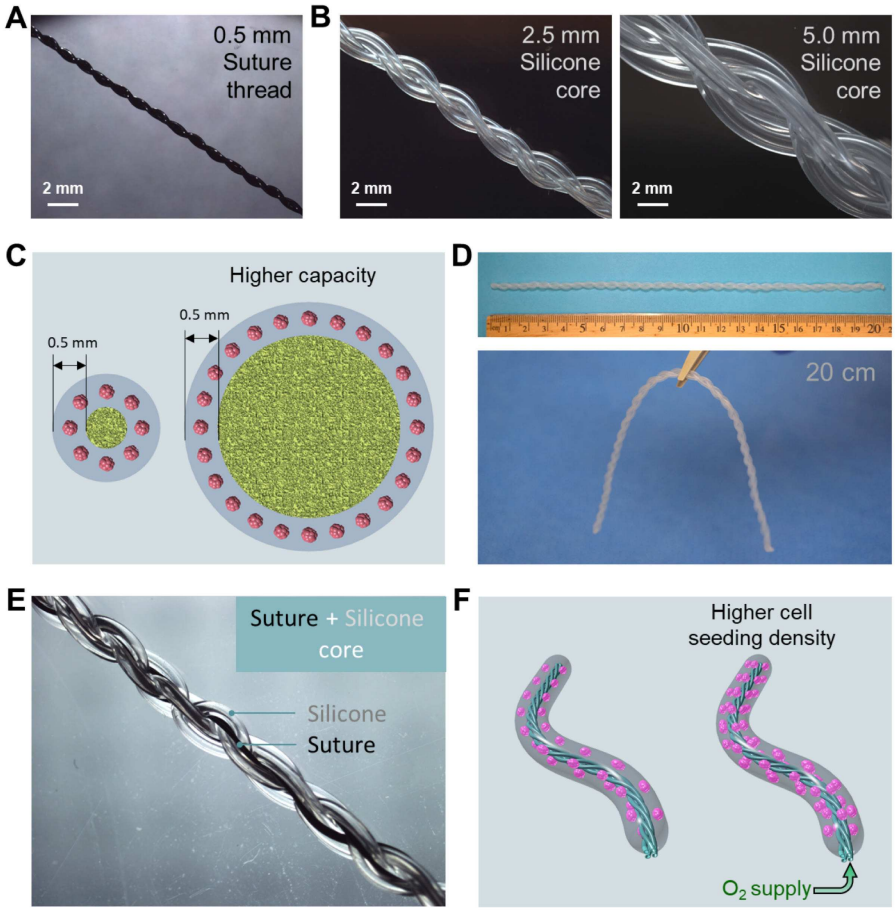
Another key aspect evaluated in this study is the potential for device retrieval and replacement. To our knowledge, this is the first study that has succeeded in achieving a smooth retrieval and *in situ* replacement of a subcutaneous macroencapsulation device for islet transplantation. This has several implications, mainly related to safety concerns and long-term outcomes, particularly when considering the case of stem cell-based β-cell replacement therapies, that are rapidly making their way into the clinic. Recently, Vertex Pharmaceuticals has reported a 91% decrease in insulin requirements with intraportal infusion of fully differentiated stem cell-derived islets in a single patient with T1D (NCT04786262). Despite this promising result, full-dose immunosuppression was still required. Two recent publications have comprehensively described the first-in-human experience with subcutaneous implantation of macroencapsulation devices containing embryonic stem cell-derived pancreatic endocrine progenitors (from ViaCyte, Inc.), together demonstrating that implanted cells are capable of meal-regulated insulin secretion, which correlated with significant (albeit marginal at this point) improvements in glycemic control and hypoglycemia unawareness.[13, 14] These recipients also required immunosuppression, since transplanted devices were perforated and not immunoisolating. Considering the immunoprotection and retrievability and replacement

capacities of the SHEATH system, we believe this approach could also be amenable for stem cell-derived β-cell transplantation into the subcutaneous site.

Despite the SHEATH system's promising features, additional challenges regarding clinical utility of the SHEATH system need to be overcome and should be acknowledged. First, while we performed experiments in large animal models, these exclusively dealt with procedures to guide scalability and identify technical issues related to clinical translation. Specifically, acceptable catheter length, identifying anatomically appropriate sites for catheter and device placement, and the need for precise localization methods for device retrieval were identified as potential roadblocks. Most notably, difficulty locating the deeply implanted device complicated its retrieval from the subcutaneous space. Ultrasound imaging (taking advantage of the echogenicity of the suture-thread[55]) could solve this problem by aiding in precise device localization, allowing for more accurate incisions and consequently smoother retrieval. Second, the current thread-like encapsulation device design should be modified to accommodate a clinically curative cell load (~500,000 IEQ human islets[56]). Specifically, replacing the current twisted nylon sutures with twisted silicone tubing produces a soft and flexible core with larger diameter that significantly increases the central core size from 0.5 mm to 5 mm (**Figure 5.2.19A and B**), while maintaining a thin islet encapsulation outer layer (500 μm, **Figure 5.2.19C**) to preserve the fast glucose-stimulated insulin secretion response. Ten of these 20 cm long devices (**Figure 5.2.19D**, at a total length of 2 meter) could support the necessary clinically relevant cell dose at current seeding density (5.6%, v/v). Furthermore, the inclusion of an O_2 supply system (**Figure 5.2.19E and F**) throughout the twisted silicone tubing would facilitate implementation by allowing for higher cell seeding densities[32], leading to even shorter devices that are more appealing for clinical translation. We anticipate moving forward initially with 'sentinel'

SHEATH-combination devices placed subcutaneously, with the major islet mass deployed intraportally, which would allow us to accrue rapid safety and preliminary efficacy data from a histologic perspective without compromising therapeutic benefit.

Figure 5.2.19. Prospective designs to increase capacity of the SHEATH core encapsulation device



Note: (A) Stereoscope image of a thin nylon suture thread (current design). (B) Stereoscope images of large-size silicone cores formed from twisted silicone tubing. (C) Schematic showing increased capacity of the device with a larger core size but with the same thin islet encapsulation outer layer thickness. (D) Digital images of a 20-cm long flexible SHEATH core made of twisted silicone tubing. (E) Stereoscope image of a combination design with nylon suture and silicone tubing, producing a flexible but non-stretchable SHEATH core. (F) Schematic showing SHEATH core designs with oxygen supply allowing higher cell seeding density.

In summary, we thoroughly characterize and test the SHEATH system to enable immunosuppression-free islet transplantation into the challenging subcutaneous space. Robust diabetes correction potential of this strategy has been demonstrated in syngeneic, allogeneic, and xenogeneic models. Additionally, we have shown that the SHEATH approach is amenable to *in situ* retrieval and replacement in small and large animal models. SHEATH introduces an efficient and reproducible platform that can accommodate future bioengineering and pharmacological interventions to realize clinically successful subcutaneous islet transplantation. Indeed, a clinical trial to evaluate the "Device-Less" prevascularization technique applied in this SHEATH system is underway at the University of Alberta (NCT05073302). Outcomes from this clinical endeavor will prove extremely informative in guiding the optimization and clinical translation of the SHEATH system.

5.2.6 – Acknowledgements

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CHAPTER 6

CHAPTER 6 - CONCLUSIONS AND INSIGHTS ON FUTURE RESEARCH PRIORITES

6.1 – Introduction

Scientific research in type 1 diabetes (T1D) has advanced greatly during the last two decades. Unfortunately, as we commemorate the 100th anniversary of the discovery of insulin, T1D remains an underdiagnosed and suboptimally treated disease. While T1D represents only 5-10% of the total population living with diabetes, however, research endeavors aimed at understanding its specific pathophysiological mechanisms could unlock the intricate phenomena occurring in other types of diabetes. Similarly, succeeding in preventing or even reverting T1D may hold promise for interventions that could benefit other patient populations, including those with other autoimmune diseases.

This thesis includes a comprehensive body of work that was planned and executed with a translational research approach in mind. Building on my background as a clinician, I conducted clinical research that could provide value to physicians and educators caring for people with T1D that have undergone either an islet (ITx) or a whole pancreas transplant (PTx). These endeavors highlight the outstanding long-term clinical outcomes that can be achieved with both therapies (**Chapter 1, Part 2 and Chapter 2, Part 2)**, but also provide data to calibrate ongoing clinical strategies to optimize islet transplantation, such as extrahepatic islet implantation, and highlight ongoing areas of opportunity that need further optimization (**Chapter 3)**. Altogether, it is clear that current β-cell replacement therapies do not represent a cure, since patients require lifelong immunosuppression to prevent immune rejection and sustain graft function. To address this major limitation, I include two novel approaches exploring immunomodulatory regulatory T cell therapies (**Chapter 4, Part 2**) and islet encapsulation (**Chapter 5, Part 2)**. These investigations showed promising results in preclinical models, however, their clinical translation remains to be evaluated.

I am confident that the work contained in this thesis contributes significantly to the field. Nevertheless, key questions are still unanswered and demand further exploration. In this section, I provide insights into research priorities and knowledge gaps that should be explored to further optimize clinical care of people undergoing ITx, but also towards improving knowledge translation from the bench to the bedside, thus paving the way to achieve a true cure for T1D.

6.2 – Priorities in Clinical Research

6.2.1 – Defining outcomes and measures of clinical success following pancreatic islet transplantation

Plasma C-peptide is the most widely used parameter to evaluate graft function following ITx. Both fasting[1, 2] and stimulated[3] C-peptide levels have been reported clinically. A typical cut-off of 0.1 nmol/L or 0.3 ng/mL has been included in most reports to define graft survival/failure, including the most recent Collaborative Islet Transplant Registry (CITR).[1] In 2018, leaders from both the International Pancreas and Islet Transplant Association (IPITA) and its European counterpart (EPITA) introduced a multi-tiered classification to define outcomes for β-cell replacement therapies in the treatment of diabetes, the Igls criteria.[4] Among other parameters, the Igls criteria recommended a C-peptide (fasting or stimulated) cut-off of 0.17 nmol/L or 0.5 ng/mL to define islet graft survival/failure. This measure was supported by approximate extrapolation from data obtained from the Diabetes Control and Complications Trial (DCCT) and small studies in people undergoing ITx showing that people with T1D having sustained stimulated C-peptide levels >0.20 nmol/L had substantially lower rates of severe hypoglycemia.[5, 6] Notably, benefits in the DCCT cohort were only observed in people treated with intensive insulin regimens, thus suggesting that remaining counter regulatory responses to

hypoglycemia (i.e., glucagon secretion) driven by preserved intra-islet paracrine signaling, more than residual insulin secretion itself, could be the leading mechanism explaining protection from severe hypoglycemia.[5] A more recent study from the Scottish Diabetes Research Network Type 1 Bioresource cohort corroborates findings from the DCCT cohort,[7] however, it is unclear whether patients included in these reports resemble those undergoing ITx. Overall, it remains unknown whether this new C-peptide cut-off level provides any clinical value compared to the previous 0.1 nmol/L, especially when considered as an isolated measure of graft function. Answering this question is a priority. Equally important is to delineate C-peptide values (fasting or stimulated) that are associated with relevant patient-centered outcomes, such as insulin independence, abrogation of hypoglycemia, or prevention of secondary diabetes-related complications, among others. This information would be extremely useful to guide public health policies, but also subsequent clinical trials, particularly those evaluating novel stem cell-derived β-cell replacement therapies.

To address limitations with the use of isolated C-peptide levels as a measure of graft function, the Igls criteria introduced a four "level" system to classify β-cell graft functional status and clinical outcomes. These include optimal, good, marginal and failure (**Table 6.1**).

Table 6.1. Igls definition of functional and clinical outcomes for β-cell replacement therapies

β-cell graft functional status	HbA_{1c} % (mmol/mol)*	Severe hypoglycemia, events per year	Insulin requirements, U/kg/day	C-peptide	Treatment Success
Optimal	≤6.5 (48)	None	None	>Baseline†	Yes
Good	≤7 (53)	None	<50% baseline ‡	>Baseline†	Yes
Marginal	Baseline	<Baseline §	≥50% baseline	>Baseline†	No¶
Failure	Baseline	Baseline**	Baseline	Baseline††	No

HbA1c, glycated hemoglobin. Baseline, pretransplant assessment.
*Mean glucose should be used to provide an estimate of the HbA_{1c} in the setting of marked anemia or administration of dapsone. †Should also be >0.5 ng/ml (>0.17 nmol/l) fasting or stimulated. ‡Should also be <0.5 U/kg/day; might include the use of noninsulin antihyperglycemic agents. §Should severe hypoglycemia occur following treatment, then continued benefit may require assessment of hypoglycemia awareness, exposure to serious hypoglycemia [<54 mg/dl (3.0 mmol/l)], and/or glycemic variability/lability with demonstration of improvement from baseline. ¶Clinically, benefits of maintaining and monitoring b-cell graft function may outweigh risks of maintaining immunosuppression. **If severe hypoglycemia was not present before b-cell replacement therapy, then a return to baseline measures of glycemic control used as the indication for treatment may be consistent with b-cell graft failure. ††May not be reliable in uremic patients and/or in those patients with evidence of C-peptide production prior to b-cell replacement therapy. *Adapted from Rickels et al. (2018).*

Beyond C-peptide levels, the authors sensibly incorporate HbA1c levels, insulin requirements, but more importantly, the incidence of severe hypoglycemia (events per year) and measures of glycemic lability and hypoglycemia awareness.[4] The Igls criteria suggest that a risk:benefit assessment should go beyond these parameters and also consider patients' preferences.[4] These recommendations align with the principles of patient-centered care, however, they introduce a more extensive spectrum of clinical outcomes that remain to be adequately characterized. A recent study by Landstra et al., evaluating outcomes with the Igls criteria found that, over 4 years of follow-up post-ITx, most recipients show good or marginal graft functional status.[8] Importantly, these authors evaluated the use of continuous glucose monitoring (CGM) and report that even patients with marginal function showed significant improvements in time in and below range compared to pre-transplant values. CGM data is currently not included in the Igls criteria. Additionally, under the Igls criteria, marginal graft function would be considered a treatment failure. Thus, the clinical benefits obtained from improving time in/below range are currently not accounted for in this classification. CGM provides valuable information on graft function post-ITx,[9, 10] thus, excluding CGM data from the Igls criteria precludes a more comprehensive evaluation of patient-centered outcomes, and introduces the possibility of unrecognized clinically meaningful effects of ITx that may justify

maintaining immunosuppression. Additionally, it limits any comparisons with automated insulin delivery systems. Identification of these areas of opportunity has prompted the Igls 2.0 criteria[8], which now incorporate time in range and below range as complementary measures of glycemic control and measures of hypoglycemia. The Igls 2.0 criteria also introduce specific cut-offs for stimulated and fasting C-peptide levels, which helps contextualizing graft function within specific clinical contexts. It should be emphasized that C-peptide levels, but also insulin requirements, now occupy a secondary level of importance, and it is now recommended they be considered in relation to the impact on measures of glucose regulation (i.e., glycemic control and hypoglycemia).[8] While insightful, the Igls 2.0 criteria requires further validation.

