Immune Risk Assessment in Pediatric Heart Transplant Patients

by

Anne M. Halpin

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Medical Sciences - Pediatrics

University of Alberta

© Anne M. Halpin, 2022

Abstract

Background: Although pediatric heart transplantation is a life-saving and effective therapy for children with cardiac disease, barriers remain due to a lack of available organs as well as transplant failure due to rejection. This dissertation aimed to increase knowledge related to immune risk assessment in pediatric heart transplant patients. There are recognised differences in immune responses across the age spectrum. Pediatric transplant patients require studies that evaluate these differences as they may alter pre- and post-transplant care. Chapters 2 and 3 evaluated HLA and AT1R immune responses in pediatric heart transplant populations. Chapter 4 involved the creation of a novel assay for use in ABO-incompatible pediatric heart transplantation for more accurate assessment of antibodies to ABO glycans specific to the heart; this assay also has applications beyond this population.

Methods: Three studies comprise this thesis. For the project described in Chapter 2, retrospective HLA antibody data were collected from pre- and post-VAD therapy patients. HLA antibody testing was performed using solid phase antibody testing methodologies. Transfusion data were also collected. Changes in HLA antibodies following VAD implantation were examined and association to blood transfusion was evaluated. AT1R antibody levels were measured in pediatric heart transplant patients and non-transplant controls (described in Chapter 3). A commercially available ELISA assay used in all recent literature in this field was employed (Cell Trend GmbH). Non-specific, non-AT1R reactivity in this assay was explored using another commercially available reagent, Adsorb Out™. Chapter 4 describes a methods development project in which a novel ABO antibody assay was created and tested. This assay was developed

by optimising coupling of ABH subtype carbohydrate structures to Luminex beads. The beads were tested using healthy control sera to demonstrate proof of concept. Detection of ABO antibodies using this assay was compared to the antibody titre detected by standard red cell agglutination.

Results: *HLA antibodies in VAD therapy*: While both pediatric and adult patients were at risk of developing HLA antibodies following VAD therapy, adult patients demonstrated a statistically significant increase in only class I PRA levels, whereas children did not; neither group showed a significant increase in class II PRA. The proportion of adult *vs* pediatric patients who developed new HLA antibodies was similar despite differences in overall PRA level. Transfusion was not associated with the development of HLA antibodies. *AT1R antibodies in pediatric heart transplant patients*: AT1R antibodies were detected in a high proportion of pediatric heart transplant recipient and control samples, 55% and 56%, respectively. Positive samples were very likely to be converted to a negative value following adsorption treatment. AT1R antibody status changes were not consistent from pre- to post-transplant for either non-adsorbed or adsorbed patient samples. Neu5Gc glycans were detected on the Adsorb Out™ microparticles, suggesting this may be a source of non-specific reactivity removed in this procedure. *ABO antibody detection*: A Luminex bead-based ABO antibody detection assay was developed, optimised, and tested. It was shown to be reproducible between laboratories. Each ABO anti-A and anti-B titre was shown to include a wide range of ABO IgG and IgM antibodies. Additionally, differences between A and B glycan subtype-specific antibody patterns were observed that will be the subject of future study.

Summary and Conclusions: The projects described in this dissertation generated new data that will assist in immune risk assessment for pediatric heart transplant patients. While larger studies are more easily conducted in adult transplant populations due to higher transplant volumes in these cohorts, findings may vary across the age spectrum. In these projects, there are measurable differences in HLA antibody development following VAD therapy as well as AT1R antibody status between children and other published studies in adults. Test method interferences may also vary between adults and children; this should be a consideration in the design (including controls), development, and validation of laboratory assays such as the AT1R assay used in Chapter 3. Differences in tissue and cell distribution of ABH glycans are, in part, a driver for the necessity for new assays such as the Luminex ABO assay presented in this thesis. Pediatric heart transplant patients will be better served by accurate determination of their immune risk status in pre- and post-transplant phases to enable improved donor selection and more precise post-transplant monitoring for rejection.

Preface

This doctoral dissertation is original work by Anne M. Halpin and consists of three research studies; each study is described in a separate chapter.

Chapter 2 is collaborative work has been published as Halpin AM, Nahirniak S, Campbell PM, Urschel S, Kim DH, West LJ, Pidorochynski T, Buchholz H, Conway J. HLA Alloimmunization Following Ventricular Assist Device Support Across the Age Spectrum. Transplantation. 2019 Dec;103(12):2715-2724. doi: 10.1097/TP.0000000000002798. PMID: 31764892. Under the mentorship of LJW and utilizing clinical patient databases from JC, laboratory data collection, data organisation, and manuscript preparation were performed by AMH. The manuscript is available at https://doi.org/10.1097/tp.0000000000002798. All participants were consented in the University of Alberta Health Research Ethics Board, Study ID Pro 00051874.

Chapter 3 is collaborative work with Anne M. Halpin, Sally Abou-Zeki, Simon Urschel, Ingrid Larsen, Bruce Motyka, and Lori J. West. Under the mentorship of LJW, AMH designed and performed this study and prepared this chapter. This work has not yet been published as a manuscript. All patients and controls were consented in the Cardiac Transplantation in Infancy (CTI) study, University of Alberta Health Research Ethics Board, Study ID Pro 00001408.

Chapter 4 is collaborative work with Anne M. Halpin, Bruce Motyka, Tess Ellis, Jean Pearce, Simon Urschel, Todd Lowary, Chris Cairo, Mylvaganam Jeyakanthan, and Lori J. West. Under the mentorship of LJW, AMH designed and performed this assay development, carried out the

analyses, and prepared this chapter. This work has not yet been published as a manuscript. All patients and controls were consented in the Cardiac Transplantation in Infancy (CTI) study, University of Alberta Health Research Ethics Board, Study ID Pro 00001408

Dedication

I dedicate this dissertation to transplant patients, their families/caregivers, and their donors. Transplant research cannot happen without their involvement.

Cardiac transplantation begins with a donor family turning tragedy into a gift for a stranger. In research we seek to honour this extreme act of kindness by making the most of that gift for our transplant patients.

Acknowledgements

I wish to thank my supervisor Dr. Lori West. I have learned so much more than I thought possible, and this is because she provided me countless opportunities and never stopped believing in me. Her support took many forms including outdoor meetings as pandemic-required and unwavering cheerleading when I couldn't do that for myself. Thanks to her leadership, I'm ending this journey with much more than a degree. I've also gained broad experience in grantsmanship, project budgeting, presentation skills, student mentoring, and acquired many skills that will serve me well in the next steps of my career. I will be forever appreciative of this experience.

I also wish to thank all my committee members who all ended up with a much longer commitment that initially expected. I am very grateful for insightful clinical perspective and honest feedback from Dr. Simon Urschel and I look forward to future collaboration on this work as well as future projects. Dr. Michael Mengel provided valuable research and laboratory medicine perspective and support in both my PhD journey and my professional role in the clinical laboratory. Dr. Troy Baldwin contributed basic science expertise, which was not my greatest strength, and entertained my many questions in his class and always did so with kindness. I also extend appreciation for support and encouragement from the staff of the Department of Pediatrics, Trish Kryzanowki and Mikhaila Skehor, and Dr. Sujata Persad for their assistance and kindness.

The entire West Lab team supported me throughout this graduate work but I was especially lifted up by Tess Ellis. She is my ABO Luminex right hand and a beautiful and strong human being. Dr. Bruce Motyka calmly took care of everything from grants to practice presentations and offered great insight to me on all things ABO on many occasions. Jean Pearcey

provided much appreciated assistance with the agglutination testing as well as a calm and helpful perspective at all times. If Jean cannot find it in the freezer, no one can. Ingrid Larsen consented many of the patients in these studies and she was always a fantastic sounding board. Dr. Stephanie Maier was a master organiser of many West Laboratory studies including the initial grant that spearheaded the Luminex bead development. Last but most certainly not least, I extend deep gratitude to Carrie Andrewes, knowledge-keeper of Dr. West's schedule; I'm not sure who else could do this with such grace and skill. She always made sure I had what I needed.

My family provided a lot of love and support through this journey. It wasn't always easy parenting through a (pandemic) PhD but I learned many valuable lessons and my children Kieran and Madeline provided a lot of love and (mostly welcome) distractions. I don't do anything without thinking of the impact to my family and I hope that I have inspired them over the past several years to not be afraid to do hard things in life. I am fortunate to have had parents who made it possible for me to achieve many goals without barriers. My dear dad is no longer here to applaud in person but he was always one of my biggest cheerleaders. My aunt Pat and my sisters Jane and Mona and their families, including 'the cousins', brought a lot of love (and sometime food) to the table.

I am also surrounded by dear friends who make up an amazing village and without whom I would be lost. PhD, and general life, ups and downs were supported and I never felt alone. There are too many people in this group to list but I hope that you feel my love and gratitude.

My HLA family is large and at its centre are my colleagues in the Alberta Precision Laboratories histocompatibility laboratory. Together we have navigated many twists and turns in our public laboratory life but we persevere to provide the best patient care possible. Core to this team is Dr. Trish Campbell with whom I have had the honour and privilege of working for over

two decades. I know of no one who is more quietly awesome. She encouraged me to take this path and listened to many rants and provided advice and encouragement on many trail run meetings. Dr. Esme Dijke, although newer to my local HLA family, is a kindred spirit and she also provided me with support in my fledgling West Laboratory days.

I received support from many professional societies along this journey including the Canadian Donation and Transplantation Research Program (CDTRP), the Alberta Transplant Institute (ATI), the Canadian Society of Transplantation (CST), the American Society of Transplantation (AST), the Women and Children's Health Research Institute (WCHRI), and the Canadian Glycomics Network (GlycoNet). The American Society for Histocompatibility and Immunogenetics (ASHI) gets special mention as ASHI is my professional home. I did not shy away from volunteering on ASHI-related activities over the course of this work and my sometimes last minute replies were received with grace. Thank you to my ASHI family for teaching me to be a better HLA person and for all the life-long personal and professional connections.

If anyone has made it to the end of these acknowledgements, I am impressed. And if you're a student reading this, there is perhaps no right or wrong way to complete a PhD. My path may not have been typical, but I wouldn't change it.

Table of Contents

Abstract ii
Preface v
Dedication…….. vii
Acknowledgements …….. viii
List of Tables……. xiv
List of Figures……. xv
List of Abbreviations ……. xviii
Chapter 1: Introduction ……. 1
1.1 Background…….. 1
1.2 Hypotheses and goals …….. 5
1.2.1 Chapter 2 hypothesis ……. 5
1.2.2 Chapter 3 hypothesis ……. 5
1.2.3 Chapter 4 goal …….. 5
1.3 Aims ……. 6
1.3.1 Chapter 2 aims …….. 6
1.3.2 Chapter 3 aims …….. 6
1.3.3 Chapter 4 …….. 6
1.4 Relevance and potential impact of research to pediatric heart transplantation ……. 7
1.5 References 8
Chapter 2: HLA Alloimmunization Following Ventricular Assist Device Support Across the Age Spectrum……. 14
2.1 Abstract……. 18
2.2 Introduction…….. 20
2.3 Materials and Methods…….. 21
2.3.1 Patients …….. 21
2.3.2 Transfusion Data……. 22
2.3.3 HLA Antibody Testing and Analysis……. 23
2.3.4 Pre-Transplant Management ……. 24
2.3.5 Post-Transplant Definitions…….. 24
2.3.6 Statistical Methods ……. 25
2.4 Results …….. 25
2.4.1 Demographics …….. 25
2.4.2 Blood Product Utilization ……. 26
2.4.3 HLA Antibody Development ……. 27
2.4.4 Factors Associated with HLA Antibody Development……. 29
2.4.5 Antibody Persistence ……. 31
2.4.6 Outcomes …….. 31
2.5 Discussion ……. 33
2.6 Conclusion …….. 39
2.7 Acknowledgements ……. 40
2.8 References ……. 41
2.9 Tables and Figures …….. 46

Chapter 3: Antibodies Detected to Angiotensin II Type 1 Receptor: Investigating Assay Interference in Pediatric Heart Transplant Recipients ……… 51
3.1 Abstract ……… 52
3.2 Introduction ……… 53
3.3 Methods ……… 54
3.3.1 Samples ……… 54
3.3.2 Anti-AT1R measurement ……… 55
3.3.3 Adsorption of non-specific reactivity using Adsorb Out ™ ……… 56
3.3.4 Neu5Gc glycan detection on Adsorb Out ™ product ……… 56
3.3.5 Statistical analysis ……… 57
3.4 Results ……… 57
3.4.1 Quality control results ……… 57
3.4.2 Patient and control populations ……… 57
3.4.3 Sera testing positive for AT1R antibodies ……… 58
3.4.4 AT1R antibody results from adsorbed sera ……… 58
3.4.5 Detection of Neu5Gc glycans on Adsorb Out™ microparticles ……… 59
3.5 Discussion ……… 59
3.6 Conclusion ……… 63
3.7 Tables and Figures ……… 64
3.8 References ……… 69
Chapter 4: ABO antibody detection: updating a century old method ……… 73
4.1 Abstract ……… 75
4.2 Introduction ……… 76
4.2 Methods/Results ……… 79
4.3.1 Overview of Luminex bead-based ABO antibody detection……… 79
4.3.2 Coupling antigens to beads ……… 79
4.3.3 Bead-antigen coupling confirmation ……… 80
4.3.4 Micro-array antibody analysis ……… 80
4.3.5 Application of assay using healthy control sera ……… 80
4.3.6 Hemagglutination ABO antibody titre testing ……… 82
4.3.7 Antibody analysis statistical methods ……… 83
4.4 Discussion ……… 84
4.5 Conclusion ……… 88
4.6 References ……… 89
4.7 Tables and Figures ……… 94
4.8 Supplemental figures ……… 100
Chapter 5: Discussion……… 102
5.1 Overview of findings ……… 102
5.1.1 Main findings from Chapter 2 ……… 103
5.1.2 Main findings from Chapter 3 ……… 103
5.1.3 Main findings from Chapter 4 ……… 104
5.2 Strengths and limitations ……… 105
5.2.1 Strengths and limitations from Chapter 2 ……… 105
5.2.2 Strengths and limitations from Chapter 3 ……… 106
5.2.3 Strengths and limitations from Chapter 4 ……… 108

5.3 Future directions and practical applications ……………………………. 114
5.3.1 Future directions and practical applications from Chapter 2 ………… 114
5.3.2 Future directions and practical applications from Chapter 3 ………… 116
5.3.3 Future directions and practical applications from Chapter 4 ………… 121
5.4 Summary of Findings…………………………………………………… 129
5.5 References ……………………………………………….................... 130
Chapter 6: Detailed Methods……………………………………………..... 137
6.1 Index and overview of methods ……………………………………... 137
6.2.1 HLA antibody screen ………………………………………………… 138
6.2. 2 HLA Luminex Single Antigen Bead Antibody Detection…………… 145
6.3.1 AT1R antibody assay ………………………………………………… 151
6.3.2 Adsorb Out™ ………………………………………………………… 157
6.3.3 Neu5Gc Detection on Adsorb Out™ ………………………………… 160
6.4.1 ABO Luminex Bead Coupling ……………………………………….. 165
6.4.2 ABO Luminex Bead Coupling Confirmation ………………………... 175
6.4.3 ABO Luminex Bead Antibody Detection in Human Samples ……… 184
6.4.4 ABO Luminex Reagent Job Aid ……………………………………... 190
Complete List of References ……………………………………………….. 196

List of Tables

Table	Description	Page
2-1	Demographics and clinical characteristics	49
2-2	Comparison of percent PRA values between pre-implant and peak value in the first year post-VAD implantation	49
2-3	Generalized linear mixed model looking at the association between patient clinical characteristics and the development of class I and II HLA antibodies following VAD insertion	50
3-1	Individual pre- and post-transplant patient data with time points of serum collection and VAD implantation date where relevant. All AT1R antibody results >40 U/mL are displayed as 41 U/mL. Sera that were positive for AT1R antibodies and treated with adsorption are included. For 4 of the 42 post-transplant samples, insufficient serum and/or reagent was available to retest AT1R antibody after adsorption	64
4-1	Parallels between HLA and ABO antibody detection challenges	94
4-2	Comparison of ABO antibody assessment methods	95

List of Figures

Figure	Description	Page
2-1	Transfusion volumes (millilitres) of platelets, PRBC, and plasma are shown by study time frame for both pediatric and adult populations (top). Number of units transfused for platelets, PRBC, and plasma are shown by study time frame for both pediatric and adult populations (bottom). PRBC, packed RBC	46
2-2	Changes in percent PRA for individual pediatric (A and C) and adult (B and D) patients pre- and post-VAD implantation for class I and class II HLA antibodies respectively. The pre-VAD %PRA was measured, on average, 27 days preimplantation for the pediatric patients and 33 days preimplantation for the adult patients. The highest %PRA detected is represented and was measured, on average, 58 days post-implantation for the pediatric patients and 70 days post-implantation for the adult patients. HLA, human leucocyte antigen; PRA, panel-reactive antibody; VAD, ventricular assist device	47
2-3	The number of patients who developed new class I or II antibodies are shown by time frame (A and B, respectively). The values in the pre-VAD time points are the patients with existing class I or II antibodies at the time of implant	48
3-1	The ages at time of sample collection are comparable between the pre-transplant and the non-transplant controls	65
3-2	The majority of the patient samples (84/154) and control samples (15/27) were positive for AT1R antibodies ≧ 17U/mL. Values over 40 U/mL are displayed as '41' U/mL	65
3-3	There was variability in pre- and post-transplant AT1R antibody levels for all 42 samples. Results are shown for each patient individually with and without adsorption. For 4 of the 42 post-transplant samples, insufficient serum and/or reagent was available to retest AT1R antibodies after adsorption. Nine patients received VAD therapy (indicated with heart icon)	66
3-4	After transplantation of the positive samples, AT1R antibody levels did not consistently increase or decrease in either the untreated sera (n=42) or the adsorbed sera (n=38)	66
3-5	Following treatment of positive samples with AdsorbOut™, 39 of 52 (75%) patient sera previously positive for AT1R antibodies decreased to <17 U/mL AT1R concentration (n=52)	67
3-6	Anti-AT1R antibodies were not removed from the assay positive control or calibrators with AdsorbOut™ treatment	68
3-7	Neu5GC glycans were detected on the AdsorbOut™ microparticles but were not detected on beads coated with human albumin	68
4-1	Individual Luminex beads were coupled to each ABO-A and ABO-B glycan subtype antigen. Negative control beads included a bead coupled to BSA as well as a bead that was not coupled with any target. A positive control bead was coupled to α-Gal. Serum or plasma was added	96

	to duplicate wells for the addition of either IgG and IgM secondary antibody. This example of an ABO antibody profile was from an ABO-A1/O genotyped individual with a negative red cell hemagglutination titre against ABO-A1 erythrocytes and an ABO-B titre of 1/32	
4-2	The Luminex assay was able to detect A glycans with more sensitivity than the microarray assay; this increased sensitivity was observed most clearly in the lower concentrations of antibody	96
4-3	There was a wide range of IgG and IgM anti-ABO antibodies in healthy adult individuals across the ABO-O, -A, and -B blood groups. ABO-O individuals (n=68) produced higher levels of IgG anti-A than ABO-B individuals (n=17), and higher IgG anti-B compared to ABO-A individuals (n=48). In contrast, IgM isotype anti-A and anti-B antibodies did not vary based on the ABO blood group	97
4-4	In sera from ABO-O individuals, there were no significant differences in the level of ABO antibodies between females (n=47) and males (n=21). Anti-A was an average of A-II, III, and IV subtype-specificities; anti-B is only B-II subtype-specificity	98
4-5	It is common for ABO-A individuals to have high levels of IgG and IgM anti-A-trisaccharide antibodies but comparatively low levels of antibody to A-II, III, and IV subtype glycans. ABO-B individuals are unlikely to have either anti-B- trisaccharide or anti-B-II antibodies	99
4-6	Sera were tested in parallel by red cell agglutination and Luminex panel analysis (n=119). There was a wide range of IgG and IgM antibody within each red cell agglutination antibody titre	99
4-S1	The A and B glycan subtype coupling was highly consistent for a beads as shown here with this bead coupling confirmation data. The presence of antigen was confirmed with Immucor anti-A and anti-B Novoclone reagents	100
4-S2	The positive control serum results from 24 individual runs shows excellent reproducibility. These results are shown as the mean +/- the 95% confidence interval	100
4-S3	There were no observed differences between IgG and IgM α-Gal antibody levels (n=132). There were also no differences by sex (n=83 females and n=49 males)	101
4-S4	The levels of IgG and IgM antiobdies were compared to titres in only the ABO-O individuals (n=54).The same variability was observed as shown in Figure 6. Hemagglutination titres were compared for ABO-O individuals (n=54)	101
5-1	Pediatric renal transplant patients have higher pre-Adsorb Out™ levels of AT1R antibodies as compared to adult renal transplant patients. All samples showed a reduction of AT1R antibody levels following adsorption although pediatric samples have a more striking reduction than adults. All samples are post-transplant. The upper red hashed line represents the 17 U/mL threshold and the lower line the 10 U/mL negative threshold	108

5-2	The positive control serum tested in nine different laboratories showed strong reproducibility of results using the Luminex bead-based method.	110
5-3	A-II and B-II subtype-specific antibody profiles for patients up to 24 months of age. Antibodies specific for A-II and B-II alone were assessed as these are the only relevant glycan subtypes in cardiac transplantation	111
5-4	Anti-B-II were compared in ABO-O *vs* ABO-A individuals. B-II antibody alone was assessed as this is the only relevant B-glycan subtype in cardiac transplantation	112
5-5	There is diversity of ABO antibody in each titre that differs from a similar adult sample comparison in Figure 4-6. High titres of antibody A-II, III, and IV antibody MFI was averaged to estimate the reactivity to ABO-A red cells used for agglutination testing while B-II alone was used for the ABO-B MFI; this is based on known representation of ABH glycans on red blood cells	113
5-6	ABO antibody data from an ABO-O infant awaiting heart transplantation. The discrepancy between anti-A and anti-B antibody titres and MFI values may be a result of passive antibody from the blood product donor or the interference of albumin infusion in either the ABO titre or the Luminex assay. Titre is shown as '0' (negative with all red cells), 'Undi' (positive only with undiluted serum), or 1/8 (positive up to and including the 1/8 serum dilution)	114
5-7	Evaluation of IVIg antibody composition	125
5-8	Sera were treated *in-vivo* with imlifidase. IgG antibodies reduced but IgM remained similar in MFI	127
5-9	Evaluation of phenotyping anti-sera	128

List of Abbreviations

ABOi	ABO-incompatible
ABOc	ABO-compatible
AHG	Anti-human globulin
AMR	Antibody-mediated rejection
ASHI	American Society for Histocompatibility and Immunogenetics
AT1R	angiotensin II type 1 receptor
BiVAD	Biventricular assist device
BSA	bovine serum albumin
CAV	Coronary allograft vasculopathy
CDC	Complement dependent cytotoxicity
CI	Confidence interval
cPRA	Calculated panel reactive antibody
DSA	Donor-specific antibody
DTT	Dithiothreitol
ECMO	Extra-corporeal membrane oxygenation
ELISA	Enzyme-linked immuno-adsorbent assay
α-Gal	Galactose-α-1,3-galactose
HLA	Human leucocyte antigen
HSCT	Hematopoietic stem cell transplant
IQR	Interquartile range
ISHLT	International Society for Heart and Lung Transplantation

Kg	Kilograms
LVAD	Left ventricular assist device
MCS	Mechanical circulatory support
MFI	Mean fluorescent intensity
Neu5Gc	N-Glycolylneuraminic acid
PE	Phycoerythrin
PRA	Panel reactive antibody
PRBC	Packed red blood cells
SD	Standard deviation
RVAD	Right ventricular assist device
TRALI	Transfusion related acute lung injury
UNOS	United Network for Organ Sharing
VAD	Ventricular assist device

Chapter 1:
Introduction

1.1 Background

Neonatal heart transplantation has been performed successfully for treatment of severe congenital heart disease since 1984.(Bailey et al. 1993) Despite the success of transplantation for congenital cardiac malformations and cardiomyopathies that would otherwise be lethal, far too few donor organs are available, leading to high waitlist mortality.(Almond et al. 2009; Singh et al. 2011) Infant heart transplant recipients have higher long-term survival than patients transplanted at any later age and are relatively protected from the development of chronic rejection in the form of graft coronary artery disease.(Almond et al. 2009; Aurora et al. 2010; Singh et al. 2011) However, despite the use of immunosuppression, there is a still a high frequency of rejection in these patients as reported in a large, multi-centre study by Ameduri et al.; this group reported over 60% rejection episodes of any kind overall and 34% late rejection episodes.(Ameduri et al. 2012) As compared to patients with no rejection, patients with rejection have increased rates of mortality and moderate to severe coronary vasculopathy.(Ameduri et al. 2012; Dipchand 2018; Dipchand and Laks 2020) Approaches to predict late graft loss are being evaluated (Almond et al. 2018) but knowledge gaps remain. The projects described in this proposal address areas of immune risk assessment that have either not been explored and/or may require a unique approach for this population.

The role of antibodies with specificities to human leukocyte antigens (HLA) in transplantation risk assessment has been recognized for decades.(Patel and Terasaki 1969) Current assessment of immunological risk is predominantly limited to detection and characterization of HLA antibodies; when present before transplant, donor-specific HLA antibodies are associated with

post-transplant rejection and poor outcomes.(Mahle et al. 2011; Mangiola et al. 2017; Rossano et al. 2010; Wright et al. 2007) While ventricular assist devices (VAD) and other forms of mechanical circulatory support are increasingly used to bridge to transplant for patients with heart failure,(Adachi et al. 2015; Intermacs n.d.; Steiner et al. 2017) VADs have also been associated with a number of complications including stimulation of HLA antibody production.(Alba et al. 2015; Askar et al. 2013; Elkind et al. 2020; Halpin et al. 2019; McKenna David H et al. 2002; O'Connor et al. 2010)

Immune risk in pediatric heart transplantation is not limited to HLA antibodies. Additional risk may be posed by the presence of autoantibodies. Specifically, autoantibodies to angiotensin II type 1 receptor (AT1R) have been reported to be relevant to transplant outcomes in some patient populations, (Carroll et al. 2016; Deltombe et al. 2017; Giral et al. 2012; Lefaucheur et al. 2019; Philogene et al. 2017; Pinelli et al. 2017) although there are also conflicting reports regarding the relevance of these antibodies.(Michielsen et al. 2016; Oaks et al. 2018; Pinelli et al. 2017) A review article by Zhang *et al* summarises many AT1R studies in a useful table.(Zhang and Reed 2016); this list includes a list of related publications but none of these studies are in pediatric heart transplant patients, leaving an unexplored question of risk.

Another area of immune risk assessment in the pediatric heart transplant population involves ABO blood group antibody detection. The use of organs from ABO-incompatible (ABOi) donors greatly expands the potential donor pool for young children.(Almond et al. 2009; Singh et al. 2011) As first reported by West *et al* in a cohort of 10 cases in 2001, there is a window of immunologic opportunity in early life during which ABOi heart transplantation can be performed

safely due to lack of pre-formed ABO antibodies.(West et al. 2001) Consideration of ABOi donors for heart transplantation in early childhood has subsequently become standard of care in many centres globally.(Dipchand et al. 2010; Dipchand and Laks 2020; Henderson et al. 2012; Urschel et al. 2013, 2020) If ABO antibodies are present, plasmapheresis can be used in ABOi organ transplantation to remove or reduce ABO antibodies pre- and post-transplant.(Massie et al. 2020; de Weerd and Betjes 2018) However, despite many advances in transplantation such as those related to surgical techniques and histocompatibility testing, the methods used to measure antibodies to structures of the ABO histo-blood group system have remained relatively unchanged for over a century, contributing to a degree of unpredictability as to the safety of ABOi transplantation.

ABO histo-blood group carbohydrate structures are complex in nature and comprise multiple subtypes.(de Mattos 2016) We previously showed that these subtype antigens are differentially expressed on erythrocytes *vs* vascular endothelium.(Jeyakanthan et al. 2015, 2016; Jeyakanthan and West 2012) The clinical method used routinely in transfusion medicine laboratories to detect antibodies to ABO structures is erythrocyte agglutination; this method is largely unchanged since Karl Landsteiner's original description of the ABO blood groups in the early 20th century. While the hemagglutination method suitable for red cell transfusion, there is abundant evidence that it is inadequate to support safe ABOi organ transplantation due to inability to distinguish antibody isotypes and A, B, and H subtype-specificities. We showed that cardiac endothelium expresses only subtype II antigens whereas erythrocytes express subtypes II, III, and IV structures.(Jeyakanthan et al. 2016) As such, using erythrocytes as surrogates to assess the presence or confirm the absence of donor-specific antibodies to ABO structures on the heart

allograft may yield false positive results. Hemagglutination may also yield false negative results due to its inability to detect IgG isotype antibodies reliably and predictably. It has been previously reported that there are differences in the expression of ABO-A subtype antigens on erythrocytes as compared to cardiac endothelium.(Clausen and Hakomori 1989; Jeyakanthan et al. 2016) In addition to limitations of hemagglutination due to specificity and isotypes, this assay is plagued by well-described lack of standardization among laboratories and observers.(AuBuchon, de Wildt-Eggen, and Dumont 2008; Kang, Lim, and Baik 2014; Khalili et al. 2017)

A clear clinical need exists for characterization of ABO antibodies with precise and predictable determination of isotype and subtype-specificity.(Lindberg et al. 2011) We created an ABH glycan microarray to fill this gap and its utility in pediatric heart transplantation has been demonstrated.(Jeyakanthan et al. 2016) While the array method has advantages over agglutination, this technology faces barriers for implementation due to the lack of microarray equipment in most clinical laboratories, as well as the time required to complete and analyse these results. The ideal method would utilize existing clinical laboratory expertise and equipment, and allow for rapid turn-around testing of individual samples. Solid phase, bead-based antibody detection methods have become the 'gold standard' for HLA antibody testing in histocompatibility laboratories for organ transplant management.(El-Awar, Lee, and Terasaki 2005; Reed et al. 2013; Tait et al. 2013; Tambur et al. 2018; Tinckam 2009) The creation of a bead-based, single antigen assay would overcome the limitations of erythrocyte agglutination methods in ABOi transplantation and would be more suitable to routine testing in the clinical laboratory than the glycan microarray.

An enhanced understanding of immune risk assessment in pediatric heart transplant recipients will contribute to improved long-term outcomes. Risk assessment of pediatric heart transplant patients requires a tailored approach specific to this population of patients. The projects described here address areas of immune risk evaluation that have either not been explored and/or may require a unique approach for these patients.

1.2 Hypotheses and goals

1.2.1 Chapter 2 hypothesis

VADs are increasingly used as a bridge to transplant. VADs are reported to be associated with HLA antibody development but the current risk for pediatric patients with current era of transfusion and HLA antibody testing practices is not well studied.

Hypothesis: The use of VADs for bridging to pediatric heart transplantation significantly increases HLA antibodies and is driven by transfusion at the time of VAD implantation

1.2.2 Chapter 3 hypothesis

There are conflicting data regarding the relevance of AT1R autoantibodies in transplantation overall. Pediatric patients may have less overall endothelial damage and development of autoantibodies than adult patients and may be less likely to have AT1R autoantibodies. The commercially available assay lacks essential controls for specificity of the antibodies detected.

Hypothesis A: Pediatric heart transplant patients will have less AT1R autoantibody as compared to adult transplant populations

Hypothesis B: The commercial ELISA assay is lacking in specificity and false positive reactivity cannot be ruled out

1.2.3 Chapter 4 Goal

Currently used ABO antibody detection methods are inadequate for ABOi pediatric heart transplant patient immune risk assessment.

Goal: Develop a precise and reproducible ABO antibody detection assay

1.3 Aims, Experimental Design, Methods, and Analysis

1.3.1 Chapter 2 aims

i. Retrospective collection of pre- and post-VAD HLA antibody and transfusion data from local pediatric and adult patients who had undergone VAD implantation
ii. Measurement of the impact of VAD implantation and transfusion on HLA antibody development
iii. Assessment of defined immunologic outcomes of pediatric heart transplantation

1.3.2 Chapter 3 aims

i. Determine the frequency of AT1R antibodies in pediatric heart transplant patients (pre- and post-transplant) as well as non-transplant controls
ii. Investigate AT1R antibody specificity using a commonly used histocompatibility adsorption procedure (Adsorb Out™, One Lambda – Thermo Fisher)

1.3.3 Chapter 4 goals

i. Develop a Luminex bead-based single antigen panel to detect ABO antibodies to A and B subtype antigens

ii. Validate the ABO bead panel in sera from healthy adult controls and compare the results to hemagglutination titres

iii. Evaluate the assay in sera from pediatric heart transplant patients

1.4 Relevance and potential impact of research in pediatric heart transplantation

The findings of studies in adult transplant recipients may not apply to pediatric populations, particularly our youngest patients. The developing immune system as well as increased challenges in organ availability necessitate a pediatric-focussed lens in evaluating immune risk assessment. The findings presented here provide important insights into the HLA sensitisation risks for pediatric patients undergoing VAD therapy. This information is important to understand additional challenges that may be faced in donor compatibility following mechanical circulatory support. AT1R antibody evaluation is becoming more common and some centres are treating patients and adjusting immunosuppression when AT1R antibodies are detected.(Carroll et al. 2019) The results here will begin to inform what practices may be relevant to pediatric heart transplant recipients and what unique challenges may apply to the interpretation of these results in pediatric populations. The novel ABO antibody method presented can be readily implemented into clinical histocompatibility laboratories as this tool is already the gold standard for HLA antibody measurement. This method for detection and full characterisation of ABO antibodies can also be used to study the role of ABO antibody isotype and, due to the reproducibility of Luminex technology, multicentre studies could employ this technique with excellent centre-to-centre concordance of results.

References

Adachi, Iki, Muhammad S. Khan, Francisco A. Guzmán-Pruneda, Charles D. Fraser, Carlos M. Mery, Susan W. Denfield, William J. Dreyer, David L. S. Morales, E. Dean McKenzie, Jeffrey S. Heinle, and Charles D. Fraser. 2015. "Evolution and Impact of Ventricular Assist Device Program on Children Awaiting Heart Transplantation." *The Annals of Thoracic Surgery* 99(2):635–40.

Alba, Ana C., Kathryn Tinckam, Farid Foroutan, Laerke M. Nelson, Finn Gustafsson, Kam Sander, Hellen Bruunsgaard, Sharon Chih, Helen Hayes, Vivek Rao, Diego Delgado, and Heather J. Ross. 2015. "Factors Associated with Anti-Human Leukocyte Antigen Antibodies in Patients Supported with Continuous-Flow Devices and Effect on Probability of Transplant and Post-Transplant Outcomes." *Journal of Heart and Lung Transplantation* 34:685–92.

Almond, C. S., R. R. Thiagarajan, G. E. Piercey, K. Gauvreau, E. D. Blume, H. J. Bastardi, F. Fynn-Thompson, and T. P. Singh. 2009. "Waiting List Mortality among Children Listed for Heart Transplantation in the United States." *Circulation* 119(5):717–27.

Almond, Christopher S., Helena Hoen, Joseph W. Rossano, Chesney Castleberry, Scott R. Auerbach, Lingyao Yang, Ashwin K. Lal, Melanie D. Everitt, Matthew Fenton, Seth A. Hollander, Elfriede Pahl, Elizabeth Pruitt, David N. Rosenthal, Doff B. McElhinney, Kevin P. Daly, and Manisha Desai. 2018. "Development and Validation of a Major Adverse Transplant Event (MATE) Score to Predict Late Graft Loss in Pediatric Heart Transplantation." *The Journal of Heart and Lung Transplantation* 37(4):441–50.

Ameduri, Rebecca K., Jie Zheng, Kenneth B. Schechtman, Timothy M. Hoffman, Robert J. Gajarski, Richard Chinnock, David C. Naftel, James K. Kirklin, Anne I. Dipchand, and Charles E. Canter. 2012. "Has Late Rejection Decreased in Pediatric Heart Transplantation in the Current Era? A Multi-Institutional Study." *The Journal of Heart and Lung Transplantation* 31(9):980–86.

Askar, Medhat, Eileen Hsich, Patrick Reville, Amy S. Nowacki, William Baldwin, Suzanne Bakdash, Jenna Daghstani, Aiwen Zhang, Lynne Klingman, Nicholas Smedira, Nader Moazami, David O. Taylor, Randall C. Starling, and Gonzalo Gonzalez-Stawinski. 2013. "HLA and MICA Allosensitization Patterns among Patients Supported by Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 32(12):1241–48.

AuBuchon, JP, J. de Wildt-Eggen, and LJ Dumont. 2008. "Reducing the Variation in Performance of Antibody Titrations." *Archives of Pathology & Laboratory Medicine* 132(7):1194–1201.

Aurora, Paul, Leah B. Edwards, Anna Y. Kucheryavaya, Jason D. Christie, Fabienne Dobbels, Richard Kirk, Axel O. Rahmel, Josef Stehlik, and Marshall I. Hertz. 2010. "Registry Series: The Registry of the International Society for Heart and Lung Transplantation: Thirteenth Official Pediatric Lung and Heart-Lung Transplantation Report—2010." *Journal of Heart and Lung Transplantation* 29:1129–41.

Bailey, L. L., S. R. Gundry, A. J. Razzouk, N. Wang, C. M. Sciolaro, and M. Chiavarelli. 1993. "Bless the Babies: One Hundred Fifteen Late Survivors of Heart Transplantation during the First Year of Life. The Loma Linda University Pediatric Heart Transplant Group." *The*

Journal of Thoracic and Cardiovascular Surgery 105(5):805.

Carroll, Robert P., Sue Deayton, Timothy Emery, Watsala Munasinghe, Eleni Tsiopelas, Adrian Fleet, Meg Lake, Ian Humphreys, Maisarah Jalalonmuhali, and Patrick Coates. 2019. "Proactive Treatment of Angiotensin Receptor Antibodies in Kidney Transplantation with Plasma Exchange and/or Candesartan Is Safe and Associated with Excellent Graft Survival at 4 years: A Single Centre Australian Experience." *Human Immunology* 80(8):573–78.

Carroll, Robert P., Michael Riceman, Christopher M. Hope, Anna Zeng, Sue Deayton, Greg D. Bennett, and P. T. Coates. 2016. "Angiotensin II Type-1 Receptor Antibody (AT1Rab) Associated Humoral Rejection and the Effect of Peri Operative Plasma Exchange and Candesartan." *Human Immunology* 77(12):1154–58.

Clausen, H. and S. Hakomori. 1989. "ABH and Related Histo-Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution." *Vox Sanguinis* 56(1):1–20.

Deltombe, Clement, Florence Gillaizeau, Daniel Anglicheau, Emmanuel Morelon, Katy Trébern-Launay, Florent Le Borgne, Marie Rimbert, Pierrick Guérif, Stéphanie Malard-Castagnet, Yohann Foucher, and Magali Giral. 2017. "Is Pre-Transplant Sensitization against Angiotensin II Type 1 Receptor Still a Risk Factor of Graft and Patient Outcome in Kidney Transplantation in the Anti-HLA Luminex Era? A Retrospective Study." *Transplant International* 30(11):1150–60.

Dipchand, A. I., S. M. Pollock BarZiv, C. Manlhiot, L. J. West, M. VanderVliet, and B. W. McCrindle. 2010. "Equivalent Outcomes for Pediatric Heart Transplantation Recipients: ABO-Blood Group Incompatible versus ABO-Compatible." *American Journal of Transplantation* 10(2):389–97.

Dipchand, Anne I. 2018. "Current State of Pediatric Cardiac Transplantation." *Annals of Cardiothoracic Surgery; Vol 7, No 1 (January 2018): Heart Transplantation* 7(1):31–55.

Dipchand, Anne I. and Jessica A. Laks. 2020. "Pediatric Heart Transplantation: Long-Term Outcomes." *Indian Journal of Thoracic and Cardiovascular Surgery* 36(2):175–89.

El-Awar, N., J. Lee, and P. I. Terasaki. 2005. "HLA Antibody Identification with Single Antigen Beads Compared to Conventional Methods." *Human Immunology* 66(9):989–97.

Elkind, Jae, Juliana Sobczyk, Oscar Ostberg-Braun, Jorge Silva Enciso, Eric Adler, and Gerald P. Morris. 2020. "Factors Influencing Transfusion-Associated HLA Sensitization in Patients Bridged to Heart Transplantation Using Ventricular Assist Device." *Clinical Transplantation* 34(1):e13772.

Giral, M., Y. Foucher, A. Dufay, J. P. D. Van Huyen, K. Renaudin, A. Moreau, A. Philippe, B. Hegner, R. Dechend, H. Heidecke, S. Brouard, A. Cesbron, S. Castagnet, A. Devys, J. P. Soulillou, D. Dragun, D. Dragun, A. Philippe, and R. Catar. 2012. "Pretransplant Sensitization Against Angiotensin II Type 1 Receptor Is a Risk Factor for Acute Rejection and Graft Loss." *CURRENT OPINION IN ORGAN TRANSPLANTATION* 13; 17(10; 4):2576; 445.

Halpin, Anne, Susan Nahirniak, Patricia Campbell, Simon Urschel, Daniel Kim, Lori West, Tara

Pidorochynski, Holger Buchholz, and Jennifer Conway. 2019. “HLA Allo-Immunization Following Ventricular Assist Device Support Across the Age Spectrum.” *Transplantation*.

Henderson, Heather T., Charles E. Canter, William T. Mahle, Anne I. Dipchand, Kelci LaPorte, Kenneth B. Schechtman, Jie Zheng, Alfred Asante-Korang, Rakesh K. Singh, and Kirk R. Kanter. 2012. “ABO-Incompatible Heart Transplantation: Analysis of the Pediatric Heart Transplant Study (PHTS) Database.” *The Journal of Heart and Lung Transplantation* 31(2):173–79.

Intermacs. n.d. “Interagency Registry for Mechanically Assisted Cirulatory Support.” Retrieved (https://www.uab.edu/medicine/intermacs/).

Jeyakanthan, M., P. J. Meloncelli, L. Zou, T. L. Lowary, I. Larsen, S. Maier, K. Tao, J. Rusch, R. Chinnock, N. Shaw, M. Burch, K. Beddows, L. Addonizio, W. Zuckerman, E. Pahl, J. Rutledge, K. R. Kanter, C. W. Cairo, J. M. Buriak, D. Ross, I. Rebeyka, and L. J. West. 2016. “ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation.” *American Journal of Transplantation* 16(5):1548–58.

Jeyakanthan, M., K. Tao, L. Zou, P. J. Meloncelli, T. L. Lowary, K. Suzuki, D. Boland, I. Larsen, M. Burch, N. Shaw, K. Beddows, L. Addonizio, W. Zuckerman, B. Afzali, D. H. Kim, M. Mengel, A. M. J. Shapiro, and L. J. West. 2015. “Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation.” *American Journal of Transplantation* (10):2602.

Jeyakanthan, M. and L. J. West. 2012. “Donor-Specific Isohemagglutinins: Measuring the Unknown.” *American Journal of Transplantation* 12(4):803–5.