Two final aspects towards improving definitions of clinical success post-ITx pertains to chronic complications and patient-reported outcomes. **Chapter 2, Part 1** provides a comprehensive discussion on the potential benefits achieved with β-cell replacement therapies, in terms of chronic complications such as retinopathy, neuropathy, nephropathy and macrovascular disease. However, most reports are limited by their small sample size and their relatively short-term assessments. Additionally, many chronic complications are evaluated by measuring biomarkers or ancillary diagnostic tests (e.g., creatinine levels, nerve conduction tests) and they do not focus on patient-centered outcomes (e.g. dialysis, kidney transplant, neuropathic pain, gastroparesis). While typical measures to evaluate the incidence and severity of chronic diabetes-related complications are useful and should not be neglected, incorporation of patient-centered outcomes to determine the clinical success of ITx is a priority. In this regard, patient-reported outcomes, such as health-related quality of life, diabetes distress, fear of hypoglycemia, and patient satisfaction should also be included in future studies as measures of clinical success. Patient-reported outcomes in people with T1D have been recognized as a

priority area of research by the American Diabetes Association[11], thus, it is only expected that this permeates to the ITx population. Multiple reports show a positive impact on health-related quality of life following ITx,[12-17] however, small samples sizes, a lack of long-term follow-up, and more detailed analyses of recipients with different levels of graft function preclude more definitive conclusions and implementation into clinical guidelines. These limitations should be addressed in the future. A way forward could be to promote and engage with patient organizations, ideally with a global reach, that could collaborate and accompany clinicians and scientists working in the field of β-cell replacement therapies to help establish both patient care and research priorities.[18] Recent studies exploring preferences for clinical outcomes[19], adjunct-to-insulin therapies[20], and devices for ITx[21] in people with T1D can provide ideas on how to tackle these important endeavors.

6.2.2 – Predicting clinical outcomes following pancreatic islet transplantation

Predicting clinical outcomes post-ITx is extremely difficult. Heterogeneity in islet isolation characteristics, patient demographics, and immune responses complicate any efforts to develop predictive models. The lack of large long-term multicenter studies using standardized islet isolation and patient care protocols introduce an additional layer of complexity. In **Chapter 1, Part 2** we introduce an exploratory analysis to identify factors associated with sustained graft survival following ITx. We identified that the use of anakinra plus etanercept for $\geq$ 1 islet infusion and a BETA-2 score $\geq$ 15 points within 1 year post-first transplant significantly increased the probabilities of achieving sustained graft survival (Page 71). The positive effect of anakinra has been previously documented by our group in a marginal islet mass transplant model using human islets in immunodeficient mice, where the combined use of anakinra and

etanercept led to engraftment in 87.5% of recipients, compared to 53.9%, 45.5% and 36.4% in anakinra alone, etanercept alone and control groups (p<0.005).[22] The use of anakinra plus etanercept was not correlated with longer total duration of insulin independence, however, the BETA-2 score did show a positive correlation with this outcome. While promising, there are a few considerations that should be discussed regarding these findings. First, validation of our findings in larger multicenter cohorts, such as the CITR, should be a priority. Although data from this cohort has been invaluable in informing clinical outcomes in the field, its use is currently centralized and the information only available to selected researchers. I propose the CITR data be open-access for institutions and researchers from all over the world. This would secure the most efficient use of such valuable information. The UK Biobank provides an example of how global access to large-scale biomedical databases can lead to contributions that significantly impact health research.[23] Second, incorporation of islet isolation morphological and functional characteristics, as well as immunological biomarkers into our predictive model (or any other) should also be considered. For instance, taking advantage of the achievements with machine learning (ML) and artificial intelligence (AI) in transplantation,[24] following a "deep phenotyping" strategy for ITx recipients, in which islet isolation characteristics, clinical data, immunological biomarkers (including antibody status and HLA phenotyping), early measures of graft function, and pharmacokinetics of immunosuppression (e.g., trough levels) could prove transformational by identifying specific scenarios in which ITx can achieve its maximum potential with the minimum risk. Such efforts have been reported in recipients of kidney[25] and liver[26] transplants. Naturally, comparison of ML/AI with more traditional statistical methods (i.e., logistic regression) is indispensable to establish their superiority and/or their potential for clinical implementation.[27] Additionally, these models may help identify

patients that would benefit from supplementary islet infusions, which could help optimize resources, but also avoid unnecessary risks.

6.2.3 – Therapies to improve or rescue graft function post-transplant

There is scarce information on pharmacological and non-pharmacological interventions beyond immunosuppression that can improve, sustain or rescue islet graft function. Herein, I focus on strategies that can be implemented after islet infusion. Approaches to enhance islet yield, function or survival pre- and peri-infusion will be revised in sections below.

There are several ways to attempt preservation or restoration of graft function post-transplant. These include β-cell self-proliferation, β-cell protection from non-immune responses, and graft protection from immune responses. While there is little evidence in clinical ITx, there are potential areas of opportunity that merit discussion. β-cell self-proliferation has been proposed as a strategy to restore or expand β-cell mass in T1D. Recent efforts evaluating inhibition of the dual specificity tyrosine-phosphorylation-regulated kinase A1 (DYRK1A) show promising results *in vitro* in terms of β-cell proliferation rates.[28] Combination with glucagon-like peptide 1 (GLP-1) agonists can improve specificity towards β-cells and synergize with DYRK1A inhibition to achieve proliferation rates (labeling indexes) of 2-6% of insulin-positive cells, without showing any functional impairments.[29] However, these strategies may be hampered by the need to halt ongoing autoimmune β-cell destruction, but also by uncertainties in their overall safety and long-term effects[30]. There are notable explorations into transdifferentiation of ductal, acinar, or other endocrine cells to β-cells and dedifferentiation/redifferentiation of β-cells themselves.[31] However, the accrued evidence for *in situ* β-cell self-replication, regeneration or transdifferentiation remains preliminary.

Some interventions have attempted to prevent or reverse graft attrition from non-immune causes. There are several trials evaluating the dipeptidyl-peptidase 4 (DPP-4) inhibitor sitagliptin to improve β-cell function in the context of islet autotransplantation,[32, 33] allo transplantation[34], and recently, in patients with cystic fibrosis.[35] While increases in incretin responses (glucagon-like peptide-1 [GLP-1] and gastric inhibitory polypeptide [GIP]) were consistently detected in these reports, no improvements on glycemic control or graft function have been achieved. Building on evidence suggesting that the use of gastrin can be additive to DPP-4 inhibitors in increasing β-cell mass and thus restoring normoglycemia in the non-obese diabetic (NOD) mouse model,[36] a study evaluating the use of sitagliptin plus pantoprazole was conducted within the Clinical Islet Transplant program at the University of Alberta. This study showed that a 6-month treatment course achieved a significant increase in both gastrin and GLP-1 levels and enabled a transient decrease in insulin requirements, however, these benefits were completely lost upon treatment discontinuation.[34] An ongoing trial (NCT03746769) exploring gastrin injections following a single islet transplant will shed more light into the role of this hormone on islet graft survival. Another related intervention is the use of GLP-1 receptor agonists, such as Exenatide. Two early reports from the Miami group showed that long-term use (up to 4 years) of Exenatide may be a feasible alternative to support graft function and achieve stable glycemic control and lower insulin doses.[37-39] However, these studies have not included proper controls and issues with tolerance (discontinuation rates >60%) substantially limit the use of Exenatide. Novel GLP-1 receptor agonists might enable similar effects with more favorable profiles and should be explored. Overall, the use of non-insulin adjunctive therapies is common following islet transplant, as shown in **Chapter 1, Part 2** (Page 84), however, there is a need for information to guide practice based on their effects on graft function

and recurrence of hypoglycemia, while accounting for potential comorbidities, such as cardiovascular disease, nephropathy or gastroparesis, but also patient preferences.[40] [20, 41]

A final comment concerns dietary interventions such as low-carbohydrate-, fasting-, and ketogenic-based interventions. A recent rapid review by Diabetes Canada suggests benefits with low- and very low-carbohydrate diets, in terms of glycemic control, insulin requirements and overall health and satisfaction with diabetes management in people with T1D.[42] Intermittent fasting is anticipated to be complicated by hypoglycemia, however, experience in people with T1D fasting during Ramadan shows that structured education and advanced glucose monitoring allow successful intermittent fasting with minimal risk of severe hypoglycemia.[43] Alternatively, calorie restriction has shown to reduce inflammation and improve the well-being of patients with multiple sclerosis.[44] Evidence from preclinical models of multiple sclerosis suggest that fasting reduces autoimmunity and multiple sclerosis symptoms by affecting the number of autoreactive dendritic cell and lymphocytes.[45] In a rat model of syngeneic islet transplantation, a post-transplant period of short-term fasting (14 days with total parenteral nutrition support) plus insulin enabled improved glucose responsiveness and supported better islet survival, as measured by total graft insulin content compared to insulin alone-treated controls.[46] Non-fasting glycemia post-transplant were similar between groups which suggests that mechanisms beyond glucose toxicity might have been involved, although this was specifically not assessed. Finally, there is evidence in clinical trials including patients with intractable childhood epilepsy that suggest that ketogenic diets might promote a significant increase in the proportions of regulatory T cells, together with a decrease in mTOR mRNA expression and IL-17 levels.[47] These changes would support a favorable environment in the post-transplant setting.

Overall, while presented from a speculative perspective and fully acknowledging the overall lack of evidence, the proposed approaches delineated herein represent safe, cost effective and clinically relevant areas of opportunity that deserve exploration in the field of ITx.

6.3 – Priorities in Preclinical Research

6.3.1 – Preclinical models

The effectiveness of therapeutic interventions relies on the level of understanding of the pathophysiological processes underlying the disease. Animal models, such as those using genetic loss or gain of function strategies,[48] have helped us dissect many illnesses up to their molecular level. This vast body of knowledge has sprouted multiple effective treatments, and even cures, for many diseases in animal models. Unfortunately, effective approaches in preclinical models are rarely followed by success in the clinic. There are general reasons to explain this dissonance. First, there is a biased perspective driven by publication and funding priorities that tend to reward implausible, low-probability, predominantly "positive" (i.e., statistically significant), and high impact experimental findings that together distort understanding of a disease. Second, most human diseases cannot be recapitulated by single animal models, thus generalization is limited. Third, most human diseases are syndromic and typically classified and stratified using diverse clinical criteria. Again, this complexity can seldom be replicated in a single animal model.[49]

Beyond the general limitations for successful knowledge translation from the bench to the bedside, there are specific caveats in T1D research. One of the most important pertains to the animal models of diabetes used in preclinical ITx research. Close to 90% of studies use chemically-induced diabetes as the background disease state, the other 10% being spontaneous

autoimmune diabetes models (e.g., non-obese diabetic [NOD]) or transgenic/knockout models (e.g., Akita mouse).[50] This partly explains why so many successful interventions in animal studies do not translate to humans. While large animals and non-human primate models are believed to better resemble human physiology, studies with these subjects are costly, unavailable to most researchers, and there are currently no models of autoimmune diabetes in these animals. Considering that ITx is almost exclusively indicated in people with T1D, an autoimmune disorder, it seems that achieving success in chemically-induced diabetes models, where there are no autoimmune responses to transplanted islets, while extremely relevant for allogeneic transplantation, does not provide a complete picture to predict successful clinical translation. Although there is conflicting evidence both against[51] and in favor[52] of autoimmune recurrence (i.e., seroconversion) as a predictor of clinical outcomes after ITx, most reports are limited by small samples sizes and short term follow-up that prevent ascertaining definitive conclusions. Beyond methodological issues, there is an intrinsic and unavoidable biological plausibility for autoimmune recurrence affecting the islet graft. Thus, I propose that preclinical studies address these concerns by evaluating interventions in both allo- and autoimmune animal models, for instance, by conducting allotransplants in NOD mice. At this point, it is worth recognizing that the NOD mouse model is not perfect, and that many non-specific stimulus (e.g., surgery, anesthesia, inflammation) can actually prevent/attenuate autoimmune responses, which potentially introduces confounding,[53] thus, careful planning and caution in interpretation of this research endeavors should be exercised.