Kang, Seon Joo, Young Ae Lim, and Sae Yun Baik. 2014. “Comparison of ABO Antibody Titers on the Basis of the Antibody Detection Method Used.” *Annals Of Laboratory Medicine* 34(4):300–306.

Khalili, Iman, Martina Koch, Friedrich Thaiss, Rosemarie Plaetke, Jan Bruegger, and Sven Peine. 2017. “Systematic Comparison of IgM and IgG ABO Antibody Titers by Using Tube and Gel Card Techniques and Its Relevance for ABO-Incompatible Kidney Transplantation.” *Clinical Laboratory* 63(09/2017).

Lefaucheur, Carmen, Denis Viglietti, Yassine Bouatou, Aurélie Philippe, Daniele Pievani, Olivier Aubert, Jean-Paul Duong Van Huyen, Jean-Luc Taupin, Denis Glotz, Christophe Legendre, Alexandre Loupy, Philip F. Halloran, and Duska Dragun. 2019. “Non-HLA Agonistic Anti-Angiotensin II Type 1 Receptor Antibodies Induce a Distinctive Phenotype of Antibody-Mediated Rejection in Kidney Transplant Recipients.” *Kidney International* 96(1):189–201.

Lindberg, Linda, Sandra M. Johansson, Jining Liu, Per Grufman, and Jan Holgersson. 2011. “Is There a Clinical Need for a Diagnostic Test Allowing Detection of Chain Type-Specific Anti-A and Anti-B?” *Transfusion* 51(3):494–503.

Mahle, William T., Margaret A. Tresler, R. E. Edens, Paolo Rusconi, James F. George, David C. Naftel, and Robert E. Shaddy. 2011. “Allosensitization and Outcomes in Pediatric Heart Transplantation.” *The Journal Of Heart And Lung Transplantation: The Official Publication Of The International Society For Heart Transplantation* 30(11):1221–27.

Mangiola, Massimo, Marilyn Marrari, Brian Feingold, and Adriana Zeevi. 2017. “Significance of Anti-HLA Antibodies on Adult and Pediatric Heart Allograft Outcomes.” *Frontiers in Immunology* 8(JAN).

Massie, Allan B., Babak J. Orandi, Madeleine M. Waldram, Xun Luo, Anh Q. Nguyen, Robert A. Montgomery, Krista L. Lentine, and Dorry L. Segev. 2020. “Impact of ABO-Incompatible Living Donor Kidney Transplantation on Patient Survival.” *American Journal of Kidney Diseases* 76(5):616–23.

de Mattos, Luiz Carlos. 2016. “Structural Diversity and Biological Importance of ABO, H, Lewis and Secretor Histo-Blood Group Carbohydrates.” *Revista Brasileira de Hematologia e Hemoterapia* 38(4):331–40.

McKenna David H, Jr., Ted Eastlund, Miriam Segall, Harriet J. Noreen, Soon Park, Jr. McKenna David H, Ted Eastlund, Miriam Segall, Harriet J. Noreen, and Soon Park. 2002. “Original Article: HLA Alloimmunization in Patients Requiring Ventricular Assist Device Support.” *Journal of Heart and Lung Transplantation* 21:1218–24.

Michielsen, Laura A., Arjan D. van Zuilen, Merle M. Krebber, Marianne C. Verhaar, and Henny G. Otten. 2016. “Clinical Value of Non-HLA Antibodies in Kidney Transplantation: Still an Enigma?” *Transplantation Reviews (Orlando, Fla.)* 30(4):195–202.

O’Connor, Matthew J., JonDavid Menteer, Maryanne R. K. Chrisant, Dimitrios Monos, Curt Lind, Selena Levine, J. William Gaynor, Brian D. Hanna, Stephen M. Paridon, Chitra Ravishankar, and Beth D. Kaufman. 2010. “Ventricular Assist Device-Associated Anti-Human Leukocyte Antigen Antibody Sensitization in Pediatric Patients Bridged to Heart Transplantation.” *The Journal of Heart and Lung Transplantation* 29(1):109–16.

Oaks, Martin, Karen Michel, Francis X. Downey, and Vinay Thohan. 2018. “Xenoreactive Antibodies and Latent Fibrin Formation in VAD and Cardiac Transplant Recipients Can Confound the Detection and Measurement of Anti-AT1R Antibodies.” *American Journal of Transplantation* 18(11):2763–71.

Patel, R. and P. I. Terasaki. 1969. “Significance of the Positive Crossmatch Test in Kidney Transplantation.” *The New England Journal of Medicine* 280(14):735–39.

Philogene, Mary Carmelle, Serena Bagnasco, Edward S. Kraus, Robert A. Montgomery, Duska Dragun, Mary S. Leffell, Andrea A. Zachary, and Annette M. Jackson. 2017. “Anti-Angiotensin II Type 1 Receptor and Anti-Endothelial Cell Antibodies: A Cross-Sectional Analysis of Pathological Findings in Allograft Biopsies.” *Transplantation* 101(3):608–15.

Pinelli, David F., John J. Friedewald, Kelley M. K. Haarberg, Shree L. Radhakrishnan, Jennifer R. Zitzner, Wendy E. Hanshew, and Anat R. Tambur. 2017. “Assessing the Potential of Angiotensin II Type 1 Receptor and Donor Specific Anti-Endothelial Cell Antibodies to Predict Long-Term Kidney Graft Outcome.” *Human Immunology* 78(5–6):421–27.

Reed, E. F., P. Rao, Z. Zhang, H. Gebel, R. A. Bray, I. Guleria, J. Lunz, T. Mohanakumar, P. Nickerson, A. R. Tambur, A. Zeevi, P. S. Heeger, and D. Gjertson. 2013. “Comprehensive Assessment and Standardization of Solid Phase Multiplex-Bead Arrays for the Detection of Antibodies to HLA.” *American Journal of Transplantation* 13(7).

Rossano, Joseph W., David L. S. Morales, Farhan Zafar, Susan W. Denfield, Jeffrey J. Kim, John L. Jefferies, and William J. Dreyer. 2010. "Impact of Antibodies against Human Leukocyte Antigens on Long-Term Outcome in Pediatric Heart Transplant Patients: An Analysis of the United Network for Organ Sharing Database." *The Journal of Thoracic and Cardiovascular Surgery* 140(3):694-699.e2.

Singh, Tajinder P., Christopher S. Almond, Gary Piercey, and Kimberlee Gauvreau. 2011. "Trends in Wait-List Mortality in Children Listed for Heart Transplantation in the United States: Era Effect Across Racial/Ethnic Groups." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11(12):2692–99.

Steiner, Jill M., Eric V Krieger, Karen K. Stout, April Stempien-Otero, Claudius Mahr, Nahush A. Mokadam, and Joshua L. Hermsen. 2017. "Durable Mechanical Circulatory Support in Teenagers and Adults with Congenital Heart Disease: A Systematic Review." *International Journal of Cardiology* 245:135–40.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. "Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation." *Transplantation* 95(1):19–47.

Tambur, Anat R., Patricia Campbell, Frans H. Claas, Sandy Feng, Howard M. Gebel, Annette M. Jackson, Roslyn B. Mannon, Elaine F. Reed, Kathryn Tinckam, Medhat Askar, Anil Chandraker, Patricia P. Chang, Monica Colvin, Anthony-Jake Demetris, Joshua M. Diamond, Anne I. Dipchand, Robert L. Fairchild, Mandy L. Ford, John Friedewald, Ronald G. Gill, Denis Glotz, Hilary Goldberg, Ramsey Hachem, Stuart Knechtle, Jon Kobashigawa, Deborah J. Levine, Joshua Levitsky, Michael Mengel, Edgar Milford, Kenneth A. Newell, Jacqueline G. O'Leary, Scott Palmer, Parmjeet Randhawa, John Smith, Laurie Snyder, Randall C. Starling, Stuart Sweet, Timucin Taner, Craig J. Taylor, Steve Woodle, Adriana Zeevi, and Peter Nickerson. 2018. "Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group Meeting Report." *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons*.

Tinckam, Kathryn. 2009. "Histocompatibility Methods." *Transplantation Reviews* 23:80–93.

Urschel, Simon, Ingrid M. Larsen, Richard Kirk, Julie Flett, Michael Burch, Nadine Shaw, Julia Birnbaum, Heinrich Netz, Elfriede Pahl, Kathleen L. Matthews, Richard Chinnock, Joyce K. Johnston, Kim Derkatz, and Lori J. West. 2013. "ABO-Incompatible Heart Transplantation in Early Childhood: An International Multicenter Study of Clinical Experiences and Limits." *The Journal of Heart and Lung Transplantation* 32(3):285–92.

Urschel, Simon, Marie McCoy, Ryan S. Cantor, Devin A. Koehl, Warren A. Zuckerman, Anne I. Dipchand, Zdenka Reinhardt, Kirk R. Kanter, Jean A. Ballweg, Joshua Sparks, James K. Kirklin, and Waldemar F. Carlo. 2020. "A Current Era Analysis of ABO Incompatible

Listing Practice and Impact on Outcomes in Young Children Requiring Heart Transplantation.” *Journal of Heart and Lung Transplantation* 39(7):627–35.

de Weerd, Annelies E. and Michiel G. H. Betjes. 2018. “ABO-Incompatible Kidney Transplant Outcomes.” *Clinical Journal of the American Society of Nephrology* 13(8):1234 LP – 1243.

West, Lori J., Stacey M. Pollock-Barziv, Anne I. Dipchand, K. Jin Lee, Carl J. Cardella, Leland N. Benson, Ivan M. Rebeyka, and John G. Coles. 2001. “ABO-Incompatible Heart Transplantation in Infants.” *New England Journal of Medicine* 344(11):793–800.

Wright, Eric J., William P. Fiser, R. Erik Edens, Elizabeth A. Frazier, W. Robert Morrow, Michiaki Imamura, and Robert D. B. Jaquiss. 2007. “Cardiac Transplant Outcomes in Pediatric Patients with Pre-Formed Anti-Human Leukocyte Antigen Antibodies and/or Positive Retrospective Crossmatch.” *The Journal of Heart and Lung Transplantation* 26(11):1163–69.

Zhang, Qiuheng and Elaine F. Reed. 2016. “The Importance of Non-HLA Antibodies in Transplantation.” *Nature Reviews Nephrology* 12(June):484–95.

Chapter 2:

HLA Allo-immunization Following Ventricular Assist Device Support Across the Age Spectrum

Anne M. Halpin[1,2,3,4], Susan Nahirniak[2], Patricia M. Campbell[2,3] , Simon Urschel[1,3,4,5], Daniel H. Kim[3, 6, 7], Lori J West[1,3,4,5,7,8], Tara Pidorochynski[1,5] , H. Buchholz[5,6,9], Jennifer Conway[1,3,5,].

[1] Department of Pediatrics, University of Alberta, Edmonton, AB, Canada

2 Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada

3 Alberta Transplant Institute (ATI), AB, Canada

4 Canadian Donation and Transplantation Research Program (CDTRP), AB, Canada

5 Stollery Children's Hospital, University of Alberta, Edmonton, AB, Canada

6 Department of Medicine, University of Alberta, Edmonton, AB, Canada

7 Department of Surgery, University of Alberta

8 Department of Medical Microbiology and Immunology, University of Alberta,7,8

[9] Mazankowski Heart Institute, University of Alberta, Edmonton, AB, Canada

AUTHORS

Anne M. Halpin: concept, design, HLA and transfusion data collection, analysis, manuscript preparation, editing

Susan Nahirniak: transfusion data collection and editing

Patricia M. Campbell: HLA data collection and editing

Simon Urschel: Review and editing

Daniel H. Kim: review and editing

Lori J West: review and editing

Tara Pidorochynski: clinical data collection, statistics and editing

H. Buchholz: review and editing

Jennifer Conway: concept, design, clinical data collection, analysis, manuscript preparation, editing

Disclosures:
The Authors have no relevant financial disclosures and or conflict of interest

Funding:
This research has been facilitated by the Women and Children's Health Research Institute through the generous support of the Stollery Children's Hospital Foundation.

The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

KEYWORDS

Human leukocyte antigen
Panel reactive antibody
Sensitization
Ventricular assist device
Transfusion
Blood products
Heart transplantation
Pediatric
Adult

ABBREVIATIONS

AMR	Antibody-mediated rejection
BiVAD	Biventricular assist device
BSA	Bovine serum albumin
CAV	Coronary allograft vasculopathy
CDC	Complement dependent cytotoxicity
CI	Confidence interval
cPRA	Calculated panel reactive antibody
DSA	Donor specific antibody
ECMO	Extra-corporeal membrane oxygenation
ELISA	Enzyme linked immuno-adsorbent assay
HLA	Human leucocyte antigen
IQR	Interquartile range
ISHLT	International Society for Heart and Lung Transplantation
Kg	(Kilograms)
LVAD	Left ventricular assist device
MFI	Mean fluorescent intensity
PRA	Panel reactive antibody
PRBC	Packed red blood cells
SD	Standard deviation
RVAD	Right ventricular assist device
TRALI	Transfusion related acute lung injury

UNOS	United Network for Organ Sharing
VAD	Ventricular assist device

2.1 ABSTRACT

Background: Ventricular assist device (VAD) therapy has become an important tool for end-stage heart failure. VAD therapy has increased survival but is associated with complications including the development of human leukocyte antigen (HLA) antibodies. We sought to determine the incidence of HLA antibody development post-VAD insertion, across the age spectrum, in patients receiving leukocyte-reduced blood products, with standardized HLA antibody detection methods and to investigate factors associated with antibody development.

Methods: This was a retrospective analysis of all patients who underwent durable VAD placement between 2005-2014. Inclusion criteria included availability of pre- and post-VAD HLA antibody results. Associations between HLA antibody development in the first year post-implant and patient factors were explored.

Results: 39 adult and 25 pediatric patients made up the study cohort. Following implant, 31% and 8% of patients developed new Class I and Class II antibodies. The proportion of newly sensitized patients was similar in adult and pediatric patients. The Class I HLA panel reactive antibody (PRA) only significantly increased in adults. Pre-VAD sensitization, age, sex (pediatrics), and transfusion were not associated with the development of HLA antibodies.

Conclusion:

In a cohort of VAD patients receiving leukocyte-reduced blood products and standardized HLA antibody testing, roughly one-third developed new Class I antibodies in the first year post-implant. Adults showed significantly increased Class I PRA following VAD support. No patient-related factors were associated with HLA antibody development. Larger prospective studies are required to validate these findings and determine the clinical impact of these antibodies following VAD insertion.

2.2 INTRODUCTION

Ventricular assist device (VAD) therapy has become an important tool for the treatment of end-stage heart failure.(Adachi et al. 2015; Steiner et al. 2017) While VAD therapy has increased the window for transplantation and survival of patients with heart failure, it has also been associated with a number of complications including the development of human leukocyte antigen (HLA) antibodies.(Alba et al. 2015; Askar et al. 2013; McKenna David H et al. 2002; O'Connor et al. 2010) A number of factors are suggested to be associated with the development of HLA antibodies in VAD patients including exposure to blood products and blood-device interaction.(George et al. 2008) However, it is unclear to what degree these factors play a role in HLA antibody development, especially given the conflicting reports of antibody development after blood product exposure.(Alba et al. 2015; Askar et al. 2013; McKenna David H et al. 2002; Moazami et al. 1998) Exposure to donor leukocytes and platelets is believed to the main stimulus in HLA antibody production. Leukocyte reduction strategies have been broadly implemented to reduce febrile transfusion reactions and reduce transmission of viruses and have resulted in decreased rates of HLA allo-immunization.(Bianchi et al. 2016; D'alessandro 2016; Murphy 2013; Seftel et al. 2004) Contradictory reports related to blood exposure and HLA antibody production may stem from inconsistent practices regarding strategies to minimize leukocytes in blood products. The investigation of allo-immunization following VAD implant has also been complicated by the evolution in HLA antibody testing from complement dependent cytotoxicity (CDC) testing, to enzyme-linked immune-adsorbent assays (ELISA), flow cytometry, and Luminex-based

testing.(Tinckam 2009) These methods have become increasingly sensitive over time; additionally, each method has potential interferences that can affect the accuracy of the testing method.(Coppage 2013; Reynolds and Tinckam 2016; Sullivan, Gebel, and Bray 2017) Studies measuring HLA antibodies post-VAD have used a combination of all of these methods. This study investigates the development of HLA antibodies in a VAD patient population receiving only leukocyte-reduced blood products, using standardized testing with solid phase, bead-based HLA antibody detection methods. We sought to determine the incidence of new HLA antibody detection across the age spectrum and to examine risk factors, including the role of blood product exposure, associated with HLA antibody production following VAD insertion.

2.3 MATERIALS AND METHODS

2.3.1 Patients

This was a retrospective analysis of all patients who underwent a durable VAD placement over a ten-year period (2005-2014) at the University of Alberta hospitals and were implanted as a bridge to transplant or transplant candidacy. Exclusion criteria included prior transplantation and lack of HLA antibody testing. Of 99 eligible patients in the study time frame, 19 were excluded due to lack of any samples submitted for HLA testing, and another 16 due to lack of either pre- or post-VAD samples submitted for testing. The final cohort of patients includes 64 patients (39 adult and 25 pediatric) for whom pre- and post-VAD sample HLA antibody data were available. Pre-implant sera collection occurred on average 31 days prior to VAD implantation (33 days for adults and 27 days for pediatric patients). Post-implant samples were collected within two months of

implant, as per protocol, with additional samples sent within the first year post-implant as per discretion of the transplant team. HLA antibody data were collected until an end-point of death, transplant, explant or minimum required observation period of one-year post-implant, with follow-up ending at December 31, 2015.

2.3.2 Transfusion Data

Complete transfusion data were collected for all patients. All blood products received were documented from 30 days pre-VAD to 12 months post-VAD. Data regarding both the number of units and the volume of each product were collected for packed red blood cells (PRBC), plasma, and platelet transfusions. Universal pre-storage leukoreduction was implemented in Canada in August of 1999; as such, all blood products utilized in the study timeframe were leukoreduced.(Seftel et al. 2004) In October of 2007, Canadian Blood Services implemented a strategy across Canada to reduce transfusion-related acute lung injury (TRALI).(Growe, Petraszko, and Bigham 2008) Because TRALI is believed to be mediated by the presence of HLA and human neutrophil antibodies in donor plasma and platelets(Bux and Sachs 2007), predominantly male donors became the source used for blood products. This strategy reduces the likelihood of HLA antibodies present in serum as female donors are more likely to have circulating HLA antibodies following exposure to paternal antigens in pregnancy.(Triulzi et al. 2009) Therefore, study patients who received pre- or post-implantation blood plasma products after October, 2007 would have received male plasma and therefore were less likely to have received blood products containing passive HLA antibodies.

2.3.3 HLA Antibody Testing and Analysis

Solid phase, bead-based antibody detection methods were used for HLA antibody testing. Antibody screening was performed using FlowPRA® or LABScreen® Single Antigen assays (One Lambda, Thermo Fisher) (n=24) and Luminex single antigen bead testing was performed for confirmation of positive screens and antibody specificity determination (n=40). As per local protocol, patient sera with high negative control bead reactivity were treated with AdsorbOut™ to reduce non-specific patterns of reactivity. A mean fluorescent intensity (MFI) of 1000 was used for reporting HLA antibody specificities, with the exception of C locus antibodies for which a 3000 MFI threshold was used. Antibody specificity patterns reflective of cryptic epitopes were not considered positive.(El-Awar, Jucaud, and Nguyen 2017; Morales-Buenrostro et al. n.d.) HLA typing was performed on all patients with antibodies detected to rule out self-reactive antibodies.

To measure the Class I or II panel reactive antibody (PRA) values from single antigen bead results, the Canadian cPRA Calculator was utilized.(Services n.d.) This tool calculates the PRA value based on donor antigen frequencies collected for the Canadian Transplant Registry and includes HLA A, B, C, (HLA Class I) as well as HLA DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 (HLA Class II). Class I and II cPRA values were calculated separately and will be referred to as 'PRA' for the purposes of this study.

Sensitization pre-VAD was defined as either Class I or Class II PRA >10%; patients were considered highly sensitized if either Class I or Class II PRA was >80%. Following VAD insertion, the PRA was considered increased and the patient was categorized as having

new antibodies if the PRA values increased by 10% or greater or if a new antibody specificity was identified. HLA antibody results were grouped by the following time frames: pre-VAD, 0-30, 31-90, 91-180, and 181-365 days post-VAD.

The development of new Class I or II HLA antibody was investigated for correlation to age, pediatric *vs*. adult, sex (pediatric only), use of pre-VAD mechanical support and transfusion information (platelets, PRBC, and plasma), as well as time.

2.3.4 Pre-Transplant Management

Practices varied over time with respect to approach to management of pre-transplant HLA antibodies. In the adult program, desensitization is not a common practice at this center. In the pediatric program starting in 2010, desensitization occurred in patients with a PRA >80% pre-transplant.

2.3.5 Post-Transplant Definitions

Acute cellular rejection was defined using the 2005 International Society for Heart and Lung Transplantation (ISHLT) standardized cardiac biopsy grading, with rejection episodes grade >1R recorded during the first year post transplant.(Stewart et al. 2005) Antibody-mediated rejection (AMR) was also defined using the 2005 or 2013 ISHLT Grading.(Berry et al. 2013; Stewart et al. 2005) Coronary allograft vasculopathy (CAV) was defined using the 2010 ISHLT grading system for allograft vasculopathy.(Mehra et al. 2010)

2.3.6 Statistical Methods

Descriptive statistics were used to report the patient characteristics at baseline and selected time intervals. Continuous data were summarized by the mean and the two-sided 95% confidence interval (CI) of the mean (normal approximation), standard deviation (SD), median, interquartile range, minimum and maximum. Categorical data were presented by absolute and relative frequencies (n and %). Change in %PRA pre- *vs* post-VAD implantation were compared using Wilcoxon signed rank test because of the small sample size and non-normally distributed data. The change in proportions of patients with Class I and Class II antibodies detected pre- and post-implantation were compared using McNemar's test. Generalized linear mixed models with binary outcome were performed to measure the effects of selected independent variables on development of new antibodies (Class I or Class II). Repeated measures were used to adjust for data from the same patient over time. SAS software, version 9.4 (SAS Institute Inc, Cary, NC, USA) was used for analysis.

2.4 RESULTS

2.4.1 Demographics

There were 39 adult and 25 pediatric patients in the study cohort; details are described in Table 1. The median age was 54.7 (IQR 48.2-60.2) years (adult) and 3.1 (IQR 0.9-8.4) years (pediatric) with the majority in both groups being male (90% adult and 60% pediatric). The average weight for adults was 82.2 ± 20.9 kilograms (kg) and 19.9 ± 17.9 kg for pediatric patients. The most common diagnosis was cardiomyopathy (n=53) and this was true for both patient populations (adults n=34, pediatrics n=19). There were four

patients with congenital heart disease all of whom were pediatric patients. Eleven patients were on mechanical support prior to VAD implantation. Five of these patients had a short-term VAD and six patients were on extra-corpeal membrane oxygenation (ECMO). The most common device type used in adults was an intra-corporeal continuous flow device (n=32, 82%) with the rest having a para-corporeal pulsatile device (n=7). Device types included Abbott Heartmate™ II (n=26), HeartWare™ HVAD™ system (n=6) and Thoratec PVAD (n=7). The majority of pediatric patients had a pulsatile para-corporeal device (Berlin Heart EXCOR®) (n=20, 80%) with the rest an intra-corporeal continuous flow device (HeartWare™ HVAD™ system). The majority of implants were an isolated LVAD (n=56, 88%) with two RVADs and six BiVADS making up the remaining devices.

2.4.2 Blood Product Utilization

All patients received blood products either during or following VAD implantation. The average number of platelets units was 1.6 ± 5.5, PRBCs 3.9 ± 11.8 and plasma 1.9 ± 6.9 in the first 30 days following implantation. During the first year post-VAD implantation the number of units transfused increased to an average number of platelets 4.5 ± 6.5, PRBC 18.0 ± 12.9 and plasma 8.9 ± 6.7. The majority of blood product use occurred within 30 days of implantation (Figures 1 and 2); this was true for both the adult and pediatric populations. Supplemental Figure 1 shows that the majority of blood products (all products combined) occur on the day of implant.

2.4.3 HLA Antibody Development

Samples were received for HLA antibody analysis in all study timeframes although not all patients had samples in each time category. All patients had pre-VAD antibody assessment; there were 43, 41, 37, and 30 samples in the 0-30, 31-90, 91-180, and 181-365 days post-VAD, respectively.

Prior to VAD therapy the average PRA for adults was 7±13% and 3±9% for Class I and II antibodies, respectively (Table 2). The proportion of adult patients classified as sensitized (PRA >10%) at the pre-VAD time point was 18% for Class I and 10% for Class II. There were no highly sensitized patients (PRA >80%) in the adult population prior to implantation. In the pediatric cohort the average pre-VAD Class I PRA was 9±24% and Class II 4±20%. Sixteen percent of the pediatric patients were classified as sensitized pre-VAD for Class I and 4% Class II; 4% of the pediatric patients were highly sensitized for Class I and Class II antibodies.

Comparing the pre-VAD to post-VAD PRA, there was a statistically significant difference only in the adult Class I PRA (7±13% vs. 23±33%, <0.0001); no significant PRA increase was observed for Class II PRA. No significant change was seen in the pediatric patients for Class I or II PRA (Table 2). There was a significant increase in the proportion of adult patients with a PRA greater than 10% from the pre- to post-VAD measurements (p=.002) but a significant difference was not detected for the proportion of adult patients with a PRA greater than 80%. No significant increase in the proportion of

sensitized patients was observed for the adult Class II PRA or the Class I or II PRA in the pediatric cohort before and after implantation.

Figure 2 demonstrates the change in %PRA for Class I and II antibodies for each individual adult (3A and 3C) and pediatric patient (3B and 3D). Many patients had 0% PRA values pre- and post-VAD. Some patients showed an increase in the %PRA post-implantation, some showed no change, and in three cases, there was an observed decrease of more than 10% PRA following implantation.

Following VAD implantation, 31.2% (n=20) of patients were categorized as developing new Class I antibodies and 12.5% (n=8) developed new Class II antibodies. Of patients with new antibodies, 27.3% (6/22) produced both new Class I and II HLA antibodies. Similar proportions of adult and pediatric patients developed Class I (33.3% vs. 28%, p-value 0.65) and Class II antibodies (12.8% vs. 12%, p-value 0.92). Although single antigen bead testing data were not available for all patients, only two of the 25 patients classified as having new antibodies were tested by FlowPRA. All other patients classified as having new HLA antibodies were tested using single antigen beads.

The majority of all new Class I and Class II HLA antibodies in adults and children were detected within 90 days of implantation, most in the first 30 days (Figure 3). In the first 90 days after implantation, 34.5% (n=19/55) of patients produced new Class I antibodies and 10.9% (n=6/55) new Class II antibodies. New Class I antibodies were produced in a similar proportion of adult and pediatric patients (36.7% *vs.* 31.8%, p=0.7). Class II

antibody development occurred less frequently than Class I in both adult and pediatric populations, with no significant difference between the two groups, (adult 12.1% vs. pediatric 9.1%, p=0.99).

2.4.4 Factor Associated with HLA Antibody Development

As described above all patients were exposed to blood products. Blood product use, age (continuous variable), sex (for pediatric patients only), pre-VAD mechanical support and time post-implantation were analyzed using logistic regression to determine the associations with Class I and Class II HLA antibody production. Age, sex, pre-VAD mechanical support and exposure to the different blood products were not associated with the development of either Class I or II HLA antibodies following VAD implantation (Table 3). The only significant variable associated with the development of HLA antibodies post-VAD was time on VAD support; with the risk of developing antibodies occurring as an early phenomenon. The odds of producing Class I antibodies in the first 30 days, was 17.5 fold higher compared to the time period of 91-180 days post-implant (OR 17.5, CI 2.1 to 145.8, p-value 0.009) (Table 3). This observation also held true when Class I antibody production was compared in the first 30 days *vs* 181-365 days post-implant (OR 14.1, CI 1.7 to 117.9, p-value 0.02). There were no associations between the development of Class II HLA antibodies with any of the factors examined.

Due to the observation that the majority of HLA antibody development in most patients occurred early post-implant, blood product exposure in the 30 days before and after VAD implantation was calculated and compared between patients who did or did not develop

new Class I or II antibodies. The average number of units of platelets transfused during this timeframe was 5.2 ± 5.2 for those who produced Class I antibodies compared to 4.0 ± 5.4, for those that did develop these antibodies. In the same time frame, the number of units of PRBC transfused was 16.8 ± 10.5 *vs* 14.0 ± 18.2 and plasma 8.5 ± 7.8 *vs* 7.9 ± 10.7. In the evaluation of Class II antibody development, the average number of platelet units a patient was exposed to was 3.2 ± 1.8 for those who made new Class II antibodies *vs.* 4.6 ± 5. for those who did not. PRBC exposure was 14.2 ± 9.3 units *vs* 15.1 ± 16.6, and plasma 6.5 ± 7.2 *vs.* 8.3 ± 10.0 in these same patients.

The impact of cardiac diagnosis on post-VAD HLA antibody development could not be examined accurately as the majority (83%) of the patients had cardiomyopathy. There was an additional limitation in examining sex-related differences in HLA sensitization in the adult population as the vast majority of patients were male. Although not part of the logistic analysis, the presence of pre-VAD HLA antibodies was examined to determine whether there was an association with production of either Class I or II antibodies at any time period in the first year post-implantation. There were 12 patients with Class I or II PRA>10% prior to VAD implantation. Of these patients, five developed either new Class I or II antibodies after implantation. There was no significant difference in the proportion of patients who developed new antibodies in the first year after implant (either Class I or II) in those who were sensitized pre-implant (Class I or II PRA>10%) (n=5/14) compared to those who did not develop antibodies (n= 14/51) (35.7% vs. 27.5%, p=0.55).

2.4.5 Antibody Persistence

Although the numbers were too small for formal statistical analysis, the persistence of HLA antibodies after first detection was examined in all patients with a post-VAD PRA ≥10%. In the adult cohort 9/10 patients with an increased Class I PRA had subsequent HLA testing and 89% of the patients' PRA remained high. For the four adults with increased Class II PRA, all subsequent values remained high in follow-up testing. In the pediatric cohort, six patients had increased Class I PRA post-VAD and five had follow-up samples. Sixty percent of the patients (3/5) had ongoing increased Class I PRA. Only three pediatric patients had increased Class II PRA and of these two had follow-up testing and both had become negative over time.

2.4.6 Outcomes

Patients were supported on device therapy for an average for 220 ± 140 days before reaching an endpoint of death (n=9), transplant (n=47), one year on VAD therapy (n=7) or weaned off device (n=1). For those patients listed for transplant, the median wait time for adults was 142.5 days (IQR 70.5-462.8) and for pediatric 137 days (IQR 53-317.5). When examined by sensitization status post-VAD, the waitlist times were as follows: in adults, the median wait time was 201 days (IQR 27-706) for those that that developed new antibodies as compared to 132 days (IQR 73-304) for those that did. For the pediatric patients, the median wait time was 308 days (IQR 176-514) for those that developed new antibodies compared to 89 days (IQR 49-262) for those that did not develop new antibodies in the first year. Only one of the pediatric patients underwent attempted desensitization but the treatment was not tolerated and therefore discontinued.

Of those that produced new HLA antibodies post-implant, thirteen were transplanted, seven died, and two patients were still on VAD support at the end of the study period. In the adult population, 26 were transplanted, seven died on the device, and six remained on the device at the end of the study period. In the pediatric population, 21 patients were transplanted, two died on the device, one patient remained on the device at the end of the study period, and one was weaned for recovery at 145 days post-VAD implant.

For those adults who underwent transplant and for whom one-year follow-up was available, 31.6% (n=6/19 patients) developed grade ≥2R cellular rejection in the year post-transplant; only two of these adults had developed new HLA antibodies following VAD implantation. There were no AMR episodes in the first year post-transplant but three patients developed CAV 1 as detected by routine angiography and none of these patients had developed new HLA antibodies after VAD implantation and all had a negative pre-transplant T and B cell flow crossmatch. In the pediatric patients who were transplanted and had one year of follow-up, 15% (n=3/20) developed grade ≥2R cellular rejection; two of these patients had developed new HLA antibodies after VAD implantation. Two of the pediatric transplant recipients had AMR; one patient had pAMR1 (H+) and the other pAMR1 (I+). Neither patients had developed HLA antibodies post-VAD implant; one did develop *de novo* weak Class II donor specific antibody (DSA) and the other Class I and II DSA post-transplant and both had negative pre-transplant T and B cell flow crossmatch. None of the pediatric transplant patients had detectable CAV within the first year.

2.5 DISCUSSION

To date studies on the proportion of patients that produce new HLA antibodies after VAD implantation have reported inconsistent results. This study focused on development of HLA antibodies in patients undergoing VAD therapy in a single center in which patients received only leukocyte-reduced blood products and standardized bead-based, solid phase HLA testing methods. In this context, we observed that 31% of all patients developed new Class I antibodies and just over 10% developed new Class II HLA antibodies following VAD implantation. These findings are consistent with some previous reports such as a recent analyses of the pre-transplant UNOS registry data.(Castleberry et al. 2016; Magdo et al. 2017) However, these large registry analyses do not compare a pre- *vs* post-VAD change in antibody status but rather compare patients who did and did not undergo mechanical circulatory support. O'Connor et al reported a much higher frequency of new HLA antibodies of 69% in a small cohort of pediatric patients (n=13) but it is not clear if non-specific patterns were ruled out or if high background sera were adsorbed, as was done here.(O'Connor et al. 2013) While our frequency of new antibodies is lower than other reports, there is one previous adult study that reported a <10% incidence of new antibody development following VAD insertion, with no patients developing a PRA >50%.(Coppage et al. 2009) This study used a comparable approach to blood products, specifically the use of leukoreduced blood products, but current bead-based solid phase HLA antibody detection methods were not used.

Discrepancies between studies may stem from different devices used, different patient populations or potentially differences in HLA antibody detection methods. Some previous studies have utilized complement dependant cytotoxicity or ELISA methods, known to be both less sensitive as well as prone to non-specific positive results compared to the methods used in our institution.(Castleberry et al. 2016; Coppage et al. 2009; Magdo et al. 2017) This inconsistency in HLA antibody detection methods has been a major criticism of many of the studies that have measured the impact of VAD therapy on HLA antibody development.(Yang et al. 2009)[,5] For this reason, only patients tested by bead-based, solid phase detection methods were included in this study, as recommended for clinical testing.(Tait et al. 2013)

Besides issues with detection methods, previous studies have shown that VAD patients developed high or non-specific reactivity to bovine serum albumin (BSA).(Newell et al. 2006; Nikaein et al. 2012) As such, the reduction of background reactivity by adsorption may be of particular relevance in this population and is a standard practice at our institution. Furthermore, the utility of bead-based antibody detection assays, as used here, has been compared to ELISA-based methods with ELISA testing having a higher likelihood of resulting in false positive results.(Yang et al. 2009) As our methods were all bead-based and included controls for background reactivity, false positive results are less likely than previous studies using less robust methods. The sensitivity of modern assays is also higher than CDC or ELISA making comparison of studies based on previous methodologies difficult from the perspective of specificity and sensitivity.(Coppage et al. 2009; Newell et al. 2006; Tait et al. 2013; Tinckam 2009) Although the sensitivity of the

modern assays may be higher, the clinical relevance of antibodies detected is less clear. While positive CDC testing is a result of complement-mediated damage to the donor cells, this is not the case with solid phase testing. Therefore the ability of these antibodies to bind complement and cause cell damage is difficult to predict.

There are few previous single center studies using only solid phase assays to evaluate the impact of VAD implantation on HLA antibody development in *both* adult and pediatric populations. In this study, similar proportions of both adult and pediatric patients developed new Class I and II HLA antibodies post-implantation. Although the detection and development of new individual antibodies is important, the PRA is a useful tool as it provides a more quantitative measure of the sensitization level and the likelihood of finding a compatible donor. As a high PRA decreases the suitable donor pool, there is increased risk of longer waiting time and increased waitlist mortality.(Mahle et al. 2011; Yang et al. 2013) We observed a statistically significant increase in the Class I PRA following VAD implant in only in adults and no change in Class I or II %PRA in children. Younger patients have previously been found to develop fewer *de novo* HLA antibodies post-transplant.(Urschel et al. 2010) This similar observation to our study may be a reflection of fewer previous sensitizing events or a less mature immune system.

It is important to consider that the absence of detectable HLA antibodies prior to implant may not be a true reflection of the degree of sensitization from prior exposure to non-self HLA antigens. The PRA provides a snapshot of detectable antibodies at the time of assessment and does not provide insight into antibodies that may have been present

previously but are no longer detectable.(Karahan, Claas, and Heidt 2015; Tambur et al. 2018) Previous sensitizing events such as prior transfusions, surgeries, and pregnancies increase the chance of exposure to non-self HLA antigens. While these exposures may be less common in the majority of pediatric patients, a unique group of patients who have undergone previous cardiac surgeries to address congenital malformations may have been exposed to non-self HLA antigens through previous transfusions and/or homograft tissue.(Meyer et al. 2005) At our center, glutaraldehyde treated homografts are used to reduce HLA sensitization in infants undergoing the Norwood procedure.(Laing et al. 2010) While it is possible the post-VAD increase in %PRA seen following implantation is driven by the inflammatory processes around VAD implantation, it also may reflect an anamnestic response from previous exposure to foreign HLA antigens. Pre-VAD HLA sensitization has previously been reported to be a risk factor for development of HLA antibody following VAD implantation.(Alba et al. 2015; Kwon et al. 2015) This pattern was not observed in our patient population. The patients for whom pre-VAD HLA were not detected were not less likely to be free of HLA antibodies post-VAD. This finding could reflect low titer HLA antibodies not detected in the pre-VAD sample that are increased in titre as a result of inflammation or contact to artificial surfaces in the context of VAD implantation. Alternatively, these antibodies may reflect new Class I and II HLA antibody development post-VAD implantation secondary to exposure to new HLA antigens. Further information on sensitizing events over the patients lifespan prior to VAD implantation as well as new assays to measure cellular immune responses may provide a better understanding of this mechanism.(Karahan et al. 2018, 2015)

The development of new antibodies in this study was an early phenomenon, with the majority of antibodies detected in the first 30 days. While this early detection of antibodies could be due to passive transfer of antibody from blood products, we did not find any association between either the average volume of, or number of, platelet, red cell, or plasma transfusions and development of HLA antibodies. This finding remained true for Class I antibody development when the adult population was analysed separately (data not shown). This lack of association of HLA antibody with use of blood products suggests that the antibodies are less likely developed in response to transfusions or passively acquired from blood products, perhaps due to the early implementation of TRALI prevention strategies.(Growe et al. 2008) In contrast to our findings, some studies previously showed that transfusion at the time of implant is associated with development of HLA antibodies.(McKenna David H et al. 2002; Moazami et al. 1998) Our center has been using universal leukoreduction practices since 1999. This practice has likely resulted in a decrease in immune stimulation from blood products. While some studies have differed from our findings, others also reported no association between blood product exposure and the development of Class I or II HLA antibodies.(Alba et al. 2015; Askar et al. 2013) This variation could stem from differences in transfusion practice between institutions over time, and may contribute to the inconsistencies reported in the literature on HLA antibody development post-VAD implantation.

In adults, most of the HLA antibodies that developed appeared persistent in nature but this was less so for the pediatric cohort. This finding further suggests that VAD sensitization in adults may be more of a memory response than in the pediatric patients.

However, post-implant sample collection was not consistent and future studies with long-term measurement at specific time points would better address whether or not HLA antibodies post -VAD are transient in nature.

Although this study is unique in its inclusion of both adult and pediatric patients, it is limited by its small sample size and under-representation of female adult patients. As such, sex-related differences in HLA sensitization in the adult population could not be measured. This limitation is consistent with most VAD studies as adult males are much more likely to undergo VAD therapy than females. Due to the known risk of allo-immunization during pregnancy, particularly multiple pregnancies(Honger et al. 2014), inclusion of higher numbers of adult females, could reveal sex as a risk factor for post-VAD sensitization, as was shown in a previous adult study.(Alba et al. 2015) Data analysis was also limited by the small patient numbers, which reduced our ability to perform multivariate logistic regression analysis; this may also have resulted in decreased statistical power and increased risk of Type II errors. In addition, because these data have been collected retrospectively, the time points for HLA antibody testing are not consistent despite having a protocol in place. Not every patient had collection of multiple serum samples in the post-VAD period thus a sustained PRA response could not be fully assessed. In addition, follow-up post-transplant was limited to the first year and therefore we are unable to comment on complications that develop following this timeframe. These limitations support the need for a larger prospective, longitudinal study.

2.6 CONCLUSION

In a VAD patient population receiving only leukocyte-reduced blood products, roughly one-third developed new Class I antibodies in the first year post-implant as detected by standardized, modern HLA antibody detection methods. Production of new HLA antibodies was an early phenomenon, occurring within most commonly within 30 days after VAD implant. In addition, only adult patients had a significant increase in Class I PRA post-implant, with no significant increase in Class II PRA and neither Class I or II PRA was significantly increased in the pediatric population. Production of new antibodies was not significantly associated with age, blood product exposure, pre-VAD mechanical support or pre-VAD sensitization. Larger, prospective studies in both the adult and pediatric populations are required to validate these findings and to develop a better understanding of risk factors for HLA sensitization, the impact on transplant outcomes, and the persistence of these antibodies over time in patients following VAD implantation.

2.7 ACKNOWLEDGEMENTS

This project was funded from the generous support of the Women and Children's Health Research Institute and the Department of Pediatrics at the University of Alberta. We gratefully acknowledge the support of Maryna Yaskina for assistance with statistical analysis as well as Morgan Sosniuk and Sudeshna Bhattacharya for assistance with transfusion data collection.

2.8 References

Adachi, Iki, Muhammad S. Khan, Francisco A. Guzmán-Pruneda, Charles D. Fraser, Carlos M. Mery, Susan W. Denfield, William J. Dreyer, David L. S. Morales, E. Dean McKenzie, Jeffrey S. Heinle, and Charles D. Fraser. 2015. "Evolution and Impact of Ventricular Assist Device Program on Children Awaiting Heart Transplantation." *The Annals of Thoracic Surgery* 99(2):635–40.

Alba, Ana C., Kathryn Tinckam, Farid Foroutan, Laerke M. Nelson, Finn Gustafsson, Kam Sander, Hellen Bruunsgaard, Sharon Chih, Helen Hayes, Vivek Rao, Diego Delgado, and Heather J. Ross. 2015. "Factors Associated with Anti-Human Leukocyte Antigen Antibodies in Patients Supported with Continuous-Flow Devices and Effect on Probability of Transplant and Post-Transplant Outcomes." *Journal of Heart and Lung Transplantation* 34:685–92.

Askar, Medhat, Eileen Hsich, Patrick Reville, Amy S. Nowacki, William Baldwin, Suzanne Bakdash, Jenna Daghstani, Aiwen Zhang, Lynne Klingman, Nicholas Smedira, Nader Moazami, David O. Taylor, Randall C. Starling, and Gonzalo Gonzalez-Stawinski. 2013. "HLA and MICA Allosensitization Patterns among Patients Supported by Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 32(12):1241–48.