Two additional aspects that contribute to incomplete translation of knowledge from preclinical research to clinical care pertain to the differences between mice and human regarding 1) immunological responses, and 2) the procedural parameters of islet transplantation. There is

a vast amount of literature delineating the differences in the immune systems of mice and humans.[54] Specific to transplantation, mice are known to have shorter recovery times and lower levels of inflammation post-surgery. Additionally, cells of the innate immune compartment differ in proportion, size and reactivity. For instance, mice have 25-40% of neutrophils in their blood (compared to 50-70% in humans), macrophages of smaller size (size positively correlating with phagocytic function), and less cytotoxic NK cells. These disparities may explain the differences in early alloimmune responses.[55] The T cell compartment also differs between mice and humans. In mice, memory T cells represent 5-10% of the total peripheral mononuclear cells, compared to 50% in humans; this is believed to be related to the pathogen-free housing of laboratory mice and the lack of exposure to exogenous antigens.[55] Again, these differences would certainly have an impact on acute and chronic rejection. Regarding autoimmune responses, while the NOD mouse shares many similarities with T1D (e.g., genetic predisposition, autoreactive memory T cells, defective regulatory T cells), some relevant aspects should be considered and addressed in future studies. First, the role of autoantibodies is essential for disease onset and progression, in contrast to humans, in which T1D can happen without autoantibody involvement.[55] It also remains unclear whether the islet autoantigens stimulating autoreactive T cells are the same as those observed in humans (which are also largely understudied). Thus, the value of the NOD mouse in studying recurrence of autoimmunity following ITx is debated and should be further evaluated. Beyond these issues, there is considerable uncertainty on whether all islets elicit the same degree of innate and adaptive immunological responses (e.g., mouse vs rat vs pig), which is extremely relevant for adequate interpretation and extrapolation of findings obtained in preclinical research. Considering the vast amount of resources spent in studying ITx and the overall lack of clinical translation

achieved, I strongly believe that refinement of the animal models is a priority. The use of humanized mouse models, although in preliminary stages, represents an attractive avenue.[56]

Finally, in terms of the procedural parameters of islet transplantation, differences compared to human ITx and their impact in selected outcomes and knowledge translation remain relatively understudied. In the clinic, islet mass is reported using the islet equivalent (IEQ), which corresponds to the tissue volume of a perfectly spherical islet with a diameter of 150 μm. While this measure is not without its flaws (e.g., islets are not typically spherical and assessment is quite subjective), the IEQ has allowed standardization of islet mass, which enables comparisons between centers.[57] Normalization of islet mass per kilogram of body weight (IEQ/kg) is also typically used. Both total and normalized islet mass have been positively correlated with better outcomes following ITx.[58] In contrast, total IEQs are reported in <25% of preclinical studies[50], whilst IEQ/kg are seldom reported. This prevents correlation and extrapolation of results, and provides little guidance for clinical translation. Additionally, while islet size in mice may differ slightly from humans, there is substantial heterogeneity among studies.[57] Considering that islet size might be a determinant of transplantation outcomes,[59] delineating the relative contributions of large or smaller islets to the total islet graft might be relevant. Moreover, the proportion of β-cells decreases as islets increase in size.[60] Together, these and other limitations regarding experimental conditions (e.g., culture time, age, viability, purity, etc.) introduce substantial uncertainty on whether the islet and transplant parameters in preclinical studies resemble those used in clinical ITx. In this regard, I would like to echo the proposals made by Poitout et al.[61] and Hart et al.[62] to include detailed reports on the characteristics of human islet preparation used in preclinical and clinical studies, and extend these recommendations to studies using animal islets.

6.3.2 – Islet sources

Clinical ITx is limited to a selected population of people with T1D experiencing from problematic hypoglycemia. Thus, in its current state, the applicability of this procedure is seldom limited by the lack of donors. However, a major caveat is that recipients typically require 2-3 islet infusions (if not more) to achieve and maintain robust graft function. Supplementary infusions increase procedural risks and may lead to alloimmune sensitization, thus restricting the possibilities of future islet (or other organ) transplants, if required. Delivering a sufficient islet mass in one single procedure would thus substantially improve the cost:benefit ratio of clinical ITx. Several potential ways of achieving this are actively being explored. Herein, I will succinctly mention some key areas that could be prioritized. A more comprehensive discussion on these issues is presented in **Chapter 1, Part 1.**

First, optimization of islet isolation protocols is still necessary. Substantial variability between centers exists, however, it is noteworthy that 50-75% of processed pancreata for islet isolation do not meet release criteria for transplantation.[63-65] Considering that islet isolation accounts for 20-30% of the costs associated with this procedure,[64, 66] improving islet isolation outcomes should be one of the top priorities in the field. While composite scores to predict isolation success have been reported,[67-69] their usefulness is questionable, particularly when compared to simpler variables (e.g., perfusion methods, cold-ischemia time).[70] Moreover, their implementation has been marginal. These prognostic scores should of course be refined; however, it is more likely that novel isolation protocols are needed. A notable recent example is the PRISM (PancReatic Islet Separation Method) developed by Doppenberg et al., from The Netherlands, in which a closed system of tissue collection, washing, buffer change and islet

purification achieved similar islet yields compared to traditional protocols. However, this system required fewer interactions and only one operator, which could help decrease costs.[71] In terms of prioritizing efforts to improve islet isolation protocols, a recent analysis has identified digestion and purification stages as the most important areas of opportunity.[72] To this extent, novel enzymes are being developed,[73] although substantial improvements in islet yield or function have not been reported. Concerning purification, a notable effort by the Miami group implementing "rescue" gradient purification (i.e., a second purification step using different gradients) successfully increased the number of islet isolations that culminated in transplantation.[74, 75] This practice, however, has not been widely adopted.

Recent reports of the first genetically-modified pig-to-human heart[76] and kidney transplants[77] are a testament to the monumental achievements in the field of xenotransplantation. With the advent of these genetically-modified pigs, I anticipate that their use for islet xenotransplantation (Xeno-ITx) will occur within the next few years. While promising, major questions on the feasibility and ethical implications of Xeno-ITx remain. The major barrier to successful xenotransplantation is immunological. Innate inflammatory responses are pivotal in xenotransplant rejection; however, they are relatively understudied. There are three major areas of research that should be evaluated in the near future to ameliorate xenograft rejection: 1) inhibition of NK cells (e.g., CD154/CD40 co-stimulation blockade), 2) inhibition of macrophages (e.g., CD47-SIRPα signalling pathways, HLA-E and G_1 expression), and 3) inhibition of neutrophils (e.g., ectopic human CD31 expression).[78] Exploration of these mechanisms could have implications for allogeneic and even stem cell-derived ITx. Adaptive immune responses also represent a major roadblock to long-term clinical success of Xeno-ITx. Cellular encapsulation has been the most recurred strategy to protect xenogeneic islets from

immune rejection, although with overall poor clinical results.[79] Further improvements in biomaterials and composite bioscaffolds with localized release of immunosuppressant or anti-inflammatory agents needs to be promoted.[80] Immunomodulatory cell therapies might represent another avenue to prevent xenotransplant rejection. A recent short report showed that porcine Xeno-ITx coupled with immunosuppression and autologous Treg infusion showed significant metabolic improvement, with a 45% decrease in insulin requirements and a 22.5% decrease in HbA1c levels at a 1-year follow-up.[81] Importantly, these efforts should be accompanied by safety tests, particularly to eliminate the risk of zoonosis. Indeed, the recently reported success of the first genetically-modified pig-to-human heart transplant might be shadowed by a porcine cytomegalovirus infection that might have contributed to the patient's demise two months after transplantation. A definitive report on this adverse event is pending, however, this issue underscores the need for more rigorous procedures to screen out this and other potential viruses and/or other infectious agents. Finally, patients' perspectives regarding xenotransplantation need further characterization. Current evidence suggests that there is variability in the perceptions that people with T1D have regarding xenotransplantation, with acceptance rates going from 46-100%.[82] Stage of the disease (e.g., chronic kidney failure), safety, and the level of physician support play a central role acceptance to xenotransplantation.[82] As Xeno-ITx becomes closer to a reality in the clinic, these considerations need further exploration.

A final remark on stem cell therapies in T1D, mainly human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), is in order. For a detailed review on the current state and future of these treatments, the reader is referred to Latres et al.[83], Bourgeois et al.[84] and Verhoeff et al.[85] Herein, I would like to elaborate on three research areas that should be prioritized in the coming years: 1) differences and similarities between hESCs

and hiPSCs, 2) optimization of differentiation protocols, and 3) immunological responses to stem cell-derived products. First, the pluripotent state of both hESCs and hiPSCs endows them with similar properties, however, which "starting material" is better to generate functional human islets is unknown. hESCs have accumulated more evidence than hiPSCs in terms of differentiation efficiency and overall safety. In fact, only hESCs-derived β-like cells are currently being tested clinically in patients with T1D (NCT03162926, NCT03163511, NCT02239354 and NCT02939118). Costs also seem to favor hESCs over hiPSCs. Current evidence suggests that hiPSCs have a higher risk of development and accumulation of genomic mutations, partly due to unsilenced reprogramming factors.[86] Additionally, hiPSCs have a variable yield in terms of the mature/terminal cell products following differentiation, which has been attributed to differences in transcriptomes and methylomes possibly originated from the epigenetic memory of hiPSCs, given their somatic origin as compared to the germ-line origin of hESCs.[87] More data to corroborate these notions with contemporary differentiation protocols is required, and the practical implications of these differences need to be established. It should be emphasized that hiPSCs are intrinsically associated with fewer ethical issues, and may be the only alternative for some patients with strong objections to the use of hESCs due to their human embryonic origin. Second, optimization of differentiation protocols is a key area of opportunity in the field. Most stem cell-derived therapies are limited by the lack of safety due to off-target growth, the costs associated with scalability, and the efficiency of differentiation. Beyond potential investigations concerning the chemical composition of differentiation media, such as the addition of components of the extracellular matrix, or the incorporation of suspension-based culture strategies,[88, 89] a major limitation pertains to the identification of cells that would yield the optimal cell product. An efficient way to select cells that are appropriately committed to

differentiation, while simultaneously excluding remaining pluripotent cells that could give rise to off-target tissues, is key. Ideally, this selection/exclusion would occur at early stages of differentiation (e.g., pancreatic endoderm stage) to optimize resources. Since no reliable markers currently exist for this purpose, I believe this should be an area of focus for future research. Alternatively, specific "expression thresholds/cut-offs" of certain early endocrine-lineage markers (e.g., genes, antigens) could be used as "continue/stop" signals for differentiation protocols to avoid unnecessary efforts and the generation of subpar cell products. Another strategy is the generation and selection of pluripotent stem cell lines that specifically respond to a protocol rather than creating protocols to control differentiation of multiple pluripotent cell lines. For instance, Southard et al. have recently reported a study in which up to 24 cell lines from 2 donor pancreata were screened for their potential to express endodermal and pancreatic progenitor markers. A specific cell line, SR1423, which showed the highest proportion of pancreatic progenitor cells, was later used to optimize differentiation protocols and generated a high proportion of insulin-producing cells with a low proportion of off-target cells.[90] This approach merits further exploration, however, while selection of stem cell lines before the differentiation protocol might be cumbersome, it could promote safety while ensuring efficiency and optimal resource utilization. Finally, immunological responses to stem cell-derived products demand more research. Although hESC-derived products are expected to elicit alloimmune responses, it is currently unknown whether hESC- or hiPSC-derived mature cell products would trigger recurrence of autoimmunity. *In vitro* generation of these cells allows introducing genetic modifications to produce immune evasive cell products. The capacity for these modifications to prevent autoimmunity remains largely understudied due to the lack of

appropriate animal models. A way to circumvent this may be the use of humanized models, as recently discussed by Flahou et al.[91]

Overall, the consistent generation of pure and fully functional islet clusters (i.e., β, α, and ϱ cells, etc.) remains elusive. The current costs of cell differentiation pose a major limitation for successful applicability of stem cell-derived β-cell replacement therapies to the general population of people with T1D. I believe that addressing the research priorities that I have exposed in this section would contribute to substantially advancing the field to ensure that safer and more effective therapeutic cell products are reliably produced.