Berry, Gerald J., Margaret M. Burke, Claus Andersen, Patrick Bruneval, Marny Fedrigo, Michael C. Fishbein, Martin Goddard, Elizabeth H. Hammond, Ornella Leone, Charles Marboe, Dylan Miller, Desley Neil, Doris Rassl, Monica P. Revelo, Alexandra Rice, E. Rene Rodriguez, Susan Stewart, Carmela D. Tan, Gayle L. Winters, Lori West, Mandeep R. Mehra, and Annalisa Angelini. 2013. "ISHLT CONSENSUS: The 2013 International Society for Heart and Lung Transplantation Working Formulation for the Standardization of Nomenclature in the Pathologic Diagnosis of Antibody-Mediated Rejection in Heart Transplantation." *Journal of Heart and Lung Transplantation* 32:1147–62.

Bianchi, Maria, Stefania Vaglio, Simonetta Pupella, Giuseppe Marano, Giuseppina Facco, Giancarlo M. Liumbruno, and Giuliano Grazzini. 2016. "Leucoreduction of Blood Components: An Effective Way to Increase Blood Safety?" *Blood Transfusion = Trasfusione Del Sangue* 14(2):214–27.

Bux, Jürgen and Ulrich J. H. Sachs. 2007. "The Pathogenesis of Transfusion-Related Acute Lung Injury (TRALI)." *British Journal of Haematology* 136(6):788–99.

Castleberry, Chesney, Farhan Zafar, Tamara Thomas, Muhammad S. Khan, Roosevelt Bryant, Clifford Chin, David L. S. Morales, and Angela Lorts. 2016. "Allosensitization Does Not Alter Post-Transplant Outcomes in Pediatric Patients Bridged to Transplant with a Ventricular Assist Device." *Pediatric Transplantation* 20(4):559–64.

Coppage, Myra. 2013. "Allosensitization and the Ventricular Assist Device: Dual Evolution of Technology." Pp. 67–79 in *Recent Advances in the Field of Ventricular*

Assist Devices, edited by K. Komamura. InTech.

Coppage, Myra, Marc Baker, Lawrence Fialkow, Danielle Meehan, Kelly Gettings, Leway Chen, H. Todd Massey, and Neil Blumberg. 2009. "Lack of Significant de Novo HLA Allosensitization in Ventricular Assist Device Recipients Transfused with Leukoreduced, ABO Identical Blood Products." *Human Immunology* 70(6):413–16.

D'alessandro, Angelo. 2016. "Leucoreduction of Blood Components: Clinical and Molecular Evidence." *Blood Transfusion = Trasfusione Del Sangue* 14(2):212–13.

El-Awar, Nadim, Vadim Jucaud, and Anh Nguyen. 2017. "HLA Epitopes: The Targets of Monoclonal and Alloantibodies Defined." *Journal of Immunology Research* 2017.

George, Isaac, Patrick Colley, Mark J. Russo, Timothy P. Martens, Elizabeth Burke, Mehmet C. Oz, Mario C. Deng, Donna M. Mancini, and Yoshifumi Naka. 2008. "Association of Device Surface and Biomaterials with Immunologic Sensitization after Mechanical Support." *The Journal of Thoracic and Cardiovascular Surgery* 135(6):1372-1379.e1.

Growe, G. H., T. R. Petraszko, and Mark Bigham. 2008. "The Approach Taken to Reducing the Risk of Transfusion Related Acute Lung Injury in Canada." *Asian Journal of Transfusion Science* 2(2):84–86.

Honger, G., H. Cardinal, M. Dieude, A. Buser, I. Hosli, D. Dragun, M. J. Hebert, and S. Schaub. 2014. "Human Pregnancy and Generation of Anti-Angiotensin Receptor and Anti-Perlecan Antibodies." *Transplant International* 27(5):467–74.

Karahan, Gonca E., Frans H. J. Claas, and Sebastiaan Heidt. 2015. "Detecting the Humoral Alloimmune Response: We Need More Than Serum Antibody Screening." *TRANSPLANTATION* 99(5):908–15.

Karahan, Gonca E., Juliette Krop, Caroline Wehmeier, Yvonne de Vaal, Janneke Langerak-Langerak, Dave L. Roelen, Neubury M. Lardy, Frederike J. Bemelman, Ineke ten Berge, Marlies E. J. Reinders, Cees van Kooten, Frans H. J. Claas, and Sebastiaan Heidt. 2018. "An Easy and Sensitive Method to Profile the Antibody Specificities of HLA-Specific Memory B Cells." *Transplantation* (Publish Ahead of Print, 9 November 2018).

Kwon, Murray H., Jennifer Q. Zhang, Joanna M. Schaenman, Martin Cadeiras, David W. Gjertson, Carolyn A. Krystal, Hillel Laks, Abbas Ardehali, Mario C. Deng, Richard J. Shemin, and Elaine F. Reed. 2015. "Characterization of Ventricular Assist Device-Mediated Sensitization in the Bridge-to-Heart-Transplantation Patient." *The Journal of Thoracic and Cardiovascular Surgery* 149(4):1161–66.

Laing, Bryce J., David B. Ross, Steven R. Meyer, Patricia Campbell, Anne M. Halpin, Lori J. West, and Ivan M. Rebeyka. 2010. "Congenital Heart Disease: Glutaraldehyde Treatment of Allograft Tissue Decreases Allosensitization after the Norwood Procedure." *The Journal of Thoracic and Cardiovascular Surgery* 139:1402–8.

Magdo, H. Sonali, Kurt R. Schumacher, Sunkyung Yu, Robert J. Gajarski, and Joshua M. Friedland-Little. 2017. "Clinical Significance of Anti-HLA Antibodies Associated

with Ventricular Assist Device Use in Pediatric Patients: A United Network for Organ Sharing Database Analysis." *Pediatric Transplantation* 21(5):e12938.

Mahle, William T., Margaret A. Tresler, R. E. Edens, Paolo Rusconi, James F. George, David C. Naftel, and Robert E. Shaddy. 2011. "Allosensitization and Outcomes in Pediatric Heart Transplantation." *The Journal Of Heart And Lung Transplantation: The Official Publication Of The International Society For Heart Transplantation* 30(11):1221–27.

McKenna David H, Jr., Ted Eastlund, Miriam Segall, Harriet J. Noreen, Soon Park, Jr. McKenna David H, Ted Eastlund, Miriam Segall, Harriet J. Noreen, and Soon Park. 2002. "Original Article: HLA Alloimmunization in Patients Requiring Ventricular Assist Device Support." *Journal of Heart and Lung Transplantation* 21:1218–24.

Mehra, Mandeep R., Maria G. Crespo-Leiro, Anne Dipchand, Stephan M. Ensminger, Nicola E. Hiemann, Jon A. Kobashigawa, Joren Madsen, Jayan Parameshwar, Randall C. Starling, and Patricia A. Uber. 2010. "International Society for Heart and Lung Transplantation Working Formulation of a Standardized Nomenclature for Cardiac Allograft Vasculopathy—2010." *The Journal of Heart and Lung Transplantation* 29(7):717–27.

Meyer, Steven R., Patricia M. Campbell, Jennifer M. Rutledge, Anne M. Halpin, Lois E. Hawkins, Jonathan R. T. Lakey, Ivan M. Rebeyka, and David B. Ross. 2005. "Use of an Allograft Patch in Repair of Hypoplastic Left Heart Syndrome May Complicate Future Transplantation." *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery* 27(4):554–60.

Moazami, N., S. Itescu, M. R. Williams, M. Argenziano, A. Weinberg, and M. C. Oz. 1998. "Platelet Transfusions Are Associated with the Development of Anti-Major Histocompatibility Complex Class I Antibodies in Patients with Left Ventricular Assist Support." *Journal of Heart and Lung Transplantation* 17(9):876–80.

Morales-Buenrostro, Luis E., Paul I. Terasaki, Lluvia A. Marino-Vazquez, Jar-How Lee, Nadim El-Awar, and Josefina Alberu. n.d. "'Natural' Human Leukocyte Antigen Antibodies Found in Nonalloimmunized Healthy Males." *TRANSPLANTATION* 86(8):1111–15.

Murphy, M. F. 2013. "Managing the Platelet Refractory Patient." *ISBT Science Series* 9(1):234–38.

Newell, H., J. D. Smith, P. Rogers, E. Birks, A. J. Danskine, R. E. Fawson, and M. L. Rose. 2006. "Sensitization Following LVAD Implantation Using Leucodepleted Blood Is Not Due to HLA Antibodies." *American Journal of Transplantation* 6(7):1712–17.

Nikaein, Afzal, Nadim El-Awar, Judson Hunt, Edward J. Rosenthal, Eric Eichhorn, Shelly Hall, James Boehrer, Mitchell Magee, Marc Pieniek, William Brinkman, and Todd Dewey. 2012. "Clinically Irrelevant Circulating Human Leukocyte Antigen Antibodies in the Presence of Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 31(5):443–47.

O'Connor, Matthew J., Terry O. Harville, Bobbie Rhodes-Clark, Sherry E. Pye, Kenneth R. Knecht, Michiaki Imamura, Elizabeth A. Frazier, and W. Robert Morrow. 2013. "Quantification, Identification, and Relevance of Anti-Human Leukocyte Antigen Antibodies Formed in Association with the Berlin Heart Ventricular Assist Device in Children." *Transplantation* 95(12):1542–47.

O'Connor, Matthew J., JonDavid Menteer, Maryanne R. K. Chrisant, Dimitrios Monos, Curt Lind, Selena Levine, J. William Gaynor, Brian D. Hanna, Stephen M. Paridon, Chitra Ravishankar, and Beth D. Kaufman. 2010. "Ventricular Assist Device-Associated Anti-Human Leukocyte Antigen Antibody Sensitization in Pediatric Patients Bridged to Heart Transplantation." *The Journal of Heart and Lung Transplantation* 29(1):109–16.

Reynolds, Ben C. and Kathryn J. Tinckam. 2016. "Sensitization Assessment before Kidney Transplantation." *Transplantation Reviews (Orlando, Fla.)*.

Seftel, Matthew D., Gershon H. Growe, Tanya Petraszko, W. Barrett Benny, Alan Le, Chao-Yong Lee, John J. Spinelli, Heather J. Sutherland, Peter Tsang, and Donna E. Hogge. 2004. "Universal Prestorage Leukoreduction in Canada Decreases Platelet Alloimmunization and Refractoriness." *Blood* 103(1):333 LP – 339.

Services, Canadian Blood. n.d. "Canadian CPRA Calculator." *CPRA Calculator Online Tool*. Retrieved (https://ctr2.transplantregistry.ca/otd-cpra-client/ctr2.jsp).

Steiner, Jill M., Eric V Krieger, Karen K. Stout, April Stempien-Otero, Claudius Mahr, Nahush A. Mokadam, and Joshua L. Hermsen. 2017. "Durable Mechanical Circulatory Support in Teenagers and Adults with Congenital Heart Disease: A Systematic Review." *International Journal of Cardiology* 245:135–40.

Stewart, Susan, Gayle L. Winters, Michael C. Fishbein, Henry D. Tazelaar, Jon Kobashigawa, Jacki Abrams, Claus B. Andersen, Annalisa Angelini, Gerald J. Berry, Margaret M. Burke, Anthony J. Demetris, Elizabeth Hammond, Silviu Itescu, Charles C. Marboe, Bruce McManus, Elaine F. Reed, Nancy L. Reinsmoen, E. Rene Rodriguez, Alan G. Rose, Marlene Rose, Nicole Suciu-Focia, Adriana Zeevi, and Margaret E. Billingham. 2005. "Revision of the 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection." *The Journal of Heart and Lung Transplantation* 24(11):1710–20.

Sullivan, Harold C., Howard M. Gebel, and Robert A. Bray. 2017. "Understanding Solid-Phase HLA Antibody Assays and the Value of MFI." *Human Immunology*.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. "Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation." *Transplantation* 95(1):19–47.

Tambur, Anat R., Patricia Campbell, Frans H. Claas, Sandy Feng, Howard M. Gebel,

Annette M. Jackson, Roslyn B. Mannon, Elaine F. Reed, Kathryn Tinckam, Medhat Askar, Anil Chandraker, Patricia P. Chang, Monica Colvin, Anthony-Jake Demetris, Joshua M. Diamond, Anne I. Dipchand, Robert L. Fairchild, Mandy L. Ford, John Friedewald, Ronald G. Gill, Denis Glotz, Hilary Goldberg, Ramsey Hachem, Stuart Knechtle, Jon Kobashigawa, Deborah J. Levine, Joshua Levitsky, Michael Mengel, Edgar Milford, Kenneth A. Newell, Jacqueline G. O'Leary, Scott Palmer, Parmjeet Randhawa, John Smith, Laurie Snyder, Randall C. Starling, Stuart Sweet, Timucin Taner, Craig J. Taylor, Steve Woodle, Adriana Zeevi, and Peter Nickerson. 2018. "Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group Meeting Report." *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons*.

Tinckam, Kathryn. 2009. "Histocompatibility Methods." *Transplantation Reviews* 23:80–93.

Triulzi, Darrell J., Steven Kleinman, Ram M. Kakaiya, Michael. P. Busch, Philip J. Norris, Whitney R. Steele, Simone A. Glynn, Christopher D. Hillyer, Patricia Carey, Jerome L. Gottschall, Edward L. Murphy, Jorge A. Rios, Paul M. Ness, David J. Wright, Danielle Carrick, and George B. Schreiber. 2009. "The Effect of Previous Pregnancy and Transfusion on HLA Alloimmunization in Blood Donors: Implications for a Transfusion-Related Acute Lung Injury Risk Reduction Strategy." *Transfusion* 49(9):1825–35.

Urschel, S., P. M. Campbell, S. R. Meyer, I. M. Larsen, J. Nuebel, J. Birnbaum, H. Netz, K. Tinckam, T. Kauke, K. Derkatz, J. Y. Coe, J. L. Platt, and L. J. West. 2010. "Absence of Donor-Specific Anti-HLA Antibodies After ABO-Incompatible Heart Transplantation in Infancy: Altered Immunity or Age?" *American Journal of Transplantation* 10(1):149–56.

Yang, J. A., Y. Naka, T. Ota, R. C. Neely, N. Uriel, P. C. Colombo, U. P. Jorde, D. M. Mancini, and H. Takayama. 2013. "Predictors of Prolonged Status 1A Wait Time Prior to Heart Transplant." *The Journal of Heart and Lung Transplantation* 32(4):S143.

Yang, Jeremy, Cynthia Schall, Doug Smith, Lisa Kreuser, Mary Zamberlan, Karen King, and Robert Gajarski. 2009. "Mechanical Circulatory Support: HLA Sensitization in Pediatric Pre-Transplant Cardiac Patients Supported by Mechanical Assist Devices: The Utility of Luminex." *Journal of Heart and Lung Transplantation* 28:123–29.

2.9 Figures and Tables

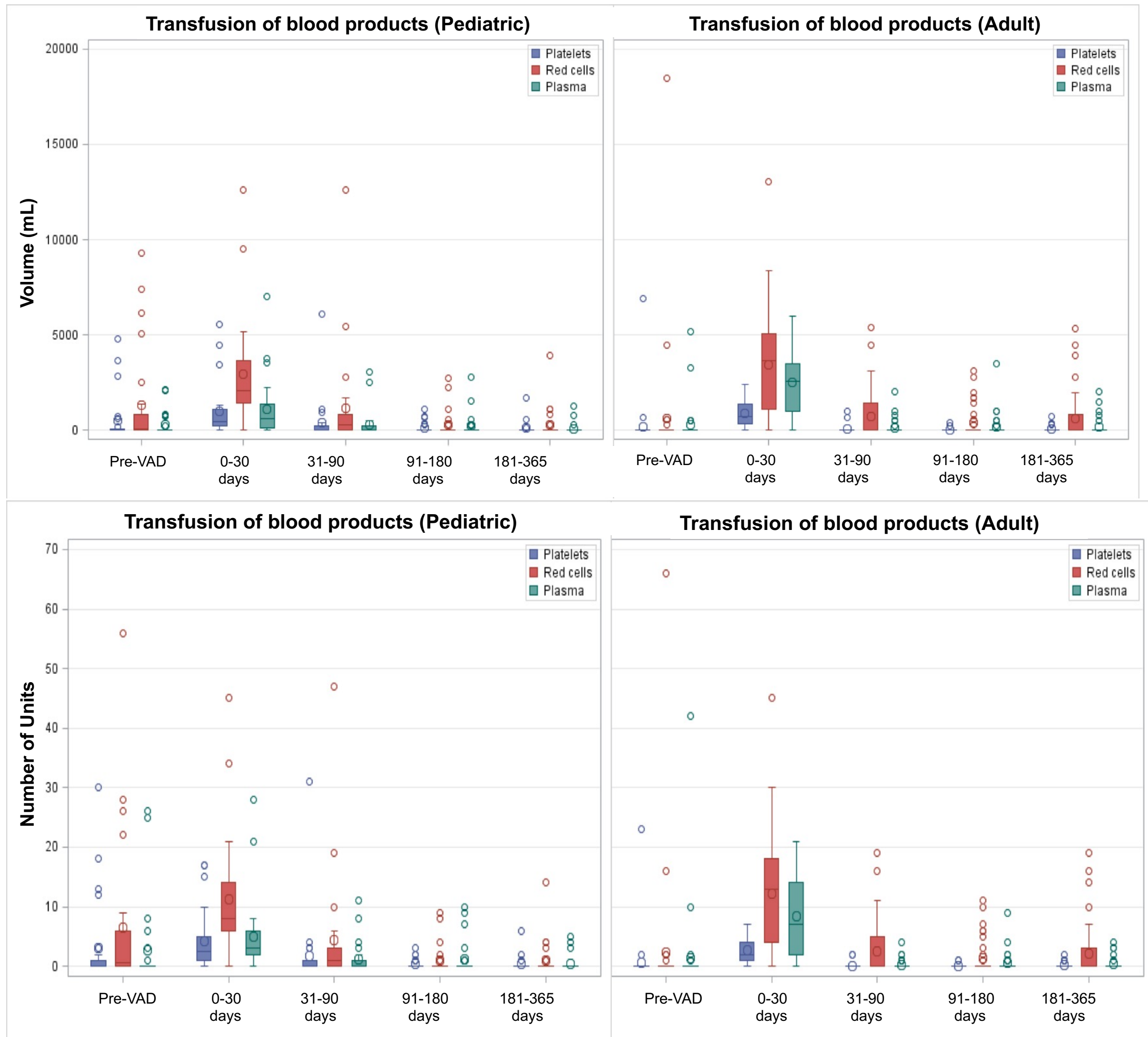


Figure 2-1: Transfusion volumes (milliliters) of platelets, PRBCs, and plasma are shown by study time frame for both pediatric and adult populations (top). Number of units transfused for platelets, PRBCs, and plasma are shown by study time frame for both pediatric and adult populations (bottom). PRBCs, packed red blood cells.

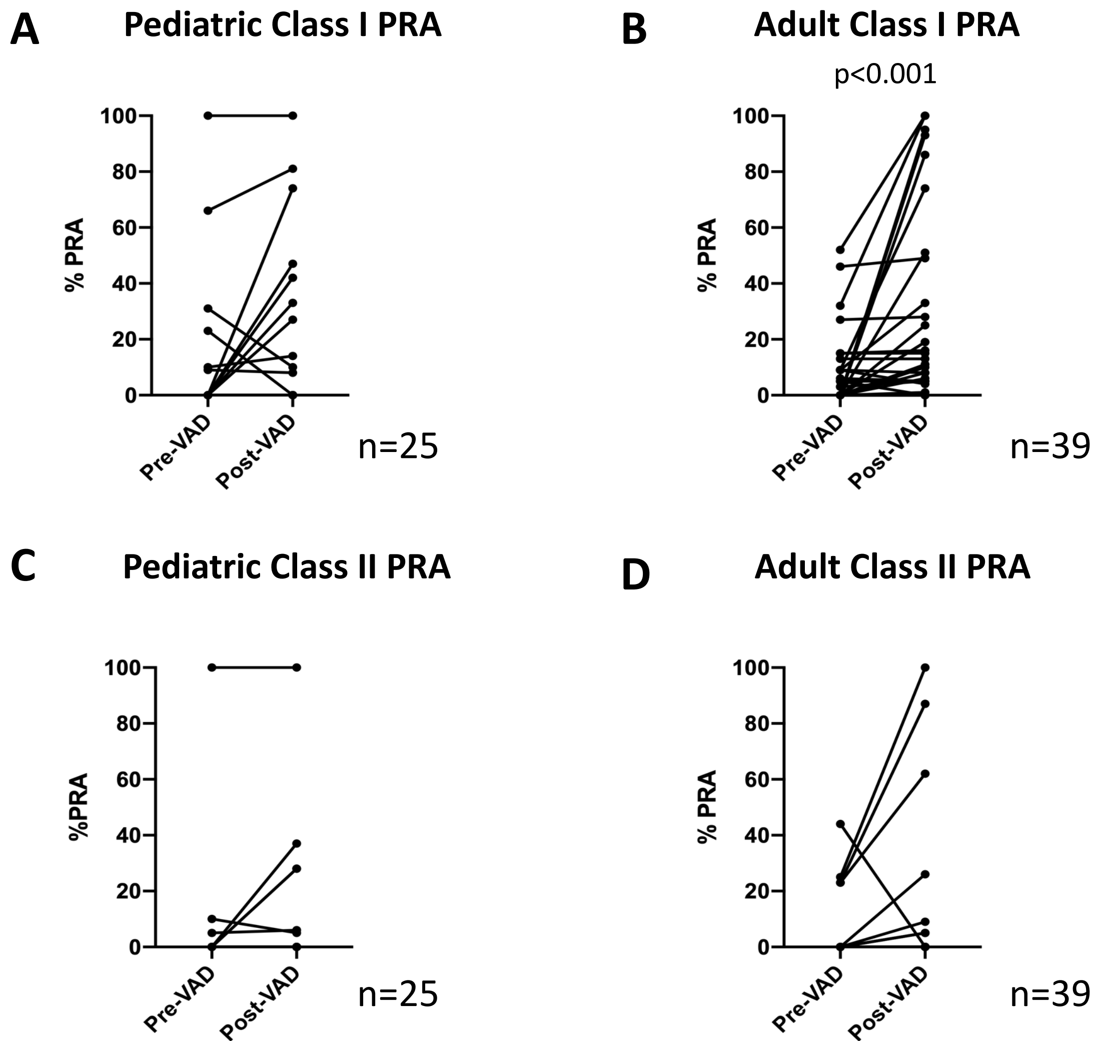


Figure 2-2: Changes in percent PRA for individual pediatric (A and C) and adult (B and D) patients pre- and post-VAD implantation for class I and class II HLA antibodies respectively. The pre-VAD %PRA was measured, on average, 27 days preimplantation for the pediatric patients and 33 days preimplantation for the adult patients. The highest %PRA detected is represented and was measured, on average, 58 days post-implantation for the pediatric patients and 70 days post-implantation for the adult patients. HLA, human leucocyte antigen; PRA, panel-reactive antibody; VAD, ventricular assist device.

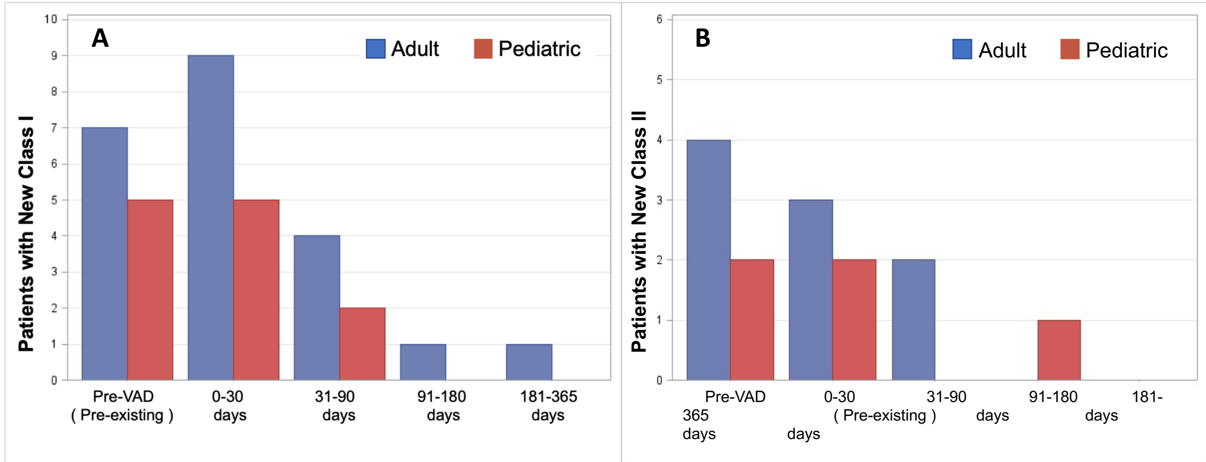


Figure 2-3: The number of patients who developed new class I or II antibodies are shown by time frame (A and B, respectively). The values in the pre-VAD time points are the patients with existing class I or II antibodies at the time of implant.

Table 2-1: Demographics and clinical characteristics

	ADULTS (n=39)	PEDIATRICS (n=25)
Mean Age (years) (±SD)	52.2 (12)	5.2 (5.9)
Median Age (years) (IQR)	54.7 (48.2-60.2)	3.1 (0.9-8.4)
Mean Weight (kg) (±SD)	82.2 (20.9)	19.9 (17.9)
Sex (Male)	35 (90%)	15 (60%)
Diagnosis		
Cardiomyopathy/myocarditis	34 (87%)	19 (76%)
Congenital	0	4 (16%)
Other	5 (13%)	2 (8%)
Previous Cardiac Surgery (yes)	7 (18%)	10 (40%)
Pump Type		
Paracorporal Pulsatile	7 (18%)	20 (80%)
Intracorporeal Continuous	32 (82%)	5 (20%)
Pump Configuration		
LVAD	37 (95%)	19 (76%)
RVAD	0	2 (8%)
BiVAD	2 (5%)	4 (16%)
Pre-Implant ECMO	2 (5%)	4 (16%)
Pre-Implant Short Term VAD	1 (3%)	4 (16%)

LVAD: left ventricular assist device
RVAD: right ventricular assist device
BIVAD: biventricular assist device
ECMO: extracorporeal membrane oxygenator
VAD: ventricular assist device

Table 2-2: Comparison of percent PRA values between preimplant and the peak value in the first year post-VAD implantation. In addition, the proportions of patients sensitized with a PRA >10% and those highly sensitized with a PRA >80% are shown

Patient Population and HLA Class	Median PRA (%) (IQR)		Average PRA (%) (+/- SD)		p-value*	Proportion of Patients with PRA>10%		p-value**	Proportion of Patients with PRA>80%		p-value**
	Pre-VAD	Post-VAD	Pre-VAD	Post-VAD		Pre-VAD	Post-VAD		Pre-VAD	Post-VAD	
Adult											
Class I	0 (0-9)	8 (0-28)	7 (13)	23 (33)	<0.0001	18% (7/39)	44% (17/39)	0.02	0% (0/39)	10% (5/39)	0.06
Class II	0 (0-0)	0 (0-5)	3 (9)	11 (28)	0.07	10% (4/39)	10% (4/39)	1.0	0% (0/39)	5% (2/39)	0.50
Pediatric											
Class I	0 (0-6)	5 (0-27)	9 (24)	20 (30)	0.06	16% (4/25)	36% (9/25)	0.06	4% (1/25)	8% (2/25)	0.32
Class II	0 (0-0)	0 (0-5)	4 (20)	9 (22)	0.19	4% (1/25)	16% (4/25)	0.08	4% (1/25)	4% (1/25)	-

PRA: Panel Reactive Antibody
*Wilcoxon signed-ranked test
**Exact McNemar's test

Table 2-3: Generalized linear mixed model looking at the association between patient clinical characteristics and the development of Class I and II HLA antibodies following VAD insertion

Independent variable		Class I New Antibody Development			Class II New Antibody Development		
		p value	OR	95% CI	p value	OR	95% CI
Age	**All population**	0.2	1.0	(0.99, 1.03)	0.6	1.0	(0.98, 1.04)
	Pediatrics	0.3	1.1	(0.9, 1.2)	0.7	1.0	(0.9, 1.3)
	Adults	0.3	1.0	(0.97, 1.1)	0.7	1.0	(0.9, 1.1)
Sex (pediatric only) Female *vs.* Male		0.8	1.2	(0.2, 6.5)	0.8	0.8	(0.06, 10.4)
Units of platelets		0.5	1.0	(0.9, 1.1)	0.7	1.0	(0.7, 1.3)
Volume of platelets (rescaled by 1000)		0.2	1.3	(0.9, 1.9)	0.9	0.9	(0.3, 2.5)
Units of red cells		0.3	1.0	(0.98, 1.1)	0.9	1.0	(0.9, 1.1)
Volume of red cells (rescaled by 1000)		0.2	1.1	(0.9, 1.2)	0.9	1.0	(0.7, 1.3)
Units of plasma		0.2	1.1	(0.97, 1.1)	0.9	1.0	(0.9, 1.2)
Volume of plasma (rescaled by 1000)		0.09	1.3	(0.96, 1.8)	0.6	1.0	(0.4, 1.6)
Pre-VAD ECMO or ST-VAD		0.93	1.1	(0.2,5.5)	0.8	1.6	(0.06,43.3)
Time post VAD	**Overall time**	0.006	-	-	0.4	-	-
	0-30 days vs. 31-90 days	0.06	2.8	(0.9, 8.4)	0.3	2.5	(0.4, 14.5)
	0-30 days vs. 91-180 days	0.009	17.5	(2.1, 145.8)	0.2	4.9	(0.5, 45.9)
	0-30 days vs. 181-375 days	0.02	14.1	(1.7, 117.9)	0.2	4.0	(0.4, 37.7)

OR: odds ratio
VAD: ventricular assist device
ECMO: extracorpeal membrane oxygenation
ST-VAD: short term VAD

Chapter 3:

Antibodies to Angiotensin II Type 1 Receptor: Investigating Assay Interference in Pediatric Heart Transplant Recipients

Authors

Anne M. Halpin
Sally Abou-Zeki
Simon Urschel
Ingrid Larsen
Bruce Motyka
Lori J. West

Abbreviations

ASHI	American Society for Histocompatibility and Immunogenetics
AT1R	angiotensin II type 1 receptor
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
HLA	human leukocyte antigen
Neu5Gc	N-Glycolylneuraminic acid
VAD	ventricular assist device

Acknowledgements

Reagent funding provided by the Edmonton Civic Employees Research Award, Dr. Lori J West
Anne Halpin received trainee funding from the Canadian Donation and Transplantation Research Program.
Anne Halpin received travel funding from the Women and Children's Health Research Institute to attend the 2017 American Transplant Congress meeting.
The American Society for Histocompatibility and Immunogenetics Proficiency Testing Program provided samples and expertise (in particular Dr. Mary Philogene and Dr. Kelley Hitchman).

Ethics

All patients were consented in the Cardiac Transplantation in Infancy (CTI) study, University of Alberta Health Research Ethics Board, Study ID Pro1408.

3.1 Abstract

Anti-AT1R antibodies have been linked to poor outcomes in some adult renal, heart, and lung transplantation, and to rejection episodes in some pediatric renal and liver transplant studies. However, there are conflicting reports in the literature overall with regard to the relevance of AT1R antibodies in solid organ transplantation. There are especially limited data regarding the frequency and impact of AT1R antibodies in pediatric heart transplantation. Using a commercial ELISA assay, we measured AT1R antibodies in pediatric heart transplant recipients at our centre as well as age- and sex-matched non-transplant controls. We found that 55% of patient sera (84/154) were positive for AT1R antibodies; this level of positivity was mirrored in controls. We also determined that many of the positive AT1R antibody results were reduced when treated with Adsorb Out™, a commercial reagent commonly used to remove non-specific reactivity in histocompatibility solid phase assays. We detected Neu5Gc glycans on the Adsorb Out™ microparticles, suggesting this may be one of the sources of non-specific reactivity removed in this procedure. Our data suggest that the commonly used ELISA AT1R antibody assay may be prone to false positive reactivity, warranting further investigation. Pediatric patients may be particularly prone to this non-specific reactivity in the AT1R commercial assay but studies in older patient populations are also needed to examine this phenomenon further.

3.2 Introduction

There is consensus that donor-specific antibodies to human leukocyte antigens (HLA) are associated with poor transplant allograft outcomes.(Patel and Terasaki 1969; Tait et al. 2013) There is also increasing evidence suggesting a negative impact of autoantibodies on transplant outcomes.(Cardinal et al. 2013; Dragun et al. 2005; Lefaucheur et al. 2019; Tiriveedhi et al. 2013). Antibodies to the angiotensin II type 1 receptor (AT1R) have been studied more widely than other autoantibodies in the context of transplantation, with a potentially detrimental influence first suggested by Dragun (Dragun et al. 2005). In this study, a bioassay was used to measure the presence of AT1R antibodies and assess their impact on neonatal rat cardiomyocytes. Subsequent studies have concurred, but the majority of these used a single commercial enzyme-linked immunosorbent assay (ELISA). There is no publication specifically documenting the development or validation of this assay beyond its description as a sandwich ELISA assay, and the derivation of its AT1R antigens from human AT1R over-expressing Chinese hamster ovary (CHO) cells. (Reinsmoen et al. 2010)

Several AT1R antibody studies in the setting of adult renal transplantation have demonstrated an association with antibody-mediated rejection (Dragun et al. 2005; Lefaucheur et al. 2019; Philogene et al. 2019), whereas others have not (Deltombe et al. 2017; Pinelli et al. 2017). Conflicting results have also been reported in adult heart transplantation: some studies demonstrated an association of AT1R antibodies with worse outcomes (Hiemann et al. 2012), while others did not find this association (Thohan et al. 2020). In pediatric populations, data from kidney and liver transplant patients suggested that AT1R antibodies were associated with

rejection; studies related to heart transplantation are lacking (Pearl and Reed 2019). In the study described herein, we measured the frequency of AT1R antibodies in pediatric heart transplant patients. We hypothesised that pediatric patients may have lower levels of AT1R antibodies than adult transplant patients. We also investigated the possibility of non-specific reactivity in the commercial AT1R ELISA assay.

The specificity of the commercial AT1R assay has been previously investigated.(Halpin et al. 2017; Oaks et al. 2018). Oaks *et al* reported that AT1R reactivity in this ELISA assay could be removed by adsorption of patient sera with CN-Glycolylneuraminic acid (Neu5Gc) and AT1R antibody levels in cardiac transplant patients in that patients with high levels of AT1R antibody also had high levels of anti-Neu5Gc.. Our finding that a very high proportion of not only pediatric heart transplant patients but also non-transplant pediatric controls contained very high levels of AT1R antibody prompted further investigation into the specificity of the ELISA AT1R assay.

3.3 Methods

3.3.1 Samples

Pediatric patients who had undergone heart transplantation between May 2010 and October 2015 for whom pre- and post-transplant serum samples were available were selected for AT1R antibody testing (n=42). Multiple serum samples were included for several patients; in total 154 serum samples were tested from the 42 patients. Samples from age- and sex-matched non-transplanted individuals (n=27) collected from the cardiac catheterization laboratory were included as controls. All samples had been stored at -70°C.

3.3.2 Anti-AT1R antibody measurement

AT1R antibodies were measured using the ELISA AT1R assay (kit lot #10) manufactured by Cell Trend GmbH, distributed by One Lambda Thermo Fisher. This assay kit uses AT1R antigens isolated from human AT1R-overexpressing Chinese hamster ovary (CHO) cells. The kit includes ELISA plate strips onto which AT1R antigens are captured, negative and positive control sera, AT1R calibration standards of 2.5, 5.0, 10.0, 20.0, and 40.0 U/mL, wash buffer, horseradish peroxidase (HP)-labelled anti-human IgG secondary antibody, and substrate. Sera were tested according to the manufacturer's recommendations. Briefly, sera from transplant patients and control patients were diluted 1/100 and added to plates in duplicate, together with calibrators and assay kit control sera, and incubated at 4 C° for two hours. After three washes, secondary antibody was added and plates were incubated at room temperature for 60 minutes. The plate was washed three times; substrate was added and incubated in the dark at room temperature for 20 minutes. The stop solution was added and plates were read on a spectrophotometer at 450nm within 30 minutes. Results were interpreted as per the manufacturer's instruction: results ≥17U/mL were considered positive, results <10 U/mL were considered negative, and results between 10 and up to 17 U/mL were classified 'at risk'. AT1R antibody values greater than 40 U/mL cannot be reported due to limitations of the calibration curve. Samples above 40 U/mL were not diluted and retested and were all reported as 41 U/mL.

Assay performance was validated by testing against previously characterized samples from the American Society for Histocompatibility and Immunogenetics (ASHI) proficiency testing exchange.

3.3.3 Adsorption of non-specific reactivity using Adsorb Out ™

The occurrence of assay reactivity not specific to AT1R ('non-specific reactivity') was investigated using Adsorb Out ™ particles (One Lambda Thermo Fisher) according to the manufacturer's instructions. Serum samples with AT1R antibodies detected in a range of low to high levels of positivity were selected to be treated in this adsorption protocol (n=52 patient sera and 3 controls in total). Aliquots of 2µL of Adsorb Out™ product were added to 20 µL of serum, vortexed, and placed on a plate shaker for 30 minutes at room temperature. Sera were centrifuged for 10 minutes at 10000 RPMs to remove the Adsorb Out™ particles. Sera were re-tested with the AT1R ELISA assay as described above. The assay kit positive control serum as well as the 20 U/mL and 40 U/mL calibrator reagents were also adsorbed using the same protocol as patient and control sera. These control and calibrator AT1R values were corrected by a factor of 1.1 to account for a 10% dilution with the adsorption reagent.

3.3.4 Neu5Gc glycan detection on Adsorb Out ™ product

The presence of Neu5Gc glycans on Adsorb Out™ particles was measured by flow cytometry using a Neu5Gc detection kit that included a chicken anti-Neu5Gc antibody and isotype control antibody (BioLegend). A FITC-labelled anti-chicken secondary antibody (BioLegend) was used to detect binding of anti-Neu5Gc antibody. Negative control latex beads coated with human albumin were stained in parallel. The Adsorb Out™ particles and human albumin control beads were each incubated with the primary antibody at 2-8°C for one hour, washed, and incubated with the secondary antibody at 2-8°C for one hour, washed and acquired on a Canto II flow cytometer (BD Biosciences).

3.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism. The Mann-Whitney test was used to compare unmatched, non-parametric continuous variables and the Fishers Exact Test was used to compare proportions of populations.

3.4 Results

3.4.1 Quality control results

The results of the ASHI proficiency testing sera were consistent with results reported by other centers, thus confirming that the test protocol and kit lot# were performing as observed in other centres. (data not shown).

3.4.2 Patient and control populations

The median age of patients at the time of transplant was 3.38 years with a range of 11 days to 16.5 years; median age of control individuals at time of collection was 4.7 years with a range of 3 days to 16.9 years; there was no significant difference in the age of patient as compared to controls. (**Figure 3-1**). Regarding sex distribution, 21 of 42 patients (50%) and 13 of 27 controls (48%) were female. The sample immediately prior to the date of transplant was selected as the pre-transplant sample. The time points of the pre- and post-transplant sera collection for all patients is shown in **Table 3-1**. Nine of the patients underwent VAD implantation prior to transplant; the time points for this therapy prior to the pre-transplant sample are also shown in **Table 3-1**.

3.4.3 Sera testing positive for AT1R antibodies

Amongst all patient samples tested, an AT1R antibody level ≥17 U/mL was detected in 84 of the 154 samples tested (55%). Many of the patient results (59 of 84 (70%) positive patient sera) were above the upper test limit of 40 U/mL. In the non-transplant control group, 8 of the 15 positive results (53%) were above 40 U/mL. A similar proportion of positive results was detected in sera from controls, with 15 of 27 samples (56%) yielding an AT1R antibody level ≥17 U/mL (**Figure 3-2**). The quantities of pre- and post-transplant AT1R antibodies for all 42 patients are included in **Table 3-1 and Figure 3-3**.

The *proportion* of positive samples was comparable for the pre-transplant samples from all patients with 24 of 42 (57%) testing positive for AT1R antibodies as compared to the combined pre- and post-transplant combined results shown in Figure 3-2 however AT1R antibody status did not always remain consistent pre- *vs* post-transplant (**Figure 3-4**). Some patients converted from positive to negative pre- *vs* post-transplant and others had the opposite change in AT1R antibody status. No sex-related association with AT1R antibodies was detected in patients or controls.

3.4.4 AT1R antibody results from adsorbed sera

Of the 52 positive samples retested following the adsorption treatment, 39 (75%) showed a drop in the AT1R antibody level below the 17 U/mL positivity threshold as shown in **Figure 3-5**. Evaluating the pre-transplant sera for AT1R antibody with and without adsorption treatment, there was a significant decrease in the proportion of samples with results ≥17U/mL (p=0.014). The same comparison of the proportions of post-transplant sera positive for AT1R antibody with

and without adsorption treatment did not show a statistically significant drop although there was a trend towards significance (p=0.06). These pre- and post-transplant sample comparisons are included in **Figure 3-4**. Three control samples with the highest levels of AT1R antibody ≥40 U/mL were selected for adsorption; all three converted to negative values following Adsorb Out™ treatment.

Adsorption did not remove AT1R antibody reactivity from the assay kit positive control serum or the 20 U/mL and 40 U/mL calibrator reagents (**Figure 3-6)**.

3.4.5 Detection of Neu5Gc glycans on Adsorb Out™ microparticles

Although Neu5Gc glycans were not detected on human albumin coated beads, they were clearly present on Adsorb Out™ particles (**Figure 3-7)**.

3.5 Discussion

AT1R antibodies are a natural consideration for relevance in transplantation and vascular rejection as these antibodies have been reported to mediate endothelial activation and to be involved in the pathology of conditions such as preeclampsia in pregnancy and systemic sclerosis. (Hubel et al. 2007; Riemekasten et al. 2011; Riemekasten, Petersen, and Heidecke 2020) Dragun *et al* elegantly set the stage in 2005 for a potential role for anti-AT1R antibodies in transplantation, preceded by their earlier work demonstrating that passive acquisition of AT1R antibodies could mediate renal allograft damage in an animal model.(Dragun et al. 2004, 2005). The neonatal rat cardiomyocyte bioassay used in the Dragun study is impractical for the clinical

laboratory thus the development of a higher throughput method such as ELISA was critical for broader study of the role of AT1R antibodies. However, as is the case with all solid phase assays in the clinical laboratory, inclusion of relevant controls is essential.

This study demonstrates that a high proportion of pediatric heart transplant recipients appear to produce antibodies to AT1R, but also shows this finding in sera from an equally high proportion of age-matched non-transplant controls. We also report that levels of AT1R antibodies detected with the commercial ELISA assay are diminished using an adsorption tool that does not decrease reactivity in the assay kit positive control or in the AT1R calibration reagents. This work does not explore all the precise mechanisms underlying the non-specific reactivity in the ELISA assay nor all mechanisms for the removal of non-specific reactivity with Adsorb Out™ but there are several possible means by which this reduction could occur.

There are common assay interferences that must be mitigated in ELISA assays. Non-specific reactivity in ELISA methods can be due to the presence of antibodies with specificities to other antigens such as the blocking agent, typically bovine serum albumin (BSA) and/or the polystyrene surface (Andersen et al. 2004; Güven et al. 2014; Pickering et al. 2010; Terato et al. 2014; Xiao and Isaacs 2012). Antibodies to BSA can also be detected in both health and disease, and appear more frequently in children than adults (Hilger et al. 2001; Rothberg and Farr 1965). These issues are known to be relevant to assays designed to detect antibodies to HLA. (Newell et al. 2006) For this reason, 'no antigen' wells were included for ELISA assays previously used for the detection of HLA antibodies. Luminex bead-based methods have replaced the ELISA assay as the gold standard for HLA antibody detection; these assays always include a 'no antigen'

bead. The Adsorb Out™ product is described by its manufacturer as microparticles without antigen treated with 'blocking solution'. While we cannot know specifically what the commercial blocking solution contains, it is very likely to include BSA; this product is widely used to reduce background reactivity in sera shown to react to the 'no antigen' bead in HLA antibody assays.

Oaks considered non-specific reactivity in the same AT1R ELISA assay in a study on AT1R antibodies in adults supported with ventricular assist devices (VAD) (Oaks et al. 2018). Investigating the possibility of xenoreactive antibodies as a source of assay interference, they adsorbed patient sera on the CHO cell line used for AT1R antigen production for the ELISA assay, then re-tested the samples on the ELISA platform. They reported reduced reactivity in many patient sera following adsorption on CHO cells. They additionally postulated that antibodies to the sialic acid Neu5Gc could be contributing to non-specific reactivity. As endogenous Neu5Gc is absent in humans, most people produce 'natural' antibodies to Neu5Gc in similar fashion to ABO blood group antibodies (Gao et al. 2017). Anti-Neu5GC antibodies develop in infancy and are also present in cord blood, demonstrating passive antibody transmission from mother to infant (Taylor et al. 2010). As CHO cells would be decorated with Neu5GC, it is possible that AT1R antigens prepared from these cells may include this glycan. BSA is assumed to be one component of the blocking solution used to create the Adsorb Out™ microparticles. Thus the finding that Neu5Gc is detected on this product but not on human albumin beads is not unexpected and identifies anti-Neu5Gc antibodies as a possible source of false positive AT1R antibody results in this ELISA assay.

No other commercial AT1R antibody assay is widely available, however, laboratory-developed assays have been reported. A large study of the Dutch Organ Transplant Registry included an AT1R antibody assay that was developed in-house.(Kamburova et al. 2019) Although the method development is not described in detail, a Luminex-based approach was used; no association was observed between the presence of AT1R antibodies and rejection episodes or reduced renal allograft survival. An additional recent report used a bioassay to measure the presence of functionally active AT1R antibodies (Bankamp et al. 2021). These authors demonstrated poor correlation of the bioassay results with both an in-house assay and the same ELISA assay used in our study. They postulated that this discrepancy may be related to the inability to fully purify AT1R antigens from the CHO cell line; they also observed that additional antigens beyond the AT1R targets may be present in the ELISA assays.

Although details of the Cell Trend commercial assay do not appear in published reports and there is no publication specific to its development and validation, it is referred to as a ‘sandwich ELISA’ by its creator Dr. Dragun and collaborators. As such, it would require the use of a capture antibody, most likely specific to an AT1R domain outside of the second extracellular loop. It has been reported that there are issues with the specificity of AT1R monoclonal antibodies (Herrera et al. 2013a, 2013b), but to investigate this possible non-specific antigen capture, further details would be needed regarding method development of the commercial ELISA assay.

Some patient populations may be more likely than others to produce antibodies with diverse specificities that interfere with the AT1R antibody ELISA assay. Here we showed not only a

high frequency of positive reactivity with this assay in sera from both pediatric heart transplant patients and age and sex-matched controls, but also that the majority of these positive reactions became negative when sera were treated with a reagent commonly used to reduce non-specific reactivity in HLA antibody assays.

3.6 Conclusion

The biologic role of AT1R, as well as mounting evidence from positive correlation studies, suggest that antibodies to this receptor may be harmful to the graft in some transplant patients. Current literature remains inconclusive, however, as to a definitive pathophysiologic impact of AT1R antibodies in organ transplantation. This discordance may be driven in part by the presence of false positive reactivity in the sole ELISA assay used. Our findings, together with lack of published information regarding the development and validation of this commercial assay against functional bioassays, make it challenging to interpret the literature. Pediatric populations may be particularly prone to non-specific reactivity in this ELISA assay; further investigation is needed across other pediatric transplant patients and controls. Here we have presented an easily performed addition to the existing assay that would enhance its specificity. The specificity of this assay could also be greatly improved by the availability of a ‘no antigen’ well. This absorption approach may offer a means to readily improve this assay in the absence of better assay controls from the commercial vendor.

3.7 Tables and Figures

Table 3-1: Individual pre- and post-transplant patient data with time points of serum collection and VAD implantation date where relevant. All AT1R antibody results >40 U/mL are displayed as 41 U/mL. Sera that were positive for AT1R antibodies and treated with adsorption are included. For 4 of the 42 post-transplant samples, insufficient serum and/or reagent was available to retest AT1R antibody after adsorption.

Patient	Days between VAD and pre-transplant sample	Days before transplant for pre-transplant sample	Pre-transplant AT1R antibody (U/mL)	Pre-transplant AT1R antibody (U/mL) ADSORB treated	Days after transplant for post-transplant sample	Post-transplant AT1R antiobdy (U/mL)	Post-transplant AT1R antibody (U/mL) ADSORB treated	Age at transplant (years)
1		6	41	25.3	364	41	19.5	1.22
2		82	17.2	5	655	20.7	2.67	10.96
3	508	17	41	21.1	492	41	8.9	5.44
4		118	41	8.1	281	41	15.1	4.07
5	141	0	41	6.4	502	41	6.5	12.10
6		0	3	3	348	2.9	2.9	0.03
7		0	2.7	2.7	374	6	6	8.10
8		23	8.4	8.4	172	4.7	4.7	16.49
9		388	37.6	5.6	464	41	26.4	1.91
10		8	8.9	8.9	404	2.9	2.9	0.04
11		1	24.3	5	360	41	8.8	0.49
12		26	5.6	5.6	249	10.8	10.8	2.35
13		21	14.4	14.4	441	8.6	8.6	12.26
14		3	41	5.7	375	41	17.1	0.64
15		1	8.5	8.5	349	41	9.3	1.16
16	761	0	41	7	385	41	4.5	15.73
17	79	0	41	24.1	376	4.2	4.2	3.85
18	638	0	41	41	373	41	3.6	8.18
19		49	1.8	NT	364	18.5	NT	0.48
20		5	24.3	4.2	776	41	41	0.76
21	6	0	20.5	4	759	41	9.7	0.93
22	242	0	41	41	49	41	20.2	6.84
23		0	15.6	15.6	26	6.5	6.5	15.58
24		145	15.4	NT	394	24	NT	1.01
25		69	9.6	9.6	114	16.1	16.1	8.52
26		0	26.4	NT	353	20.9	NT	4.55
27		0	41	3.8	453	41	3.9	11.78
28		0	6.1	6.1	734	41	15	0.56
29	140	24	26	8.4	423	2.9	2.9	5.42
30	218	1	41	4.4	775	41	3.3	2.64
31		1	2.8	2.8	368	2.8	2.8	0.71
32		0	24.1	1.9	414	13.6	13.6	5.43
33		0	41	36.4	24	41	13	2.91
34		113	26	3.5	218	41	3	2.22
35		47	41	34.3	660	27.4	2.6	7.89
36		1	2.4	2.4	366	2.2	2.2	2.41
37		51	6.9	6.9	370	41	18.6	0.19
38		0	24.5	1.8	283	41	4.7	10.59
39		1	9.5	9.5	220	6.7	6.7	15.22
40		0	25.5	NT	167	23.8	NT	0.84
41		16	1.7	1.7	75	1.5	1.5	0.12
42		91	7.3	7.3	263	30.2	1.5	7.17
Median	**218**	**1**	**24.2**	**6.65**	**369**	**28.8**	**6.5**	**3.38**
Range	**6-755**	**0-388**	**1.7-41**	**1.7-41**	**24-752**	**1.5-39.5**	**1.5-39.5**	**0.03-16.49**
95% CI (median)	**79-638**	**0-17**	**9.6-26.4**	**5.0-8.5**	**349-394**	**16.1-41**	**3.9-9.7**	**1.22-6.84**

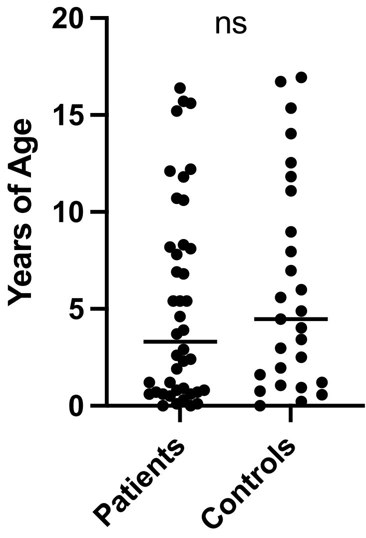


Figure 3-1: The ages at time of sample collection are comparable between the pre-transplant patient sera and the non-transplant controls.

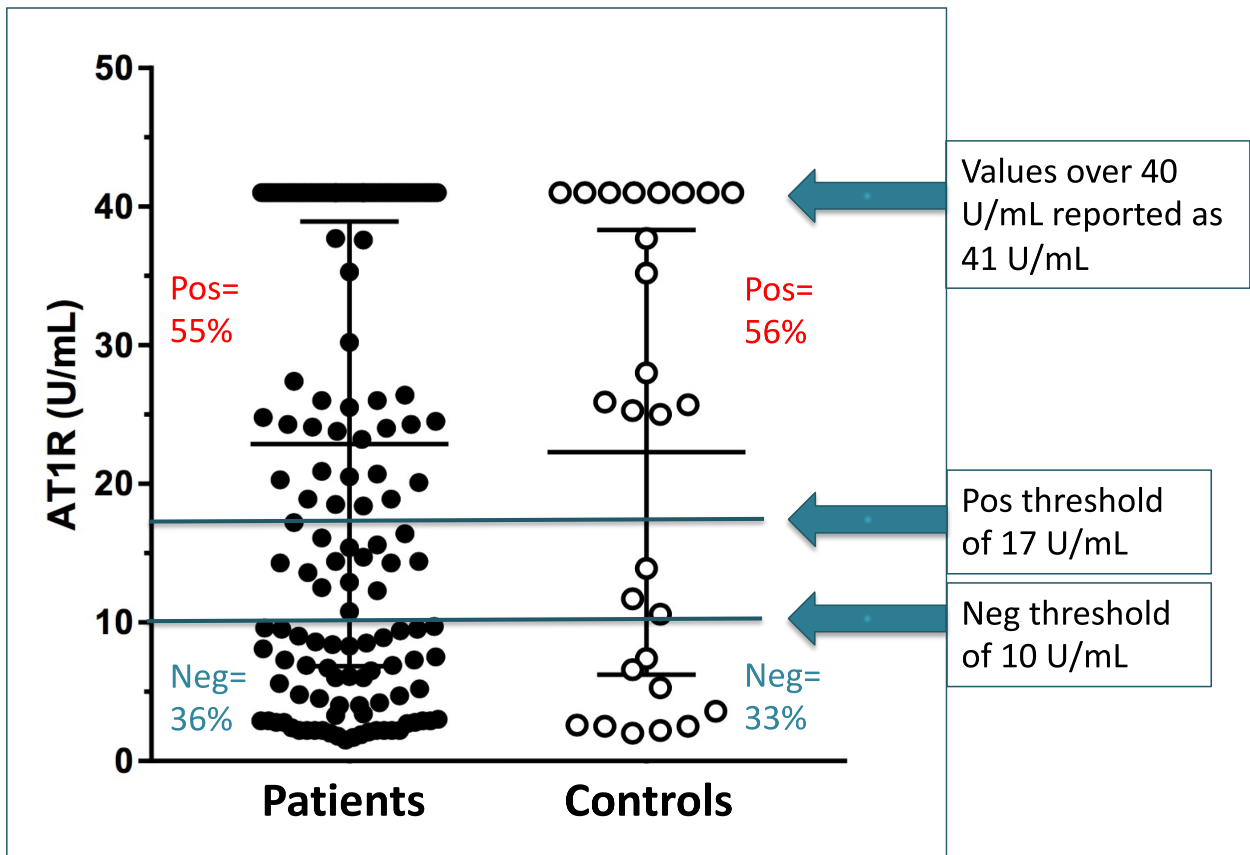


Figure 3-2: The majority of patient (84 out of 154) and control (15 out of 27) sera were positive for AT1R antibodies ≥17 U/mL. Values >40 U/mL are displayed as '41' U/mL.

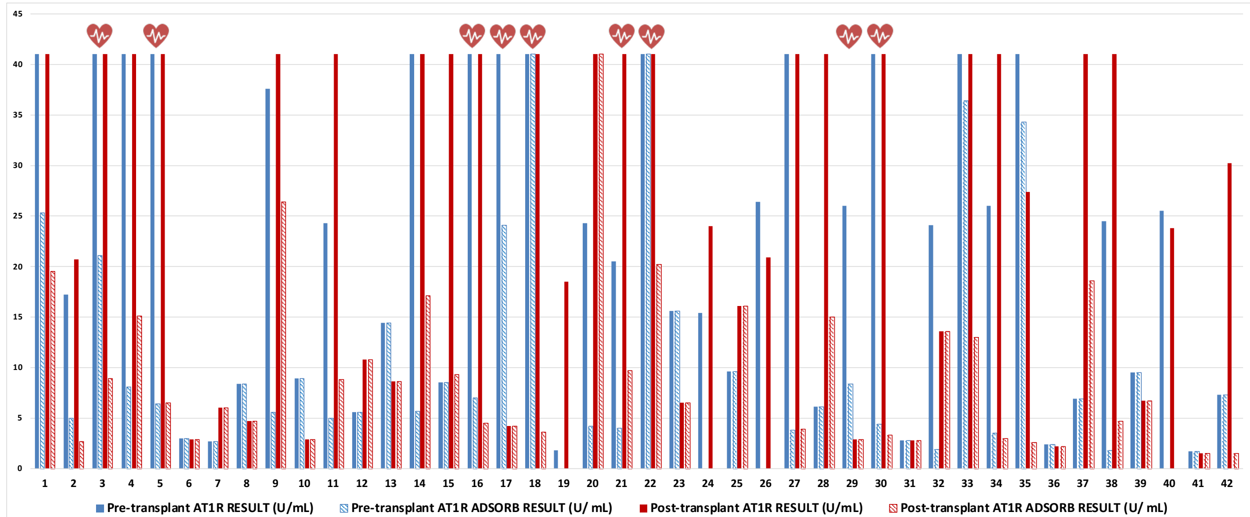


Figure 3-3: There was variability in pre- and post-transplant AT1R antibody levels for all 42 samples. Results are shown for each patient individually with and without adsorption. For 4 of the 42 post-transplant samples, insufficient serum and/or reagent was available to retest AT1R antibodies after adsorption. Nine patients received VAD therapy (indicated with heart icon).

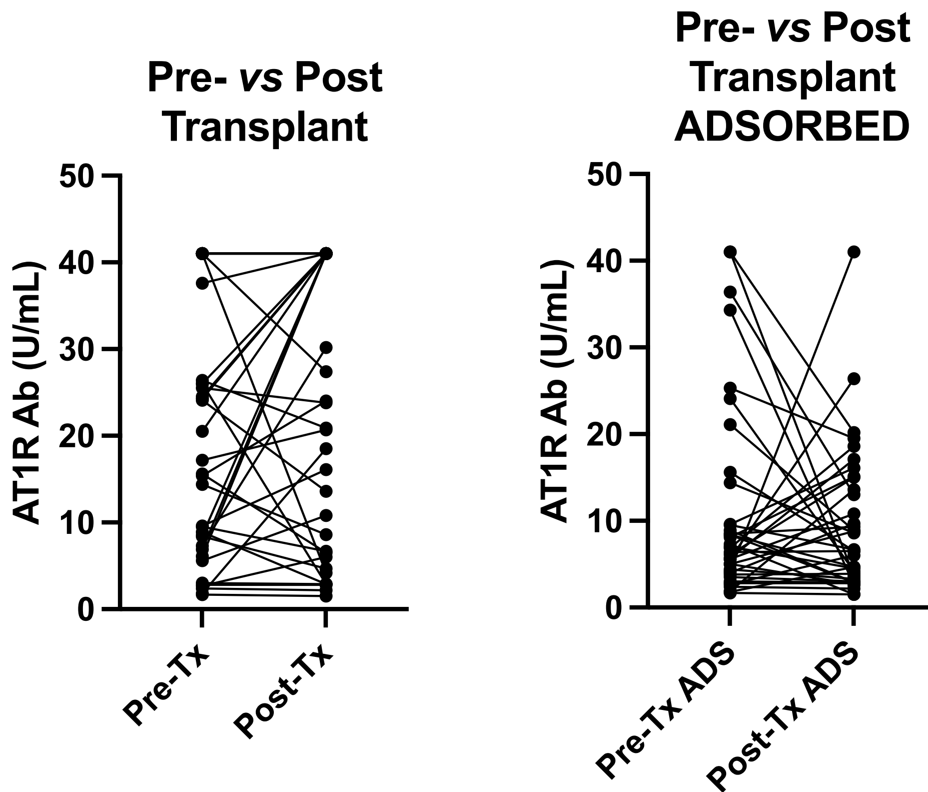


Figure 3-4: After transplantation of the positive samples, AT1R antibody levels did not consistently increase or decrease in either the untreated sera (n=42) or the adsorbed sera (n=38).

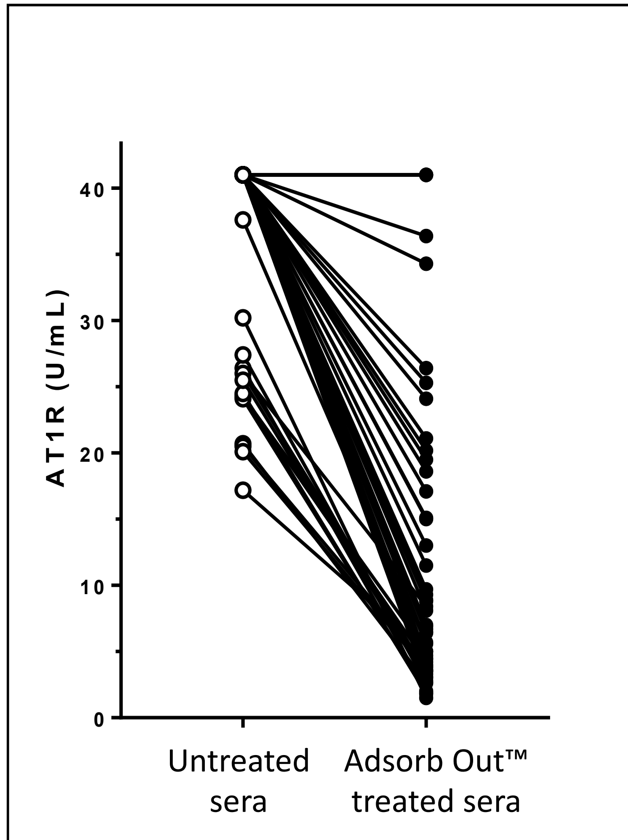


Figure 3-5: Following treatment of positive samples with AdsorbOut™, 39 of 52 (75%) patient sera previously positive for AT1R antibodies decreased to <17 U/mL AT1R concentration (n=52).

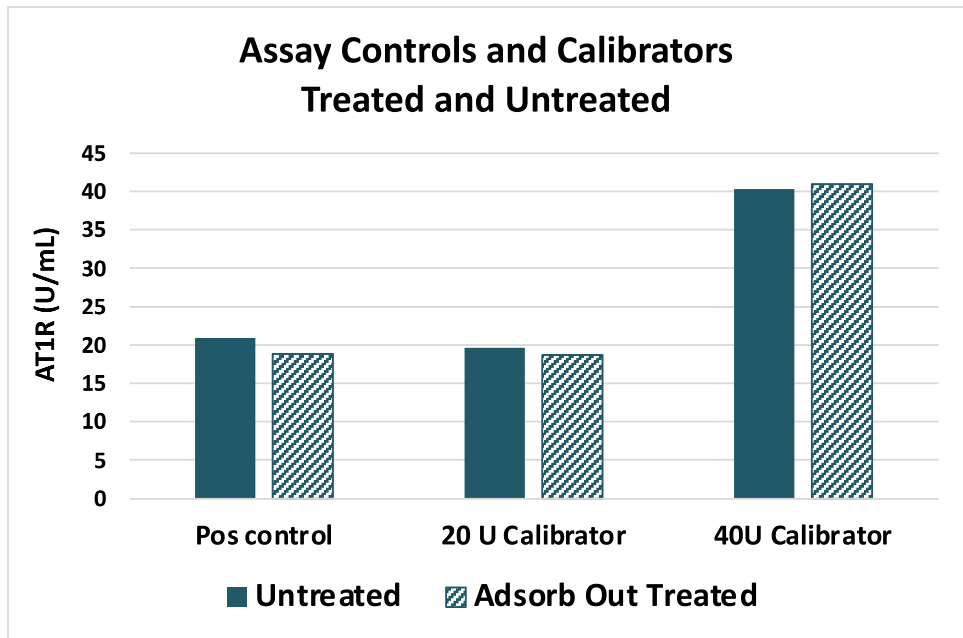


Figure 3-6: Anti-AT1R antibodies were not removed from the assay positive control or calibrators with AdsorbOut™ treatment

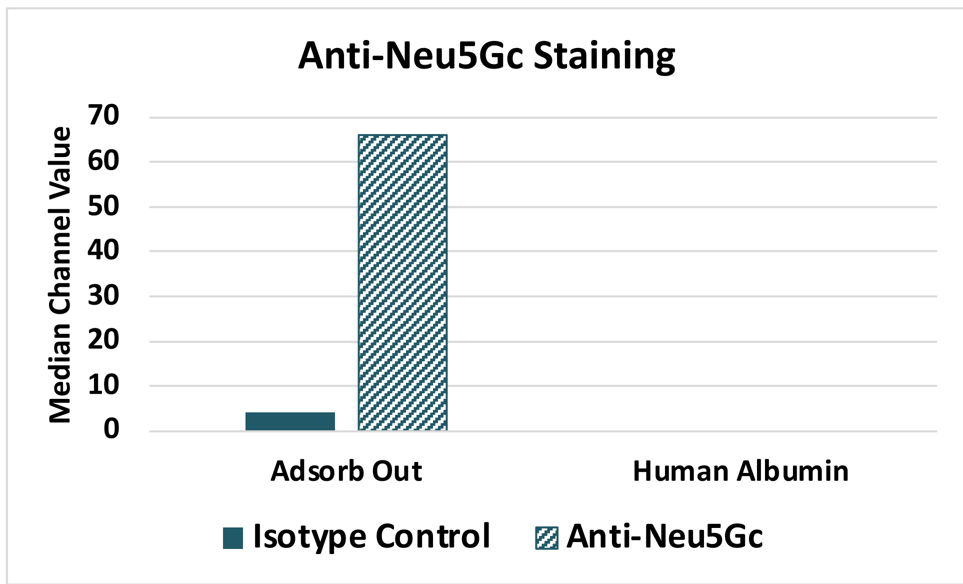


Figure 3-7: Neu5Gc glycans were detected on the AdsorbOut™ microparticles but were not detected on beads coated with human albumin

3.8 References

Andersen, Ditte C., Claus Koch, Charlotte H. Jensen, Karsten Skjødt, Jette Brandt, and Børge Teisner. 2004. "High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies." *Journal of Immunoassay and Immunochemistry* 25(1):17–30.

Bankamp, Lukas, Beate Preuß, Ann Christin Pecher, Nicola Beucke, Jörg Henes, and Reinhild Klein. 2021. "Functionally Active Antibodies to the Angiotensin II Type 1-Receptor Measured by a Luminometric Bioassay Do Not Correlate With Clinical Manifestations in Systemic Sclerosis: A Comparison With Antibodies to Vascular Receptors and Topoisomerase I Detected B." *Frontiers in Immunology* 12(December):1–13.

Cardinal, H., M. Dieudé, N. Brassard, S. Qi, N. Patey, M. Soulez, D. Beillevaire, F. Echeverry, C. Daniel, Y. Durocher, F. Madore, and M. J. Hébert. 2013. "Antiperlecan Antibodies Are Novel Accelerators of Immune-Mediated Vascular Injury." *American Journal of Transplantation* 13(4):861–74.

Deltombe, Clement, Florence Gillaizeau, Daniel Anglicheau, Emmanuel Morelon, Katy Trébern-Launay, Florent Le Borgne, Marie Rimbert, Pierrick Guérif, Stéphanie Malard-Castagnet, Yohann Foucher, and Magali Giral. 2017. "Is Pre-Transplant Sensitization against Angiotensin II Type 1 Receptor Still a Risk Factor of Graft and Patient Outcome in Kidney Transplantation in the Anti-HLA Luminex Era? A Retrospective Study." *Transplant International* 30(11):1150–60.

Dragun, D., M. Nieminen-Kelha, J. H. Bräsen, L. Fritsche, D. Eckert, R. Dechend, R. Plehm, G. Wallukat, H. H. Neumayer, F. C. Luft, and D. N. Muller. 2004. "Passive Transfer of Human Non-HLA Antibodies Targeting Angiotensin II Type 1 Receptor Causes Vascular Rejection and Hypertension in Rat Renal Allografts." *Kidney & Blood Pressure Research* 27(5):338.

Dragun, Duska, Lutz Fritsche, Jan Hinrich Brasen, Dominik N. Muller, Melina Nieminen-Kelha, Birgit Rudolph, Ulrich Kintscher, Ralf Dechend, Johan Hoebeke, Ralph Plehm, Istvan Mazak, Diana Eckert, Constanze Schonemann, Klemens Budde, Thomas Unger, Hans-Hellmut Neumayer, Gerd Wallukat, and Friedrich C. Luft. 2005. "Angiotensin II Type 1-Receptor Activating Antibodies in Renal-Allograft Rejection." *The New England Journal of Medicine* (6):558.

Gao, Bingsi, Cassandra Long, Whayoung Lee, Zhongqiang Zhang, Xiaotian Gao, Doug Landsittel, Mohamed Ezzelarab, David Ayares, Yuliang Huang, David K. C. Cooper, Yi Wang, and Hidetaka Hara. 2017. "Anti-Neu5Gc and Anti-Non-Neu5Gc Antibodies in Healthy Humans." *PLOS ONE* 12(7):e0180768.

Güven, Esin, Karen Duus, Magnus Christian Lydolph, Charlotte Sværke Jørgensen, Inga Laursen, and Gunnar Houen. 2014. "Non-Specific Binding in Solid Phase Immunoassays for Autoantibodies Correlates with Inflammation Markers." *Journal of Immunological Methods* 403(1–2):26–36.

Halpin, Anne, Sally Abou-Zeki, Ingrid Larsen, Patricia M. Campbell, Simon Urschel, Bruce Motyka, and Lori West. 2017. "OR52 AT1R Antibody in Pediatric Heart Transplant Patients: What Are We Really Detecting." *Human Immunology* 78(Supplement):48–49.

Herrera, Marcela, Matthew A. Sparks, Adolfo R. Alfonso-Pecchio, Lisa M. Harrison-Bernard, and Thomas M. Coffman. 2013a. "Lack of Specificity of Commercial Antibodies Leads to Misidentification of Angiotensin Type 1 Receptor Protein." *Hypertension* 61(1):253–58.

Herrera, Marcela, Matthew A. Sparks, Adolfo R. Alfonso-Pecchio, Lisa M. Harrison-Bernard, and Thomas M. Coffman. 2013b. "Response to Lack of Specificity of Commercial Antibodies Leads to Misidentification of Angiotensin Type-1 Receptor Protein." *Hypertension* 61(4):e32–e32.

Hiemann, N. E., R. Meyer, E. Wellnhofer, C. Schoenemann, H. Heidecke, N. Lachmann, R. Hetzer, and D. Dragun. 2012. "Non-HLA Antibodies Targeting Vascular Receptors Enhance Alloimmune Response and Microvasculopathy After Heart Transplantation." *Transplantation* 94(9):919–24.

Hilger, C., F. Grigioni, C. De Beaufort, G. Michel, J. Freilinger, and F. Hentges. 2001. "Differential Binding of IgG and IgA Antibodies to Antigenic Determinants of Bovine Serum Albumin." *Clinical and Experimental Immunology* 123(3):387–94.

Hubel, C. A., G. Wallukat, M. Wolf, F. Herse, A. Rajakumar, J. M. Roberts, N. Markovic, R. Thadhani, F. C. Luft, and R. Dechend. 2007. "Agonistic Angiotensin II Type 1 Receptor Autoantibodies in Postpartum Women With a History of Preeclampsia." *HYPERTENSION -DALLAS-* 49(3):612–17.

Kamburova, Elena G., Maartje L. Gruijters, Tineke Kardol-Hoefnagel, Bram W. Wisse, Irma Joosten, Wil A. Allebes, Arnold van der Meer, Luuk B. Hilbrands, Marije C. Baas, Eric Spierings, Cornelis E. Hack, Franka E. van Reekum, Arjan D. van Zuilen, Marianne C. Verhaar, Michiel L. Bots, Adriaan C. A. D. Drop, Loes Plaisier, Rowena C. A. Melchers, Marc A. J. Seelen, Jan Stephan Sanders, Bouke G. Hepkema, Annechien J. A. Lambeck, Laura B. Bungener, Caroline Roozendaal, Marcel G. J. Tilanus, Christina E. Voorter, Lotte Wieten, Elly M. van Duijnhoven, Mariëlle A. C. J. Gelens, Maarten H. L. Christiaans, Frans J. van Ittersum, Shaikh A. Nurmohamed, Neubury M. Lardy, Wendy Swelsen, Karlijn A. M. I. van der Pant, Neelke C. van der Weerd, Ineke J. M. ten Berge, Andries Hoitsma, Paul J. M. van der Boog, Johan W. de Fijter, Michiel G. H. Betjes, Sebastiaan Heidt, Dave L. Roelen, Frans H. Claas, Frederike J. Bemelman, and Henny G. Otten. 2019. "Antibodies against ARHGDIB Are Associated with Long-Term Kidney Graft Loss." *American Journal of Transplantation* 19(12):3335–44.

Lefaucheur, Carmen, Denis Viglietti, Yassine Bouatou, Aurélie Philippe, Daniele Pievani, Olivier Aubert, Jean-Paul Duong Van Huyen, Jean-Luc Taupin, Denis Glotz, Christophe Legendre, Alexandre Loupy, Philip F. Halloran, and Duska Dragun. 2019. "Non-HLA Agonistic Anti-Angiotensin II Type 1 Receptor Antibodies Induce a Distinctive Phenotype of Antibody-Mediated Rejection in Kidney Transplant Recipients." *Kidney International* 96(1):189–201.

Newell, H., J. D. Smith, P. Rogers, E. Birks, A. J. Danskine, R. E. Fawson, and M. L. Rose. 2006. "Sensitization Following LVAD Implantation Using Leucodepleted Blood Is Not Due to HLA Antibodies." *American Journal of Transplantation* 6(7):1712–17.

Oaks, Martin, Karen Michel, Francis X. Downey, and Vinay Thohan. 2018. "Xenoreactive Antibodies and Latent Fibrin Formation in VAD and Cardiac Transplant Recipients Can Confound the Detection and Measurement of Anti-AT1R Antibodies." *American Journal of Transplantation* 18(11):2763–71.

Patel, R. and P. I. Terasaki. 1969. "Significance of the Positive Crossmatch Test in Kidney Transplantation." *The New England Journal of Medicine* 280(14):735–39.

Pearl, Meghan H. and Elaine F. Reed. 2019. "Angiotensin II Type I Receptor Antibodies in Pediatric Solid Organ Transplant." *Human Immunology*.

Philogene, Mary Carmelle, Tory Johnson, Arthur Jason Vaught, Sammy Zakaria, and Neal Fedarko. 2019. "Antibodies against Angiotensin II Type 1 and Endothelin A Receptors: Relevance and Pathogenicity." *Human Immunology*.

Pickering, Jerry W., Matthew T. Larson, Thomas B. Martins, Susan S. Copple, and Harry R. Hill. 2010. "Elimination of False-Positive Results in a Luminex Assay for Pneumococcal Antibodies." *Clinical and Vaccine Immunology* 17(1):185 LP – 189.

Pinelli, David F., John J. Friedewald, Kelley M. K. Haarberg, Shree L. Radhakrishnan, Jennifer R. Zitzner, Wendy E. Hanshew, and Anat R. Tambur. 2017. "Assessing the Potential of Angiotensin II Type 1 Receptor and Donor Specific Anti-Endothelial Cell Antibodies to Predict Long-Term Kidney Graft Outcome." *Human Immunology* 78(5–6):421–27.

Reinsmoen, N. L., C. H. Lai, H. Heidecke, M. Haas, K. Cao, G. Ong, M. Naim, Q. Wang, J. Mirocha, J. Kahwaji, A. A. Vo, S. C. Jordan, and D. Dragun. 2010. "Anti-Angiotensin Type 1 Receptor Antibodies Associated With Antibody Mediated Rejection in Donor HLA Antibody Negative Patients." *Transplantation* 90(12):1473–77.

Riemekasten, Gabriela, Frank Petersen, and Harald Heidecke. 2020. "What Makes Antibodies Against G Protein-Coupled Receptors so Special? A Novel Concept to Understand Chronic Diseases." *Frontiers in Immunology* 11:564526.

Riemekasten, Gabriela, Aurélie Philippe, Melanie Näther, Torsten Slowinski, Dominik N. Müller, Harald Heidecke, Marco Matucci-Cerinic, László Czirják, Ivo Lukitsch, Mike Becker, Angela Kill, Jacob M. van Laar, Rusan Catar, Friedrich C. Luft, Gerd R. Burmester, Björn Hegner, and Duska Dragun. 2011. "Involvement of Functional Autoantibodies against Vascular Receptors in Systemic Sclerosis." *Annals of the Rheumatic Diseases* 70(3):530–36.

Rothberg, Richard M. and Richard S. Farr. 1965. "ANTI-BOVINE SERUM ALBUMIN AND ANTI-ALPHA LACTALBUMIN IN THE SERUM OF CHILDREN AND ADULTS." *Pediatrics* 35(4):571 LP – 588.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. "Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation." *Transplantation* 95(1):19–47.

Taylor, Rachel E., Christopher J. Gregg, Vered Padler-Karavani, Darius Ghaderi, Hai Yu, Shengshu Huang, Ricardo U. Sorensen, Xi Chen, Jaime Inostroza, Victor Nizet, and Ajit Varki. 2010. “Novel Mechanism for the Generation of Human Xeno-Autoantibodies against the Nonhuman Sialic Acid N-Glycolylneuraminic Acid.” *Journal of Experimental Medicine* 207(8):1637–46.

Terato, Kuniaki, Christopher T. Do, Dawn Cutler, Takaki Waritani, and Hiroshi Shionoya. 2014. “Preventing Intense False Positive and Negative Reactions Attributed to the Principle of ELISA to Re-Investigate Antibody Studies in Autoimmune Diseases.” *Journal of Immunological Methods* 407:15–25.

Thohan, Vinay, Karen Michel, Anil Purohit, Owais Malick, Francis X. Downey, and Martin Oaks. 2020. “The Effect of Pre-Transplant and Post-Transplant Anti-AT1R Antibodies in Heart Transplant Recipients.” *Transplantation Reports* 5(4):100069.

Tiriveedhi, Venkataswarup, Baskaran Gautam, Nayan J. Sarma, Medhat Askar, Marie Budev, Aviva Aloush, Ramsey Hachem, Elbert Trulock, Bryan Myers, Alexander G. Patterson, and Thalachallour Mohanakumar. 2013. “Pre-Transplant Antibodies to Kα1 Tubulin and Collagen-V in Lung Transplantation: Clinical Correlations.” *The Journal of Heart and Lung Transplantation* 32(8):807–14.

Xiao, Yuhong and Stuart N. Isaacs. 2012. “Enzyme-Linked Immunosorbent Assay (ELISA) and Blocking with Bovine Serum Albumin (BSA)—Not All BSAs Are Alike.” *Journal of Immunological Methods* 384(1):148–51.

Chapter 4:

ABO antibody detection: updating a century old method

Authors:

Anne M. Halpin
Bruce Motyka
Tess Ellis
Jean Pearce
Simon Urschel
Todd Lowary
Chris Cairo
Mylvaganam Jeyakanthan
Lori J. West

Acknowledgements

Reagent funding provided by GlycoNet and Enduring Hearts Foundation

Ethics

All patients and controls were consented in the Cardiac Transplantation in Infancy (CTI) study, University of Alberta Health Research Ethics Board, Study ID Pro1408.

Abbreviations:

ABOi	ABO-incompatible
AHG	Anti-human globulin
BSA	Bovine serum albumin
α-Gal	Galactose-α-1,3-galactose
BSA	Bovine serum albumin
DTT	Dithiothreitol
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplant
MFI	Mean fluorescence intensity
PE	Phycoerythrin

4.1 Abstract

Reliable risk assessment in ABO-incompatible transplantation relies on accurate and reproducible ABO antibody measurement. We have relied on red cell agglutination titres for the determination of ABO antibody levels for over a century and there are known issues with reproducibility in this method. Furthermore, there are many different iterations of this method that lead to poor inter-laboratory and inter-study comparability. ABO-A and B glycan representation is known to differ between red cells and tissues making the red cell a poor surrogate assessment of alloimmune risk in ABO-incompatible transplantation. The use of solid phase, single antigen antibody detection assays has revolutionized HLA antibody detection. The same methodology principles can be applied to ABO antibody detection. In this study we created a Luminex panel to measure A and B subtype and isotype specific antibodies. We tested this method in panel of healthy controls and compared the findings to a traditional hemagglutination determination of ABO antibody titre. These results show that there is a great deal of variability in ABO antibody profiles within an individual titre as well as within the same ABO blood groups of healthy individuals. This assay lends itself to rapid implementation into clinical histocompatibility laboratories which have the required equipment and expertise to perform this testing.

4.2 Introduction

It has been over a century since Karl Landsteiner first described the ABO blood groups(Landsteiner 2001). Additional knowledge of A, B, and H antigen structures (H antigen defining blood group O) has since been gained, but detection of ABO antibodies in clinical practice and related research is still reliant on variations of the erythrocyte agglutination method described in 1901. Hemagglutination is known to be plagued by a lack of sensitivity and poor reproducibility. (Datta et al. 2021; Denomme and Anani 2020; Kang, Lim, and Baik 2014) It is also cumbersome to distinguish between IgG and IgM isotypes by agglutination and it is not possible to define ABH glycan-subtype specificities. Yet ABO hemagglutination titres are routinely used to inform clinical management of transfusion and ABO-incompatible (ABOi) transplantation.

Knowledge of ABH glycobiology has evolved including the sequencing of the genes that encode for the glycosyltransferases that decorate human cells and tissues with these carbohydrate structures(Lane 2016; de Mattos 2016; Oriol et al. 1992; Pendu et al. 1989). It is known that there are six glycan structure subtypes (I-VI) of each A, B, and H antigens but These subtype glycans are not equally represented on various cells and tissues. The most biologically relevant antigens are reported to be A-II,III,IV and B-II. (Bentall et al. 2021; Clausen and Hakomori 1989; Jeyakanthan et al. 2016; de Mattos 2016). In the case of ABO-A individuals, assuming the more common A1 subgroup, subtype A-II is the only A antigen found on vascular endothelium, but epithelial cells are additionally decorated with A-III and A-IV glycans. Tissues of ABO-B individuals appear to have only B-II glycans although not all tissues have been well studied; this

work is hampered by the limited availability of B subtype-specific monoclonal antibodies. (Clausen et al. 1985; Jeyakanthan et al. 2015; Ravn and Dabelsteen 2000) Some glycobiology studies use A- and B-trisaccharides as surrogates for A- and B-subtype glycans however, since these surrogate glycans are not biologically relevant, anti-A- and B-trisaccharide-specific antibodies are of uncertain significance. (Pochechueva et al. 2011; Stussi et al. 2005)

Naturally occurring antibodies to non-self ABH glycans present a major immunologic barrier in transplantation and transfusion. As the hemagglutination assay does not distinguish glycan subtype-specificities of ABO antibodies, it remains challenging to fully characterise antibody profiles, resulting in suboptimal risk assessment. The subtype-specificity is critical to assessment of ABO antibodies in ABOi transplantation due to tissue-specific glycan representation on endothelial and epithelial cells. (Bentall et al. 2021; Jeyakanthan et al. 2016) Previously we developed an ABH-glycan microarray to improve precision and accuracy of ABO antibody detection. This method has not been widely implemented, however, due in part to lack of readily available instrumentation and expertise in the clinical laboratory setting.(Bentall et al. 2021; Daga et al. 2021; Jeyakanthan et al. 2015, 2016) It is also a challenge to achieve intra- and inter-laboratory standardization when scanning microarray slides on different array readers. (Jeyakanthan et al. 2016; Muthana and Gildersleeve 2016).

In contrast, Luminex methodologies are now widely used in antibody detection assays. Individual polystyrene beads are coupled with target antigens; up to 500 beads can be distinguished from one another by their different hue intensities. Following incubation of patient serum (or plasma) with antigen-coupled beads, phycoerythrin (PE)-labelled secondary antibodies

are used to detect serum antibodies bound to each individual bead, with the Luminex instrument reporting PE fluorescence intensity of each bead. The instrument settings are automatically determined in its calibration, making it a highly reproducible method within and between clinical laboratories. Indeed, this more sophisticated antibody detection technique has become the clinical standard for histocompatibility laboratories worldwide in the detection of antibodies to human leukocyte antigens (HLA) to support solid organ and hematopoietic stem cell transplantation (HSCT) (El-Awar, Lee, and Terasaki 2005; Sullivan, Gebel, and Bray 2017; Tait et al. 2013). The presence of Luminex instruments and expertise in HLA-histocompatibility laboratories makes this highly standardized method a natural fit for ABO-histocompatibility antibody profiling. We developed a Luminex assay to characterize A and B glycan subtype-specific antibodies in human serum and define antibody isotypes, overcoming the many limitations of the hemagglutination assay. This new assay will enable much needed refinement of ABH-histocompatibility assessment for organ and cell transplantation and transfusion medicine, and will facilitate new advances in the field of glycoimmunology.

4.3 Methods/Results

4.3.1 Overview of Luminex bead-based ABO antibody detection

Figure 4-1 provides a broad overview of the ABO antibody detection assay and an example of the results from one ABO-A individual. Briefly, we coupled individual A- and B-subtype glycans to individual beads. The glycan-coupled beads were incubated with patient plasma/serum then washed to remove unbound antibodies. PE-labelled IgG- and IgM-specific secondary antibodies were used to detect bound antibodies. The mean fluorescence intensity (MFI) for each bead was detected using Luminex instrumentation. Details on the development of this assay are as follows.