6.3.3 – Implantation sites and transplant strategies

Research into novel implantation sites for ITx has advanced substantially in the last two decades. Unfortunately, results from animal studies have not successfully translated to the clinic. In **Chapter 3**, we have included the largest cohort of patients that have undergone extrahepatic ITx and show that these strategies, in their current state, consistently fail to support islet engraftment. Thus, more research is needed. In this section, I will expose new key research priorities that should be explored, as well as novel implantation sites that could be evaluated in the upcoming years. For a detailed review on the progress of implantation sites for ITx the reader is referred to Cayabiab et al.[92]

Myriad extrahepatic sites have been evaluated in both preclinical and clinical studies. Examples include the anterior chamber of the eye, the subcutaneous space, the spleen, the epididymal fat, the greater omentum, the kidney subcapsular space, the muscle, the bone marrow, among others. There are two extrahepatic sites where I believe deserve special attention for future research endeavors: the subcutaneous space and the kidney subcapsular

space. The subcutaneous space remains attractive as an implantation site due to the possibility of minimally invasive (potentially ambulatory) implantation procedures, its capacity to accommodate large tissue volumes, and its accessibility for graft monitoring and retrieval, if required. However, its relatively avascular nature limits nutrient and oxygen delivery, which severely impacts graft survival and function. In **Chapter 5, Part 2** we explore the potential of a previously developed neovascularization technique that harnesses the foreign body response to create a vascularized subcutaneous cavity that was later used to implant an islet encapsulation device. This sequential approach enabled immunosuppression-free subcutaneous islet transplantation in allo- and xenogeneic models of islet transplantation. While we present promising data, several questions are left unanswered in our body of work. Most are open questions in the field and reflect key areas of opportunity for future research. First, it is unclear whether foreign body responses leading to increased vascularization in mice would lead to the same degree of vascularization in large animal models, which better resemble human physiology. Second, the biocompatibility and immune protective properties of most biomaterials are still largely uncharacterized. Indeed, evidence suggests that modification of commonly available biomaterials is necessary to prevent fibrosis and enhance immunoisolation to enable long-term diabetes reversal in large animal models (e.g., non-human primate models).[80, 93] These models used implantation into the omentum, which is relatively favorable for islet engraftment in animal models, however, a recent report by Yu et al., showed that subcutaneous implantation of non-encapsulated islets admixed with a device-free islet viability matrix composed of human collagen-1, L-glutamine, fetal bovine serum, sodium bicarbonate and medium 199 supported engraftment and diabetes reversal in porcine and non-human primate models.[94] These efforts set up a potential pathway to move forward on how to generate

biomaterials or bioscaffolds with enhanced properties to support optimal islet survival and function. Finally, it should be emphasized that the glucose responsiveness of subcutaneously implanted islets, even if non-encapsulated, would be intrinsically non-physiological, thus, it is expected that delays in both glucose sensing and insulin secretion would occur with these grafts, even if optimal engraftment is achieved. Whether increased vascularization or other strategies would solve this remains unknown in large animal models.

The kidney subcapsular space represents the gold standard implantation site for most preclinical models of islet transplantation, and it is the most effective in reversing diabetes, even when directly compared to the intraportal circulation, the muscle and the omentum.[95, 96] Early clinical reports suggested similar efficacy to intraportal transplantation,[97] however, since protocols have improved the latter site disproportionately, these results cannot be extrapolated to the present. With the relative success of intraportal ITx, the kidney subcapsular approach has been widely neglected. Nevertheless, there are several considerations that merit discussion. First, the human kidney capsule is believed to be more rigid compared to mice, and thus less able to accommodate sufficient packed cell volumes. These notions were put forward when islet isolation protocols resulted in large volume impure preparations,[98] and recent evidence with non-human primate models shows that the kidney subcapsular space can accommodate clinically relevant islet masses.[99] In our center, the median packed cell volume is 3 mL (interquartile range 2.5 - 4 mL), which might be adequately fitted into the human kidney subcapsular space, even if it requires 2 or 3 different locations within the same kidney. Second, implantation into this site would require more invasive surgical procedures. While this is true, surgical innovations might allow safer implantation techniques. Conversely, this site could allow better access to non-invasive and invasive graft monitoring. Third, early inflammatory

responses, such as the instant blood-mediated inflammatory reaction (*see below),* typically cause 50-70% islet loss within 24 hours of intraportal islet infusion. Islet implantation into the kidney subcapsular space could reduce the degree of inflammation as direct exposure to blood would be limited. This could transform ITx by decreasing the number of infusions required to achieve durable clinical success. A concern for hypoxia would not be justified given the wealth of data supporting the kidney subcapsular space as the optimal implantation site in preclinical models, however, more information in humans is certainly required. Fourth, subcapsular islet transplantation may protect the islets from high intraportal concentrations of immunosuppressants (compared to systemic venous levels) that could impair β-cell function.[98] While ITx into the kidney subcapsular space might require refinement of current surgical procedures to justify its implementation as an alternative to intraportal islet infusion, I strongly believe that research into the properties that explain the success of this implantation site in preclinical models of ITx should be a priority.

Innovative implantation strategies have been proposed recently and deserve discussion. Recently, the intrapleural space was tested in a diabetic (chemically-induced) non-human primate.[100] In this report, robust glycemic control and graft survival was attained for >90 days with a background immunosuppression of thymoglobulin, rituximab, and sirolimus and an islet mass of 15,500 IEQ/kg. Importantly, intrapleural islet infusion did not lead to any surgical complications or pneumothorax.[100] Limitations regarding purity of the islet preparations, and the potential effect of pancreatic enzyme release in a closed space could hamper widespread applications and merit further study. An additional strategy for ITx is the generation of pre-vascularized composite kidney-islet grafts, in which islets are implanted into the kidney subcapsular space and transplanted along with the kidney. While promising results have been

consistently reported in non-human primate models[99], this approach is limited by many technical considerations. First, this strategy is inherently reserved to people with diabetes and end-stage renal disease. Second, the composite kidney-islet grafts are generated by implantation of autologous islets (obtained from partial pancreatectomy) into the kidney subcapsular space. In other words, these pre-vascularized composite grafts need to be established *in vivo*, a process that requires 2 months on average. This would require that live donors (non-diabetic) have a partial pancreatectomy, islet isolation and implantation into the kidney subcapsular space, and subsequently undergo nephrectomy of the composite graft, which would then be transplanted in a recipient with diabetes and end-stage renal disease.[99] This strategy is unlikely to translate to the clinic, and it might be only feasible as an approach for xenogeneic islet-kidney transplantation. However, much value can be obtained from studying the process of islet engraftment using this composite islet-kidney graft.

As a final comment, I would like to briefly propose an alternative implantation approach that might merit exploration. Safe intraportal islet infusion requires a balance between introducing low packed cell volumes to avoid portal vein thrombosis,[101] while ensuring that sufficient islets with the highest purity are delivered. Unfortunately, achieving the highest islet purity, compromises islet yield, and many islets are discarded with the less pure fractions. In this regard, rescue gradient purification of low purity preparations (e.g., <30%) has shown to increase islet isolation yield and purity, with no negative impact on viability and/or function.[74, 75] However, this approach consumes additional resources and might not be sufficient to rescue trapped islets. To address this, I propose that the less pure islet fractions be transplanted into extrahepatic sites. This proposal builds on experience with islet autotransplantation after total pancreatectomy, in which any remaining islet product not going into the intraportal circulation

(due to large packed cell volumes or increased portal pressures) are implanted in the peritoneal cavity or omentum. Considering that no adverse effects related to intraabdominal infusion of the islet preparations have been reported,[102] I believe this strategy might be a cost:effective way of increasing islet mass and potentially improving clinical outcomes. However, the degree of success and the efficiency of engraftment of less pure islets in the peritoneal cavity should be thoroughly studied in preclinical models to generate evidence to support clinical implementation of this proposal.

6.3.4 – Immunological responses

In its current form, clinical success following ITx remains challenging due to strong innate and adaptive immune responses, the latter being two-fold: allo- and autoimmune. This section summarizes current knowledge on these phenomena and proposes several avenues for future research.

6.3.4.1 – Anti-inflammatory therapies

A primary factor for islet death during and immediately after an islet infusion is the instant blood-mediated inflammatory response (IBMIR). As previously discussed in **Chapter 1, Part 1**, IBMIR leads to a loss of over 25% of the infused islet mass within 24 hours,[103] some reports suggesting over 50% of loss as early as 1 hour post-infusion.[104] Considering that most ITx recipients require 2-3 islet infusions to achieve optimal outcomes (**Chapter 1, Part 2**), ameliorating or preventing IBMIR should be a priority. IBMIR is a multi-layered innate immune response triggered by direct exposure of islets (and tissue factor) to the recipients' blood. IBMIR is not limited to allo- or xenotransplantation, as evidence in autologous human ITx exists.[105]

Components of this inflammatory response include activation of the coagulation cascade, the complement pathway, cytokine secretion and acute cell-mediate injury (i.e., via neutrophils, monocytes and macrophages).[106] These responses synergize with hypoxia-mediated cell death, activation of inflammatory pathways within islets themselves (e.g., MAP and JNK kinases, IL-1β and NFAT/NF-*k*B- signaling), local innate immune responses exerted by Kupffer cells in the liver, and toxicity from pharmacological immunosuppression.[106] Several strategies targeting components of IBMIR, such as heparin,[107] TNF-α and IL-1 inhibitors[108-110] have shown to improve clinical outcomes in ITx, however, several aspects of IBMIR remain to be characterized. First, the influence of the liver immune microenvironment has not been completely unraveled, which prevents biologically-informed therapies to control or eliminate IBMIR. The use of intraportal islet transplant models in small animal models might preclude any evaluations that can be later extrapolated to humans, given the differences in islet to hepatic sinusoid size ratios compared to humans. Indeed, one reason the murine intraportal islet transplant model cannot achieve the same degree of success as clinical intraportal islet transplantation might be related to the degree and extent of embolization that occurs with intraportal infusion in these small animal models.[111] A pathway towards addressing these unanswered questions might be the use of autologous pig islet transplant models or *ex vivo* liver perfusion models. Second, Xeno-ITx and stem cell-derived islet transplantation (SC-ITx) demands investigation into potential similarities and difference in IBMIR when using primary human vs pig and stem cell-derived islets. The third aspect relates to implantation sites for ITx. It is currently unknown whether extrahepatic ITx is associated with a lower degree of IBMIR. These knowledge gaps should be explored in the future.

In parallel to unraveling the pathophysiological mechanisms of IBMIR, novel therapeutic agents recently proposed in preclinical studies could be considered in clinical trials moving forward. Cibinetide, an agonist of the innate repair receptor (a.k.a. as common β-subunit of the erythropoietin receptor) shows a favorable safety profile in clinical trials[112] and has been recently reported to preserve human islet survival and function in a pro-inflammatory *in vitro* environment.[113] Moreover, using an *in vitro* model of IBMIR, where human islets were exposed to autologous blood for 60 minutes, cibinetide significantly decreased platelet consumption, and showed synergy with heparin treatment. *In vivo* studies transplanting human islets intraportally in immunocompromised mice showed that cibinetide-treated subjects had higher plasma C-peptide levels and graft insulin content. Finally, macrophage infiltration was decreased in cibinetide-treated mice, compared to controls.[113] Another potential approach to tackle IBMIR is to strive for a more complete inhibition of cytokine responses early post-transplant. To this extent, tocilizumab, a competitive inhibitor of IL-6 has shown to preserve function of human islet in a cytokine-based *in vitro* pro-inflammatory assays.[114] Tocilizumab displayed equal potency to anakinra, which is currently used in clinical ITx. Additionally, tocilizumab-treated immunocompromised mice showed better glycemic profiles post-transplant of human islets compare to untreated controls, but also to anakinra-treated subjects.[114] While promising, anti-inflammatory effects of tocilizumab should be weighed against evidence suggesting delayed revascularization of transplanted islets (although with no detrimental effects on islet graft survival)[115], and a potentially increased rate of opportunistic infections in clinical trials.[116] Tofacitinib, a JAK inhibitor that has been reported to inhibit the production of macrophage-mediated inflammatory cytokines has also shown positive results in an allogeneic cynomolgus macaque islet transplant model that used anakinra, adalimumab, thymoglobulin (basiliximab

for 2nd infusion) and sirolimus as immunosuppression regime.[117] Another option is to explore canonical anti-inflammatory agents, such as dexamethasone. A single, low dose (e.g., 4-6 mg) course of dexamethasone, particularly through localized release (e.g., intraportal infusion), might mitigate IBMIR by promoting macrophage polarization towards an anti-inflammatory phenotype (M2).[118-120] Indeed, there is recent preliminary evidence in a Landrace pig model of intraportal transplantation showing that systemic plus intraportal heparin infusion improves IBMIR as compared to systemic alone delivery.[121] These effects may be additive or synergistic with intraportal infusion of anti-inflammatory agents. However, more *in vitro* and *in vivo* preclinical studies using human islets are needed to fully support this intervention. *In vitro* modification of islets to withstand innate immune responses might be another way to tackle IBMIR. A recent study engineering primary islets with CD47, a transmembrane glycoprotein that inhibits myeloid cells, showed protection from IBMIR, mainly through inhibition of macrophage activation and phagocytosis, but also by prevention of adhesion and activation of platelets and neutrophils.[122] These effects translated into improved engraftment and graft function in a syngeneic marginal mass intraportal islet transplant rat model.[122] Other potential interventions to mitigate IBMIR that can be implemented at the pre-transplant/culture stage include mytomicin C[123], inhibitors of the pro-inflammatory c-Jun NH_2-terminal kinase (JNK) pathway[124], co-culture with mesenchymal stromal cells[125] or pancreatic stellate cells[126], among others. Although potentially effective, *in vitro* strategies to ameliorate the impact of IBMIR might be difficult to approve in countries such as the U.S.A, where regulatory agencies have raised concerns (perhaps unjustified)[127] on the biological effect of pre-transplant culture time on islets.

6.3.4.2 – Cellular encapsulation and bioengineering approaches

As delineated in **Chapter 5, Part 1**, the field of cellular encapsulation lies at a complex intersection between chemistry, physics, bioengineering, cell biology and immunology, and clinical medicine. This section does not provide an in-depth review of cellular encapsulation and bioengineering approaches, as this has been included in **Chapter 5, Part 1.** However, few key areas of opportunity for future research will be briefly exposed.