4.3.2 Coupling antigens to beads

Luminex MagPlex® Microspheres beads were coupled to individual ABO-A and ABO-B subtype glycans that were manufactured as previously described.(Jeyakanthan et al. 2015, 2016; Meloncelli and Lowary 2009, 2010; Meloncelli, West, and Lowary 2011) Briefly, A and B glycan subtype I-VI tetrasaccharide antigens were synthesized; bovine serum albumin (BSA) was linked to amines in these carbohydrate structures. Additional beads were coupled to galactose-α-1,3-galactose (α-Gal) and to BSA as positive and negative control beads, respectively (Dextra, UK). Blood group A- and B-trisaccharides were also coupled to individual beads (Dextra UK). The coupling procedure was performed as per the protein coupling procedure recommended by Luminex bead manufacturers(Angeloni et al. 2016). The ratio of antigen to bead was optimized for this glycan-protein antigen target using titration studies. The xMAP Antibody Coupling (AbC) Kit® was used for further standardization of the coupling procedure. Refer to Coupling and Coupling Confirmation SOP for additional details.

4.3.3 Bead-antigen coupling confirmation

Coupling of antigens to beads was confirmed using a panel of monoclonal antibodies either pan-reactive to ABO-A and ABO-B glycan structures or with specificities to individual ABO-A and -B glycan-subtype structures. The amount of antigen coupled to beads was consistent and comparable bead-to-bead as shown in Supplemental **Figure 4-S1**. Additional details, including the specific monoclonal antibodies employed, are provided in Coupling and Coupling Confirmation standard operating protocols (SOPs) in Chapter 6.

4.3.4 Micro-array antibody analysis

The monoclonal antibodies used in the bead coupling confirmation protocol were also run in parallel on the ABH-glycan microarray assay as previously described (Jeyakanthan et al. 2015, 2016). Antigens bound to glass arrays slides were identical to those coupled to the Luminex beads. The Luminex assay detected antibodies at low concentrations with greater sensitivity than the glycan array (**Figure 4-2**).

4.3.5 Application of assay using healthy control sera

Using antigen-coupled beads, profiles of anti-A and anti-B antibodies (and related antibodies) were characterized in serum or plasma samples from healthy adult individuals (n=143; ABO-O: n=68; ABO-A: n=48; ABO-B: n=17; ABO-AB: n=10) as described in the Luminex ABO Antibody Detection SOP. Briefly, 50uL of diluted serum or plasma was incubated with pooled single antigen beads then incubated at room temperature with gentle agitation. The beads were washed three times prior to addition of either anti-human IgG or IgM secondary antibody. Plates

were incubated again at room temperature with gentle agitation and washed three more times. The beads were resuspended in 80uL of buffer and acquired on a Luminex 200 instrument or a FlexMap 3D Luminex instrument. When acquired on the FlexMap 3D, the mean fluorescence intensity (MFI) results were divided by 1.67 to achieve MFI comparability between instruments. Each run included a positive and negative control serum.

The results were highly reproducible as shown in Supplemental **Figure 4-S2** demonstrating the results of the positive control sample tested over 24 individual runs.

A comparison of IgG and IgM antibodies by blood group is shown in **Figure 4-3**. **Here and throughout the results, antibody analysis was focused on antibodies specific to A-II, -III, and -IV and B-II glycan subtypes as these are reported to be the most biologically relevant glycan targets.** (Bentall et al. 2021; Clausen and Hakomori 1989; Jeyakanthan et al. 2016) Levels of antibodies with specificities to these A- and B- subtypes were highly variable between individuals. Additionally, sera from ABO-O individuals contained significantly higher levels of IgG isotype antibodies to A-II, -III, -IV and to B-II glycans than sera from ABO-B and ABO-A individuals, respectively, whereas there was no difference in levels of IgM isotype antibodies amongst sera from individuals of the different ABO blood groups. There were no significant sex-related differences observed (**Figure 4-4**).

Antibodies to A and B trisaccharide targets were compared to antibodies with specificities for the cell and tissue relevant tetrasaccharide glycan subtypes A-II, -III, and -IV and B-II. Over half of the ABO-A individuals had detectable IgG and IgM antibodies to A-trisaccharide whereas less

than 10% of the ABO-A blood group healthy controls exhibited reactivity to A-tetrasaccharide target beads (**Figure 4-5**). This same finding was not observed for B-trisaccharide glycan vs B-II subtype glycan.

Similar levels of α-Gal antibodies of IgG *vs* IgM isotypes were detected and this observation remained true when analysing α-Gal antibodies by sex as shown in Supplemental **Figure 4-S3.**

4.3.6 Hemagglutination ABO antibody titre testing

Traditional ABO titre testing was performed on individuals also tested by the Luminex ABO antibody assay. Sufficient sample for agglutination testing was available for 119 of the 143 healthy controls. (ABO-O n=54, ABO-A, n=43, ABO-B n=16, ABO=AB, n=6). Serially diluted sera/plasma (50uL) were incubated with 25uL of 1% ABO-A1 and ABO-B reagent red cells (Referencells®, Immucor) at room temperature in a 96-well tray. The plate was mixed and allowed to incubate for one hour. Agglutination was read on an ELISPOT reader (CTL). The agglutination titre was reported as the last dilution showing visual agglutination. These agglutination scores were compared to the levels of tetrasaccharide-specific antibodies detected in the Luminex assay.

Within each anti-A and anti-B ABO hemagglutination titre, there was a high degree of variability of IgG and IgM antibodies detected by the Luminex assay. There were increasing overall levels of IgM antibodies in each titre as shown in the IgM alone data in **Figure 4-6**, but the levels of IgG and IgM antibodies in each titre are overlapping. There are two cases in which anti-A and anti-B tetrasaccharide antibodies were detected when a negative antibody titre had been reported;

these results were repeated to confirm. Supplemental **Figure 4-S4** demonstrates this same comparison for ABO-O individuals only.

4.3.7 Antibody analysis statistical methods

All data were tested for normality using the Shapiro-Wilk, Anderson-Darling, and Kolmogorov-Smirnov tests. Non-parametric analysis of Mann-Whitney test and Wilcoxon test were used for paired and non-paired data, respectively when results were not normally distributed. The only normally distributed data were the positive control comparisons shown in Supplemental **Figure 4-S2**. GraphPad Prism 9.3.1 was used for analysis and data graphing.

4.4 Discussion

Here we describe a novel assay to measure serum antibodies with specificities for ABO-A and -B glycans and demonstrate its reproducibility and utility in a study of samples from healthy adults. It has been widely reported that there is a need for better methods for the detection of ABO antibodies.(Denomme and Anani 2020). The field of histocompatibility has used a similar tool for almost 2 decades. **Table 4-1** describes several of the lessons learned through the widespread use of the Luminex assay for HLA antibody risk assessment can be applied to the validation and implementation of this ABO antibody detection platform. (El-Awar et al. 2005)

Clinical laboratories currently lack a reproducible assay for detection and precise characterisation of glycan subtype-specific IgG and IgM antibodies to support ABOi transplantation and transfusion. It is widely acknowledged that red cell agglutination methods are poorly standardized and that inter-laboratory comparisons of titre data are challenging(Bentall et al. 2016; Daga et al. 2021; Denomme and Anani 2020; JP, J, and LJ 2008; Kang et al. 2014). Our Luminex assay is highly reproducible and allows for accurate characterization of isotype and subtype-specificity of anti-A and anti-B antibodies, thus offering the potential to overcome these barriers in clinical ABO antibody assessment. This assay has already been tested in a multi-centre exercise. Well characterised samples, reagents, and SOPs were sent to nine international histocompatibility laboratories for blind testing. The results were highly comparable thus demonstrating the strength of this assay as a highly reproducible tool for use in multi-centre trials as well as to ensure consistency of patient results within each centre. (Halpin et al. 2020)

The ease of differentiating the isotype of ABO antibodies also creates the potential to understand the risk of IgG vs IgM ABO antibodies in clinical ABO-incompatible (ABOi) organ and cell transplantation. While some studies have employed the use of dithiothreitol (DTT) and the addition of anti-human globulin (AHG) in an attempt to distinguish IgG from IgM ABO antibodies, this practice is inconsistent. While DTT is known to disassociate the IgM pentamer, it is not specific for IgM antibodies alone. There is also inconsistency in AHG titration methods, with some laboratories including DTT treatment and others not performing this treatment prior to ABO antibody titre testing.(Kahlyar et al. 2022)

It is widely recognized that there are discrepancies in organ transplant waitlist times based on ABO blood group of the patient. Blood group O and B patients wait longer to be transplanted with an ABO-compatible donor compared to A and AB patients. There is also an increasing need to consider ABOi transplantation as transplant registries globally are seeing increased numbers of patients with high levels of HLA antibodies resulting in poor access to HLA-compatible organs. (Hussey, Parameshwar, and Banner 2007) The success of ABOi transplantation suggests that disadvantaged blood groups ABO-O and ABO-B as well as HLA-sensitised patients would benefit from access to ABOi donors(Fan et al. 2004; de Weerd and Betjes 2018; West et al. 2001). However the well-recognized limitations of red cell agglutination titre testing are a barrier for consistent and reproducible ABO antibody assessment within and between programs as well as for national transplant registry use.

Increased knowledge of subtype-specific antibodies in the setting of ABOi transplantation will facilitate advancements in other areas of transplant clinical practice and research. Improved

histopathology studies of ABOi grafts could be carried out as antibodies to endothelial *vs* epithelial cells can be readily characterized. The pathology of transplant rejection is constantly evolving and this additional knowledge could be applied to retrospective and prospective studies. (Bruneval et al. 2017; Haas et al. 2017; Kobashigawa et al. 2018) Immune memory has been identified as an as a critical gap in transplant risk assessment but one that can be challenging to study (Tambur et al. 2018). Our Luminex tool can be readily incorporated into immune memory assays for simultaneous assessment of HLA and ABO B cell memory(Karahan et al. 2018).

While the field of ABOi transplantation would clearly benefit from the use of this assay, there are also potential transfusion medicine applications. There is a growing demand for whole blood transfusion from ABO-O donors but naturally occurring anti-A and anti-B antibodies may cause transfusion reactions. (Yazer and Spinella 2018) Currently female whole blood donors are excluded due to reports that females have more ABO antibodies than males but we did not observe this sex-based difference in our study population. The ABO blood group barrier is often crossed with platelet transfusions but transfusion medicine laboratories lack a consistent approach to measuring ABO antibodies in this blood product.

The ABO glycobiology field is hampered by lack of readily available monoclonal antibodies with specificity for individual glycan subytypes. This Luminex tool allows for clear characterization of A and B glycan monoclonal antibodies as shown in the coupling confirmation data from this assay development. This bead panel can also be readily expanded to include H disaccharide and tetrasaccharide targets. In fact, H panel beads have already been manufactured as part of this work (data not shown) and can readily be included with the A and B beads for

simultaneous antibody determination. The specificity of commonly used monoclonal antibodies in clinical transfusion medicine is also not well defined and this Luminex panel could be used for this purpose.

It is possible that there are *in-vivo* glycan modifications to A and B tetrasaccharides that are not represented in this panel. If additional relevant glycans are determined to be biologically relevant, this panel could easily be expanded to include such targets. While we have shown that IgG and IgM ABO-A and B antibodies vary widely within each ABO-A and B titre, it would be prudent to carry out additional hemagglutination studies in parallel with Luminex panel antibody profiling as the cell may continue to shed light on the glycobiology of these antigen systems. Erythrocytes will always be in an inadequate surrogate for tissues and organs but molecular profiling of the RNA regulation of A and B glycosyltransferases in health and all transplant outcomes, performed in parallel with Luminex antibody profiles would provide valuable new information.

We did not assess the secretor status of the subjects in this study. Subtype I glycans are reported to be secreted in individuals with the corresponding fucosylsyl transferase 2 (FUT2) genotype. We observed A-I and B-I subtype specific antibody patterns that suggest the FUT2 non-secretor genotype in some individuals (data not shown). Secretor status is not known to be relevant in transplantation or routine transfusion practice but this tool could be used to study this further. Similarly, the H panel mentioned above could be used to study A, B, and H antibody profiles in individuals with Bombay phenotype who lack the normal fucosyltransferase 1 (FUT1) gene.

As transplantation has recently observed steps forward in the in the field of xenotransplantation. (Cowan and Tector 2017; Montgomery et al. 2022; Porrett et al. 2022) This Luminex tool could be incorporated in the recipient risk assessment algorithms as ABO-O swine may have different ABO-H and glycans decorating their cells and tissues as compared to humans. (Milland and Sandrin 2006) Although the xenografts used in these recent transplants were from α-Gal knockout animals, there are other potential carbohydrate targets of interest and this reproducible assay could be expanded to include other xeno-glycans of interest.

4.5 Conclusion

In summary, we present a novel, highly reproducible ABO antibody detection assay that can be readily incorporated into clinical histocompatibility laboratories (**Table 4-2**). The initial data presented demonstrate a high degree of variability in ABO antibody profiles and the inadequacy of red cell titration assays to report the complexity of these antibodies. Our Luminex assay can characterize A and B glycan subtype-specific antibodies in human serum and define antibody isotypes, overcoming the many limitations of the hemagglutination assay. This glycan-specific antibody will allow allograft-specific determination of ABO antibody risk evaluation for use in ABOi transplantation as well as transfusion and has the potential to significantly advance these fields. This method also lends itself readily to basic glycobiology research in multi-centre studies.

4.6 References

Angeloni, Stephan, Robert Cordes, Sherry Dunbar, Carlos Garcia, Grant Gibson, Charles Martin, and Valerie Stone. 2016. *XMAP Cookbook: A Collection of Methods and Protocols for Developing Multiplex Assays with XMAP Technology*. 3rd ed. Austin, TX: Luminex Corporation.

Bentall, Andrew, Mylvaganam Jeyakanthan, Manjit Braitch, Christopher W. Cairo, Todd L. Lowary, Stephanie Maier, Anne Halpin, Bruce Motyka, Lu Zou, Lori J. West, and Simon Ball. 2021. "Characterization of ABH-Subtype Donor-Specific Antibodies in ABO-A-Incompatible Kidney Transplantation." *American Journal of Transplantation* 21(11):3649–62.

Bentall, Andrew, A. Nicholas R. Barnett, Manjit Braitch, Nicos Kessaris, Will McKane, Chas Newstead, Gavin McHaffie, Alison Brown, Sian Griffin, Nizam Mamode, David Briggs, and Simon Ball. 2016. "Clinical Outcomes with ABO Antibody Titer Variability in a Multicenter Study of ABO-Incompatible Kidney Transplantation in the United Kingdom." *Transfusion* 56(11):2668–79.

Brian, Duncan Tait. 2016. "Detection of HLA Antibodies in Organ Transplant Recipients-Triumphs and Challenges of the Solid Phase Bead Assay." *Frontiers in Immunology, Vol 7 (2016)*.

Bruneval, P., A. Angelini, D. Miller, L. Potena, A. Loupy, A. Zeevi, E. F. Reed, D. Dragun, N. Reinsmoen, R. N. Smith, L. West, S. Tebutt, T. Thum, M. Haas, M. Mengel, P. Revelo, M. Fedrigo, J. P. Duong Van Huyen, and G. J. Berry. 2017. "The XIIIth Banff Conference on Allograft Pathology: The Banff 2015 Heart Meeting Report: Improving Antibody-Mediated Rejection Diagnostics: Strengths, Unmet Needs, and Future Directions." *American Journal of Transplantation* 17.

Clausen, H. and S. Hakomori. 1989. "ABH and Related Histo-Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution." *Vox Sanguinis* 56(1):1–20.

Clausen, H., S. B. Levery, E. Nudelman, S. Tsuchiya, and S. Hakomori. 1985. "Repetitive A Epitope (Type 3 Chain A) Defined by Blood Group A1-Specific Monoclonal Antibody TH-1: Chemical Basis of Qualitative A1 and A2 Distinction." *Proceedings of the National Academy of Sciences of the United States of America* 82(4):1199–1203.

Cowan, P. J. and A. J. Tector. 2017. "The Resurgence of Xenotransplantation." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 17(10):2531–36.

Daga, Sunil, Shimon Hussain, Nithya Krishnan, David Lowe, Manjit Braitch, Prashanth Patel, Andrew Bentall, Simon Ball, Daniel A. Mitchell, Robert Higgins, Ian Skidmore, Daniel Zehnder, and David Briggs. 2021. "Immunoglobulin Isotype Compositions of ABO Specific Antibodies Are Dependent on the Individual Patient Blood Group and Blood Group Specificity: Results from a Healthy Donor Cohort." *Journal of Immunological Methods* 494:113053.

Datta, S. S., S. Basu, M. Reddy, K. Gupta, and S. Sinha. 2021. "Comparative Evaluation of the Conventional Tube Test and Column Agglutination Technology for ABO Antibody

Titration in Healthy Individuals: A Report from India." *Immunohematology* 37(1):25–32.

Denomme, Gregory A. and Waseem Q. Anani. 2020. "ABO Titers: Harmonization and Identifying Clinically Relevant ABO Antibodies." *Transfusion* 60(3):441–43.

El-Awar, N., J. Lee, and P. I. Terasaki. 2005. "HLA Antibody Identification with Single Antigen Beads Compared to Conventional Methods." *Human Immunology* 66(9):989–97.

Fan, Xiaohu, Andrew Ang, Stacey Pollock-BarZiv, Anne I. Dipchand, Phillip Ruiz, Gregory Wilson, Jeffrey L. Platt, and Lori J. West. 2004. "Donor-Specific B-Cell Tolerance after ABO-Incompatible Infant Heart Transplantation." *Nature Medicine* 10(11):1227–33.

Haas, M., A. Loupy, C. Lefaucheur, C. Roufosse, D. Glotz, D. Seron, B. J. Nankivell, P. F. Halloran, R. B. Colvin, N. Alachkar, S. Bagnasco, Y. Bouatou, J. U. Becker, L. Cornell, J. P. Duong van Huyen, I. Gibson, R. Mannon, M. Naesens, V. Nickeleit, P. Nickerson, D. L. Segev, H. K. Singh, M. Stegall, P. Randhawa, L. Racusen, K. Solez, and M. Mengel. 2017. "The Banff 2017 Kidney Meeting Report: Revised Diagnostic Criteria for Chronic Active T Cell-Mediated Rejection, Antibody-Mediated Rejection, and Prospects for Integrative Endpoints for Next-Generation Clinical Trials." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*.

Halpin, Anne M., Robert A. Bray, Chris W. Cairo, Patricia M. Campbell, Dongfeng\ Chen, Esme Djike, Tess Ellis, Howard M. Gebel, Anna Greenshields, Sebastiaan Heidt, Annette M\ Jackson, Gonca Karahan, Robert Liwksi, Todd L. Lowary, Stephanie Maier, Bruce Motyka, Cathi L. Murphey, Jean Pearcey, Harold C. Sullivan, Anat R. Tambur, Paul R. Warner, and Lori J. West. 2020. "ABO Antibody Detection: Comparing Apples to Apples in a Multisite Luminex Tool Evaluation." *Human Immunology* 81(Supplementao):36.

Hussey, J. C., J. Parameshwar, and N. R. Banner. 2007. "Influence of Blood Group on Mortality and Waiting Time before Heart Transplantation in the United Kingdom: Implications for Equity of Access." *The Journal of Heart and Lung Transplantation : The Official Publication of the International Society for Heart Transplantation* 26(1):30–33.

Irure, Juan, Esther Asensio, Emilio Rodrigo, igo Romn, Javier Gmez, Manuel Arias, Marcos Lpez-Hoyos, and David San Segundo. 2017. "Improvement in the Definition of Anti-HLA Antibody Profile in Highly Sensitized Patients." *PLoS ONE* 12(2):1–13.

Jeyakanthan, M., P. J. Meloncelli, L. Zou, T. L. Lowary, I. Larsen, S. Maier, K. Tao, J. Rusch, R. Chinnock, N. Shaw, M. Burch, K. Beddows, L. Addonizio, W. Zuckerman, E. Pahl, J. Rutledge, K. R. Kanter, C. W. Cairo, J. M. Buriak, D. Ross, I. Rebeyka, and L. J. West. 2016. "ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation." *American Journal of Transplantation* 16(5):1548–58.

Jeyakanthan, M., K. Tao, L. Zou, P. J. Meloncelli, T. L. Lowary, K. Suzuki, D. Boland, I. Larsen, M. Burch, N. Shaw, K. Beddows, L. Addonizio, W. Zuckerman, B. Afzali, D. H. Kim, M. Mengel, A. M. J. Shapiro, and L. J. West. 2015. "Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation." *American Journal of Transplantation* (10):2602.

JP, AuBuchon, de Wildt-Eggen J, and Dumont LJ. 2008. “Reducing the Variation in Performance of Antibody Titrations.” *Archives of Pathology & Laboratory Medicine* 132(7):1194–1201.

Kahlyar, Htar, David Roxby, Tony Badrick, and Thiru Vanniasinkam. 2022. “Challenges in Antibody Titration for ABO-Incompatible Renal Transplantation.” *Vox Sanguinis* 117(1):109–18.

Kang, Seon Joo, Young Ae Lim, and Sae Yun Baik. 2014. “Comparison of ABO Antibody Titers on the Basis of the Antibody Detection Method Used.” *Annals Of Laboratory Medicine* 34(4):300–306.

Karahan, Gonca E., Juliette Krop, Caroline Wehmeier, Yvonne de Vaal, Janneke Langerak-Langerak, Dave L. Roelen, Neubury M. Lardy, Frederike J. Bemelman, Ineke ten Berge, Marlies E. J. Reinders, Cees van Kooten, Frans H. J. Claas, and Sebastiaan Heidt. 2018. “An Easy and Sensitive Method to Profile the Antibody Specificities of HLA-Specific Memory B Cells.” *Transplantation* (Publish Ahead of Print, 9 November 2018).

Kobashigawa, Jon, Monica Colvin, Luciano Potena, Duska Dragun, Maria G. Crespo-Leiro, Juan F. Delgado, Michael Olymbios, Jayan Parameshwar, Jignesh Patel, Elaine Reed, Nancy Reinsmoen, E. Rene Rodriguez, Heather Ross, Randall C. Starling, Dolly Tyan, Simon Urschel, and Andreas Zuckermann. 2018. “The Management of Antibodies in Heart Transplantation: An ISHLT Consensus Document.” *Journal of Heart and Lung Transplantation* 1–11.

Landsteiner, K. 2001. “[Agglutination phenomena of normal human blood].” *Wiener klinische Wochenschrift* 113(20–21):768–69.

Lane, William. 2016. “Next Generation Sequencing for Blood Group Antigen Profiling.” *Blood* 128(22):SCI-24 LP-SCI-24.

de Mattos, Luiz Carlos. 2016. “Structural Diversity and Biological Importance of ABO, H, Lewis and Secretor Histo-Blood Group Carbohydrates.” *Revista Brasileira de Hematologia e Hemoterapia* 38(4):331–40.

Meloncelli, Peter J. and Todd L. Lowary. 2009. “Synthesis of ABO Histo-Blood Group Type V and VI Antigens*.” *Australian Journal of Chemistry* 62(6):558–74.

Meloncelli, Peter J. and Todd L. Lowary. 2010. “Synthesis of ABO Histo-Blood Group Type I and II Antigens.” *Carbohydrate Research* 345(16):2305–22.

Meloncelli, Peter J., Lori J. West, and Todd L. Lowary. 2011. “Synthesis and NMR Studies on the ABO Histo-Blood Group Antigens: Synthesis of Type III and IV Structures and NMR Characterization of Type I–VI Antigens.” *Carbohydrate Research* 346(12):1406–26.

Milland, J. and M. S. Sandrin. 2006. “ABO Blood Group and Related Antigens, Natural Antibodies and Transplantation.” *Tissue Antigens* 68(6):459–66.

Montgomery, Robert A., Jeffrey M. Stern, Bonnie E. Lonze, Vasishta S. Tatapudi, Massimo Mangiola, Ming Wu, Elaina Weldon, Nikki Lawson, Cecilia Deterville, Rebecca A. Dieter,

Brigitte Sullivan, Gabriella Boulton, Brendan Parent, Greta Piper, Philip Sommer, Samantha Cawthon, Erin Duggan, David Ayares, Amy Dandro, Ana Fazio-Kroll, Maria Kokkinaki, Lars Burdorf, Marc Lorber, Jef D. Boeke, Harvey Pass, Brendan Keating, Adam Griesemer, Nicole M. Ali, Sapna A. Mehta, and Zoe A. Stewart. 2022. "Results of Two Cases of Pig-to-Human Kidney Xenotransplantation." *New England Journal of Medicine* 386(20):1889–98.

Muthana, Saddam M. and Jeffrey C. Gildersleeve. 2016. "Factors Affecting Anti-Glycan IgG and IgM Repertoires in Human Serum." *Scientific Reports* 6(1):19509.

Oriol, R., R. Mollicone, P. Coullin, A. M. Dalix, and J. J. Candelier. 1992. "Genetic Regulation of the Expression of ABH and Lewis Antigens in Tissues." *APMIS. Supplementum* 27:28–38.

Pendu, J. Le, A. M. Dalix, R. Mollicone, K. Crainic, and R. Oriol. 1989. "EXPRESSION OF ABH, LEWIS AND RELATED TISSUE ANTIGENS IN THE HUMAN THYMUS." *International Journal of Immunogenetics* 16(1):19–32.

Pochechueva, Tatiana, Alexander Chinarev, Marianne Spengler, Elena Korchagina, Viola Heinzelmann-Schwarz, Nicolai Bovin, and Robert Rieben. 2011. "Multiplex Suspension Array for Human Anti-Carbohydrate Antibody Profiling." *Analyst* 136(3):560–69.

Porrett, Paige M., Babak J. Orandi, Vineeta Kumar, Julie Houp, Douglas Anderson, A. Cozette Killian, Vera Hauptfeld-Dolejsek, Dominique E. Martin, Sara Macedon, Natalie Budd, Katherine L. Stegner, Amy Dandro, Maria Kokkinaki, Kasinath V Kuravi, Rhiannon D. Reed, Huma Fatima, John T. Killian Jr., Gavin Baker, Jackson Perry, Emma D. Wright, Matthew D. Cheung, Elise N. Erman, Karl Kraebber, Tracy Gamblin, Linda Guy, James F. George, David Ayares, and Jayme E. Locke. 2022. "First Clinical-Grade Porcine Kidney Xenotransplant Using a Human Decedent Model." *American Journal of Transplantation* 22(4):1037–53.

Ravn, V. and E. Dabelsteen. 2000. "Tissue Distribution of Histo-Blood Group Antigens." *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 108(1):1–28.

Stussi, G., K. Huggel, H. U. Lutz, U. Schanz, R. Rieben, and J. D. Seebach. 2005. "Isotype-Specific Detection of ABO Blood Group Antibodies Using a Novel Flow Cytometric Method." *British Journal of Haematology* 130(6):954–63.

Sullivan, Harold C., Howard M. Gebel, and Robert A. Bray. 2017. "Understanding Solid-Phase HLA Antibody Assays and the Value of MFI." *Human Immunology*.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. "Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation." *Transplantation* 95(1):19–47.

Tambur, A. R., N. D. Herrera, K. M. K. Haarberg, M. F. Cusick, R. A. Gordon, J. R. Leventhal, J. J. Friedewald, and D. Glotz. 2015. "Assessing Antibody Strength: Comparison of MFI,

C1q, and Titer Information." *American Journal of Transplantation* 15(9).

Tambur, Anat R., Patricia Campbell, Frans H. Claas, Sandy Feng, Howard M. Gebel, Annette M. Jackson, Roslyn B. Mannon, Elaine F. Reed, Kathryn Tinckam, Medhat Askar, Anil Chandraker, Patricia P. Chang, Monica Colvin, Anthony-Jake Demetris, Joshua M. Diamond, Anne I. Dipchand, Robert L. Fairchild, Mandy L. Ford, John Friedewald, Ronald G. Gill, Denis Glotz, Hilary Goldberg, Ramsey Hachem, Stuart Knechtle, Jon Kobashigawa, Deborah J. Levine, Joshua Levitsky, Michael Mengel, Edgar Milford, Kenneth A. Newell, Jacqueline G. O'Leary, Scott Palmer, Parmjeet Randhawa, John Smith, Laurie Snyder, Randall C. Starling, Stuart Sweet, Timucin Taner, Craig J. Taylor, Steve Woodle, Adriana Zeevi, and Peter Nickerson. 2018. "Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group Meeting Report." *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons*.

de Weerd, Annelies E. and Michiel G. H. Betjes. 2018. "ABO-Incompatible Kidney Transplant Outcomes." *Clinical Journal of the American Society of Nephrology* 13(8):1234 LP – 1243.

West, Lori J., Stacey M. Pollock-Barziv, Anne I. Dipchand, K. Jin Lee, Carl J. Cardella, Leland N. Benson, Ivan M. Rebeyka, and John G. Coles. 2001. "ABO-Incompatible Heart Transplantation in Infants." *New England Journal of Medicine* 344(11):793–800.

Yazer, Mark H. and Philip C. Spinella. 2018. "The Use of Low-Titer Group O Whole Blood for the Resuscitation of Civilian Trauma Patients in 2018." *Transfusion* 58(11):2744–46.

4.7 Tables and Figures

Table 4-1: Parallels between HLA and ABO antibody detection challenges

HLA and ABO antibody assessment challenge	Proposed resolution of this challenge in ABO antibody analysis by use of a Luminex bead-based antibody assay
Clinical significance of cell-based compatibility assessment *vs* virtual compatibility	There is need to determine clinical relevance of positive Luminex bead-based solid phase antibody detection in the presence of low or negative antibody titre in the cell based platform(s). Flow cytometry evaluation of ABO antibodies using red cell targets will not address the antibody subtype specificity limitation. Much like the HLA antibody evaluation by single antigen bead where antibody can only be determined to alleles or in the setting of high levels of HLA antibody, the ABH subtype specificity is only possible on platforms such as the Luminex system.
Reproducibility of results	The superior reproducibility of ABO antibody detection on the Luminex platform has already been established in these studies. Further evaluation of this antibody method could easily be carried out in the Canadian Blood Services HLA working group with national exercises involving all HLA laboratories across Canada as has already been done for HLA antibody assessment. This type of national standardization of the assessment of antibody fits within the mandate of the Canadian Transplant Registry.
Determination of positive threshold	An effective way to determine the biologically relevant levels of ABO antibodies in ABOi transplantation would be to perform retrospective studies where stored samples from ABOi transplant recipients and short and long-term outcomes are known. There is evidence that ABOi transplants can be safely performed in several large meta-analyses but there are also cases in which ABOi transplants had a poor outcome. These cases could begin to inform the clinically relevant levels of ABO antibody.
Test method interferences	Solid phase assays are known to be plagued by the test method interferences created by therapies used in transplantation. The use of intravenous immune globulin (IVIg) in desensitization is an example of such an interference. IVIg preparations have been tested using the Luminex panel and it was observed that they all have high levels of detectable ABO antibody (data not shown). This information is critical to the accurate interpretation of both ABO and HLA antibody results. Anti-thymocyte globulin could also contain ABO antibodies which could be investigated on the Luminex ABO antibody platform.
Detection of antibodies to cryptic epitopes	A challenge in Luminex bead-based HLA antibody detection been the detection of antibodies to cryptic epitopes present on beads. While glycans are not likely to have the same challenges as have been experienced with complex MHC protein targets as the protein folding is not an issue, it is possible that *in-vivo* modifications of ABH glycans in tissues such as those by sialic acids may create tissue glycotopes that differ from the tetra-saccharide structures bound to the Luminex beads. Monoclonal antibody flow and immunohistochemistry studies suggest that may not be the case but it cannot be ruled out as a possibility. It is also possible that polymorphisms in the H glycosyltransferase FUT1 will result in downstream modifications to the A and B glycans resulting from each of these respective transferases. Further development of monoclonal antibodies as well as flow cytometry studies of cells, immunohistochemistry studies of multiple tissues, and sequencing of the ABH-related genes will provide discovery in this area.
Complement interference	Complement interference has been shown to be a cause of false negative antibody results in the measurement of HLA antibodies in Luminex single antigen bead-based methodologies which can be addressed through the use of serum dilution or use of EDTA treatment of serum. (Brian 2016; Irure et al. 2017; Sullivan et al. 2017; Tambur et al. 2015) The presence of sometimes high levels of IgM ABO antibodies suggests that complement should in evaluated as a potential source of falsely low ABO antibody detection. We compared several high MFI sera samples to EDTA plasma and did not observe this phenomenon (data not shown). The dilution of the serum samples prior to incubation on the ABO beads may mitigate this technical issue.

	Further studies using Melon column to remove IgM antibodies could be performed to confirm that IgM antibodies are not blocking the ability of IgG antibodies to bind to the beads and/or binding C1q which also could block IgG antibody binding.

Table 4-2: Comparison of ABO antibody assessment methods.

	Hemagglutination	ABH Glycan MicroArray	Bead-Based Solid Phase Assay
Determination of ABH subtype antibody specificity	No	Yes	Yes
Readily distinguish IgM vs IgG antibodies	No	Yes	Yes
Read-out is documented for future review	No	Yes	Yes
Reproducibility	Low	High	Very High
Equipment required exists in the clinical laboratory	Yes	No	Yes
Expertise to perform assay exists in the clinical laboratory	Yes	No	Yes
Time to perform assay	1.5 hours	4.75 hours	2.5 hours
Test can be easily run as STAT	Yes	No	Yes
Cost	Low	Moderate	Moderate
Summary of methodology:	**Inexpensive method suited to transfusion support but inadequate in ABOi transplantation**	**Excellent research tool for the fine characterization of ABO antibodies**	**Best suited tool for clinical laboratory measurement of ABO antibodies in ABOi transplantation**

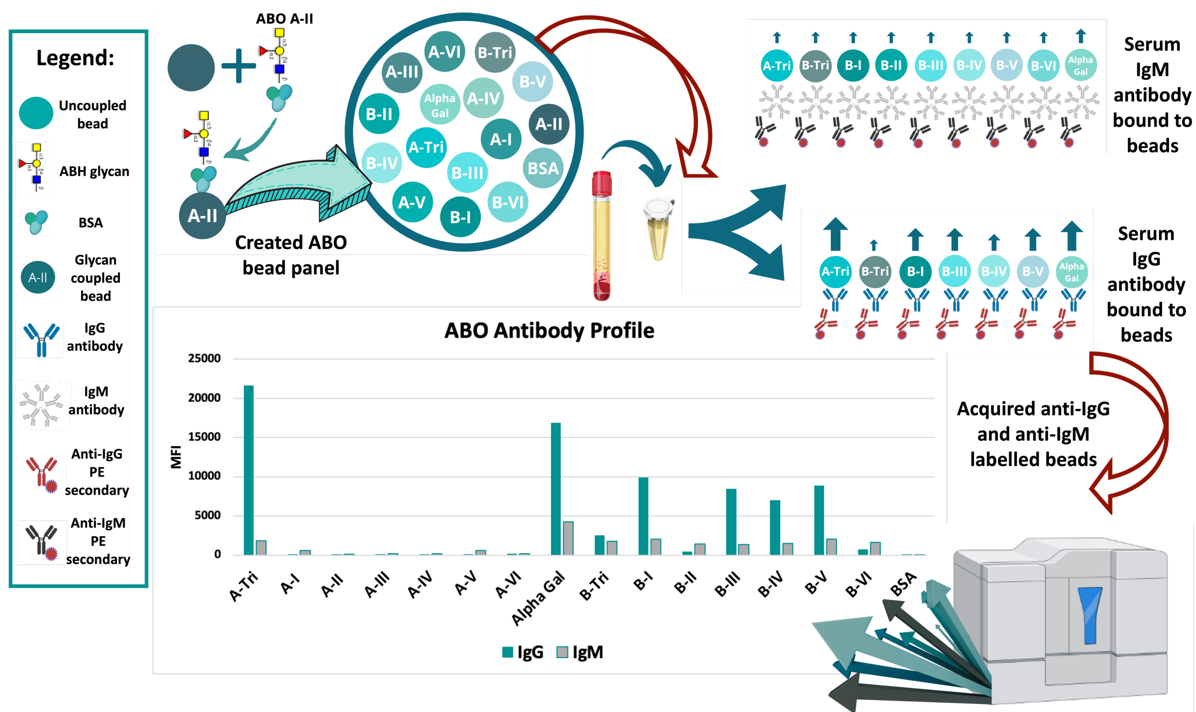


Figure 4-1. Individual Luminex beads were coupled to each ABO-A and ABO-B glycan subtype antigen. Negative control beads included a bead coupled to BSA as well as a bead that was not coupled with any target. A positive control bead was coupled to α-Gal. Serum or plasma was added to duplicate wells for the addition of either IgG and IgM secondary antibody. This example of an ABO antibody profile was from an ABO-A1/O genotyped individual with a negative red cell hemagglutination titre against ABO-A1 erythrocytes and an ABO-B titre of 1/32.

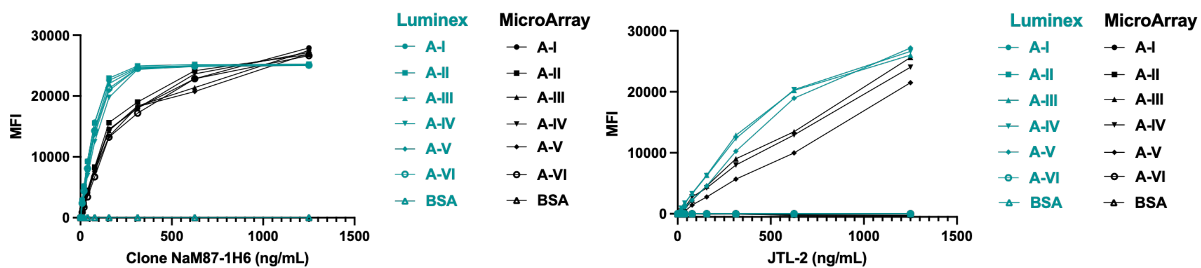


Figure 4-2. The Luminex assay was able to detect A glycans with more sensitivity than the microarray assay; this increased sensitivity was observed most clearly in the lower concentrations of antibody.

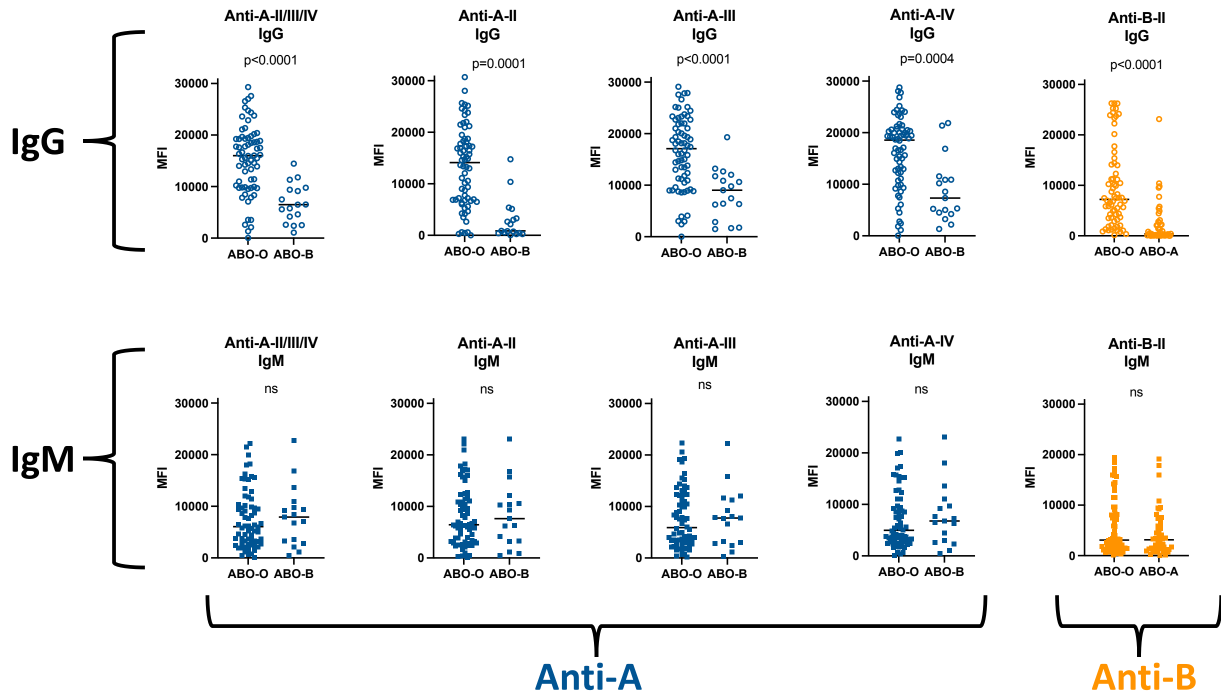


Figure 4-3. There was a wide range of IgG and IgM anti-ABO antibodies in healthy adult individuals across the ABO-O, -A, and -B blood groups. ABO-O individuals (n=68) produced higher levels of IgG anti-A than ABO-B individuals (n=17), and higher IgG anti-B compared to ABO-A individuals (n=48). In contrast, IgM isotype anti-A and anti-B antibodies did not vary based on the ABO blood group.

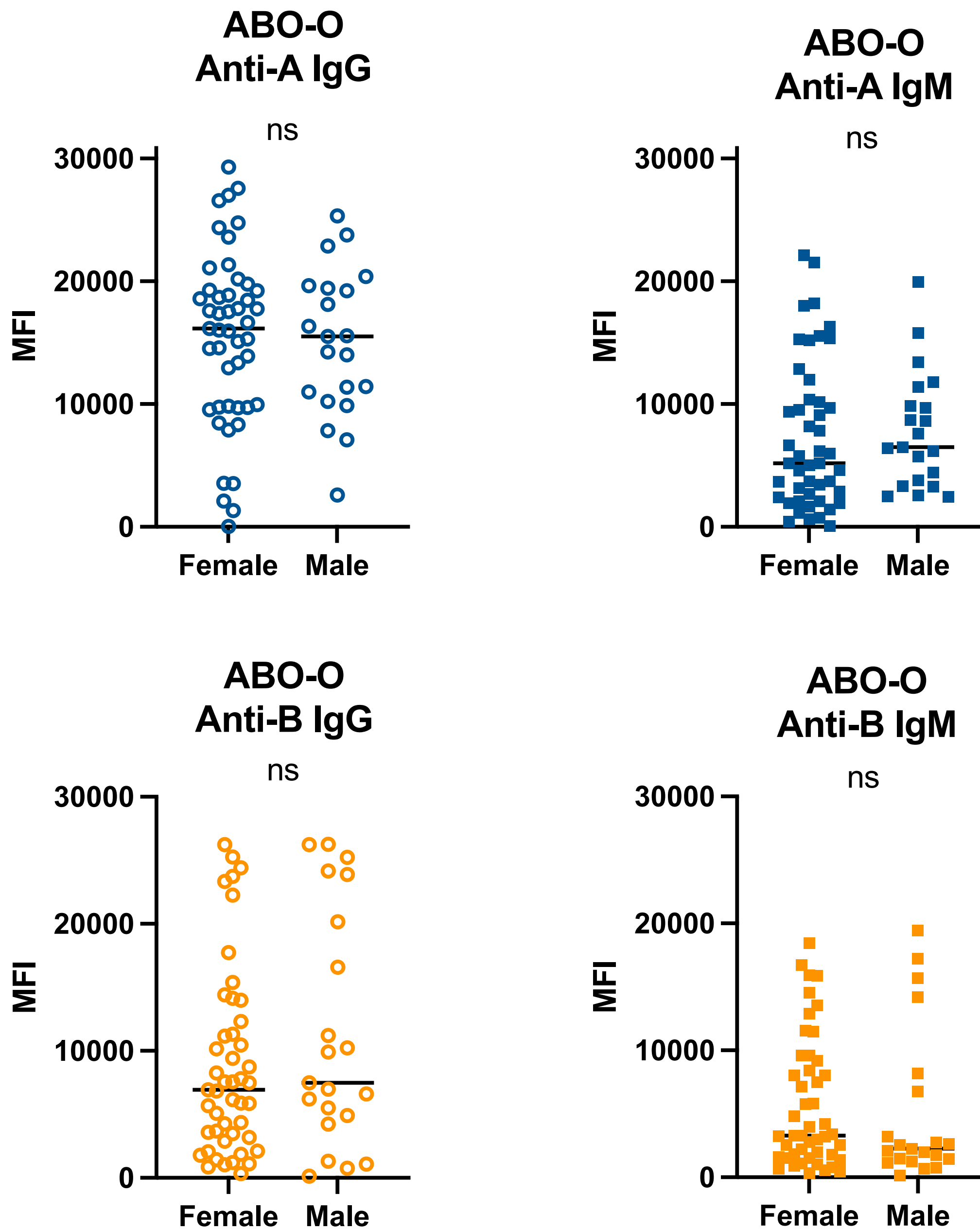


Figure 4-4. In sera from ABO-O individuals, there were no significant differences in the level of ABO antibodies between females (n=47) and males (n=21). Anti-A was an average of A-II, III, and IV subtype-specificities; anti-B is only B-II subtype-specificity.