Preclinical studies frequently show efficacy in achieving immunosuppression-free islet transplantation using cellular encapsulation. Unfortunately, clinical translation remains limited to a little over 100 patients, with outcomes being less than encouraging. Echoing with **Chapter 5, Part 1** of this thesis, I propose establishing the following research priorities: 1) Standardizing current reporting practices for novel encapsulation methods and devices, 2) Promoting large-scale efforts analogous to high-throughput drug screening to find optimal biomaterials for cellular encapsulation, 3) exploring novel techniques and strategies to optimize immunoprotection as well as molecule diffusion properties of encapsulation structures, and 4) characterizing the specific conditions for optimal encapsulation and long-term safety of potentially limitless cell sources for β-cell replacement therapies such as xeno- and stem cell-derived islets.

Specific themes that demand immediate exploration to improve the success of cellular encapsulation include characterization of the peri-implant environment post-implantation and the differences between biomaterials but also between animal models, the role of islet size, the importance of purity and cell seeding density in hypoxia-mediated cell death, the efficacy of hypoxia mitigating strategies, and the relevance of biomaterial immunoengineering. First, there is evidence suggesting that the foreign body response differs between small and large animal

models, with fibrosis occurring substantially earlier in the latter.[128] Achieving similar degrees of control on the foreign body response with common pharmacological interventions (e.g., dexamethasone) may also require significant adjustments in posology in small vs large animals.[128] Second, hypoxia remains a major limitation for cell survival within encapsulation devices, particularly those using macroencapsulation. Islets are particularly prone to hypoxia-mediated cell death, compared to other tissues.[129] Following islet isolation, these structures are devoid of any vasculature and rely on passive oxygen diffusion for survival. Cell encapsulation, while providing a physical barrier to protect from cell-mediated immune responses, increases oxygen gradients and limits passive oxygen and nutrient diffusion. Large islets are more vulnerable to these conditions compared to smaller islets. Islet purity and seeding density are additional factors positively correlated with hypoxia-mediated cell death.[129, 130] Hence, islet preparations containing large islets and with a high content of exocrine tissue (i.e., low purity), might not be optimal for cell encapsulation. Hypoxia-mediated cell death may further aggravate local innate immune responses through the release of inflammatory biomolecules (i.e., danger-associated molecular patterns), which may precipitate or reinforce adaptive immune responses.[131-133] Thus, investigations that shed light into optimal conditions for islet survival within encapsulation devices, including the potential for delivering lower doses of pure, smaller islets vs higher doses of impure, larger islets are necessary. In parallel, approaches to mitigate hypoxia-induced cell death including prevascularization, oxygen generating/transporting materials, and external oxygen delivery should be further pursued.[134] Optimizing the physical properties (e.g., size, shape, texture and charge) and introducing chemical modifications (e.g., zwitterionic materials, modified alginates) to biomaterials in order to decrease the foreign body response has shown efficacy in preclinical studies, including those in non-human primates.[135]

Drug-based approaches incorporating pro- and anti-inflammatory molecules into diverse biomaterials also demonstrate utility in mitigating fibrotic deposition.[136] While a vast number of stand-alone immunoengineering strategies show promise, it is more likely that a synergistic multi-layered approach will be required to achieve functional and durable encapsulated islet transplantation.

Overall, clinical translation of cellular encapsulation and bioengineering strategies to treat T1D has been limited and mostly unsuccessful. However, I anticipate that thanks to the tremendous collaborative network of multidisciplinary researchers, encapsulated immunosuppression-free islet transplantation (a potential functional cure for T1D) is within reach.

6.3.4.3 – Immunomodulatory cellular therapies

Co-adjuvant cellular therapies that protect islet grafts from immune rejection represent a very active area of research. **Chapter 4, Part 1** summarized current knowledge and experience with regulatory T cells in T1D and islet transplantation, thus only a brief commentary on future research priorities will be included in this section. For a concise and recent review providing a balanced analysis of other immunomodulatory cell therapies in solid organ transplantation, including mesenchymal stem cells, tolerogenic dendritic cells, regulatory macrophages, myeloid-derived suppressor cells and regulatory B cells, the reader is referred to Bottomley et al.[137]

Clinical translation of Treg-based therapies in transplantation has begun. Recent multicenter studies show promise with autologous Tregs in kidney transplantation in terms of rejection-free and patient survival compared to reference cohorts, with minimal infusion-related

adverse effects.[138, 139] In these trials, some patients were also able to transition to single-agent immunosuppression without experiencing rejection or graft failure.[138, 139] A recent pilot clinical trial including 5 patients undergoing co-transplant of allogeneic islets with autologous Tregs also reported no infusion-related adverse effects, and adequate graft function at day 75 post-transplant in all patients.[140] Unfortunately, no control or reference groups were included in this study, thus conclusions regarding the efficacy of this intervention on improving outcomes post-ITx cannot be made. While this pilot clinical trial (and others in other solid organ transplant setting) have shown safety with Treg-based therapies in transplantation, several questions remain unanswered. First, the optimal dose of Treg doses is still unknown. The highest dose in clinical trials has been 5×10^9 cells, which showed no adverse infusion-related side effects or a higher risk of infection up to two years post-kidney transplant, however, no significant correlations between clinical outcomes and higher Treg doses were observed and 100% patient and graft survival was observed in all groups (0.5, 1.0 and 5.0×10^9 Treg doses).[141] Thus, it may be that dose is not the key parameter determining clinical success with autologous Treg therapies. Second, the impact of Treg specificity has not been adequately characterized. It has been described that donor-antigen specific Tregs can achieve similar results with lower doses. While this suggest a greater potency, there is no evidence on whether antigen-specific or monoclonal Tregs are superior to polyclonal, which can be cheaper and more reliably manufactured.[137] Chimeric antigen receptor (CAR) Tregs might provide an alternative to consistently obtain clinically relevant quantities of antigen-specific Tregs. A clinical trial evaluating this strategy in living donor renal transplantation is ongoing (NCT04817774), and might inform the field on these matters. Third, the optimal timing for Treg infusion, particularly in relation to induction immunosuppression, needs to be further characterized. In animal studies,

it has been suggested that Treg infusion close to T cell depletion (using thymoglobulin) is associated with worse outcomes, however, the evidence is not conclusive and further research with other induction immunosuppression regimes is needed.[142] Treg infusion close to the surgical procedure might also determine their efficiency and phenotype stability, and infusions when the graft is relatively quiescent and inflammation has subsided might be more beneficial.[143] Additionally, the optimal induction and maintenance immunosuppression regimens to be combined with Treg therapies remains unknown. Evidence in the kidney and pancreas transplant setting suggests that alemtuzumab-treated patients show lower ratios of circulating T follicular regulatory to helper cells after induction compared to basiliximab. This could put them at a higher risk for *de novo* donor-specific antibody formation.[144] On the contrary, other studies have shown that alemtuzumab has a more Treg-preserving profile compared to daclizumab and thymoglobulin, at least in terms of the relative frequency among the total population of $CD4^+$ T cells.[145] In terms of maintenance immunosuppression, it has been suggested that both sirolimus and mycophenolate mofetil have a more Treg-sparing profile, however, the use of sirolimus in clinical ITx has been limited by its adverse effect profile.[146, 147] Renovated efforts exploring Treg-based therapies in ITx might renew interest in using these two immunosuppressants to promote a more Treg-friendly environment, but also in improving their pharmacokinetics to ameliorate adverse effects, while still benefiting from their Treg-preserving properties. Alternatively, a novel delivery strategy evaluating subcutaneous rapamycin-loaded polymersomes (SC-rPS) was shown to improve rapamycin's tissue biodistribution and induce a predominant suppressive macrophage phenotype, coupled with a significant increase in Tregs.[148] Additionally, using an allogeneic mouse model of intraportal islet transplantation, SC-rPSC treatment enabled significantly better graft survival compared to oral and subcutaneous

rapamycin-treated controls, and promoted antigen-specific tolerance.[148] Most importantly, RNA-sequencing analysis of splenic T cells demonstrated that SC-rPS administration mitigated expression of genes associated with rapamycin-induced adverse effects (e.g., malignancy, infection, metabolic). Additionally, no local effects of SC-rPS administration (i.e., alopecia) were observed, which were universally prevalent in subcutaneous rapamycin-treated subjects. These preliminary results, together with tremendous advances in nanoparticle-based drug delivery systems,[149, 150] are promising and should be further pursued in large-animal models.

6.4 – Concluding Remarks

Throughout the last five decades, we have witnessed a monumental amount of research being generated in the field of pancreatic islet transplantation. The collaborative efforts of clinicians, scientists, students and technicians in concert with the courage of patients that have decided to participate in innumerable clinical trials cannot be understated. The research environment in the field of islet transplantation is vibrant and never short of innovative ideas to push the boundaries between the possible and the impossible. Collaborative and multidisciplinary efforts have substantially improved clinical outcomes following ITx. It is now extremely common to attain complete elimination of severe hypoglycemia, durable improvements in glycemic control, and in many cases, years of insulin independence. Comparative analyses with other β-cell replacement therapies, such as whole pancreas transplantation (PTx), show that clinical ITx is safer in terms of procedural complications and hospital readmissions, however, there are still areas of opportunity to reach equiparable metabolic benefits. Overall, it is clear that β-cell replacement therapies in their current form are not a true cure for T1D, since patients require lifelong immunosuppression to sustain graft

function. It is striking that, while it is now extremely frequent to prevent or cure T1D in many animal models (e.g., using immunomodulatory cellular therapies or cellular encapsulation), we have yet to achieve this in a single patient. It seems that knowledge translation is not efficient enough. It is our responsibility to keep trying. It is our responsibility to do better. In this thesis, I have explored central themes regarding the current state and potential future for β-cell replacement therapies. As expected, generating new knowledge always begets more questions. In this final chapter, I have included a set of proposals to answer some of these questions and attempted to set up a potential framework to continue moving the field forward. It is my strong conviction that β-cell replacement therapies will provide a true cure for T1D. Achieving this would be a major turning point in medicine, but it could also mark the beginning of a new era for humankind.

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APPENDIX A.

APPENDIX A – SUPPLEMENTARY MATERIAL TO CHAPTER 5, PART 2

Title: Immunosuppression-free islet transplantation into a vascularized subcutaneous site with a replaceable and scalable encapsulation device

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The appendix includes:

Supplementary Methods

Supplementary Tables A.1 and A.2

Supplementary Figures A.1-A.5

Supplementary References (1-14)

A.1 - Supplementary Methods

A.1.1 - Perifusion simulation model

A.1.1.1 - Transport physics. Glucose-stimulated insulin release kinetics in a dynamic perifusion experiment were compared in non-encapsulated ("free") versus device-encapsulated human islets. The physical problem was considered time-dependent and 3-dimensional. The free islet perifusion model considered two domains: the media (subscript m), treated as a fluid, and the human islets (subscript τ) (**Figure A.3**), while the encapsulation device model featured two additional cylindrical domains for the internal scaffold thread (subscript s) and the islet-encapsulating alginate hydrogel layer (subscript h) (**Figure A.3**). Media was treated as flowing within a rectangular channel of dimensions 2.5 × 2.5 × 3 mm, representing just the relevant portion of a hypothetical perifusion tubing. Islet diameters and positions were determined by random selection from the quantified size distribution up to an islet volume of ~100 IEQ, representing a volumetric density of 5.6% in a representative one tenth portion of the alginate layer of the device (1.5 mm outer diameter, 0.5 mm inner diameter, 2 mm length). The internal thread was represented as a cylinder (0.5 mm diameter, 2 mm length). The islet positions in the free and encapsulated models were identical, despite the absence of any material which would fix the islet positions in the free situation. This is obviously nonphysical but allows us to precisely evaluate the effect of device encapsulation on insulin release dynamics.

The concentrations of oxygen (c_{O_2}), glucose (c_G), and insulin (c_I) were studied. All three components are at relatively low concentrations and thus may be modeled as dilute species governed by the diffusion equation in the nonconservative formulation

$$\frac{\partial c_i}{\partial t} = D_{i,j}\nabla^2 c_i + V_{i,j} - \mathbf{u}\nabla c_i \tag{S1}$$

where c_i is the concentration of species i (in mol m^{-3}), t is time (in seconds), $D_{i,j}$ is the diffusion coefficient of species i in subdomain j (in m^2 s^{-1}), $V_{i,j}$ is the production or consumption rate of species i in subdomain j, $\mathbf{u}$ is the velocity field (in m s^{-1}), ∇^2 is the Laplacian operator, and ∇ is the gradient operator. There is no flow in the islets, thus in the cell cluster subdomains the governing equation resolves to

$$\frac{\partial c_i}{\partial t} = D_{i,\tau}\nabla^2 c_i + V_{i,\tau} \tag{S2}$$

Furthermore, there is no production or consumption of oxygen, glucose, or insulin in domains other than the islets, thus in the internal scaffold and alginate hydrogel the governing equation is

$$\frac{\partial c_i}{\partial t} = D_{i,j}\nabla^2 c_i \tag{S3}$$

though we note that the scaffold is only permeable to oxygen and thus glucose and insulin are not defined in this subdomain.

We may relate the concentration of oxygen to the partial pressure, p (in mmHg), via Henry's law, which states that the concentration and partial pressure are proportional

$$c_{O_2} = \alpha_{O_2,j} p \tag{S4}$$

where $\alpha_{O_2,j}$ is the partial pressure-dependent solubility of oxygen in subdomain j (in mol m^{-3} mmHg^{-1}).

The equations relating the consumption of oxygen and glucose to the production of insulin followed the approach developed for a similar problem described elsewhere.[1-3] We summarize them here. Oxygen consumption is governed according to Michaelis-Menten kinetics modulated by an additional term which increases the consumption rate in sigmoidal proportion to the local glucose concentration

$$V_{O_2,\tau} = \begin{cases} 0, & c_{O_2} < \alpha_{O_2,\tau} p_N \\ -\dfrac{c_{O_2} V_{O_2,\max}}{c_{O_2} + \alpha_{O_2,\tau} K_{O_2|O_2}} \times A_G \left(\dfrac{1}{2} + \dfrac{1}{2} \left(\dfrac{c_G^{n_{O_2|G}}}{c_G^{n_{O_2|G}} + K_{O_2|G}^{n_{O_2|G}}} \right) \right), & c_{O_2} \geq \alpha_{O_2,\tau} p_N \end{cases} \quad \text{(S5)}$$

Where the first term of the second case is the Michaelis-Menten oxygen consumption and the second is the glucose modulation term. Above, $V_{O_2,\max}$ is the basal maximum oxygen consumption rate (OCR) of human islets (in mol m^{-3} s^{-1}), $K_{O_2|O_2}$ is the half-maximal coefficient of the oxygen-modulated oxygen consumption term (in mmHg), $K_{O_2|G}$ is the half-maximal coefficient of the glucose-modulation term (in mol m^{-3} glucose), $n_{O_2|G}$ is the Hill coefficient of the glucose-modulation term (unitless), and A_G is an amplitude coefficient (unitless). The conditionality of Equation S5 represents the lack of oxygen consumption for regions of tissue below a threshold level required for cellular survival, p_N (in mmHg).

Glucose consumption in the islet tissue was also modeled according to Michaelis-Menten kinetics:

$$V_{G,\tau} = \begin{cases} 0, & c_{O_2} < \alpha_{O_2,\tau} p_N \\ -\dfrac{c_G V_{G,\max}}{c_G + K_{G|G}}, & c_{O_2} \geq \alpha_{O_2,\tau} p_N \end{cases} \quad \text{(S6)}$$

where $V_{G,\max}$ is the maximum consumption rate of glucose (in mol m^{-3} s^{-1}) and $K_{G|G}$ is the half-maximal coefficient of glucose consumption (in mol m^{-3}).

Insulin production is divided into biosynthesis and then secretion. Insulin biosynthesis itself is modeled as two separate mechanisms, consisting of a glucose-rate-of-change-dependent first phase (V_{I_1}) and a glucose-dependent second phase (V_{I_2}). First-phase insulin biosynthesis is given primarily by a Hill relationship modulated by the rate of glucose change and the glucose concentration but only for when the former is increasing:

$$V_{I_1} = \begin{cases} 0, & \frac{\partial c_G}{\partial t} < 0 \\ \frac{\left(\frac{\partial c_G}{\partial t}\right)^{n_{I_1|G'}} V_{I_1,\max}}{\left(\frac{\partial c_G}{\partial t}\right)^{n_{I_1|G'}} + K_{I_1|G'}^{n_{I_1|G'}}} \times 2.5 \left(\frac{c_G^{2.5} K_{I_1|G}^{2.5}}{c_G^{2.5} + K_{I_1|G}^{2.5}}\right), & \frac{\partial c_G}{\partial t} \geq 0 \end{cases} \quad \text{(S7)}$$

Where the first term of the second case describes the Hill relation to the rate of glucose change and the second term is a modulator which accentuates biosynthesis at certain glucose ranges. In Equation S7, $V_{I_1,\max}$ is the maximum rate of first-phase insulin biosynthesis (in mol m^{-3} s^{-1}), $K_{I_1|G'}$ (in mol m^{-3} s^{-1}) and $n_{I_1|G'}$ are the half-maximal and Hill coefficients, respectively, and $K_{I_1|G}$ is the glucose concentration (in mol m^{-3}) for which first-phase insulin biosynthesis is highest. Second-phase biosynthesis is described by

$$V_{I_2} = \frac{c_G^{n_{I_2|G}} V_{I_2,\max}}{c_G^{n_{I_2|G}} + K_{I_2|G}^{n_{I_2|G}}} \quad \text{(S8)}$$

where $V_{I_2,\max}$ is the maximum second-phase insulin secretion rate (in mol m^{-3} s^{-1}) and $K_{I_2|G}$ (in mol m^{-3}) and $n_{I_2|G}$ (unitless) are the half-maximal and Hill coefficients, respectively. The net biosynthesis rate, $V_{I,b}$ (in mol m^{-3} s^{-1}) is the sum of the first- and second-phase secretion rates modulated by the oxygen availability via a Hill relationship

$$V_{I,b} = \begin{cases} 0, & c_{O_2} < \alpha_{O_2,\tau} p_N \\ \frac{c_{O_2}^{n_{I|O_2}} \left(V_{I_1} + V_{I_2}\right)}{c_{O_2}^{n_{I|O_2}} + \left(\alpha_{O_2,\tau} K_{I|O_2}\right)^{n_{I|O_2}}}, & c_{O_2} \geq \alpha_{O_2,\tau} p_N \end{cases} \quad \text{(S9)}$$

where $K_{I|O_2}$ (in mmHg) and $n_{I|O_2}$ (unitless) are the half-maximal and Hill coefficients, respectively. Finally, the concentration of biosynthesized insulin ($c_{I,b}$) is related to the insulin secretion rate by:

$$V_{I,\tau} = k_{b\tau}\left(c_{I,b} - c_I\right) \tag{S10}$$

where $k_{b\tau}$ is the first-order rate constant (in s^{-1}). The values of all parameters in Equations S5–S10 are provided in **Table A.1**.

The physics describing flow in the media also followed the approach described in references 1-3.[1-3] The media (essentially water) is an incompressible fluid with constant viscosity thus transport governed by the Navier-Stokes equations for Newtonian flow:

$$\rho_m \frac{\partial \mathbf{u}}{\partial t} = -\eta \nabla^2 \mathbf{u} + \rho_m (\mathbf{u} \cdot \nabla)\mathbf{u} + \nabla P = \mathbf{F} \tag{S11}$$

$$\nabla \cdot \mathbf{u} = 0 \tag{S12}$$

Here, Equation S11 is the momentum balance and Equation S12 relates mass continuity. Parameter ρ_m is the density (in kg m^{-3}), η the viscosity (in Pa s^{-1}), P is the absolute pressure (in Pa), and **F** is the volume force (in N $m^{—3}$). The solution to these equations yields the velocity field **u**, which is coupled to the species transport relations via Equation S1.

A.1.1.2 - Model implementation. Boundary conditions (on both external and interior interfaces) are illustrated schematically in **Figure A.3**. At all internal interfaces (*e.g.*, between the alginate hydrogel and the media), concentration fluxes, $J_{i,j}$ (in mol m^{-2} s^{-1}), denoted as

$$J_{i,j} = -D_{i,j}\nabla c_i + c_i \mathbf{u} \tag{S13}$$

were equal. The concentration of glucose and insulin were also equal at all internal interfaces (recalling that insulin and glucose were not defined in the scaffold). For oxygen, concentration discontinuities were imposed, the magnitude of the difference given by the partition coefficients, $\chi_{j_1 j_2}$ (unitless), defined as the solubility ratios in subdomains j_1 and j_2:

$$\chi_{j_1 j_2} = \frac{\alpha_{O_2,j_1}}{\alpha_{O_2,j_2}} \tag{S14}$$

The boundary walls (*i.e.*, all channel surfaces excluding the inlet and outlet) were considered impermeable ($J_{i,m} = 0$). At the outlet, the convective flux is the product of the velocity field and the local concentration. At the inlet, inward fluxes ($J_{i,m} = -D_{i,j}\nabla c_i + c_i \nabla \mathbf{u}$) were implemented. For oxygen, the partial pressure was assumed to be in equilibrium with the atmosphere at 160 mmHg, which can be converted into concentration using Equation S4, and the insulin concentration is 0. The inlet concentration for glucose is alternated in three 60-min phases according to:

$$V_{I,b}(t) = \begin{cases} 2.8 \text{ mM}, 0 \leq \text{t} \leq 60 \text{ min} \\ 16.7 \text{ mM}, 60 \leq \text{t} \leq 120 \text{ min} \\ 2.8 \text{ mM}, 120 \leq \text{t} \leq 180 \text{ min} \end{cases} \tag{S15}$$

Furthermore, no slip ($\mathbf{u} = 0$) conditions were imposed at all islet/media or device/media interfaces for the free islet and encapsulated islet simulations, respectively, and at all walls excluding the inlet and the outlet. A constant inlet velocity in the x direction (*i.e.*, from the inlet towards the outlet) was implemented ($\mathbf{u} = v_{in}\mathbf{i}$) where $\mathbf{i}$ is the unit vector in the x direction and the initial inlet speed v_{in} is 1.0×10^{-3} m s^{-1} according to previously used experimental settings.[4]

In the perifusion simulations, we measured the average glucose concentration within the islets over time, as well as the insulin surface flux at the outlet, normalized to the total IEQ used in the study. The average glucose concentration, $\bar{c}_{G,\tau}$ (in mol m^{-3}) is

$$\bar{c}_{G,\tau} = \sum_{n=1}^{n_c} \frac{1}{V_c(n)} \iiint_{c(n)} c_G \, dV \tag{S16}$$

where n_c is the number of simulated cell clusters, $V_c(n)$ is the volume of the n^{th} cell cluster, and the region of the integral $c(n)$ is simply the n^{th} simulated cell cluster. The normalized insulin mass surface outflux, $N_{I,\text{out}}$ (in g m^{-2} s^{-1} IEQ^{-1}) is

$$N_{I,\text{out}} = \left(\frac{M_{w,I}}{\text{IEQ}_{\text{tot}}}\right) J_{I,m} = \left(\frac{M_{w,I}}{\text{IEQ}_{\text{tot}}}\right) \mathbf{i}\left(-D_{I,m}\nabla c_I + \mathbf{u}\nabla c_I\right) \quad \text{(S17)}$$

where $M_{w,I}$ is the molecular weight of monomeric insulin (5,734 g mol^{-1}) and IEQ_{tot} (~100) is the total islet volume.

Model constants were obtained from the literature and are provided in **Table A.1**. Unlisted therein, the threshold oxygen level required for cell survival was 0.08 mmHg. Furthermore, we assumed that the media was warmed to body temperature during the perifusion. Finally, a dummy variable for the diffusivity of biosynthesized islets (nevertheless, only defined in the islets) of 1.0×10^{-16} m^2 s^{-1} was used. The governing equations (and associated boundary conditions) were solved using COMSOL Multiphysics, wherein a "Transport of Diluted Species" module was coupled to a "Laminar Flow" module. A custom mesh was generated using COMSOL's "Free Tetrahedral" program for each simulation, with a maximum element size of 2.5×10^{-3} m, a minimum element size of 4.0×10^{-7} m, a "resolution of narrow regions" parameter of 2.50 (unitless), and a maximum element growth rate of 1.13 (unitless). It was ensured that the solutions were independent of the mesh. A strict time step of 20 s was used for the transient study.

A.1.2 - Simulation models for oxygen transport in transplanted rat and human islet-containing devices

A.1.2.1 - Transport physics. A model of oxygen transport in rat and human islet-containing devices was developed. Three 3-dimensional subdomains were considered: a cylinder (0.5 mm diameter) representing the internal scaffold with two spherical (1.1 mm diameter) knots positioned 1.775 mm from each end, a concentric outer cylinder (1.5 mm diameter) representing the hydrogel, and finally randomly non-overlapping spheres representing the rat or human islets. In the experiments, rat islet devices were about 1 cm in length and contained 250 IEQ per device, and human islet devices were about 2 cm in length and contained 1,000 IEQ per device (in both cases, two devices were transplanted into one mouse recipient). These dimensions were applied in the computational model (**Figures A.4 and A.5**). The target islet size distributions were obtained by trace data or the tabular method described in.[5]

Our aim was to simulate device oxygenation after transplantation in the unmodified or vascularized sites several weeks or months after transplantation. The time scale for relaxing gradients in diffusion driven systems is approximated by L^2/D, where L is the length scale and D is the diffusion coefficient. Applying a length scale of 500 μm (the hydrogel thickness) and a conservative order-of-magnitude value for the diffusion coefficient of 1.0×10^{-9} m^2 s^{-1} yields a characteristic time of 250 s (about 4 min). As this is significantly lower than our time of interest we may consider the system at steady state, with the oxygen partial pressure described by the conventional mass balance equation (in terms of p):

$$\alpha_{O_2,i} D_{O_2,i} \nabla^2 p = V_{O_2,i} \tag{S18}$$

where $V_{O_2,i}$ is zero in all domains except the cell clusters, wherein it is equal to

$$V_{O_2,i} = \begin{cases} 0, & p < p_N \\ -\dfrac{pV_{O_2,\max}}{p + K_{O_2|O_2}}, & c_{O_2} \geq p_N \end{cases} \quad \text{(S19)}$$

Note, this is analogous to Equation S5 without the inclusion of the glucose modulation term and provided in the partial pressure rather than the concentration.

A.1.2.2 - Model implementation. As previously, model constants were obtained from the literature. Unreported in **Table S2**, the maximum oxygen consumption rate for rat islets ($V_{O_2,\max}$) was retrieved at value of 0.034 mol m^{-3} s^{-1}.[6] At all external boundaries, we assumed that the pO_2 was constant at a value p_{ext} (in mmHg). Monte Carlo simulations were performed where, for each iteration, the selected islet sizes and positions were randomized anew. In most simulations, the average value of the pO_2 (obtained from EPR imaging) was used as the p_{ext} values (*i.e.*, 10 mmHg in the control versus 40 mmHg in the vascularized site). In other simulations, p_{ext} was treated as another random variable along with the islet sizes and positions. The distributions of p_{ext} were assumed to be normal and described by the mean and standard deviation of the EPR imaging measurements. Solutions to the governing equations (and associated boundary conditions) were obtained using COMSOL Multiphysics with MATLAB.