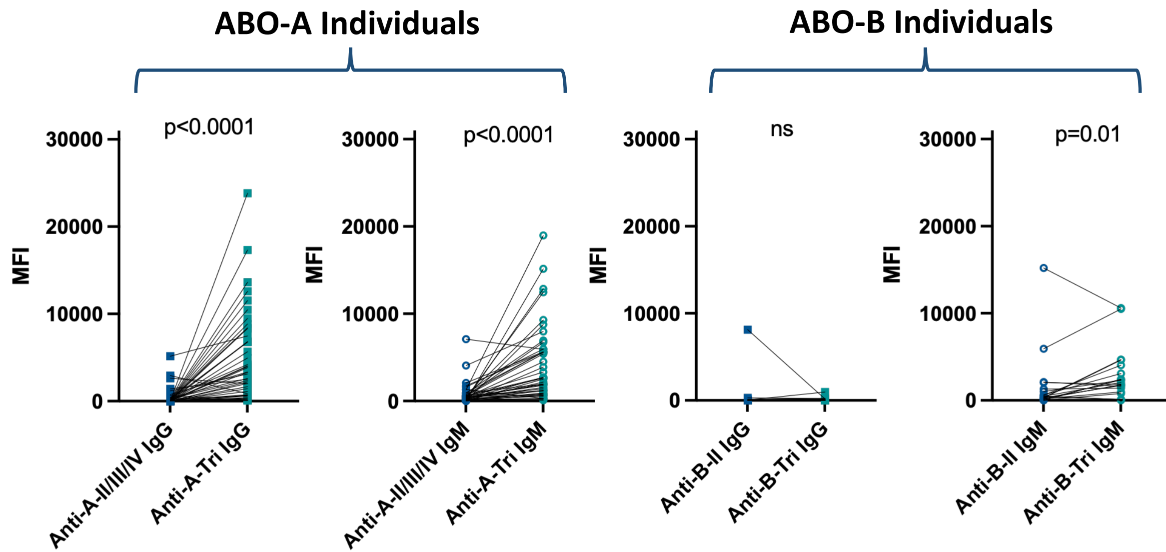


Figure 4-5. It is common for ABO-A individuals to have high levels of IgG and IgM anti-A-trisaccharide antibodies but comparatively low levels of antibody to A-II, III, and IV subtype glycans. ABO-B individuals are unlikely to have either anti-B- trisaccharide or anti-B-II antibodies.

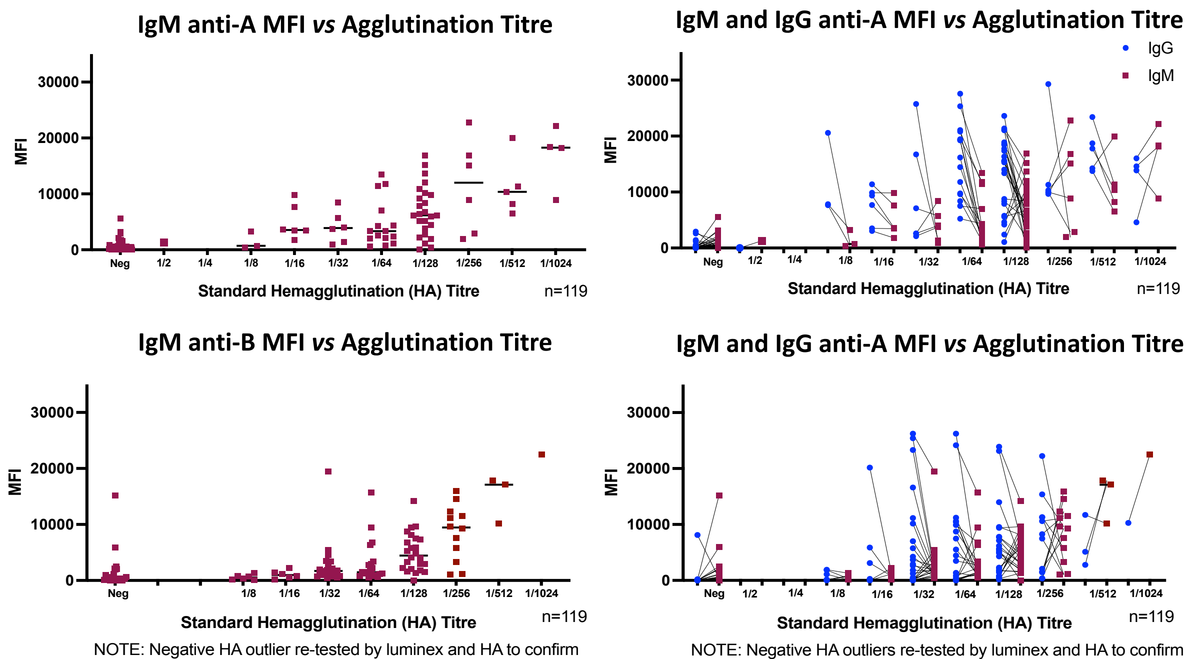
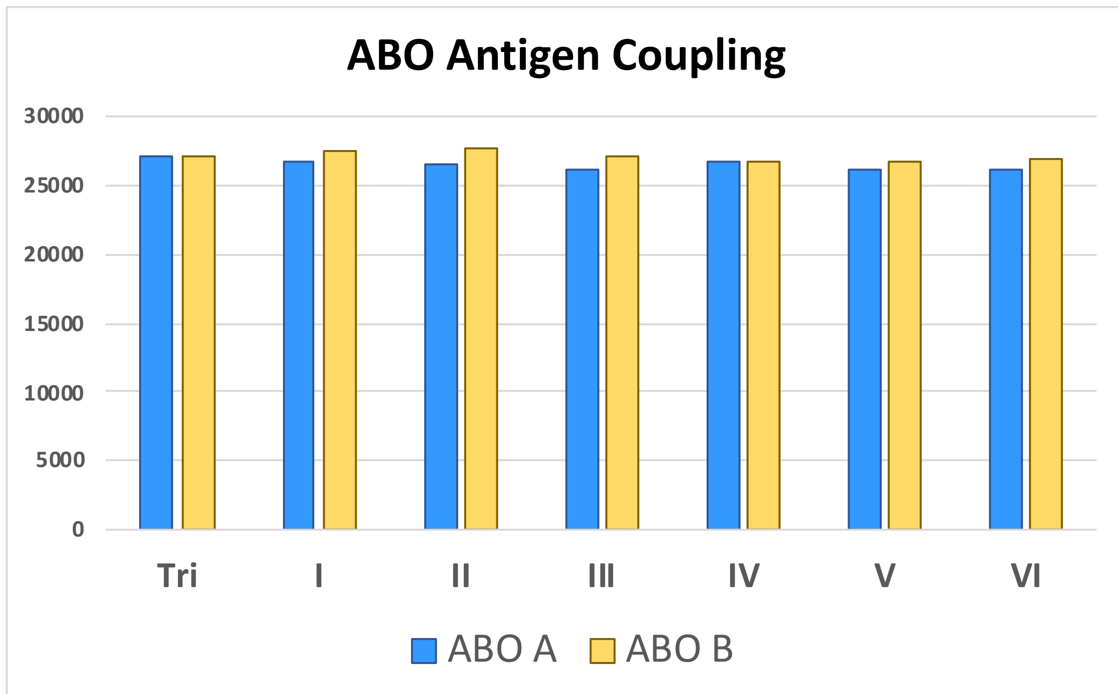
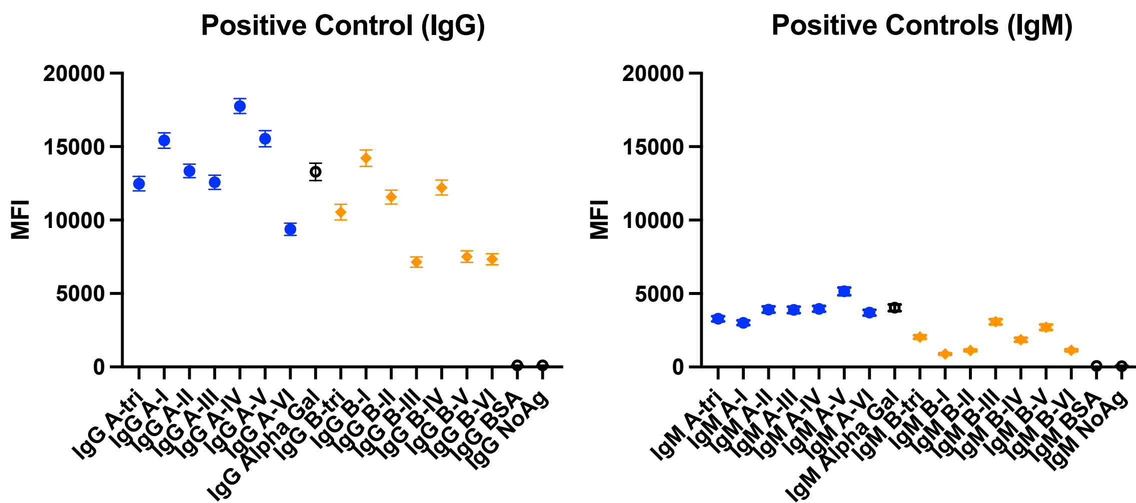


Figure 4-6. Sera were tested in parallel by red cell agglutination and Luminex panel analysis (n=119). There was a wide range of IgG and IgM antibody within each red cell agglutination antibody titre.

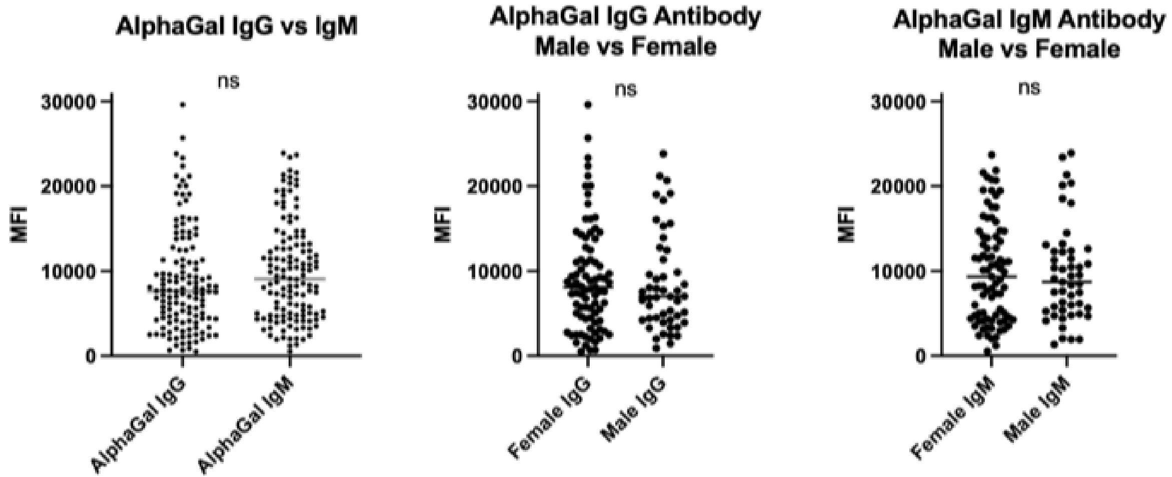
4.8 Supplemental figures



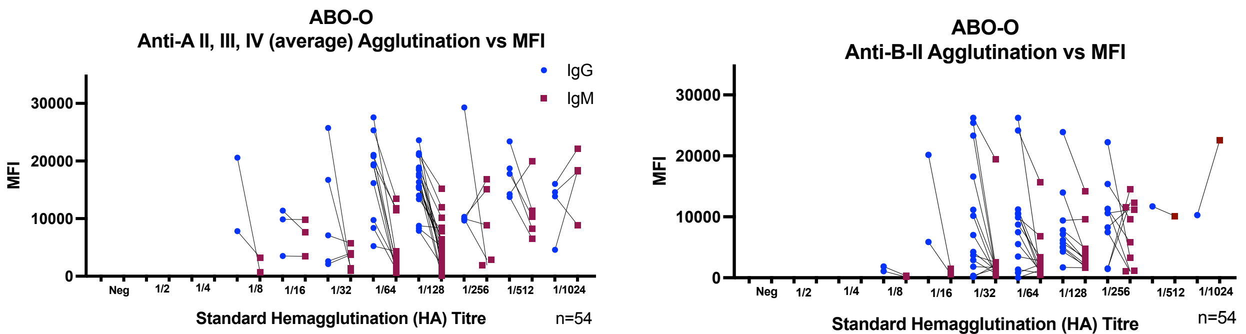
Supplemental Figure 1 (4-S1): The A and B glycan subtype coupling was highly consistent for a beads as shown here with this bead coupling confirmation data. The presence of antigen was confirmed with Immucor anti-A and anti-B Novoclone reagents.



Supplemental Figure 2 (4-S2): The positive control serum results from 24 individual runs shows excellent reproducibility. These results are shown as the mean +/- the 95% confidence interval.



Supplemental Figure 3 (4-S3): There were no observed differences between IgG and IgM α-Gal antibody levels (n=132). There were also no differences by sex (n=83 females and n=49 males)



Supplemental Figure 4 (4-S4): The levels of IgG and IgM antiobdies were compared to titres in only the ABO-O individuals (n=54).The same variability was observed as shown in Figure 6. Hemagglutination titres were compared for ABO-O individuals (n=54)

Chapter 5:

Discussion

5.1 Overview of findings

The unique immunology of young children allows transplant opportunities such as ABOi heart transplantation and simultaneously creates a compelling need for pediatric studies. The data presented in this thesis highlight three distinct areas in which a focus on pediatric transplant research is of key importance. First, we demonstrated a different risk for HLA antibody production following VAD implementation in children as compared to adults. Second, we demonstrated the apparent production of AT1R antibodies in a higher proportion of children than previously reported in adults, both transplant patients and non-transplant controls. Importantly, however, we also demonstrated potential interference in the most commonly used commercial assay. While this work did not include adult populations as a comparison group, there are known differences in potentially interfering substances between children and adults that may explain this finding. Finally, the developmental inability of infants to mount immune responses to glycan targets provides an opportunity to cross ABO barriers to expand donor availability, but further advances are limited by current techniques for ABO antibody detection. The Luminex bead-based ABO antibody characterisation method developed here provides many advantages over hemagglutination; this assay can be readily implemented into the clinical histocompatibility laboratory as the expertise, equipment, and patient samples are already in place.

5.1.1 Main Findings from Chapter 2

Previous studies on the degree of HLA sensitization in patients undergoing VAD support have reported variable results.(Alba et al. 2015; Askar et al. 2013; McKenna David H et al. 2002; O'Connor et al. 2010) This inconsistency may be impacted by the evolution of HLA antibody detection methods, and thus the sensitivity of testing, as well as advances in transfusion practices such as leukocyte reduction strategies. A role for blood product exposure during VAD therapy on the development of HLA antibodies has also not been definitively established or refuted.(Alba et al. 2015; Askar et al. 2013; McKenna David H et al. 2002; Moazami et al. 1998). Our retrospective study compared adult and pediatric patients from the same centre with identical histocompatibility testing practices and transfusion practices. We found that pediatric VAD patients were significantly less likely than adults to produce HLA antibodies. Additionally, we observed that antibodies specific to class I HLA structures were not elevated in either VAD population. The vast majority of *de novo* antibodies with VAD therapy were detected within 30 days of implantation, the same timeframe during which the largest quantities of blood products were administered.

5.1.2 Main findings from Chapter 3

Although AT1R antibodies have emerged as a potentially damaging factor in adult organ transplantation, the frequency of AT1R antibody production in pediatric populations has not been widely reported. Our study showed an apparent high frequency of AT1R antibodies in pediatric heart transplant patients as well as pediatric non-transplanted controls compared to historical studies in adult kidney transplant recipients. Importantly, however, our additional findings in this study call into question the specificity of the commercial ELISA assay used in all

but the original paper reporting the relevance of AT1R antibodies in transplantation. When sera were retested following adsorption with a reagent commonly used in histocompatibility laboratories to reduce non-specific binding, 75% of samples positive for AT1R antibodies converted to negative. This same adsorption protocol did not remove the AT1R reactivity from the positive control or the assay calibrators. Neu5Gc glycans were detected on the adsorption microparticles and this same glycan is known to be expressed on CHO cells used to generate the AT1R antigens used in the assay. Non-specific binding in the commercial AT1R assay may be due, at least in part, to anti-Neu5Gc antibodies; use of this common adsorption reagent will improve the assay specificity.

5.1.3 Main findings from Chapter 4

ABOi heart transplantation is possible in infancy and early childhood due to the normal delay in development of immune responses to glycan antigens. The clinical method currently used to determine eligibility for ABOi pediatric cardiac transplantation (and all ABOi transplantation) is the red cell agglutination test. This assay is plagued by low reproducibility. (Datta et al. 2021; Kahlyar et al. 2022) Additionally, erythrocyte agglutination cannot distinguish antibodies with specificities for ABH glycan subtypes. This method limitation is relevant to cardiac transplantation because red cells are decorated with a different ABH glycan repertoire than vascular endothelium of the heart graft. Thus, an ABO-A erythrocyte agglutination titre could be high due to antibody specificities that are irrelevant to the cardiac allograft. Access to transplantation from ABOi donors increases options for these children for whom too few ABOc donor organs are available and waitlist mortality is high.(Almond et al. 2009; Singh et al. 2011) A Luminex bead-based single glycan subtype-specific antibody assay was developed and

validated as an alternative to the red cell agglutination method of assessing ABO antibodies in pediatric heart transplant candidates.

5.2 Strengths and limitations

5.2.1 Strengths and limitations from Chapter 2

The single-centre design of this study with a highly active VAD program offered some advantages. The pediatric and adult patients were collected at similar time points and the HLA antibody assessment was performed by a single histocompatibility laboratory. Similarly, a single transfusion medicine laboratory provided all blood products, which simplified the collection of extensive transfusion data.

It has been reported that following VAD implantation, non-specific antibody patterns in HLA solid phase antibody analysis can be observed. (See et al. 2017) These antibody results were analysed as per standard clinical practice, reviewing for epitopes and ruling out non-specific patterns. Some of the retrospective data were from an earlier time point in the use of this solid phase, single antigen bead antibody testing and these data were all reviewed for consistency in reporting of antibody patterns.

For patients who proceeded to transplantation, all transplants were performed at the same transplant centre, which facilitated the collection of pre- and post-transplant outcomes. Another finding of this data collection that was not reported in the Chapter 2 publication was that it provided an opportunity to cross-reference the information regarding sensitizing events of transplant patients from the local VAD registry as compared to the information provided to the

histocompatibility laboratory. It is the practice of our facility that a sample is sent to the HLA laboratory 30 days following VAD implant to allow for detection of *de-novo* HLA antibodies or any change in antibody status following this event and/or the accompanying transfusions. Despite this practice, our study noted that a post-VAD sample was not sent to the HLA laboratory for more than 10% of pediatric patients; additionally, 30% of samples that were collected post-VAD were sent with no indication of the VAD implant. Furthermore, 43% of patients proceeded to transplant with the HLA laboratory unaware of the VAD implant. This information is critical to appropriate sample handling and antibody testing algorithms as well as sample selection and crossmatch at the time of a donor offer. As a direct result of our study, communication with the transplant program was improved. Specifically, the form used at the time of patient activation was edited to add questions related to VAD history. This important system improvement process was also published. (Khoury et al. 2021)

There were also limitations to this study. As is the case for most adult VAD studies, the number of adult female subjects is low as compared to men, which did not allow for sex-based analysis of the adult study population. Pregnancy history information was also not available for two female subjects who did not proceed to transplant. As the study was retrospective in nature, the time collection points were slightly variable despite pre-determined orders for post-VAD sample collection.

5.2.2 Strengths and limitations from Chapter 3

The AT1R antibody study had several strengths. This work benefitted from the use of one single lot number of reagents, which removed lot-to-lot variability in testing. At the initiation of the study, stored American Society for Histocompatibility and Immunogenetics (ASHI) external

proficiency testing samples were used to confirm that test results were comparable to those from another centre. The availability of age- and sex-matched non-transplant control samples for comparison to the transplant patient population was an additional strength.

It is a limitation of this study that the median age of patients and controls was less than five years. This cohort was not an unreasonable reflection of the age range of children undergoing cardiac transplantation but it did not enable specific study of adolescent patients. We did not see an association with sex but it has been reported in a large prospective study by Lefaucheur et al that adult female kidney transplant recipients are more likely to develop AT1R antibodies than males. (Lefaucheur et al. 2019) It has also been shown that AT1R antibodies are not associated with pregnancy. (Honger et al. 2014) It is known that females develop more autoimmune disease than males (Klein and Flanagan 2016; Ngo, Steyn, and McCombe 2014). Therefore, it is possible that females could be more likely than males to produce AT1R antibodies in pediatric populations as well. Sex differences in AT1R antibody status may emerge during adolescence, as reported for sex-based differences in allo-immunity (Foster et al. 2021; Lepeytre et al. 2017).

One of the limitations of the adsorption aspect of this study is that only cardiac transplant pediatric patients and controls were included. We have developed collaborations with Dr. Kelley Hitchman, University of Texas Health San Antonio, whose laboratory performed the same adsorption protocol in pediatric and adult renal transplant patients (preliminary results shown here in **Figure 5-1**). It is reassuring that similar findings have been observed by others utilizing this adsorption method in AT1R antibody testing with this commercial assay. Our comparison between adult and pediatric patients revealed that children were more likely to have AT1R

antibodies, but these decreased to a negative status post-adsorption in the majority of cases. A previous study of pediatric renal transplant patients, which also included non-transplant controls, showed that there was a high proportion of AT1R antibody positive pediatric patients and also that the pediatric controls had higher levels of AT1R antibody as compared to adult controls. (Bjerre et al. 2016) However, this study did not consider pediatric-specific issues with the ELISA antibody detection method.

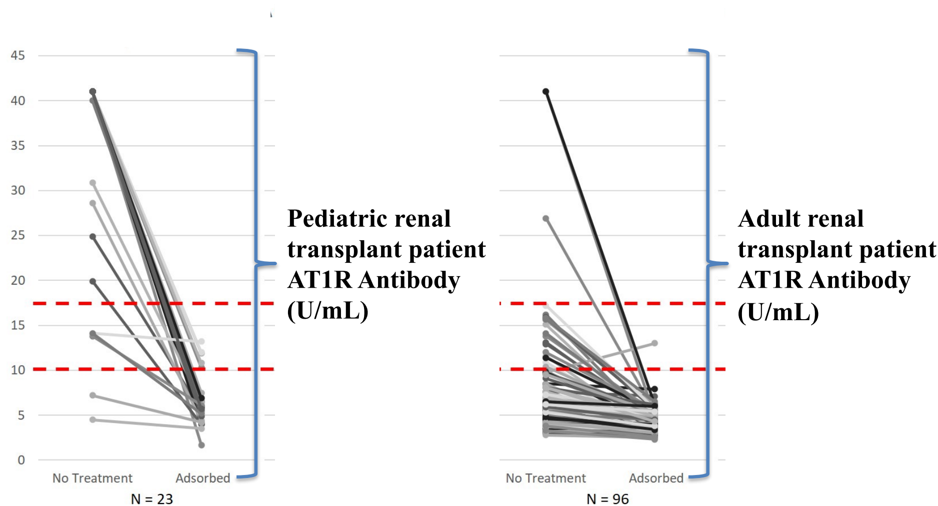


Figure 5-1. Pediatric renal transplant patients have higher pre-Adsorb Out™ levels of AT1R antibodies as compared to adult renal transplant patients. All samples showed a reduction of AT1R antibody levels following adsorption although pediatric samples have a more striking reduction than adults. All samples are post-transplant. The upper red hashed line represents the 17 U/mL threshold and the lower line the 10 U/mL negative threshold.
Figure and data shared with permission from Dr. Kelley Hitchman.

5.2.3 Strengths and limitations from Chapter 4

The bead-based Luminex ABO-antibody assay overcomes several limitations of the erythrocyte agglutination method of ABO-antibody assessment, most notably antibody subtype specificity, isotype determination, and reproducibility. This assay builds on the glycan array method that was

evaluated in a pediatric heart transplant population. (Jeyakanthan et al. 2016). The same source of A and B subtype glycans was used, but the antigens were coupled to Luminex beads rather than applied onto glass slides. Bead manufacturing could be done in the lab, avoiding the need to send antigens to a third party to produce the assay. **Figure 2-2** demonstrates that the sensitivity of the Luminex assay is comparable to or better than the array, particularly in the lower range of antibody detection. The bead-based assay is also quicker to perform than the microarray and easier to run as a stand-alone test. The ability to run a single, rapid test is important in the clinical laboratory as transplant patients may need to be listed urgently for transplant and/or assessed rapidly for antibody-mediated rejection.

Many of the strengths of the Luminex ABO antibody assay mirror those of solid phase, single antigen bead HLA antibody testing. HLA antibody testing evolved from a cell-based, complement-dependent, cytotoxicity assay to an ELISA assay, then a flow cytometry-based method to the multiplex Luminex assay currently used globally. (Gebel and Bray 2010; Tinckam 2009) The many lessons learned historically through this process can be applied to the validation and implementation of the ABO Luminex antibody assay, and the HLA community is well-versed in use of Luminex technologies.

Another strength of this assay is the reproducibility of the results. In addition to the positive control data shared in Supplemental Figure 2.2, a multicentre validation trial was performed. Well-characterized sera (n=10) as well as the ABO bead panel, all reagents, and a standard operating procedure was sent to eight histocompatibility laboratories across Canada, the US, and Europe. Excellent concordance was observed amongst the MFI results. The results of the testing

of the positive control serum from the 8 sites as well as our local testing of this control are shown in **Figure 5-2**. Despite this being the first use of this assay, and variations in wash methods as well as Luminex machines, the results showed high concordance amongst the centres. (Halpin et al. 2018) These findings are especially relevant to pediatric heart transplant studies as multi-centre trials are particularly needed for ABOi pediatric heart transplant research due to low numbers of patients transplanted at each centre.

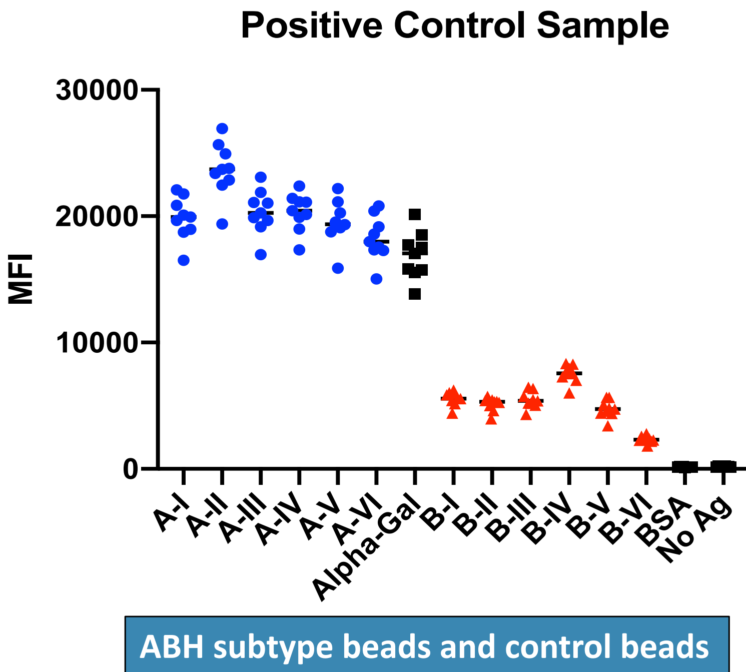


Figure 5-2: The positive control serum tested in nine different laboratories showed strong reproducibility of results using the Luminex bead-based method.

The study presented here was focused on analysis of healthy adult sera as a first step in assay development. Our data provide valuable information about ABO antibody patterns and comparison to standard agglutination titres but this is not the ultimate intended population for this thesis. Thus, this assay was also used to measure ABO antibodies in 31 patients under the age of 24 months awaiting heart transplantation. These data are preliminary as a complete

transfusion history must be collected before ABO antibody levels and isotypes can be fully interpreted, but the results are presented here for discussion.

The ABO antibody patterns in these children were diverse. The presence of passive maternal IgG antibodies was seen in sera from very early time points (**Figure 5-3**). In ABO-O individuals IgG anti-A antibody levels were significantly higher than anti-B levels ($p<0.0001$) although IgM anti-A and anti-B levels were not significantly different. IgM isotype antibodies did not appear to precede IgG antibodies. The evaluation of anti-B antibodies in both O and A individuals revealed higher levels of anti-B in ABO-O as compared to ABO-A patients (**Figure 5-4**).

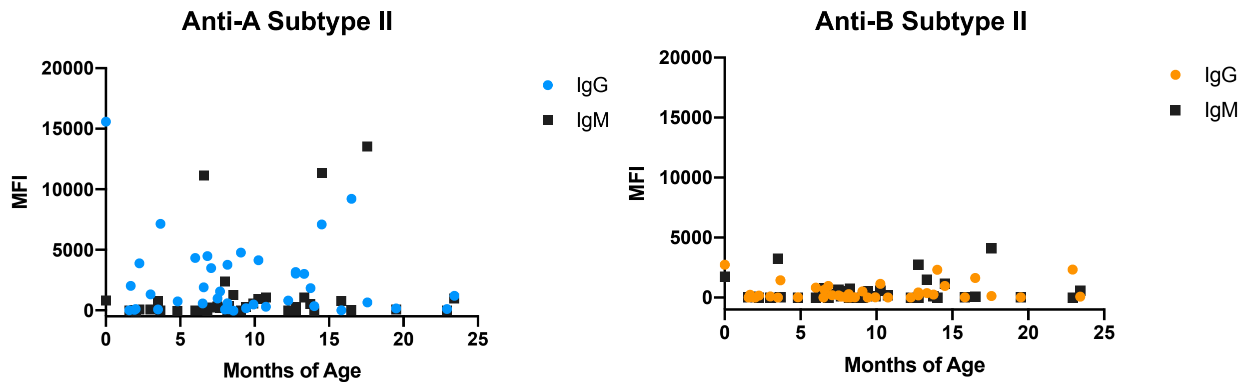


Figure 5-3: A-II and B-II subtype-specific antibody profiles for patients up to 24 months of age. Antibodies specific for A-II and B-II alone were assessed as these are the only relevant glycan subtypes in cardiac transplantation.

Anti-B Subtype II Antibody Development Over Time

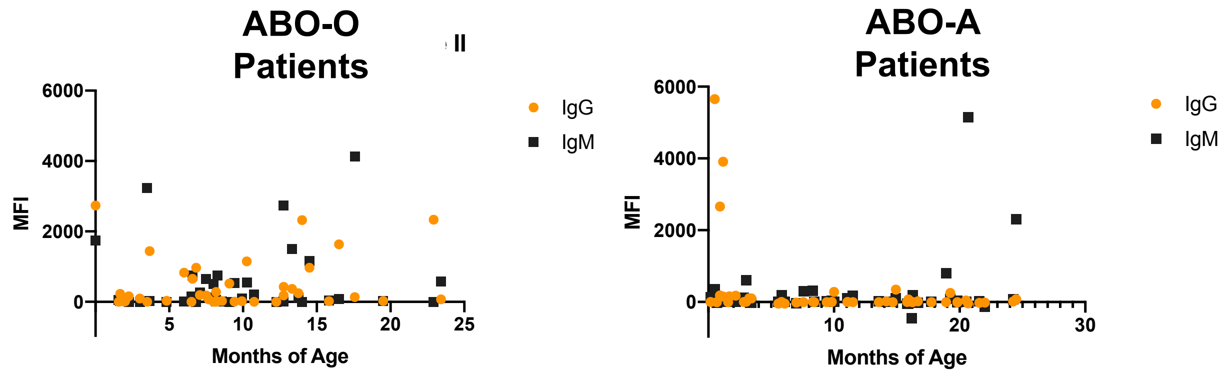


Figure 5-4: Anti-B-II were compared in ABO-O *vs* ABO-A individuals.
B-II antibody alone was assessed as this is the only relevant B-glycan subtype in cardiac transplantation.

Hemagglutination titres *vs* Luminex MFI values were compared in 56 samples from this patient cohort (**Figure 5-5)**. There were unexpected findings in that some high titre samples had low MFI values detected. Testing of sera from these outliers was repeated with the same results. Red cell allo-antibodies other than ABO antibodies, such as anti-M, can react at room temperature in the absence of anti-human globulin.(Yudin and Heddle 2014) The presence of other erythrocyte allo-antibodies was ruled out using a three-cell red cell screening panel (PanoCell, Immucor) in 17 outlier samples with sufficient available sera; these results were all negative suggesting that the ABO titre is not falsely increased by other known erythrocyte allo-antibodies.

Conversely, there were several sera with high MFI values and low anti-A and/or anti-B titres. For example, the anti-A titre for one serum sample was negative yet IgM anti-A antibodies were detected by Luminex at a MFI level greater than 15,000. This specific patient had also received a blood transfusion on the day of sample collection. It is possible that passively acquired

antibodies were detected by the bead-based assay, but it is unclear why blood donor anti-A antibodies would not also be detected by the agglutination test.

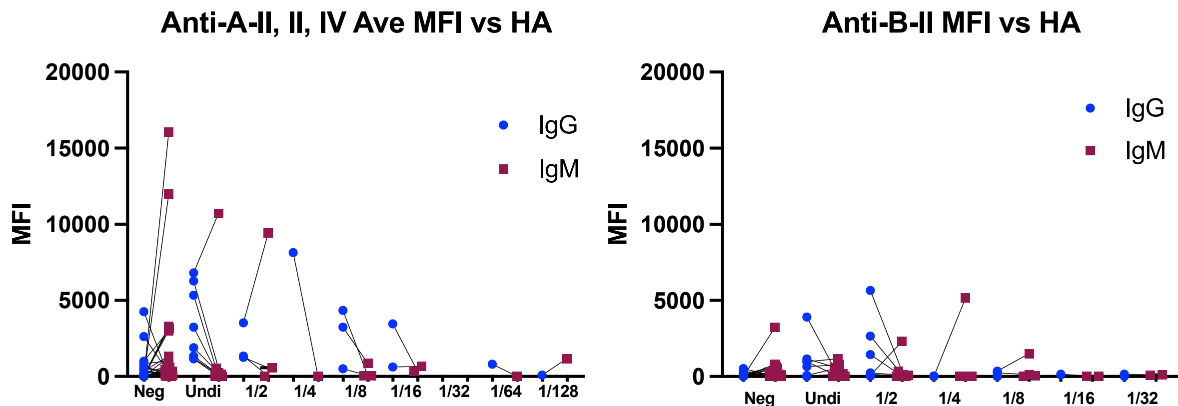


Figure 5-5: There is diversity of ABO antibody in each titre that differs from a similar adult sample comparison in Figure 4-6. High titres of antibody A-II, III, and IV antibody MFI was averaged to estimate the reactivity to ABO-A red cells used for agglutination testing while B-II alone was used for the ABO-B MFI; this is based on known representation of ABH glycans on red blood cells.

To explore the impact of transfusion on detection of ABO antibodies, a single patient with several documented transfusion events as well as multiple time points of Luminex antibody detection was examined more closely (**Figure 5-6**). This patient received transfusion of 560 mLs of red cell product and 150 mLs of platelets from ABO-O donors within 7 days of the final ABO antibody data shown here. These data suggest that passive antibody from erythrocyte transfusion may be more readily detected by the Luminex bead-based assay than the agglutination test. This patient also received many albumin transfusions but the impact of this blood product on detection of ABO antibodies by the agglutination test or the Luminex assay is unknown; the Luminex assay BSA control bead was negative for these samples suggesting that there is not a high background due to albumin reactivity. These passive antibodies shown in Figure 5-6 may be

clinically relevant therefore transfusion history may be essential to the interpretation of Luminex ABO-antibody results. As noted above, it is unclear why ABO antibodies in transfused blood products would not also be detected by the agglutination test.

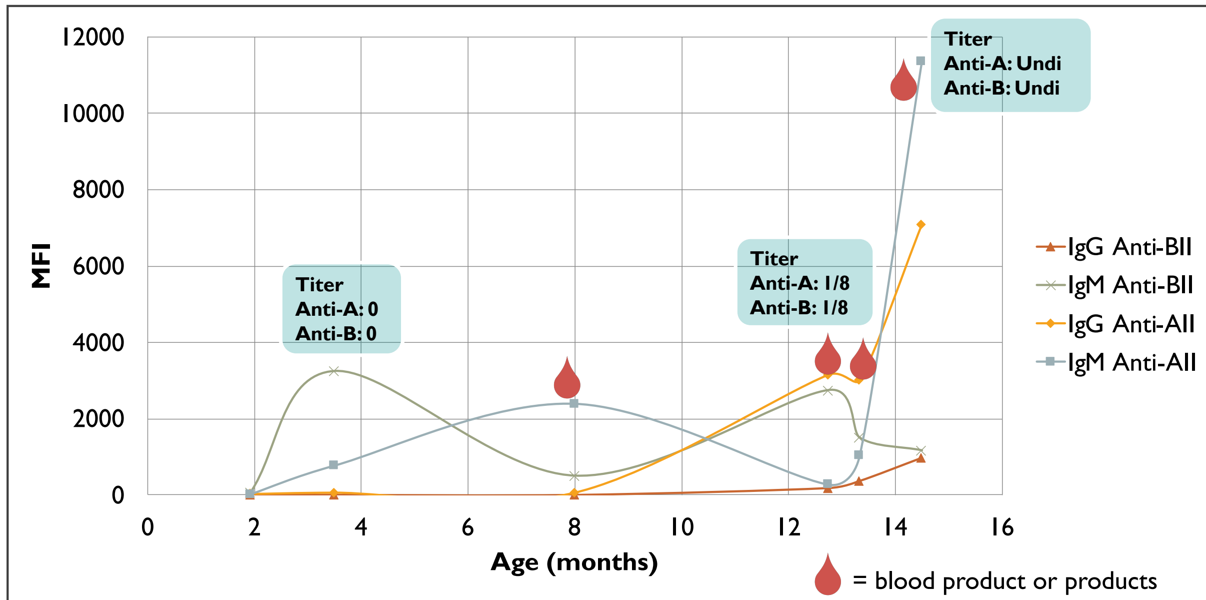


Figure 5-6: ABO antibody data from an ABO-O infant awaiting heart transplantation. The discrepancy between anti-A and anti-B antibody titres and MFI values may be a result of passive antibody from the blood product donor or the interference of albumin infusion in either the ABO titre or the Luminex assay. Titre is shown as ‘0’ (negative with all red cells), ‘Undi’ (positive only with undiluted serum), or 1/8 (positive up to and including the 1/8 serum dilution).

5.3 Future Directions and Practical Applications

5.3.1 Future Directions and Practical Applications for Chapter 2

The use of VAD therapy continues to be an important bridge to transplantation(Javier Delmo, Javier, and Hetzer 2020). As such, it is important to understand the impact on the development of HLA antibodies as these antibodies complicate donor selection. A similar retrospective study with methods similar to ours, but in an adult cohort, reported that presence of HLA antibodies before VAD implantation was the biggest predictor for the development of HLA antibodies after

VAD implantation but like our study had few adult female subjects (15% female) therefore the effect of sex and/or pregnancy in an adult cohort could not be measured.(Elkind et al. 2020)

Larger, prospective studies will enable more consistent sample collection time points, and for adult studies, could be designed to be more sex-balanced. In addition, memory B cell assays could be incorporated into study protocols to provide new insight into the role of immune memory and the development of HLA antibodies in the setting of VAD therapy. Assays such as those developed in Leiden utilize smaller volumes of blood and can be done using Luminex beads to test B cell supernatants.(Karahan et al. 2018). More thorough sensitization history would also enhance these VAD alloimmunization studies to include data such as pregnancy history, detailed historical and VAD-related transfusion data, as well as a history of transplanted tissue such the homografts used in pediatric cardiac surgeries.

Of note, the adolescent pediatric VAD patient population may have unique immune function and may warrant specific study in this area. Although no sex-based differences were seen in the data presented in Chapter 2, the median age was only 3.38 years. A multi-centre trial may be required to study this specific population due to low numbers in individual centres.

As was done in the work presented in Chapter 2, consideration for non-specific reactivity in the HLA panels following VAD therapy is important in these studies. In addition to seeking HLA-specific patterns, further investigation to the cause of non-specific reactivity is needed. It has been proposed that high background in HLA solid phase antibody assays is due to exposure to biomaterials and T cell activation.(Itescu and John 2003). Further study would provide the

clinical laboratory with more insight into how to mitigate non-specific HLA antibody patterns and may also shed some light on test method interferences in the AT1R antibody assay used in Chapter 3.

5.3.2 Future Directions and Practical Applications for Chapter 3

Conclusions amongst studies investigating the role of AT1R antibodies in transplant outcomes have been discordant. Some studies reported an association with antibody-mediated rejection whereas others found an association with cellular rejection, and others reported no association. (Fichtner et al. 2018; Kim et al. 2018; Lefaucheur et al. 2019; Pinelli et al. 2017). One centre initially reported an association between AT1R antibody and rejection but went on to publish a follow-up study where this association was no longer observed. (Deltombe et al. 2017; Giral et al. 2012). Inclusion of additional method controls such as a 'no antigen' or 'blank' well may reduce these types of conflicting reports. Routine use of the AdsorbOut treatment protocol may also provide a valuable tool to explore the true clinical relevance of AT1R antibodies pre- and post-transplantation. Pediatric populations may especially require these types of controls due to the presence of higher levels of anti-BSA antibodies as well as other naturally occurring antibodies *vs* adult populations that may cause interference in solid phase assays. (Gao et al. 2017; Rothberg and Farr 1965)

There is overlap between AT1R antibody and HLA antibody assays in this regard as VAD studies have also reported a role of anti-BSA antibodies driving non-specific reactivity in solid phase HLA antibody assays. (Newell et al. 2006) This finding may be especially relevant to pediatric heart transplant patients who have undergone VAD therapy; inclusion of anti-BSA antibody testing in these studies may assist with interpretation of the results.