A custom mesh was generated using COMSOL's "Free Tetrahedral" program for each simulation, with a maximum element size of 2.5×10^{-3} m, a minimum element size of $4.0\times10-7$ m, a "resolution of narrow regions" parameter of 2.50 (unitless), and a maximum element growth rate of 1.13 (unitless). It was ensured that the solutions were independent of the mesh.

Appendix table A.1. Parameter values of oxygen, glucose, and insulin consumption or production rate equations

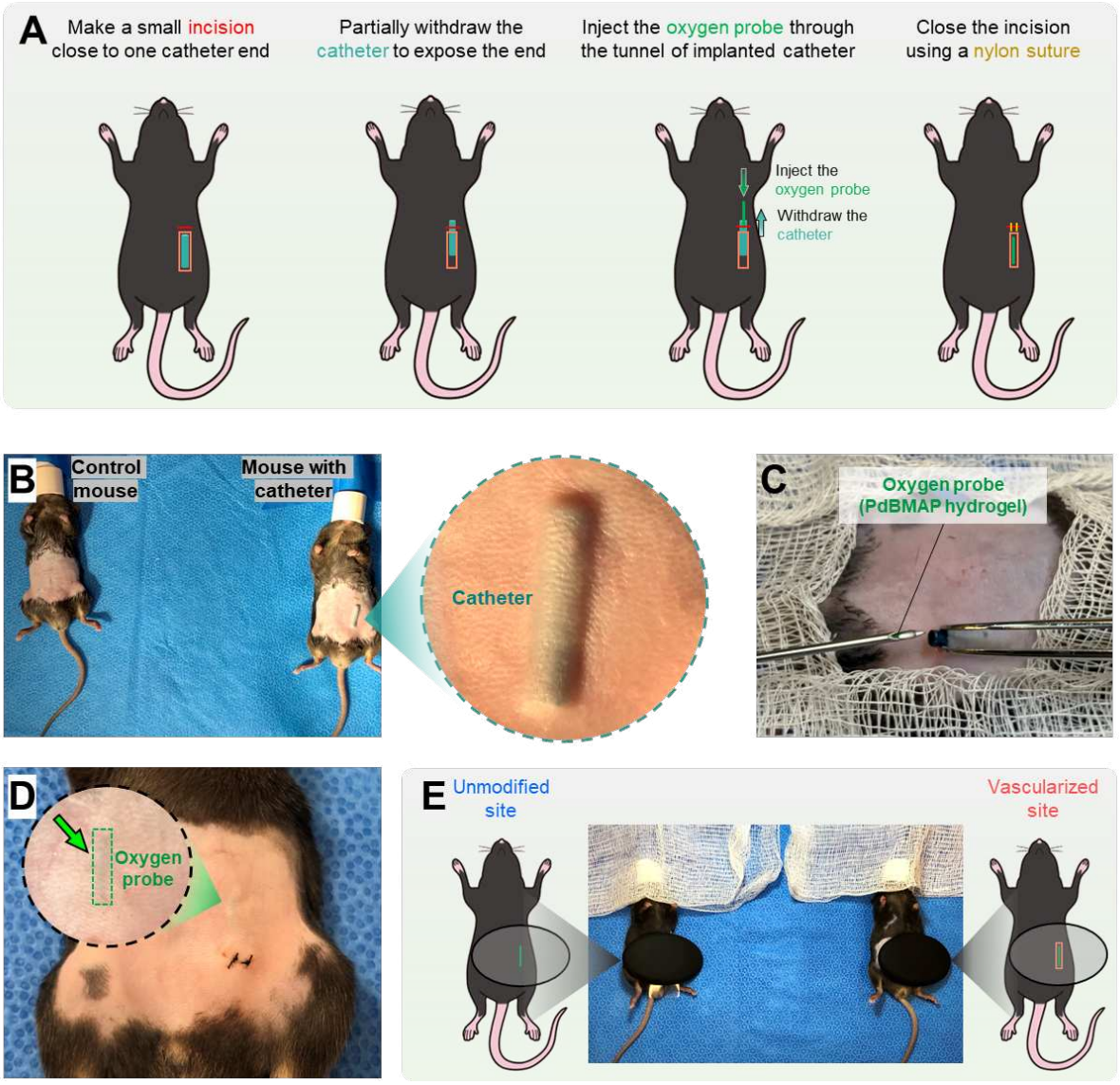
Description	Symbol	Value (units)
Oxygen consumption, $V_{O_2,\tau}$ (Equation S5)		
Maximum consumption rate*	$V_{O_2,\max}$	0.0134 (mol m^{-3} s^{-1})
Half-maximal coefficient	$K_{O_2\|O_2}$	0.80 (mmHg)
Glucose modulation, amplitude	A_G	1.8
Glucose modulation, half-maximal coefficient	$K_{O_2\|G}$	7.0 (mol m^{-3})
Glucose modulation, Hill coefficient	$n_{O_2\|G}$	2.5
Glucose consumption, $V_{G,\tau}$ (Equation S6)		
Maximum consumption rate	$V_{G,\max}$	0.0128 (mol m^{-3} s^{-1})
Half-maximal coefficient	$K_{G\|G}$	1.0×10^{-5} (mol m^{-3})
First-phase insulin biosynthesis, V_{I_1} (Equation S7)		
Maximum production rate	$V_{I_1,\max}$	1.0×10^{-4} (mol m^{-3} s^{-1})
Glucose rate change modulation, half-maximal coefficient	$K_{I_1\|G'}$	0.03 (mol m^{-3} s^{-1})
Glucose rate change modulation, Hill coefficient	$n_{I_1\|G'}$	2.0
Glucose modulation, half-maximal coefficient	$K_{I_1\|G}$	5.0 (mol m^{-3})
Second-phase insulin biosynthesis, V_{I_2} (Equation S8)		
Maximum production rate	$V_{I_2,\max}$	1.8×10^{-5} (mol m^{-3} s^{-1})
Glucose modulation, half-maximal coefficient	$K_{I_2\|G}$	7.0 (mol m^{-3})
Glucose modulation, Hill coefficient	$n_{I_2\|G}$	2.5
Net insulin biosynthesis, $V_{I,b}$ (Equation S9)		
Oxygen modulation, half-maximal coefficient	$K_{I\|O_2}$	2.0 (mmHg)
Oxygen modulation, Hill coefficient	$n_{I\|O_2}$	3.0
Insulin secretion, $V_{I,\tau}$ (Equation S10)		
First-order rate constant	$k_{b\tau}$	0.003 (s^{-1})

*Maximum oxygen consumption rate of human islets is used.[7] All other parameter values were obtained from references 1-3.[1-3]

Appendix table A.2. Perifusion model parameter values

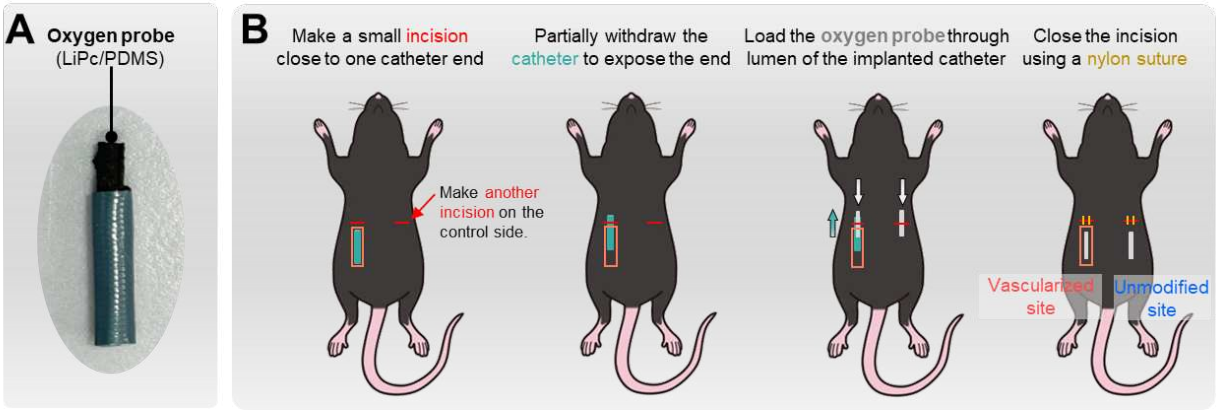
Description	Symbol	Value (units)	Reference/Source
Oxygen solubility coefficients			
Media (m)	$\alpha_{O_2,m}$	1.27×10^{-3} (mol m^{-3} mmHg^{-1})	(*8*)[8]
Islets (τ)	$\alpha_{O_2,\tau}$	1.02×10^{-3} (mol m^{-3} mmHg^{-1})	(*9*)[9]
Hydrogel (h)	$\alpha_{O_2,h}$	1.24×10^{-3} (mol m^{-3} mmHg^{-1})	(*10*)[10]
Scaffold (s)	$\alpha_{O_2,s}$	1.90×10^{-3} (mol m^{-3} mmHg^{-1})	(*11*)[11]
Oxygen partition coefficients			
Media/islets	$\chi_{m\tau}$	1.25	$\alpha_{O_2,m}/\alpha_{O_2,\tau}$
Media/hydrogel	χ_{mh}	1.02	$\alpha_{O_2,m}/\alpha_{O_2,h}$
Hydrogel/islets	$\chi_{h\tau}$	1.22	$\alpha_{O_2,h}/\alpha_{O_2,\tau}$
Hydrogel/scaffold	χ_{hs}	0.65	$\alpha_{O_2,h}/\alpha_{O_2,s}$
Diffusion coefficients			
Oxygen in media	$D_{O_2,m}$	2.78×10^{-9} (m^2 s^{-1})	(*12, 13*)[12, 13]
Oxygen in islets	$D_{O_2,\tau}$	2.00×10^{-9} (m^2 s^{-1})	1-3
Oxygen in hydrogel	$D_{O_2,h}$	2.70×10^{-9} (m^2 s^{-1})	(*8, 13*)[8, 13]
Oxygen in scaffold	$D_{O_2,s}$	5.00×10^{-13} (m^2 s^{-1})	(*11*)[11]
Glucose in media	$D_{G,m}$	9.00×10^{-10} (m^2 s^{-1})	1-3
Glucose in islets	$D_{G,\tau}$	2.60×10^{-10} (m^2 s^{-1})	(*14*)[14]
Glucose in hydrogel	$D_{G,h}$	6.00×10^{-10} (m^2 s^{-1})	1-3
Insulin in media	$D_{I,m}$	1.50×10^{-10} (m^2 s^{-1})	1-3
Insulin in islets	$D_{I,\tau}$	5.00×10^{-11} (m^2 s^{-1})	1-3
Insulin in hydrogel	$D_{I,h}$	1.00×10^{-10} (m^2 s^{-1})	1-3
Fluid (media) properties			
Inlet velocity	v_{in}	1.00×10^{-4} (m s^{-2})	1-3
Initial temperature	T_O	310.15 (K)	1-3
Dynamic viscosity	η	0.70×10^{-3} (Pa s)	1-3
Density	ρ_m	993 (kg m^{-3})	1-3
Heat capacity	c_p	4,200 (J kg^{-1} K^{-1})	1-3
Expansion coefficient	ε	2.10×10^{-4} (K^{-1})	1-3
Thermal conductivity	$k_{c,m}$	0.634 (J m^{-1} s^{-1} K^{-1})	1-3

Figure A.1. Schematic illustrating the placement of the Lumee™ oxygen sensor for obtaining pseudo-quantitative local oxygen measurements using the Lumee™ Oxygen Platform



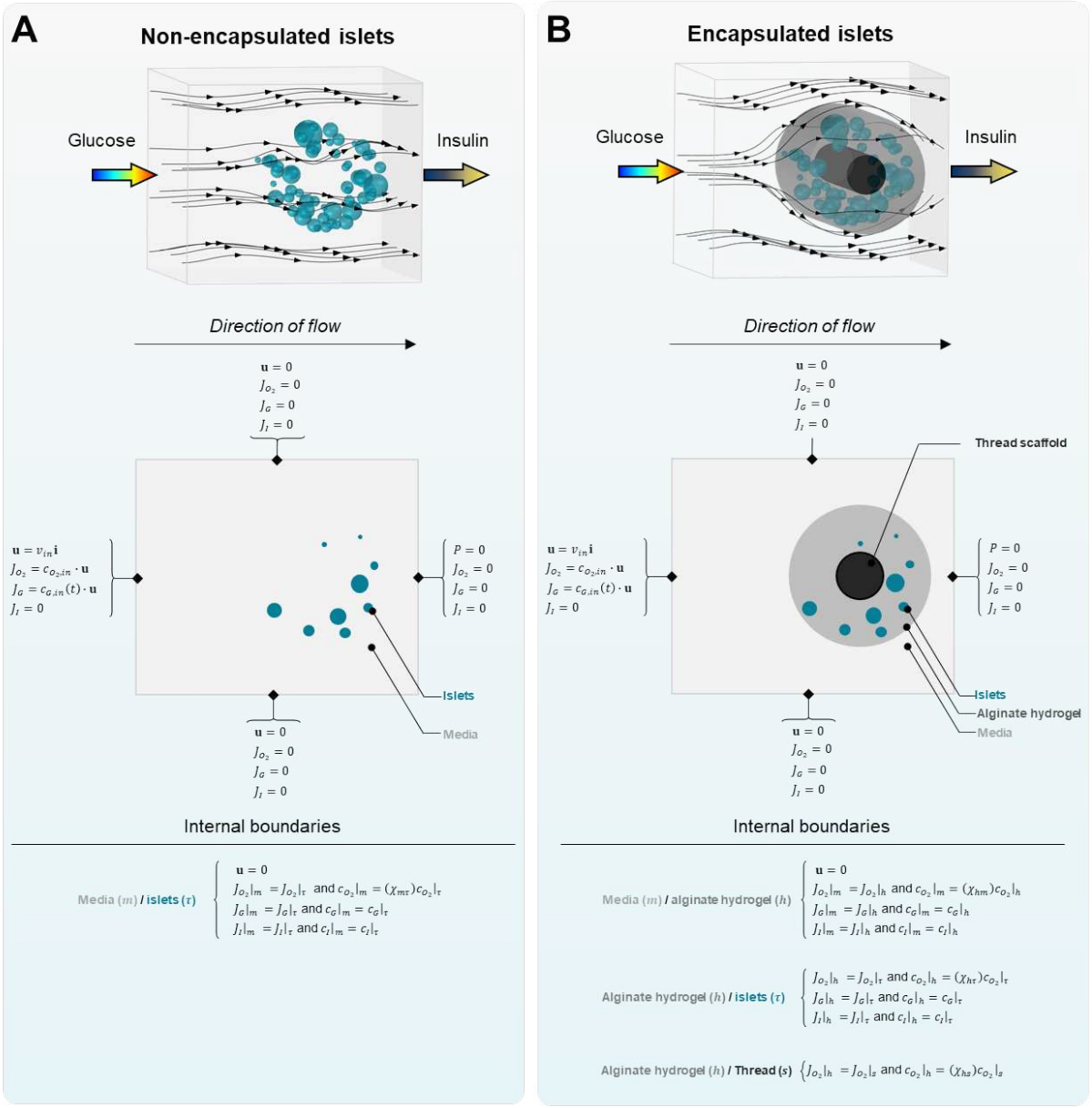
Note: (**B**) A digital photo of two C57BL/6 mice simultaneously under anesthesia prior to measurements. The mouse the viewer's left was for measurements in the native (control) subcutaneous site and the mouse on the right was for measurements in the catheter-modified site. (**C**) A digital photo showing the coincident procedure for removing the catheter and injecting oxygen probe into the vascularized site. (**D**) A digital photo showing the injected oxygen probe at the vascularized site. (**E**) A digital photo taken during the simultaneous recording of the oxygen measurements on both unmodified and vascularized sites one day after the implantation of the oxygen sensor. Both mice were anesthetized using 3% isoflurane in air simultaneously to ensure that the oxygen conditions were equivalent for both subjects.

Figure A.2. Schematic illustrating the placement of probes used for Electron Paramagnetic Resonance (EPR) oxygen measurement



Note: (**A**) A digital photo showing that the custom EPR oxygen probe can fit through the catheter for the probe placement. (**B**) Schematic illustrating the placement of the EPR oxygen probe for oxygen measurement.

Figure A.3. Model settings for the perifusion simulation with non-encapsulated (A) and encapsulated (B) islets.



Note: The variable $c_i|_j$ denotes the concentration species i in subdomain. Top images show the 3-dimensional model; bottom images show the boundary conditions applied in the simulation on a representative 2-dimensional cross section.

Figure A.4. (A) Schematic of a two-dimensional representation of the geometry dimensions applied in simulations of rat islet-containing devices. (B) Mathematical representation of the external and internal boundary conditions

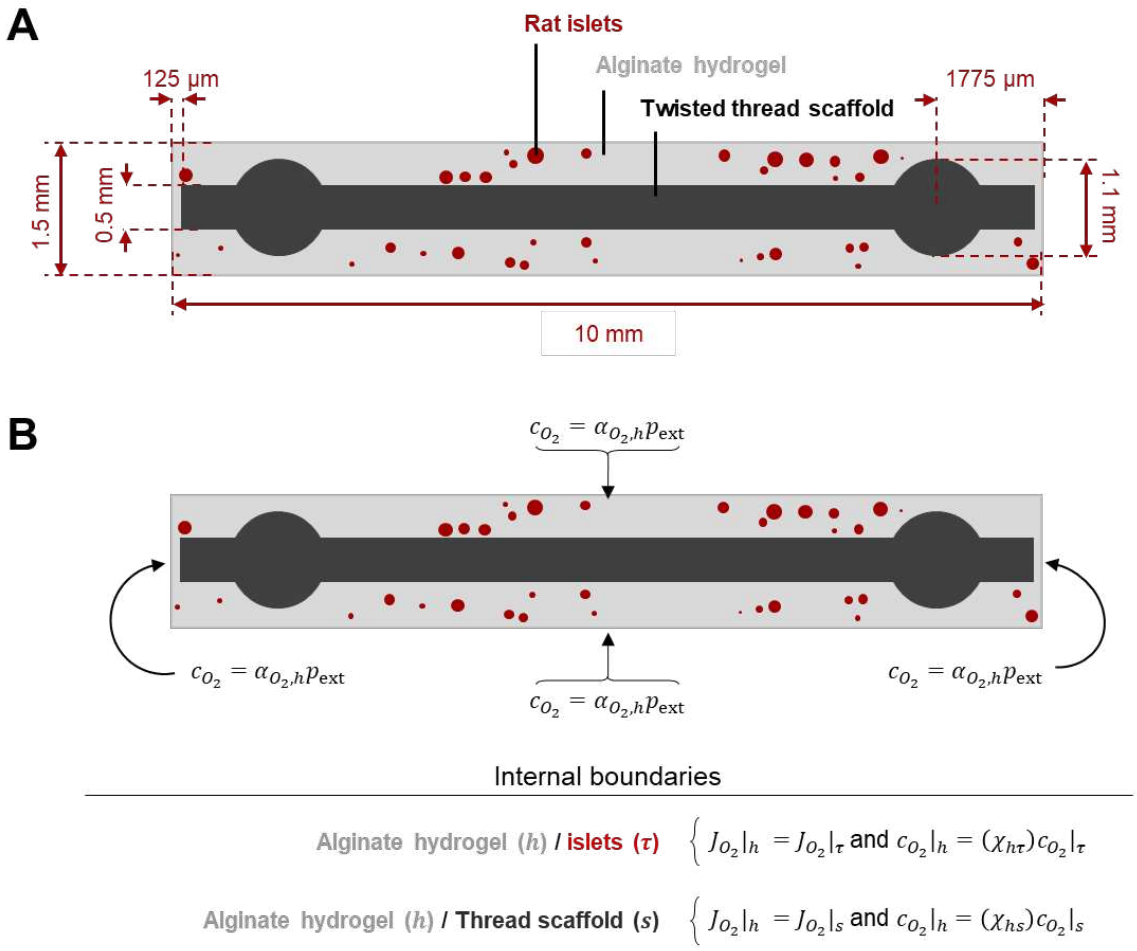
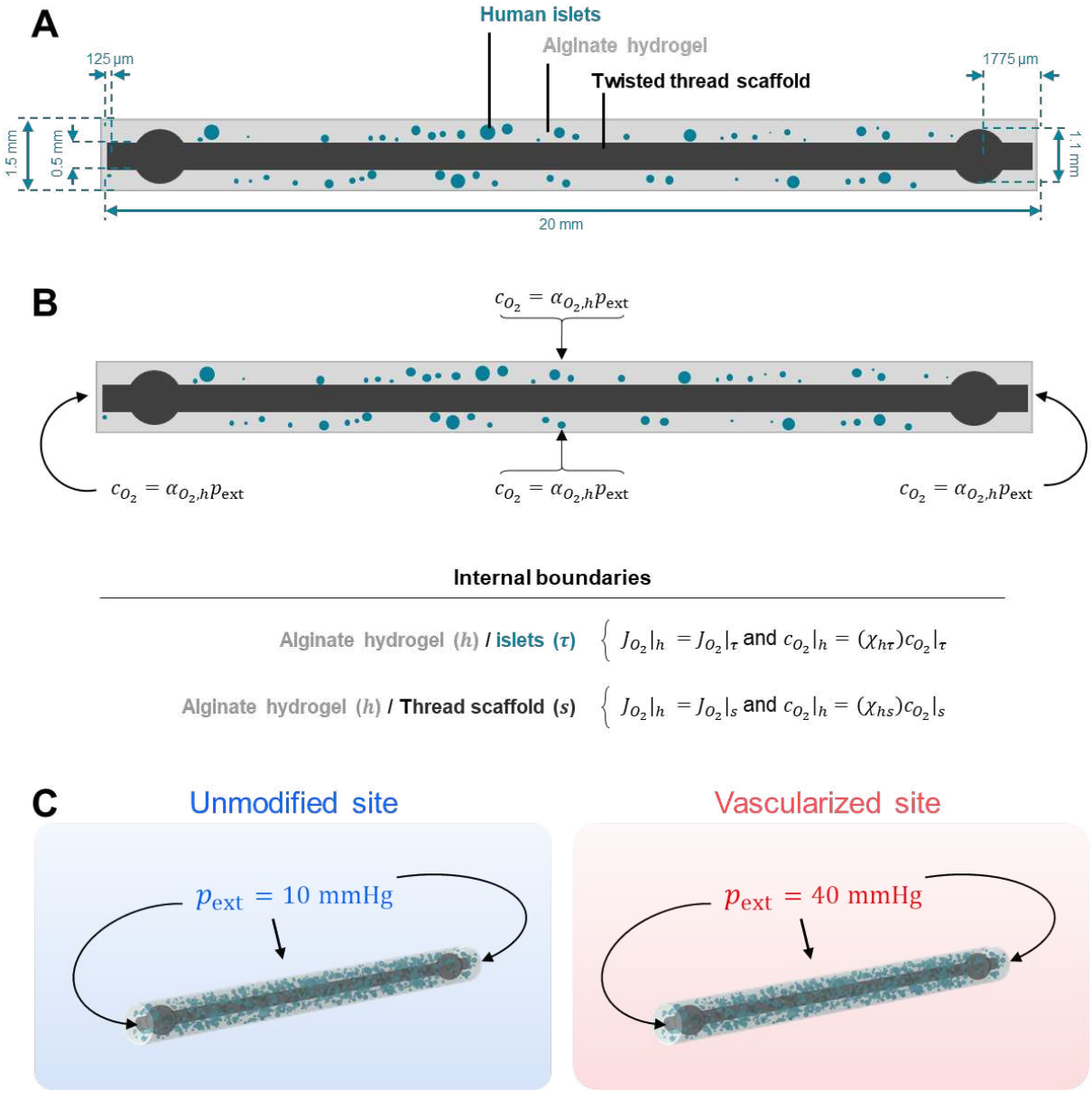


Figure A.5. (A) Schematic of a two-dimensional representation of the geometric dimensions applied in simulations of rat islet-containing devices. (B) Mathematical representation of the external and internal boundary conditions. (C) Values of the external boundary oxygen tension applied in simulations (based on the average value from EPR measurements) for devices at the unmodified control and vascularized site, respectively.



A.2 – References

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APPENDIX B.

APPENDIX B – OTHER PUBLISHED WORKS

In this appendix, I include a reference list of other published works that were not a main part of my thesis work, but in which I collaborated as a co-author during my PhD.

1. Kim R, **Marfil-Garza BA**, Shapiro AMJ, Kin T. Circumportal pancreas accompanied with pancreas divisum in a deceased donor for islet transplantation. ***Surg Radiol Anat***. 2018;40(11):1323-5.
2. Bral M, Pawlick R, **Marfil-Garza B**, Dadheech N, Hefler J, Thiesen A, et al. Pan-caspase inhibitor F573 mitigates liver ischemia reperfusion injury in a murine model. ***PLoS One***. 2019;14(11):e0224567.
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6. Verhoeff K, **Marfil-Garza BA**, Shapiro AMJ. Update on islet cell transplantation. ***Current Opinion in Organ Transplantation***. 2021;26(4).
7. Hefler J, **Marfil-Garza BA**, Pawlick RL, Freed DH, Karvellas CJ, Bigam DL, et al. Preclinical models of acute liver failure: a comprehensive review. ***PeerJ***. 2021;9:e12579.

8. Marfil-Garza BA, Hefler J, Dajani K, Kin T, James Shapiro AM. Total pancreatectomy with islet cell autotransplantation in a 2-year-old child with hereditary pancreatitis due to a PRSS1 mutation. ***Am J Transplant***. 2021;21(11):3790-3.

9. Verhoeff K, **Marfil-Garza BA**, Cuesta-Gomez N, Jasra I, Dadheech N, Shapiro AMJ. Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell-Based Islet Cell Transplant. ***Adv Exp Med Biol***. 2022.

10. Verhoeff K, **Marfil-Garza BA**, Dajani KZ, Bigam D, Anderson B, Kin T, Lam A, O'Gorman D, Senior PA, Shapiro AMJ. C-peptide Targets and Patient-Centered Outcomes of Relevance to Cellular Transplantation for Diabetes. ***Transplantation (In press).***

11. Verhoeff K, Cuesta-Gomez N, Albers P, Pawlick R, **Marfil-Garza BA**, Jasra I, West LJ, Shapiro AMJ. Evaluating the Potential for ABO-Incompatible Islet Transplantation: Expression of ABO-Antigens on Human Pancreata, Isolated Islets, and Embryonic Stem Cell-Derived Islets. ***Transplantation (In press).***