We did not report on the impact of VAD implantation on AT1R antibody levels because the number of patients who had received an implant was low (n=9 of 42 patients, 21%) and the date of implant relative to AT1R antibody testing varied. However, 100% of the VAD patients had detectable AT1R antibodies pre-transplant when no adsorption was performed; the majority of the patients had AT1R antibodies ≧ 40 U/mL. When analysing sera without serum adsorption, there was a statistically significantly higher proportion of VAD patients with AT1R antibodies as compared to patients who did not receive a VAD ($p<0.01$). Following adsorption, four of the nine patients remained positive which is a slightly higher proportion of the patients tested with and without adsorption overall although this is not a statistically significant difference. It is possible that VAD implantation increases the presence of interfering substances and/or true AT1R antibodies but more study is needed.

The presence of non-specific reactivity to solid phase HLA platforms following VAD implant has been reported and was mitigated in the Chapter 2 HLA antibody study. (Nikaein et al. 2012) In the HLA antibody study it was possible to evaluate for the presence of non-specific reactivity due to the presence of negative antigen beads as well as known true HLA epitope patterns of reactivity. With the advent of additional commercial assays for non-HLA antibodies, similar consideration must be given to assay specificity. It has been reported that broad, high levels of reactivity were observed in Luminex panels for non-HLA antibodies after VAD implantation. (Askar et al. 2020) This study concluded that VAD patients often develop high levels of antibodies to many non-HLA targets and proposed that there may be an increased inflammatory state triggered by these devices. Perhaps patients who have undergone VAD therapy are particularly prone to non-AT1R reactivity in the commercial assay. This effect may be driving

the positive AT1R antibody status in VAD patient cohorts and may explain the large increase in the proportion of patients found to be positive for AT1R antibody following VAD implant with no reported difference in cellular or antibody-mediated rejection post-transplant.(Urban et al. 2016). It is also possible that VAD implantation does result in development of true AT1R antibodies via the production of microvesicles as was shown to occur in the development of anti-perlecan antibodies in transplant recipients. (Cardinal et al. 2013; Yang et al. 2016). The inability to distinguish true from non-specific AT1R antibodies in the commercial assay used in every transplant study may be masking this antibody as an analyte of importance. This issue may be exacerbated in pediatric populations due to the increased levels of anti-BSA, as well as other potentially assay-interfering antibodies, in children as compared to adults. (Gao et al. 2017; Rothberg and Farr 1965)

Clinical laboratories are required to participate in external proficiency testing programs for all areas of testing reported. Although the ASHI Proficiency Testing Program has included AT1R as a reportable analyte since 2016, it is still only included as an ungraded, education component of the antibody survey. Approximately 10-12 laboratories have reported result over the past 4 years. There has been a very low frequency of positive results from these samples with only 2/40 (5%) of samples reported as positive over the past 3 years. This is a curious finding given that 20% of healthy adults have been reported to be AT1R positive (Honger et al. 2014). The samples shared for this proficiency testing exercise are not serum samples but rather recalcified and filtered plasma collected from healthy donors (personal communication with ASHI Proficiency Program vendor). It is possible that the sample handling protocols used for these samples removes non-specific antibodies and thus removes non-specific binding in the ELISA AT1R assay; there are

clear, high MFI HLA antibodies detectable by solid phase and cell-based assays in these same samples. Parallel testing of positive AT1R results using recalcified, filtered plasma from the same individuals would be another approach to investigate non-specific reactivity in this commercial ELISA AT1R assay.

There is controversy regarding the nature of this AT1R ELISA assay. Many papers, including those from the co-creator of this assay, Dr. Duska Dragun, reference this as a sandwich ELISA method (Dragun and Philippe 2018; Reinsmoen et al. 2010) but the vendor of this assay (Cell Trend) states that it is not a sandwich assay but rather a direct binding assay (personal communication). Because there is no published paper describing the assay development, it may be challenging to definitively clarify the method. But if it is, in fact, a sandwich ELISA assay, there may be additional assay interferences such as antibody to the capture antibody and/or increased relevance of anti-BSA antibodies. (Andersen et al. 2004; Datta 2013).

An alternate approach to detecting AT1R antibodies would be to use an assay that focusses on peptides that are specific to the second extracellular loop of the AT1R target. There are known peptides from this portion of the receptor which could be explored for this purpose. Reported issues in monoclonal antibody specificities that would need to be considered for coupling confirmation of these peptides to solid phase platforms. (Herrera et al. 2013). This approach may also be advantageous as the specificity of the assay would be limited to the second extracellular loop of this G-coupled protein receptor which has been reported to be the relevant portion of the antigen vascular endothelial activation. (Luft 2013). These assays should also be validated against bioassays and in all transplant populations, including pediatric patient cohorts.

There has been variability in the positive threshold used to stratify results as positive and negative in this commercial ELISA assay. There is no rationale for any given threshold in the assay's product insert as both studies referenced in this document claim to report cases with no pre-transplant HLA antibody detection; one study used only complement dependent cytotoxicity method to detect pretransplant antibodies (Reinsmoen et al. 2010) and the other used an ELISA HLA antibody detection method (Kelsch et al. 2011). The second reference from Kelsch et al includes retrospective HLA antibody testing by the more sensitive solid phase Luminex HLA antibody method that do detect donor specific antibody to class II HLA targets thus making it challenging to determine the role of the AT1R antibody in this patient's post-transplant outcome. Many studies have used the 17 U/mL threshold to define positive reactivity although a large, prospective kidney transplant study used a threshold of 10 U/ml to define positive reactivity. (Lefaucheur et al. 2019). The lack of threshold studies has been criticized in the past but not this limitation has not been resolved. (Tinckam and Campbell 2013) and to date there remains a lack of consensus on the clinical relevant AT1R antibody threshold. Given that there appear to be differences between pediatric and adult patient reactivity using this assay, future threshold studies should include pediatic populations as a specific subset.

It is recognised that solid phase antibody detection assays should include 'no antigen well' to determine background of individual samples (Terato et al. 2014) Indeed, these wells were available in the era of ELISA HLA antibody assessment. These controls serve a similar purpose to the negative control beads in our current Luminex bead-based assays. Clinical laboratories are required to meet a minimum standard as part of the accreditation process. The American Society for Histocompatibility and Immunogenetics (ASHI) is an internationally recognized leader in the

accreditation of clinical histocompatibility laboratories. It is now, and has always been, an ASHI standard that laboratories are required to rule out test method interferences in all assays. The widely available assay to measure AT1R antibodies does not currently include that control therefore does not meet ASHI accreditation standards. This assay could be adapted to include such a control well which would greatly improve its design and allow clinical laboratories to meet accreditation requirements.

5.3.3 Future Directions and Practical Applications for Chapter 4

Antibody analysis in the histocompatibility field was revolutionised by the advent of solid phase antibody testing. ELISA and flow cytometry bead-based assays paved the way for Luminex bead-based assays which quickly became the gold standard for HLA antibody determination. (El-Awar, Lee, and Terasaki 2005; Gebel and Bray 2010; Gebel, Bray, and Kerman 2000; Patel and Terasaki 1969; Reed et al. 2013; Tait et al. 2013). While debate persists regarding the most accurate interpretation of these beads with regard to clinically relevant thresholds as well as standardization and modifications to this assay, there is no denying that single antigen HLA beads moved transplant risk assessment into a new era. (Fontaine et al. 2012; Kiernan, Ellison, and Tinckam 2018; Middleton, Jones, and Lowe 2014; Picascia et al. 2014; Reynolds and Tinckam 2016; Sullivan, Gebel, and Bray 2017; A. R. Tambur et al. 2018; A. Tambur, DMD, and Wiebe 2018).

As was and is the case in HLA antibody transplant risk assessment, we will likely need to continue to perform cell-based ABO antibody detection in parallel with solid phase assays. Flow cytometry red cell ‘titre’ or crossmatch has been performed and may be a superior alternative to

red cell agglutination titres for ABOi transplant risk assessment.(Kang, Lim, and Baik 2014; Stussi et al. 2005) This method could also be performed in the histocompatibility laboratory where this testing is already performed for lymphocyte crossmatching; the samples, equipment, and expertise already exists in this area of the clinical laboratory.

All the same questions and lessons learned in the realm of HLA antibody detection will exist when considering the application of this new ABO assay:

i. Cell based *vs* virtual crossmatch result and clinical significance of each
ii. Reproducibility
iii. Threshold for positive results
iv. Knowledge of and mitigation of interfering substances such as IVIg
v. Non-specific antibody due to antigen confirmation differences from biologic state
vi. Complement interference

i) Regardless of the cell-based method of ABO antibody determination, there will be a need to determine clinical relevance of positive Luminex bead-based solid phase antibody detection in the presence of low or negative antibody titre in the cell based platform(s). But flow evaluation of ABO antibodies using red cell targets will not address the subtype specificity limitation of using cells. Much like the HLA antibody evaluation by single antigen bead, the subtype specificity is only possible on platforms such as the Luminex system.

ii) The superior reproducibility of ABO antibody detection on the Luminex platform has already been established in these studies. Further evaluation of this antibody method

could easily be carried out in the Canadian Blood Services HLA working group with national exercises involving all HLA laboratories across Canada. This type of national standardization of the assessment of antibody is already part of the mandate of our transplant registry programs.

iii) The most efficient way to determine the biologically relevant levels of ABO antibodies in ABOi transplantation is to perform retrospective studies where stored samples are available from ABOi transplant recipients and short and long-term outcomes are known. There is evidence that ABOi transplants can be safely performed in several large meta-analyses but there are also cases in which ABOi transplants had a poor outcome. These cases could begin to inform the clinically relevant levels of ABO antibody.

iv) Solid phase assays are known to be plagued by the test method interferences created by therapies used in transplantation. The use of intravenous immune globulin (IVIg) in desensitization is an example of such an interference. We have tested several IVIg preparations and found that they all have high levels of detectable ABO antibody. **Figure 5-7** shows the results from a vendor that is purported to reduce the level of ABO antibodies. There are clearly remaining anti-A antibodies although antibodies to some A targets such as A-trisaccharide and A-II appears to have been reduced. The A-III and A-IV antibodies are relevant to renal epithelial cells. (Bentall et al. 2021) Anti-thymocyte globulin could also contain ABO antibodies but this antibody preparation has not yet been tested on the Luminex ABO antibody platform.

v) A challenge in Luminex bead-based HLA antibody detection been the detection of antibodies to cryptic epitopes on the antigens bound to the beads. While glycans will

not have quite the same challenges as have been experienced with complex MHC protein targets, it is possible that modifications of ABH glycans in tissues such as those by sialic acids may create tissue glycotopes that differ from the tetrasaccharide structures bound to the Luminex beads. Monoclonal antibody flow and immunohistochemistry studies suggest that may not be the norm but it cannot be ruled out as a possibility. It is also possible that polymorphisms in the H glycosyltransferase FUT1 will result in downstream modifications to the A and B glycans resulting from each of these respective transferases. Further development of monoclonal antibodies as well as flow cytometry studies of cells, immunohistochemistry studies of multiple tissues, and sequencing of the ABH-related genes will provide discovery in this area.

vi) Complement interference has been shown to be a cause of false negative antibody results in the measurement of HLA antibodies in Luminex single antigen bead-based methodologies which can be addressed through the use of serum dilution or use of EDTA treatment of serum. (Brian 2016; Irure et al. 2017; Sullivan et al. 2017; Tambur et al. 2015) The presence of sometimes high levels of IgM ABO antibodies suggests that complement should in evaluated as a potential source of falsely low ABO antibody detection. We compared several high MFI sera samples to EDTA plasma and did not observe this phenomenon. Perhaps the 1/25 dilution of the serum samples prior to incubation on the beads mitigates this technical issue. Further studies using Melon column to remove IgM antibodies could be performed to confirm that IgM antibodies are not blocking the ability of IgG antibodies to bind to the beads and/or binding C1q which also could block IgG antibody binding.

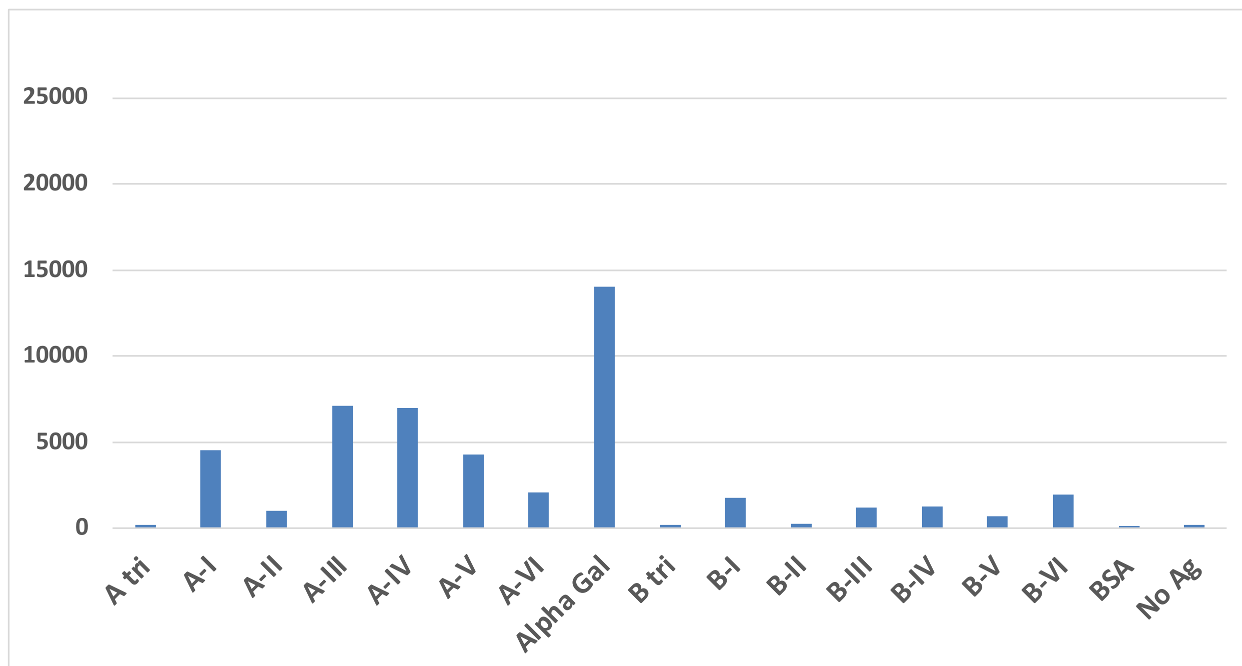


Figure 5-7: Evaluation of IVIg antibody composition

Currently pediatric heart transplant recipients are considered for ABOi transplant if anti-A and anti-B titres are low. Use of this tool could potentially increase the window of opportunity for ABOi transplantation and provide allograft-relevant, glycan A-II and B-II subtype specific antibody data. Patients who are undergoing desensitisation therapy to reduce HLA antibody levels will also experience a decrease in ABO antibody levels as these therapies are not specific to HLA antibodies. This Luminex ABO antibody tool could be used in tandem with HLA antibody detection tools during the course of desensitisation to monitor both HLA and ABO antibody levels. If ABO antibodies have decreased along with the HLA antibodies, this could provide excellent opportunity for ABOi transplant to increase the donor pool for patients who will struggle to find an HLA-compatible donor. This bead panel could also be used in the setting of ABO-A2 into ABO-O and ABO-BB patients. ABO-A2 donors have lower levels of A glycans

therefore the ABO-A1 reagent red cells used for the titre may detect clinically irrelevant antibodies that could rule out ABOi transplant unnecessarily.

When crossing antibody barriers in the setting of pre-formed ABO antibody (*i.e.* non-infant ABOi transplants) it would be valuable to better understand memory responses to ABH glycans. This Luminex tool could be used to assess ABO memory B cells in the setting of a polyclonal stimulation of memory B cells. (Karahan et al. 2018; Karahan, Claas, and Heidt 2015) This bead panel could also be used to investigate the IgG subclasses of ABO antibodies. (Jackson et al. 2020)

As even in young children there can be detectable ABO IgG antibody. For patients with predominantly IgG antibody pattern, use of imlifidase (Ides) could allow this antibody barrier to be safely crossed. This tool works well with imlifidase as is shown in **Figure 5-8**

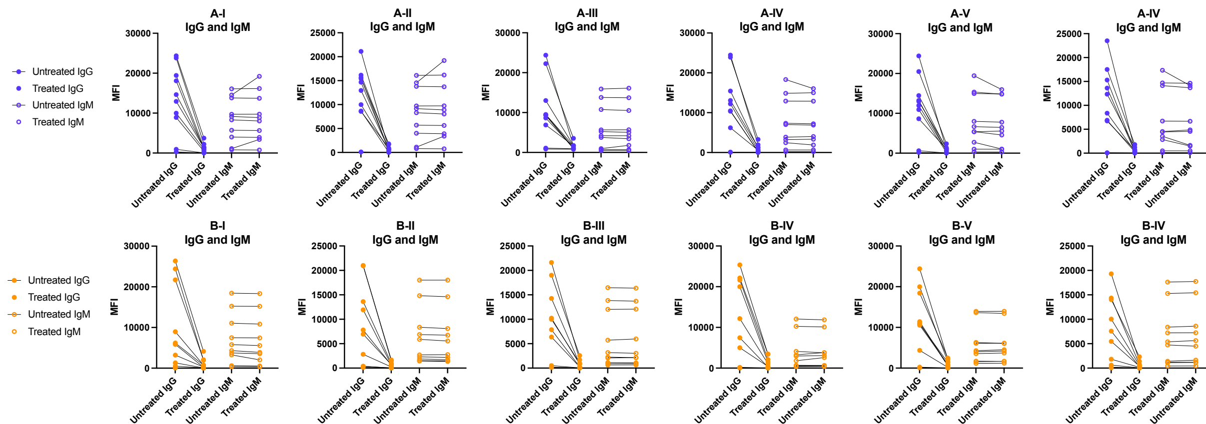


Figure 5-8: Sera were treated *in-vivo* with imlifidase. IgG antibodies reduced but IgM remained similar in MFI.

Anti-sera reagent vendors could utilize this bead panel as part of manufacturing quality control. As an example, **Figure 5-9** shows that the ES-15 clone, reported to be anti-A,(B) does not actually detect ABO-B targets as expected. (Moore et al. 1984) Rather, this monoclonal appears to have higher reactivity with A glycans as compared to a commonly used anti-A anti-sera (Novoclone) which may explain its ability to agglutinate red cells with low 'expression' of A glycans such as ABO-Ax. It is also possible that this clone reacts with H targets as well as the A targets; this theory could be explored by including H targets in the bead panel. Additional ABO antisera and monoclonal antibodies could be tested on this platform. These beads could also be useful in the characterisation of new monoclonal antibodies and define ABH glycotopes in absorption studies. Accuracy of ABO typing of the cardiac donors in pediatric ABOi transplantation is critical for transplant outcomes and long-term post-transplant monitoring.

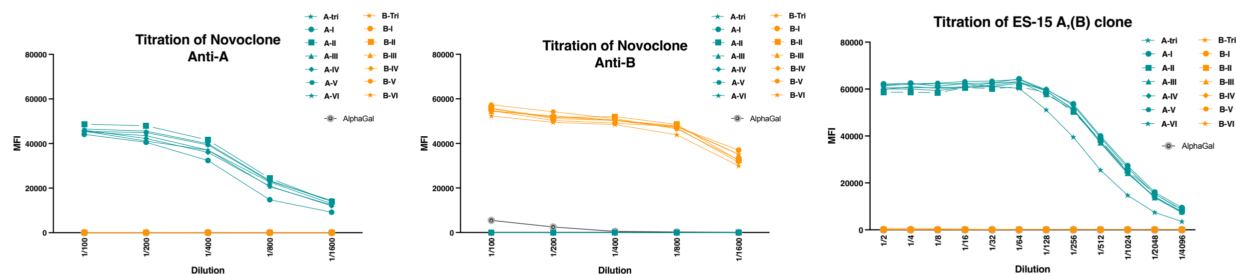


Figure 5-9: Evaluation of phenotyping anti-sera

ABO antigen typing is routinely performed by phenotyping with anti-A and anti-B antisera. ABO genotyping assays are commercially available both at low to medium resolution typing by qPCR as well as by next-generation sequencing. As shown in Chapter 4, there considerable variation between ABO antibody levels, even between individuals with the same ABO blood group (and the same agglutination titre). Correlation between ABO genotype and ABO antibody patterns could be performed by combining genotyping and fine characterisation of ABO antibodies using the Luminex ABO panel.

The relevance of secretor status is not known in pediatric cardiac ABOi transplantation. The A-I and B-I coupled beads included in this Luminex panel allow for reporting of antibodies to these secreted antigens. Genotyping of the FUT2 gene should also accompany the anti-subtype I antibody data to confirm secretor status.

5.4 Summary of Findings

The projects presented here support immune risk assessment for pediatric heart transplant patients. While larger studies are more easily conducted in adult transplant populations due to higher transplant volumes in these cohorts, findings may vary across the age spectrum. Pediatric heart transplant patients are better served by accurate determination of their immune risk status in pre- and post-transplant phases to enable donor selection and post-transplant monitoring for rejection.

References

Alba, Ana C., Kathryn Tinckam, Farid Foroutan, Laerke M. Nelson, Finn Gustafsson, Kam Sander, Hellen Bruunsgaard, Sharon Chih, Helen Hayes, Vivek Rao, Diego Delgado, and Heather J. Ross. 2015. "Factors Associated with Anti-Human Leukocyte Antigen Antibodies in Patients Supported with Continuous-Flow Devices and Effect on Probability of Transplant and Post-Transplant Outcomes." *Journal of Heart and Lung Transplantation* 34:685–92.

Almond, C. S., R. R. Thiagarajan, G. E. Piercey, K. Gauvreau, E. D. Blume, H. J. Bastardi, F. Fynn-Thompson, and T. P. Singh. 2009. "Waiting List Mortality among Children Listed for Heart Transplantation in the United States." *Circulation* 119(5):717–27.

Andersen, Ditte C., Claus Koch, Charlotte H. Jensen, Karsten Skjødt, Jette Brandt, and Børge Teisner. 2004. "High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies." *Journal of Immunoassay and Immunochemistry* 25(1):17–30.

Askar, M., D. Bhakta, H. Zafar, A. Alam, L. Pittmon, S. Kang, L. Kllingman, J. Felius, SM Joseph, D. Wencker, DM Meyer, and SA Hall. 2020. "Multiplicity of Non-HLA Targets for Antibodies Developed in Association with Ventricular Assist Device Implantation." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 20 Suppl 3:5–1166.

Askar, Medhat, Eileen Hsich, Patrick Reville, Amy S. Nowacki, William Baldwin, Suzanne Bakdash, Jenna Daghstani, Aiwen Zhang, Lynne Klingman, Nicholas Smedira, Nader Moazami, David O. Taylor, Randall C. Starling, and Gonzalo Gonzalez-Stawinski. 2013. "HLA and MICA Allosensitization Patterns among Patients Supported by Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 32(12):1241–48.

Bentall, Andrew, Mylvaganam Jeyakanthan, Manjit Braitch, Christopher W. Cairo, Todd L. Lowary, Stephanie Maier, Anne Halpin, Bruce Motyka, Lu Zou, Lori J. West, and Simon Ball. 2021. "Characterization of ABH-Subtype Donor-Specific Antibodies in ABO-A-Incompatible Kidney Transplantation." *American Journal of Transplantation* 21(11):3649–62.

Bjerre, Anna, Trine Tangeraas, Harald Heidecke, Duska Dragun, Ralf Dechend, and Anne Cathrine Staff. 2016. "Angiotensin II Type 1 Receptor Antibodies in Childhood Kidney Transplantation." *Pediatric Transplantation* 20(5):627–32.

Brian, Duncan Tait. 2016. "Detection of HLA Antibodies in Organ Transplant Recipients-Triumphs and Challenges of the Solid Phase Bead Assay." *Frontiers in Immunology, Vol 7 (2016)*.

Cardinal, H., M. Dieudé, N. Brassard, S. Qi, N. Patey, M. Soulez, D. Beillevaire, F. Echeverry, C. Daniel, Y. Durocher, F. Madore, and M. J. Hébert. 2013. "Antiperlecan Antibodies Are Novel Accelerators of Immune-Mediated Vascular Injury." *American Journal of Transplantation* 13(4):861–74.

Datta, Pradip. 2013. *Immunoassay Design and Mechanisms of Interferences*. First Edit. Elsevier

Inc.

Datta, S. S., S. Basu, M. Reddy, K. Gupta, and S. Sinha. 2021. “Comparative Evaluation of the Conventional Tube Test and Column Agglutination Technology for ABO Antibody Titration in Healthy Individuals: A Report from India.” *Immunohematology* 37(1):25–32.

Deltombe, Clement, Florence Gillaizeau, Daniel Anglicheau, Emmanuel Morelon, Katy Trébern-Launay, Florent Le Borgne, Marie Rimbert, Pierrick Guérif, Stéphanie Malard-Castagnet, Yohann Foucher, and Magali Giral. 2017. “Is Pre-Transplant Sensitization against Angiotensin II Type 1 Receptor Still a Risk Factor of Graft and Patient Outcome in Kidney Transplantation in the Anti-HLA Luminex Era? A Retrospective Study.” *Transplant International* 30(11):1150–60.

Dragun, Duska and Aurélie Philippe. 2018. “Tempest in a Sugar-Coated Lab Vial.” *American Journal of Transplantation* 18(11):2622–23.

El-Awar, N., J. Lee, and P. I. Terasaki. 2005. “HLA Antibody Identification with Single Antigen Beads Compared to Conventional Methods.” *Human Immunology* 66(9):989–97.

Elkind, Jae, Juliana Sobczyk, Oscar Ostberg-Braun, Jorge Silva Enciso, Eric Adler, and Gerald P. Morris. 2020. “Factors Influencing Transfusion-Associated HLA Sensitization in Patients Bridged to Heart Transplantation Using Ventricular Assist Device.” *Clinical Transplantation* 34(1):e13772.

Fichtner, Alexander, Caner Süsal, Claudia Schröder, Britta Höcker, Susanne Rieger, Rüdiger Waldherr, Jens H. Westhoff, Anja Sander, Duska Dragun, and Burkhard Tönshoff. 2018. “Association of Angiotensin II Type 1 Receptor Antibodies with Graft Histology, Function and Survival in Paediatric Renal Transplant Recipients.” *Nephrology Dialysis Transplantation* 33(6):1065–72.

Fontaine, Magali J., Jenny Kuo, Ge Chen, Susan A. Galel, Evelyn Miller, Flavia Sequeira, Maurene Viele, Lawrence T. Goodnough, Dolly B. Tyan, G. D. Wool, M. Moayeri, and S. Pandey. 2012. “Complement (C1q) Fixing Solid-Phase Screening for HLA Antibodies Increases the Availability of Compatible Platelet Components for Refractory Patients.” *Transfusion* 51; 52(12):2618; 192A.

Foster, Bethany J., Xun Zhang, Alexia de Simone, Mourad Dahhou, Ruth Sapir-Pichhadze, Heloise Cardinal, and Lori West. 2021. “Differences in Heart Graft Survival by Recipient Sex.” *Transplantation Direct* 1–10.

Gao, Bingsi, Cassandra Long, Whayoung Lee, Zhongqiang Zhang, Xiaotian Gao, Doug Landsittel, Mohamed Ezzelarab, David Ayares, Yuliang Huang, David K. C. Cooper, Yi Wang, and Hidetaka Hara. 2017. “Anti-Neu5Gc and Anti-Non-Neu5Gc Antibodies in Healthy Humans.” *PLOS ONE* 12(7):e0180768.

Gebel, H., B. Bray, and R. Kerman. 2000. “FlowPRA(TM) Detects Clinically Relevant Pretransplant HLA Antibodies in Renal Allograft Recipients.” *VISUALS OF THE CLINICAL HISTOCOMPATIBILITY WORKSHOP* 29–30.

Gebel, Howard M. and Robert A. Bray. 2010. “The Evolution and Clinical Impact of Human Leukocyte Antigen Technology.” *Current Opinion in Nephrology and Hypertension* 19(6):598–602.

Giral, M., Y. Foucher, A. Dufay, J. P. D. Van Huyen, K. Renaudin, A. Moreau, A. Philippe, B. Hegner, R. Dechend, H. Heidecke, S. Brouard, A. Cesbron, S. Castagnet, A. Devys, J. P. Soulillou, D. Dragun, D. Dragun, A. Philippe, and R. Catar. 2012. "Pretransplant Sensitization Against Angiotensin II Type 1 Receptor Is a Risk Factor for Acute Rejection and Graft Loss." *CURRENT OPINION IN ORGAN TRANSPLANTATION* 13; 17(10; 4):2576; 445.

Halpin, Anne, Janet Zhou, Jean Pearcey, Todd L. Lowary, Christopher W. Cairo, Gour Daskhan, Bruce Motyka, Stephanie Maier, and Lori J. West. 2018. "OR34 Modernizing ABO Antibody Detection: Development of a Bead-Based ABO Antibody Detection Assay." *Human Immunology*.

Herrera, Marcela, Matthew A. Sparks, Adolfo R. Alfonso-Pecchio, Lisa M. Harrison-Bernard, and Thomas M. Coffman. 2013. "Lack of Specificity of Commercial Antibodies Leads to Misidentification of Angiotensin Type 1 Receptor Protein." *Hypertension* 61(1):253–58.

Honger, G., H. Cardinal, M. Dieude, A. Buser, I. Hosli, D. Dragun, M. J. Hebert, and S. Schaub. 2014. "Human Pregnancy and Generation of Anti-Angiotensin Receptor and Anti-Perlecan Antibodies." *Transplant International* 27(5):467–74.

Irure, Juan, Esther Asensio, Emilio Rodrigo, igo Romn, Javier Gmez, Manuel Arias, Marcos Lpez-Hoyos, and David San Segundo. 2017. "Improvement in the Definition of Anti-HLA Antibody Profile in Highly Sensitized Patients." *PLoS ONE* 12(2):1–13.

Itescu, Silviu and Ranjit John. 2003. "Interactions between the Recipient Immune System and the Left Ventricular Assist Device Surface: Immunological and Clinical Implications." *The Annals of Thoracic Surgery* 75:S65.

Jackson, Annette M., Sai Kanaparthi, Bryna E. Burrell, Donna P. Lucas, Renato M. Vega, Anthony J. Demetris, and Sandy Feng. 2020. "IgG4 Donor-Specific HLA Antibody Profile Is Associated with Subclinical Rejection in Stable Pediatric Liver Recipients." *American Journal of Transplantation* 20(2):513–24.

Javier Delmo, Eva Maria, Mariano Francisco del Maria Javier, and Roland Hetzer. 2020. "The Role of Ventricular Assist Device in Children." *Cardiovascular Diagnosis and Therapy; Vol 11, No 1 (February 2021): Cardiovascular Diagnosis and Therapy*.

Jeyakanthan, M., P. J. Meloncelli, L. Zou, T. L. Lowary, I. Larsen, S. Maier, K. Tao, J. Rusch, R. Chinnock, N. Shaw, M. Burch, K. Beddows, L. Addonizio, W. Zuckerman, E. Pahl, J. Rutledge, K. R. Kanter, C. W. Cairo, J. M. Buriak, D. Ross, I. Rebeyka, and L. J. West. 2016. "ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation." *American Journal of Transplantation* 16(5):1548–58.

Kahlyar, Htar, David Roxby, Tony Badrick, and Thiru Vanniasinkam. 2022. "Challenges in Antibody Titration for ABO-Incompatible Renal Transplantation." *Vox Sanguinis* 117(1):109–18.

Kang, Seon Joo, Young Ae Lim, and Sae Yun Baik. 2014. "Comparison of ABO Antibody Titers on the Basis of the Antibody Detection Method Used." *Annals Of Laboratory Medicine* 34(4):300–306.

Karahan, Gonca E., Frans H. J. Claas, and Sebastiaan Heidt. 2015. "Detecting the Humoral

Alloimmune Response: We Need More Than Serum Antibody Screening." *TRANSPLANTATION* 99(5):908–15.

Karahan, Gonca E., Juliette Krop, Caroline Wehmeier, Yvonne de Vaal, Janneke Langerak-Langerak, Dave L. Roelen, Neubury M. Lardy, Frederike J. Bemelman, Ineke ten Berge, Marlies E. J. Reinders, Cees van Kooten, Frans H. J. Claas, and Sebastiaan Heidt. 2018. "An Easy and Sensitive Method to Profile the Antibody Specificities of HLA-Specific Memory B Cells." *Transplantation* (Publish Ahead of Print, 9 November 2018).

Kelsch, Reinhard, Anne Schulze Everding, Eberhard Kuwertz-Bröking, Eva Brand, Bernd M. Spriewald, Walter Sibrowski, Martin Konrad, and Duska Dragun. 2011. "Accelerated Kidney Transplant Rejection and Hypertensive Encephalopathy in a Pediatric Patient Associated With Antibodies Against Angiotensin Type 1 Receptor and HLA Class II." *Transplantation* 92(10).

Khoury, Michael, Tara Pidborochynski, Anne Halpin, Patricia Campbell, Simon Urschel, Daniel Kim, Lori West, Holger Buchholz, and Jennifer Conway. 2021. "Human Leukocyte Antigen Antibody Sampling in Ventricular Assist Device Recipients: Are We Talking?" *Transplantation Proceedings* 53(7):2377–81.

Kiernan, Jeffrey J., Cynthia A. Ellison, and Kathryn J. Tinckam. 2018. "Measuring Alloantibodies." *Current Opinion in Organ Transplantation* 1.

Kim, D., I. Gimferrer, P. Warner, K. Nelson, L. Sibulesky, R. Bakthavatsalam, and N. Leca. 2018. "Preformed Angiotensin II Type-1 Receptor Antibodies Are Associated With Rejection After Kidney Transplantation: A Single-Center, Cohort Study." *Transplantation Proceedings* 50(10):3467–72.

Klein, Sabra L. and Katie L. Flanagan. 2016. "Sex Differences in Immune Responses." *Nature Reviews Immunology* 16(10):626–38.

Lefaucheur, Carmen, Denis Viglietti, Yassine Bouatou, Aurélie Philippe, Daniele Pievani, Olivier Aubert, Jean-Paul Duong Van Huyen, Jean-Luc Taupin, Denis Glotz, Christophe Legendre, Alexandre Loupy, Philip F. Halloran, and Duska Dragun. 2019. "Non-HLA Agonistic Anti-Angiotensin II Type 1 Receptor Antibodies Induce a Distinctive Phenotype of Antibody-Mediated Rejection in Kidney Transplant Recipients." *Kidney International* 96(1):189–201.

Lepeytre, Fanny, Mourad Dahhou, Xun Zhang, Julie Boucquemont, Ruth Sapir-Pichhadze, Heloise Cardinal, and Bethany J. Foster. 2017. "Association of Sex with Risk of Kidney Graft Failure Differs by Age." *Journal of the American Society of Nephrology* 28(10):3014 LP – 3023.

Luft, Friedrich C. 2013. "Activating Autoantibodies and Cardiovascular Disease." *Physiology (Bethesda, Md.)* 28(4):254–61.

McKenna David H, Jr., Ted Eastlund, Miriam Segall, Harriet J. Noreen, Soon Park, Jr. McKenna David H, Ted Eastlund, Miriam Segall, Harriet J. Noreen, and Soon Park. 2002. "Original Article: HLA Alloimmunization in Patients Requiring Ventricular Assist Device Support." *Journal of Heart and Lung Transplantation* 21:1218–24.

Middleton, D., J. Jones, and D. Lowe. 2014. "Nothing's Perfect: The Art of Defining HLA-Specific Antibodies." *Transplant Immunology* 30(4):115–21.

Moazami, N., S. Itescu, M. R. Williams, M. Argenziano, A. Weinberg, and M. C. Oz. 1998. "Platelet Transfusions Are Associated with the Development of Anti-Major Histocompatibility Complex Class I Antibodies in Patients with Left Ventricular Assist Support." *Journal of Heart and Lung Transplantation* 17(9):876–80.

Moore, S., A. Chirnside, L. R. Micklem, D. B. McClelland, and K. James. 1984. "A Mouse Monoclonal Antibody with Anti-A,(B) Specificity Which Agglutinates Ax Cells." *Vox Sanguinis* 47(6):427–34.

Newell, H., J. D. Smith, P. Rogers, E. Birks, A. J. Danskine, R. E. Fawson, and M. L. Rose. 2006. "Sensitization Following LVAD Implantation Using Leucodepleted Blood Is Not Due to HLA Antibodies." *American Journal of Transplantation* 6(7):1712–17.

Ngo, S. T., F. J. Steyn, and P. A. McCombe. 2014. "Gender Differences in Autoimmune Disease." *Frontiers in Neuroendocrinology* 35(3):347–69.

Nikaein, Afzal, Nadim El-Awar, Judson Hunt, Edward J. Rosenthal, Eric Eichhorn, Shelly Hall, James Boehrer, Mitchell Magee, Marc Pieniek, William Brinkman, and Todd Dewey. 2012. "Clinically Irrelevant Circulating Human Leukocyte Antigen Antibodies in the Presence of Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 31(5):443–47.

O'Connor, Matthew J., JonDavid Menteer, Maryanne R. K. Chrisant, Dimitrios Monos, Curt Lind, Selena Levine, J. William Gaynor, Brian D. Hanna, Stephen M. Paridon, Chitra Ravishankar, and Beth D. Kaufman. 2010. "Ventricular Assist Device-Associated Anti-Human Leukocyte Antigen Antibody Sensitization in Pediatric Patients Bridged to Heart Transplantation." *The Journal of Heart and Lung Transplantation* 29(1):109–16.

Patel, R. and P. I. Terasaki. 1969. "Significance of the Positive Crossmatch Test in Kidney Transplantation." *The New England Journal of Medicine* 280(14):735–39.

Picascia, Antonietta, Chiara Sabia, Vincenzo Grimaldi, Maria Lourdes Montesano, Linda Sommese, Concetta Schiano, and Claudio Napoli. 2014. "Lights and Shadows of Anti-HLA Antibodies Detected by Solid-Phase Assay." *Immunology Letters* 162(1, Part A):181–87.

Pinelli, David F., John J. Friedewald, Kelley M. K. Haarberg, Shree L. Radhakrishnan, Jennifer R. Zitzner, Wendy E. Hanshew, and Anat R. Tambur. 2017. "Assessing the Potential of Angiotensin II Type 1 Receptor and Donor Specific Anti-Endothelial Cell Antibodies to Predict Long-Term Kidney Graft Outcome." *Human Immunology* 78(5–6):421–27.

Reed, E. F., P. Rao, Z. Zhang, H. Gebel, R. A. Bray, I. Guleria, J. Lunz, T. Mohanakumar, P. Nickerson, A. R. Tambur, A. Zeevi, P. S. Heeger, and D. Gjertson. 2013. "Comprehensive Assessment and Standardization of Solid Phase Multiplex-Bead Arrays for the Detection of Antibodies to HLA." *American Journal of Transplantation* 13(7).

Reinsmoen, N. L., C. H. Lai, H. Heidecke, M. Haas, K. Cao, G. Ong, M. Naim, Q. Wang, J. Mirocha, J. Kahwaji, A. A. Vo, S. C. Jordan, and D. Dragun. 2010. "Anti-Angiotensin Type 1 Receptor Antibodies Associated With Antibody Mediated Rejection in Donor HLA Antibody Negative Patients." *Transplantation* 90(12):1473–77.

Reynolds, Ben C. and Kathryn J. Tinckam. 2016. "Sensitization Assessment before Kidney Transplantation." *Transplantation Reviews (Orlando, Fla.)*.

Rothberg, Richard M. and Richard S. Farr. 1965. "ANTI-BOVINE SERUM ALBUMIN AND

ANTI-ALPHA LACTALBUMIN IN THE SERUM OF CHILDREN AND ADULTS.” *Pediatrics* 35(4):571 LP – 588.

See, Sarah B., Kevin J. Clerkin, Peter J. Kennel, Feifan Zhang, Matthew P. Weber, Kortney J. Rogers, Debanjana Chatterjee, Elena R. Vasilescu, George Vlad, Yoshifumi Naka, Susan W. Restaino, Maryjane A. Farr, Veli K. Topkara, Paolo C. Colombo, Donna M. Mancini, P. Christian Schulze, Bruce Levin, and Emmanuel Zorn. 2017. “Ventricular Assist Device Elicits Serum Natural IgG That Correlates with the Development of Primary Graft Dysfunction Following Heart Transplantation.” *The Journal of Heart and Lung Transplantation* 36(8):862–70.

Singh, Tajinder P., Christopher S. Almond, Gary Piercey, and Kimberlee Gauvreau. 2011. “Trends in Wait-List Mortality in Children Listed for Heart Transplantation in the United States: Era Effect Across Racial/Ethnic Groups.” *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11(12):2692–99.

Stussi, G., K. Huggel, H. U. Lutz, U. Schanz, R. Rieben, and J. D. Seebach. 2005. “Isotype-Specific Detection of ABO Blood Group Antibodies Using a Novel Flow Cytometric Method.” *British Journal of Haematology* 130(6):954–63.

Sullivan, Harold C., Howard M. Gebel, and Robert A. Bray. 2017. “Understanding Solid-Phase HLA Antibody Assays and the Value of MFI.” *Human Immunology*.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. “Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation.” *Transplantation* 95(1):19–47.

Tambur, A. R., N. D. Herrera, K. M. K. Haarberg, M. F. Cusick, R. A. Gordon, J. R. Leventhal, J. J. Friedewald, and D. Glotz. 2015. “Assessing Antibody Strength: Comparison of MFI, C1q, and Titer Information.” *American Journal of Transplantation* 15(9).

Tambur, Anat, PhD DMD, and Chris Wiebe. 2018. “HLA Diagnostics: Evaluating DSA Strength by Titration.” *Transplantation* 102(1S):S23–30.

Tambur, Anat R., Patricia Campbell, Frans H. Claas, Sandy Feng, Howard M. Gebel, Annette M. Jackson, Roslyn B. Mannon, Elaine F. Reed, Kathryn Tinckam, Medhat Askar, Anil Chandraker, Patricia P. Chang, Monica Colvin, Anthony-Jake Demetris, Joshua M. Diamond, Anne I. Dipchand, Robert L. Fairchild, Mandy L. Ford, John Friedewald, Ronald G. Gill, Denis Glotz, Hilary Goldberg, Ramsey Hachem, Stuart Knechtle, Jon Kobashigawa, Deborah J. Levine, Joshua Levitsky, Michael Mengel, Edgar Milford, Kenneth A. Newell, Jacqueline G. O’Leary, Scott Palmer, Parmjeet Randhawa, John Smith, Laurie Snyder, Randall C. Starling, Stuart Sweet, Timucin Taner, Craig J. Taylor, Steve Woodle, Adriana Zeevi, and Peter Nickerson. 2018. “Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group Meeting Report.” *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The*

American Society Of Transplant Surgeons.

Terato, Kuniaki, Christopher T. Do, Dawn Cutler, Takaki Waritani, and Hiroshi Shionoya. 2014. "Preventing Intense False Positive and Negative Reactions Attributed to the Principle of ELISA to Re-Investigate Antibody Studies in Autoimmune Diseases." *Journal of Immunological Methods* 407:15–25.

Tinckam, K. and P. Campbell. 2013. "Angiotensin II Type 1 Receptor Antibodies: Great Expectations?" *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons* 13(10):2515–16.

Tinckam, Kathryn. 2009. "Histocompatibility Methods." *Transplantation Reviews* 23:80–93.

Urban, Marian, Antonij Slavcev, Tomas Gazdic, Peter Ivak, Josef Besik, and Ivan Netuka. 2016. "The Impact of Angiotensin II Type 1 Receptor Antibodies on Post-Heart Transplantation Outcome in Heart Mate II Bridged Recipients." *Interactive Cardiovascular And Thoracic Surgery* 22(3):292–97.

Yang, B., M. Dieudé, K. Hamelin, M. Hénault-Rondeau, N. Patey, J. Turgeon, S. Lan, L. Pomerleau, M. Quesnel, J. Peng, J. Tremblay, Y. Shi, J. S. Chan, M. J. Hébert, and H. Cardinal. 2016. "Anti-LG3 Antibodies Aggravate Renal Ischemia–Reperfusion Injury and Long-Term Renal Allograft Dysfunction." *American Journal of Transplantation* 16(12):3416–29.

Yudin, Jovana and Nancy M. Heddle. 2014. "A 13-Question Approach to Resolving Serological Discrepancies in the Transfusion Medicine Laboratory." *Laboratory Medicine* 45(3):193–206.

Chapter 6

Index and Overview of Methods

Relevant to Chapter	Method Name	Method Description*
2	HLA Antibody Screen	This method was used to screen for class I and II HLA antibodies. This testing was performed in the University of Alberta, Alberta Precision Laboratories Histocompatibility Laboratory as standard of care testing for patients listed for cardiac transplantation.
2	HLA Luminex Single Antigen Bead Antibody Detection	This method was used to identify specificities to class I and II HLA antibodies. This testing was performed in the University of Alberta, Alberta Precision Laboratories Histocompatibility Laboratory as standard of care testing for patients listed for cardiac transplantation.
3	AT1R Antibody Assay	This method describes the steps used to perform the AT1R ELISA assay testing.
3	Adsorb Out™	This method describes the protocol used to treat the serum samples with Adsorb Out™ microparticles.
3	Neu5Gc Detection on Adsorb Out™	This method details the labelling of the Adsorb Out™ with anti-Neu5Gc antibodies and flow detection of this labelling.
4	ABO Luminex Bead Coupling	This protocol describes the process for coupling the A and B glycans to the Luminex beads.
4	ABO Luminex Bead Coupling Confirmation	This procedure is used to confirm that the glycans were successfully coupled to the Luminex beads using monoclonal antibodies.
4	ABO Luminex Bead Antibody Detection in Human Samples	This method describes how the prepared ABO single glycan beads are used to measure ABO antibodies in human serum
4	ABO Luminex Reagent Job Aid	This document lists the reagents used in the ABO antibody protocols. The Luminex coupling kit provides all but the TBN-PBS wash buffer and can be used in place of the in house prepared reagents. The Luminex coupling kit is recommended for greater reproducibility of the coupling process and ease of use.

*** A detailed purpose for each protocol is included in the SOP documents.**

Flow PRA Screen - Staining

Purpose This procedure provides information for how to perform a Flow PRA screen for Class I and Class II antibodies.

Materials

Reagents	Supplies	Equipment
• Flow PRA Class I/II Screening Kit • Control Beads • 4% Paraformaldehyde • Distilled water from white tap • One Lambda negative control • In-house positive control	• Long pipette tips • 0.5 mL tubes • 1.5 mL tubes • 5 mL tubes • U-bottom plate	• Beckman Allegra 6 Centrifuge • 96 Well Plate VP 177A-1 Aspiration Manifold • Vortex

Specimen Patient previously frozen serum from red top or gold top may be used in this assay. An absolute minimum serum volume is 25µL however, 50µL or greater is the preferred aliquot volume. Sera samples should NOT be heat inactivated as it may give a high background. Fresh serum received on stat or priority samples should be flash-frozen and thawed before using. Previously frozen EDTA plasma is acceptable if patient is on ECMO.

Quality Control Positive and negative control sera must be included with each flow PRA screen. The One Lambda negative control is used to determine the background reactivity of the PRA beads. The in-house positive control serum ensures that all reagents were added, etc. Control beads are also included with each tube; these beads are the same beads used for Class I and II beads however HLA antigen is NOT present. Control beads allow detection of non-specific reactivity such as may be seen with anti-plastic/latex antibodies.

One Lambda negative control is stable for 1 week at 2-8°C after thawing.
In house positive control is stable for 2 weeks at 2-8°C after thawing.

Procedure

Step	Detail	Information
1. Thaw reagent kit and warm wash buffer	1.1. Thaw reagent kit 1.2. Warm stock wash buffer in 37°C waterbath for a minimum of 10 minutes 1.3. Mix well	If not already done Ensures there are no crystals
2. Prepare control and patient sera for testing	2.1. Pull negative and positive control sera aliquots from fridge or freezer 2.2. Thaw and/or aliquot patient sera into 0.5mL tubes as per Test Batch Worksheet 2.3. Freeze any aliquots not previously frozen for 5 minutes at -70°C 2.4. Centrifuge control and patient sera • 10,000 rpm • 5 minutes	Neg control: One Lambda NC Pos control: In house positive sera 50µL or greater is the preferred volume To remove immunoglobulin aggregates
3. Label tray	3.1. Document tray wells on the Test Batch Worksheet 3.2. Label side of tray with bath name and date 3.3. Circle wells to be used with colored marker	
NOTE: The Class II beads and control beads are stained with a fluorescent dye (PE) and **must** be protected from light.		
4. Prepare bead mixture	4.1. Label a 1.5mL tube as bead mixture 4.2. Vortex Class I, Class II and control beads 4.3. Prepare bead mixture as per Table 1 4.4. Vortex to mix 4.5. Store in the dark until ready to use	Reagents from 2 kits can be combined IF the lot # and dot # are the same Control beads = 1µL per well Class I/II beads = 5µL per well + 5µL correction factor

Step	Detail	Information
5. Add bead mixture and sera to tray	5.1. Add 10µL of the bead mixture to each well 5.2. Add 20µL of each control or patient sera to designated well as per tray map 5.3. Cover tray with adhesive seal 5.4. Gently vortex tray	Class I + Class II + Control beads Ensure all samples were previously frozen At setting 4-5
6. Protect tray from light and incubate on shaker for 30 minutes.	6.1. Wrap tray in foil 6.2. Incubate on shaker for 30 minutes	Shaker settings: Form = 28 Amplitude = 5 Time = 30
7. Prepare working wash buffer (WWB)	7.1. Label an autoclaved 100 mL glass bottle 7.2. Prepare working wash buffer as per Table 2	1/10 dilution If insufficient wash buffer in kit, wash buffer from other kits may be used 1.8 mL dH_2O and 200 µL of wash buffer per well
NOTE: Prepare conjugate antibody dilution near the end of the incubation.		
8. Make working dilution of the FITC labeled conjugate antibody	8.1. Prepare a 1/100 dilution of the FITC labeled conjugate antibody as per Table 3 8.2. Vortex to mix 8.3. Store protected from the light until ready to use	**Working FITC dilution** = **FITC conjugate required** + **WWB required** **FITC Conjugate required** = 1µL x (samples to test +1 for volume correction) **WWB required** = 99µL x (samples to test + 1 for volume correction)

Step	Detail	Information
9. Wash tray three times	9.1. Remove foil and adhesive seal from tray 9.2. Add 150µL of WWB to each well 9.3. Place adhesive seal on tray 9.4. Centrifuge in the swing out tray adapter • 2700 rpm • 2 minutes 9.5. Remove seal from plate 9.6. Aspirate supernatant with aspiration manifold 9.7. Vortex tray gently 9.8. Repeat step 9.1 to 9.4 for a total of three washes	It is very important to have a thorough wash at this step or weak antibodies may be missed Beckman Allegra 6 Centrifuge 1500g 96-Well Plate VP 177A-1 Aspiration Manifold
10. Add FITC labeled conjugate antibody	10.1. Add 100µL of working FITC labeled conjugate antibody to each well 10.2. Cover tray with adhesive seal 10.3. Gently tap tray	
11. Protect tray from light and incubate on shaker for 30 minutes.	11.1. Protect tray from light and incubate on shaker for 30 minutes as per step 6	
12. Store reagent kit	12.1. Document the date and number of tests used on the kit box 12.2. Document the following on the Test Batch Worksheet: • Flow kit lot, dot and expiry date • Control bead lot, dot and expiry 12.3. Return kit box to 2-5°C for storage	
13. Prepare 0.5% paraformaldehyde fixative	13.1. Prepare 0.5% paraformaldehyde fixative 13.2. Vortex	1/8 dilution As per Table 4

Step	Detail	Information
14. Wash tray three times	14.1. Wash as per step 9	
15. Add fixative	15.1. Add 250µL of 0.5% fixative to each well	
16. File sera	16.1. File control and patient sera 16.2. Document 'Sera filed' on worksheet	

Table 1: Bead Mixture

Wells	Class I Beads (µL)	Class II Beads (µL)	Control Beads (µL)
1	10	10	1
2	15	15	2
3	20	20	3
4	25	25	4
5	30	30	5
6	35	35	6
7	40	40	7
8	45	45	8
9	50	50	9
10	55	55	10
11	60	60	11
12	65	65	12
13	70	70	13
14	75	75	14
15	80	80	15
16	85	85	16
17	90	90	17
18	95	95	18
19	100	100	19
20	105	105	20
21	110	110	21
22	115	115	22
23	120	120	23
24	125	125	24
25	130	130	25
26	135	135	26
27	140	140	27
28	145	145	28
29	150	150	29
30	155	155	30

Table 2: Working Wash Buffer

Wells	dH2O (mL)	Stock Wash Buffer (mL)
1	1.8	0.2
2	5.4	0.6
3	7.2	0.8
4	9.0	1.0
5	10.8	1.2
6	12.6	1.4
7	14.4	1.6
8	16.2	1.8
9	18.0	2.0
10	19.8	2.2
11	21.6	2.4
12	23.4	2.6
13	25.2	2.8
14	27.0	3.0
15	28.8	3.2
16	30.6	3.4
17	32.4	3.6
18	34.2	3.8
19	36.0	4.0
20	37.8	4.2
21	39.6	4.4
22	41.4	4.6
23	43.2	4.8
24	45.0	5.0
25	46.8	5.2
26	48.6	5.4
27	50.4	5.6
28	52.2	5.8
29	54.0	6.0
30	55.8	6.2

Table 3: FITC Labeled Conjugate Antibody

Wells	FITC Conjugate (µL)	Wash Buffer (µL)
1	2	198
2	3	297
3	4	396
4	5	495
5	6	594
6	7	693
7	8	792
8	9	891
9	10	990
10	11	1089
11	12	1188
12	13	1287
13	14	1386
14	15	1485
15	16	1584
16	17	1683
17	18	1782
18	19	1881
19	20	1980
20	21	2079
21	22	2178
22	23	2277
23	24	2376
24	25	2475
25	26	2574
26	27	2673
27	28	2772
28	29	2871
29	30	2970
30	31	3069

Table 4: 0.5% Paraformaldehyde

Wells	4% Paraformaldehyde (µL)	Wash Buffer (µL)
1	35	245
2	70	490
3	105	735
4	140	980
5	175	1225
6	210	1470
7	245	1715
8	280	1960
9	315	2205
10	350	2450
11	385	2695
12	420	2940
13	455	3185
14	490	3430
15	525	3675
16	560	3920
17	595	4165
18	630	4410
19	665	4655
20	700	4900
21	735	5145
22	770	5390
23	805	5635
24	840	5880
25	875	6125
26	910	6370
27	945	6615
28	980	6860
29	1015	7105
30	1050	7350

Method Limitations Not Applicable

Interpretation/ Results Not Applicable

Procedural

Notes

The Flow PRA™ Screening Test is designed for flow cytometric screening of panel reactive antibody (PRA) against HLA using a panel of Flow PRA™ beads, which are microparticles (2-4µm in diameter) coated with purified HLA antigens

The Flow PRA™ Screening Test provides precalibrated reagents for rapid flow cytometric detection of PRA in human serum. Reactivity of each of these Flow PRA™ has been confirmed by flow cytometry with human allosera and specific HLA monoclonal antibodies. HLA antibodies in the serum react specifically to the Flow PRA™ beads. After incubation of serum with Flow PRA™ beads, followed by a staining with a fluorescent-labeled anti-human IgG antibody, the anti-HLA IgG positive serum shows a fluorescent channel shift as compared to the negative serum. Percent PRA is represented is represented by the percentage of beads that react positively with the serum

Flow PRA™ I beads are non-fluorescent particles, whereas, Flow PRA™ II beads are fluorescent particles. The latter can be excited at 488nm, generating a maximum emission of approximately 580nm, which is similar to phycoerythrin (PE) and can be detected by the FL2 channel. This allows Flow PRA™ II beads to be separated from Flow PRA™ I beads when they are run together

Additional Information

Positive and negative control sera for the Flow PRA™ Screening Test should be tested daily as standards for sample analysis. The percentage of reaction of the positive serum should be consistent if the same lot of the Flow PRA™ beads and control sera is used.

References

Current One Lambda PRA Screening beads product insert

Luminex Antibody Assays - Staining

Purpose This procedure provides instruction for how to stain antibody assays using the LABScreen method for the Luminex.

Materials

Reagents	Supplies	Equipment
• One Lambda LABScreen • PE goat conjugated anti-human IgG (OLI Cat#LS-AB2) • PBS • Negative Control, OLI Cat# LS-NC • Pos Pool 1/512 • 0.1625M EDTA working Solution	• Polystyrene V bottom 96 well trays (Whatman, cat # 7701-2250) • Millipore vacuum trays (Durapore membrane Cat # MSBVN1210) • Pipettes (P5, P20, P100, multichannel P300, P10) • 96 Well U-bottom plates • Reagent boats	• Luminex cytometer system • Centrifuge with swinging bucket rotor • Plate shaker • Vortex mixer • Vacuum manifold (QIAVac) with gauge

Specimen Frozen patient serum from red top or gold top may be used in this assay. An absolute minimum serum volume is 25µL, however 50µL or greater is the preferred aliquot volume. Sera samples should NOT be heat inactivated as it may give high background. Fresh serum received on stat or high priority samples should be flash-frozen and thawed before using. Previously frozen EDTA or heparin plasma is acceptable if patient is on ECMO.

Safety Precautions See MSDS for detailed information.

Quality Control Negative Control serum must be included with each assay run. The One Lambda Negative Control serum is used to determined background reactivity of the LABScreen beads. Negative and positive control beads are also contained with the bead mixture; the negative control bead allows for detection of non-specific reactivity as may be seen with anti-plastic antibodies. The positive control bead ensures that all reagents were added, proper washing etc.

Negative Control serum is stable for 1 month at 2-8°C after thawing.

The Negative Control should be analyzed immediately after acquisition to ensure that the MFI is acceptable before >3 hours have elapsed after staining. Should the test run need to be re-acquired, the plate can be vacuumed and the beads re-suspended with 80µL PBS.

Procedure

Step	Detail	Information
1. Thaw kit and warm wash buffer	1.1. Thaw reagent kit (if not already done) 1.2. Warm wash buffer in 37°C waterbath and mix well to ensure there are no crystals	Kits are stored at -65°C or colder until first use
2. Create test batch and print Tray Printout	2.1. Create a test batch and Tray Worksheet as per 'Creating a Batch' SOP 2.2. Initial Tray Worksheet and document box#	
3. Prepare controls and patient sera for testing	3.1. Pull LS-NC and Pos Pool aliquots from fridge or thaw from freezer if necessary 3.2. Thaw and/or aliquot patient sera into 0.4 mL Beckman tubes as per Test Batch Worksheet 3.3. Freeze any 0.4mL Beckman tubes not previously frozen for 5 minutes at -70°C 3.4. Centrifuge control and patient sera • 10,000 rpm • 10 minutes	When a new box of LS-NC is thawed, aliquot into **three** aliquots and refreeze two Beckmans To remove serum aggregates Eppendorf 5417C Centrifuge
4. It a LSA run being prepared?	Yes → 5 No → 6	
Ensure test wells used and tray layout in U-Bottom plate correspond to test wells used and tray layout on filter plate		
5. Mix EDTA and sera in 96 Well U-Bottom plate.	5.1. Add 500 µL of 0.1625M EDTA to reagent boats 5.2. Add 1.5µL of 0.1625M EDTA **U-Bottom plate** as per Tray Worksheet 5.3. Add 28.5µL of sera and control to **U-Bottom plate** as per Tray Worksheet 5.4. Mix tray by gently tapping 5.5. Discard unused EDTA in boat	Prepare 0.1625M EDTA if necessary as per 'Reagent Preparation' SOP Unused EDTA are never poured back into tube to avoid contamination

Step	Detail	Information
6. Make working dilution of wash buffer	6.1. Make working dilution of wash buffer using 'Luminex Antibody Job Aid'	Dilute 1/10
7. Prepare and soak filter plate	7.1. Ensure plate is not chipped or cracked before using 7.2. Document start well on Tray Worksheet if not beginning in 1A (ie. re-using a tray) 7.3. Cover any unused wells with adhesive plastic cover 7.4. Add 300µL of wash buffer to test wells using a multi-channel pipette and Biohit 300µL pipette tips 7.5. Incubate for 10 minutes at room temperature.	Always start at the top of the tray (row A)
8. Check vacuum manifold while filter plate is soaking	8.1. Using a practice tray and tap water, ensure vacuum manifold has been set to the correct pressure. 8.2. Make adjustments if necessary	Pressure should not exceed 135mbar(100mmHg) and it should take approximately 5-6 seconds for liquid to go through the filter
9. Prepare beads	9.1. Mix bead gently vial by inversion 9.2. Quick spin the vial • 5 seconds • 2600 rpm 9.3. Vortex beads for 5-10 seconds prior to aliquoting.	To remove beads from lid
10. Dilute LSA beads **ONLY**	10.1. Make up **LSA** bead dilution in a 200µL tube as per 'Luminex Antibody Job Aid' 10.2. Gently vortex to mix	70% dilution Vortex setting 2 - 3
NOTE: The plate cannot be allowed to dry out between steps once the soaking procedure is underway		

Step	Detail	Information
11. Aspirate buffer from test plate	11.1. Aspirate wash buffer from test plate using vacuum manifold 11.2. Clamp vacuum tubing after all liquid has gone through the filter	Pressure should not exceed 135mbar(100mmHg) Vacuum should take approximately 5-6 seconds
12. Add beads	12.1. Add 5µL of (diluted) beads to each test well 12.2. Mark the # of tests used on the kit box	Add beads to the side of well to avoid puncture of filter
13. Add sera to tray	13.1. **LumPRA:** Add 20µL of neat sera to the test well as per Tray Worksheet 13.2. **LSA:** Transfer 20µL of EDTA treated sera to the test well in the **filter plate** as per Tray Worksheet	
14. Cover and incubate	14.1. Cover plate with adhesive foil strip and wrap in foil to protect from light 14.2. Incubate tray for 30 minutes on a rotating platform at 200 rotations per minute 14.3. Document temperature on tray worksheet 14.4. Store unused reagents in the dark at 2-8°C	Acceptable Temp=20-25°C Form = 28 Amplitude(Mode) = 5 Time = 30
15. File control and patient sera	15.1. File control and patient sera 15.2. Document 'Sera filed' on worksheet	
Perform Step 16 near the end of the incubation in step 14		
16. Prepare working dilution of antibody conjugate	16.1. Retrieve stock conjugate from walk-in fridge 16.2. Prepare 1/100 working dilution of conjugate as per 'Luminex Antibody Job Aid' 16.3. Mix well 16.4. Store in the dark at RT until use 16.5. Return stock conjugate to walk-in fridge	Store at 2-8°C. Do not freeze. Adust the volume of antibody conjugate made depending on the number of samples tested Room temperature

Step	Detail	Information
17. Wash the tray five times	17.1. Remove tray from the rotating platform and carefully remove the adhesive cover 17.2. Add 150µL of wash buffer to each test well 17.3. Mix by gently tapping the side of the tray 17.4. Aspirate the wash buffer using the vacuum manifold. 17.5. Clamp vacuum tubing after all liquid has gone through the filter 17.6. Repeat steps 17.2 to 17.5 four times, adding 275µL of wash buffer (instead of 150µL) for a total of 5 washes	Do **not** add wash buffer to unused wells Pressure should not exceed 135mbar. Vacuum for approximately 5-6 seconds *Do NOT let plate dry out*
18. Add diluted antibody conjugate and incubate	18.1. Add 100µL of diluted conjugate to each assigned well 18.2. Cover the plate with adhesive cover then wrap in foil to protect from light 18.3. Incubate tray for 30 minutes on a rotating platform at 200 rotations per minute	
19. Prepare Luminex for Acquisition	19.1. Import test batch worklist into Luminex software as per 'Luminex Antibody Assays- Acquisition' SOP.	
20. Wash the tray	20.1. Repeat step 17	
21. Re-suspend beads in 80uL PBS	21.1. Add 80µL PBS to each test well 21.2. Carefully mix each well without disturbing the filter. Use a clean pipette tip for each well.	Remixing is required if more than 4 minutes before acquisition begins
22. Turn plate shaker off	22.1. Ensure plate shaker is turned off	
NOTE: Test run should be acquired immediately after staining to ensure that re-acquisition can be performed within 3 hours should there be a technical failure.		
23. Acquire on Luminex.	23.1. Acquire plate immediately as per 'Luminex Antibody Assay Acquisition' SOP	Store plate in the dark if there is a delay in acquisition

Interpretation/ Results

Not Applicable

Additional Information

LABScreen products are used for antibody detection tests that utilize a panel of color-coded beads, which are coated with purified HLA antigens. Up to 100 different beads may be combined in one suspension for a single test.

Test serum is first incubated with LABScreen beads. Any HLA antibodies present in the test serum bind to the antigens and then are labeled with R-Phycoerythrin (PE)-conjugated Goat anti-human IgG. The LABScan100 flow analyzer detects the fluorescent emission of PE from each bead, allowing almost real-time data acquisition. The reaction pattern of the test serum is compared to the lot-specific antigen array to assign PRA and HLA specificity.

References

Current version of One Lambda LABScreen Product Insert

AT1R Antibody ELISA assay

Purpose

This ELISA assay is used to measure and quantitate antibody to angiotensin II receptor type I (AT1R)

Angiotensin II receptor is pre-coated onto a microtiter plate. During the first incubation, the anti-angiotensin II receptor 1 antibodies in the sample are immobilized on the plate. In a second incubation with a peroxidase (POD) labeled anti-human IgG antibody, enzymatic substrate reaction creates a color intensity correlated with concentration and/or avidity of the anti-angiotensin II receptor 1 antibody.

Materials

Reagents	Supplies	Equipment
• One Lambda AT1R EIA (cat# EIA-AT1RX) • Deionized or distilled water	• Pipettes • Pipette tips • Bottle for 1X wash buffer • Graduated cylinder • 1.5mL eppendorf tubes	• ELISA spectrophotometer Infinite 200, Tecan • Microplate shaker

Specimen

Serum or plasma may be used; samples should be frozen at -20°C or stored at 2-8°C for up to 4 days. Repeat freeze/thaw should be avoided.

Safety Precautions

See MSDS for detailed information.

Quality Control

Negative and positive controls are included in the kit and included in each run. Calibration standards are also included in each run. These concentrations are:
2.5 U/mL
5 U/mL
10 U/mL
20 U/mL
40 U/m

Procedure

Step	Detail	Information
Bring all reagents to room temperature before use.		
1. Prepare worksheet	1.1. Record sample ID on the worksheet 1.2. Label the worksheet with the test date, lot #, and document any relevant comments on the samples 1.3. Document in Run# and details in the AT1R lab manual	Use electronic or paper version Leave room for the worksheet to be pasted in
2. Remove the required number of strips	2.1. Remove excess strips from the breakable frame 2.2. Reseal the package with the desiccant 2.3. Store at 2-8°C	Push from the bottom to avoid snapping the plastic tabs
Controls and calibrators are ready to use; DO NOT DILUTE		
3. Prepare samples	3.1. Label 1.5mL eppendorf tube with the sample ID 3.2. Add 495uL of sample diluent to each tube 3.3. Add 5uL of serum sample to each tube 3.4. Vortex to mix 3.5. Repeat steps 3.1 to 3.4 for all samples	Samples are diluted 1/100

Step	Detail	Information
4. Add sample to wells	4.1. Pipette 100uL of each in duplicate and as per worksheet o Neg control o Pos control o Each calibrator: 2.5, 5.0, 10.0, 20.0, 40.0 o Each sample 4.2. Seal tray with adhesive strip 4.3. Using a sharpie, write well A1 over the A1 location 4.4. Incubate tray for 2 hours at 2-8°C 4.5. Set second timer to perform steps 5 and 6 immediately before wash step 7	See QC section above for more detail on calibrators In HLA lab, use walk in fridge, in West lab, use 'Anne' shelf
5. Prepare 1X Wash Buffer	5.1. Determine the required volume of working wash buffer 5.2. Prepare the 1/10 (1X) working wash buffer as described in Table 1 of the Procedural Notes 5.3. Label the solution with the date prepared and the expiry date of 30 days. 5.4. Store unused 1X wash buffer at 2-8°C	Thimerosal may precipitate in solution. If crystals have formed, mix gently until the crystals have completely dissolved. Dilute the wash buffer with deionized or distilled water 1:10 (e.g., 50 mL + 450 mL water). The diluted solution is stable for 30 days at 2-8°C.
6. Prepare HRP conjugate	6.1. Determine the required volume of working HRP conjugate 6.2. Prepare the 1/10 (1X) working wash buffer as described in Table 1 of the Procedural Notes	The final dilution is 1/100

Step	Detail	Information
7. Wash tray 3X	**7.1. Flick the samples from the wells** 7.2. Add 300uL of working wash buffer 7.3. Flick tray 7.4. Repeat steps 6.2 and 6.3 twice for a total of 3 washes 7.5. After the third wash, invert the plate and place it on a clean paper towel 7.6. Tap to remove excess fluid	If you do not flick the samples out of the well, they will overflow when the 300uL of wash buffer is added
8. Add conjugate	8.1. Add 100uL of diluted HRP conjugate to each well 8.2. Seal tray with adhesive strip 8.3. Incubate tray for 60 minutes on plate shaker at room temperature	
9. Wash tray 3X	9.1. Repeat wash steps as in Step 7	
10. Add substrate	10.1. Add 100uL of substrate to each well 10.2. Incubate at room temperature for 20 minutes **in the dark**	Substrate comes ready to use
11. Turn on ELISA reader	11.1. Turn on the ELISA reader and create tray for reading	Allow sufficient time for this step. The absorbance is 450 nm. Reference wavelength of 620 nm/690 nm is recommended.
Tray must be read within 30 minutes		
12. Add stop solution and read tray	12.1. Add 100uL of stop solution to each well 12.2. Read tray on ELISA reader 12.3. Save results to flash drive and save to another computer	Stop solution comes ready to use

Step	Detail	Information
13. Determine the results	13.1. Create a four parameter logistics fit curve: standard curve: o x-axis: linear, anti-AT1R-Ab standard concentrations (2.5 U/mL, 5 U/mL, 10 U/mL,20 U/mL, 40 U/mL) o y-axis: linear, absorbance 13.2. Sample concentrations can be calculated from this standard curve 13.3. Negative control must be less than 10U/mL for results to be valid 13.4. Samples with results higher than the calibration curve are greater than 40U/mL	Samples may be diluted at a higher concentration (such as 1/500) and re-tested if exact concentration over 40 U/mL is to be determined

Part B Reading and Analysing the Tray

Step	Detail	Information
1. Turn on ELISA reader	1.1. Check if ELISA PC is on 1.2. Turn on ELISA reader	Login='guest' Password='guest'
2. Open iControl program and connect to the instrument	2.1. Double click on the 'iControl (for infinite reader) icon 2.2. Connect by double clicking on the 'Infinite 200' selection	
3. Open the AT1R protocol	3.1. Click on the 'Open' icon 3.2. Select the C: West Lab C/Anne/AT1R Plate	Protocol details: Plate: Corning 96 well flat transparent Measuring wavelength: 450 Reference wavelength: 690

Step	Detail	Information
4. Load plate	4.1. Click on the 'open tray' icon 4.2. Place tray in holder with A1 in top left 4.3. Click on the 'close tray' icon	Red arrow with plate Green arrow with plate
5. Read plate	5.1. Click 'start'	
6. Save results	6.1. Select 'Save as' 6.2. Name the tray with the run # and the date	
7. Save to flash drive	7.1. Click 'Save as' and save the run with the same name as in step 6 above	Do this immediately in case data are lost from PC

Procedural Notes

All test samples and standards should be assayed in duplicate.

Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

Additional Information

Number of Patient Samples	Volume of 10X Wash Buffer	Volume of Water	Total Volume of Working Wash Buffer	Volume of Stock HRP Conjugate	Volume of Conjugate Diluent	Total Volume of Working Conjugate
9	6.4mL	57.6mL	64mL	36uL	3.564mL	3.6mL
17	9.6mL	86.4mL	96mL	52uL	5.148mL	5.2mL
25	12.8mL	115.2mL	128mL	100uL	9.9mL	10.0mL
33	16.0mL	144.0mL	160mL	164uL	16.236mL	16.4mL
40	19.2mL	172.8mL	192mL	200uL	19.8mL	20.0mL

Note that each run includes duplicates of Neg and Pos controls as well as 5 calibrators (ie 14 wells). These calculations were made assuming that 1mL of wash buffer are required per well, allowing 100uL extra per well.

References Current version of Product Insert

Adsorb Out Serum Treatment Protocol

Purpose

The Adsorb Out™ reagent is used to reduce high background in the One Lambda Thermo Fisher FlowPRA® or LabScreen® HLA antibody assays to reduce background binding with patient sera; high background in these HLA solid phase assays is determined by fluorescence on the no antigen, negative control bead. In these experiments this reagent was used in conjunction with the AT1R ELISA assay, distributed by One Lambda Thermo Fisher, to investigate non-specific binding in this ELISA assay that lacks a no antigen well.

Materials

Reagents	Supplies	Equipment
• Adsorb Out™ (cat# ADSORB), One Lambda Thermo Fisher	• Pipettes, P10, P200, P1000 • Pipette tips • 1.5mL microcentrifuge tubes tubes	• Microcentrifuge

Specimen

Serum or plasma may be used; samples should be be previously frozen at -20°C or colder and once thawed can be stored at 2-8°C for up to 4 days. Repeat freeze/thaw should be avoided.

Safety Precautions

See SDS for detailed information.

Quality Control

Samples will be tested with and without the adsorption protocol.

Procedure

Step	Detail	Information
Bring all reagents to room temperature before use.		
1. Prepare worksheet	1.1. Create a worksheet for the samples to be adsorbed 1.2. Record sample ID and serum date on the worksheet 1.3. Label the worksheet with the test date, lot #, and document any relevant comments on the samples	

Step	Detail	Information
2. Thaw and centrifuge the serum samples	2.1. Thaw the serum samples to be adsorbed 2.2. Centrifuge the sera for 2 minutes at 10,000 rpm	Sera have been aliquoted in microcentrifuge vials
3. Add serum to labelled microcentrifuge tubes	3.1. Label microcentrifuge tubes with the sample ID and the serum date from the worksheet 3.2. Aliquot 30uL of patient serum into each tube	
4. Vortex and dispense Adsorb Out beads	4.1. Vortex the Adsorb Out™ beads 10 seconds 4.2. Add 3 uL of beads to each aliquoted serum sample 4.3. Vortex the serum and beads for 10 seconds	Note that this is a 1:10 dilution.
5. Incubate sera and Adsorb Out for 30 minutes at room temperature	5.1. Place cryovials in rack on plate shaker 5.2. Set shaker to setting '2' 5.3. Incubate for 30 minutes	
6. Centrifuge samples	6.1. Remove plate from shaker 6.2. Centrifuge samples for 10 minutes at 10,000 rpm	
7. Transfer serum to labelled microcentrifuge tubes and proceed to AT1R antibody assay	7.1. Label microcentrifuge tubes with the sample ID and the serum date from the worksheet 7.2. Carefully transfer the treated serum into the new tube 7.3. Proceed to testing in the AT1R ELISA immediately, store at 2-8° C for up to 4 days, or store at -20° C or colder	If Adsorb Out microparticles are transferred with serum, repeat step 6.2 and transfer again

Procedural Notes NA

Additional Information NA

References Current version of Product Insert

Neu5Gc Detection on Adsorb Out™ Microparticles

Purpose The purpose of the protocol is to assess the Adsorb Out™ microparticles for the presence of Neu5Gc glycan.

Materials

Reagents	Supplies	Equipment
• Adsorb Out™ microparticles (Cat# ADSORB, One Lambda Thermo Fisher) • Human-Albumin latex coated beads (in-house manufactured, further details in Additional Information section) • Anti-Neu5Gc Antibody Kit (Cat# 146901, BioLegend) • FITC Goat anti-chicken IgY antibody (Cat # 410802, BioLegend)	• dH^{2}0 • PBS (Gibco) • 1.5mL lidded tubes • Gilson style pipettes and tips	• Swing-out microcentrifuge • BD BioSciences Canto II Flow Cytometer

Specimen No specimens are required for this protocol

Safety Precautions Refer to SDS for detailed information.

Quality Control Human albumin lacks Neu5Gc; in-house human albumin coated latex beads are labelled in parallel as a negative control.

A chicken isotype control antibody is run in parallel to the chicken anti-Neu5Gc antibody to assess background binding

Procedure

Step	Detail	Information
Turn on and calibrate the Canto II cytometer as per standard protocol Remove Anti-Neu5Gc antibody kit from -80°C storage to thaw		
1. Prep work area and instruments	1.1. Prepare an ice bath 1.2. Reconstitute the antibodies with 25uL of dH^2O if the kit is being used for the first time 1.3. Store antibody reagents from kit and PBS on ice 1.4. Label all tubes for experiment as per the experiment worksheet	Do not store FITC-labelled secondary in the light
2. Prepare dilutions of the anti-Neu5Gc antibody and isotype control	2.1. Prepare dilutions of the anti-Neu5Gc antibody as well as the isotype control • 1/200 • 1/500 • 1/1000 2.2. Store the dilutions on ice	These dilutions were recommended by the manufacturer for flow cytometer Primary antibodies do not need to be protected from light
3. Add Adsorb Out and human albumin beads	3.1. Add 5ul of Adsorb Out™ to designated tubes as per tray worksheet 3.2. Add 5uL of Human Albumin beads to designated tubes as per tray worksheet	
4. Wash 1x	4.1. Add 1000uL cold PBS to each tube 4.2. Centrifuge at 10,000 rpm for 10 minutes 4.3. Using a P1000 pipette, carefully remove as much supernatant as possible without removing beads/particles 4.4. Vortex each tube	Pellets will be visible If pellet is disturbed, resuspend and centrifuge again as per step 4.2

Step	Detail	Information
5. Add primary antibody/isotype controls and incubate	5.1. To each tube add the designated primary antibody or isotype control antibody as per the worksheet 5.2. Vortex tubes 5.3. Incubate tubes for 60 minutes as 2-8°C	
During the Step 5 incubation, prepare the secondary antibody as per Step 6		
6. Prepare FITC labelled secondary antibody	6.1. Prepare antibody as per worksheet 6.2. Wrap the vial in foil 6.3. Store on ice	Final concentration is 0.5uL/100 uL
7. Wash 1x	7.1. Add 1000uL cold PBS to each tube 7.2. Centrifuge at 10,000 rpm for 10 minutes 7.3. Using a P1000 pipette, carefully remove as much supernatant as possible without removing beads/particles 7.4. Vortex each tube	Pellets will be visible If pellet is disturbed, resuspend and centrifuge again as per step 4.2
8. Add FITC labelled secondary antibody controls and incubate	8.1. Add 100uL of secondary antibody to each tube 8.2. Vortex tubes 8.3. Incubate tubes for 60 minutes as 2-8°C IN THE DARK	
9. Wash 1x	9.1. Add 1000uL cold PBS to each tube 9.2. Centrifuge at 10,000 rpm for 10 minutes 9.3. Using a P1000 pipette, carefully remove as much supernatant as possible without removing beads/particles 9.4. Vortex each tube	Pellets will be visible If pellet is disturbed, resuspend and centrifuge again as per step 4.2

Step	Detail	Information
10. Resuspend beads/particles	10.1. Add 400uL of PBS to each tube 10.2. Vortex	
11. Acquire beads/particles on the flow cytometer	11.1. Acquire microparticles and beads on the cytometer	Information on flow settings is provided in the Procedural Notes section. Viewing unlabelled beads and microparticles on a hemocytometer will provide some guidance for FSS and SSC settings

Procedural Notes

Wash Buffer and diluent note: The use of PBS or wash buffer with fetal bovine/calf serum was avoided to not introduce other sources of potential xeno-antigens into the staining protocol.

Flow Cytometry Settings Details
The 1/200 primary antibody dilution was selected for analysis. Higher dilutions stained but with less intensity.

Adsorb Out product instrument settings: The forward scatter setting was increased to the maximum voltage (999) and the FS threshold was dropped as low as possible (200). Biexponential scales for FSS and SSC were used to see smaller sized particles more readily.
These settings were required because the microparticles are diverse in size and the smallest ones are very small. There are also larger particles. In order to capture all particles, no gating on the particles was performed. Sub-gating on a larger size population of particles was possible and this population was used for analysis.
The FITC settings were set to place the isotype reactivity in the first decade of the log scale. No colour compensation was required as this is a single stain experiment
The median channel value of these larger sized particles was used rather than the mean value to reduce interference from outlier values however the mean MCV values were slightly higher for the Neu5Gc staining were higher for both the isotype control as well as the antibody therefore the overall shift was the same. Gating all events also showed staining with Neu5Gc but the shift was 42 channels over the isotype rather than 62.

Human Albumin product instrument settings: The gating for the human albumin beads was straight-forward as there are a typical bead population

with a single size; doublets were present but were less than 5% of events and were not included in the analysis. The FITC settings were set to place the isotype reactivity in the first decade of the log scale.

Future flow experiments: Use of a small particle flow cytometer or a newer model cytometer may allow more discrimination of the very small Adsorb Out participles. Use of a higher concentration of the primary antibody and isotype (such as 1/50 and 1/100) could be compared in future experiments. The suggested dilutions from the vendor were likely for cells and therefore higher dilutions may be needed to reduce background staining.

Additional Information

Human Albumin Bead Manufacturing and Use: There are beads manufactured for clinical use in the UAH Histocompatibility laboratory. This product is routinely used to treat transplant patient sera with MFI reactivity over 500 mean fluorescence intensity (MFI) to the negative control bead in HLA antibody solid phase Luminex bead-based assays. This product is produced by incubating latex beads (Sigma) with human albumin (Canadian Blood Services).

References Product Inserts for all commercial reagents

Coupling Procedure for ABO-BSA-Antigen to Luminex Beads

Purpose This SOP outlines the procedure for the coupling of ABO subtype antigens (BSA-linked) to Luminex beads. These coupled beads are not for same day use. This SOP is based on the August 2016 Rev E guidelines from Luminex's User Manual for the xMAP® Antibody Coupling Kit.

NOTE: The limiting reagent in this kit is the activation buffer. A maximum of 10 individual beads can be coupled using one kit.

Materials

Reagents	Supplies	Equipment
• xMAP 40-50016 Antibody Coupling Kit *all kit components are to be stored at 2-8°C, EDC in original packaging may be stored at -30°C for longest shelf life; all other kit components should never be frozen *Kit is SINGLE USE and **EDC cannot be made in advance** • Luminex MagPlex® microspheres (1 mL vials) such as MagPlex-C Microspheres, Region 012	• Axygen MCT-150-C 1.5 mL microfuge tubes, autoclaved • Foil cut in small squares for 1.5 mL tubes	• Vortex • MIKRO 22R swing out microcentrifuge in Molecular Core or magnetic separator • Tube rotator for 1.5mL tubes • Pipettes/tips, autoclaved

Specimen Specimens are not used in this antigen coupling procedure.

Safety Precautions See chemical specific MSDS for detailed information.

Procedure

<table>
<tr><th>Step</th><th>Detail</th><th>Information</th></tr>
<tr><td colspan="3">Arrange use of microcentrifuge with Li Ka Shing Core
Remove xMAP Coupling Kit from refrigerator and allow to equilibrate to room temperature
for 20-30 minutes</td></tr>
<tr><td>1. Start Coupling Excel Worksheet</td><td>1.1. Prepare and print a Coupling Worksheet (Excel)
1.2. Fill in:
• date
• bead address
• bead lot #
• calculations
• required bead volumes</td><td>Note that the bead vial and the quality control document for that specific bead lot # may have slightly different bead concentrations. USE THE BEAD CONCENTRATION FROM THE QUALITY CONTROL DOCUMENT
Required bead volumes are calculated on Excel Coupling Worksheet: WORKSHEET_ABO Bead Coupling_[DATE OF COUPLING] ABO Beads</td></tr>
<tr><td>2. Resuspend required volume of Luminex microspheres and add to microcentrifuge tube</td><td>2.1. Document calculations and volume on the Excel worksheet
2.2. Label Axygen microfuge tubes as per the worksheet. DO NOT USE the kit-provided tubes as the lids do not seal well.
2.3. Vortex the 1 mL stock microspheres vial for 30 seconds (in lieu of 10 second vortex + 10 second sonication)
2.4. Transfer designated number of microspheres into its labeled designated Axygen microfuge tube.</td><td>As per the worksheet, to calculate the volume of microspheres use the formula:
Volume of microspheres required = X.X x 10^6 beads / concentration of beads indicated on the product insert in beads per mL
(Formula is present in Excel sheet as a calculation so will auto-populate</td></tr>
</table>

Step	Detail	Information
3. Wash the microspheres twice	3.1. Centrifuge all tubes at 8000g for 2 minutes 3.2. Using a P1000, remove and discard supernatant 3.3. Add 500 µL of Activation Buffer to each tube 3.4. Vortex each tube for 30 seconds 3.5. Centrifuge all tubes at 8000g for 2 minutes 3.6. Using a thin tipped transfer pipette, remove and discard supernatant	Refer to Procedural Notes for instructions on using magnetic separator for wash steps if centrifuge is unavailable
4. Activate beads - Part I	4.1. Add 480 µL of Activation Buffer to each tube 4.2. Vortex the reaction tube for 30 seconds 4.3. Vortex the provided Sulfo-NHS tube from the kit for 20 seconds 4.4. Add 10 µL of Sulfo-NHS to the reaction tube	
5. Prepare EDC	5.1. Add 250 µL of Activation Buffer to the 10 mg vial of EDC 5.2. Invert the EDC vial and then vortex the vial for 12 seconds to dissolve the EDC	**DO NOT MAKE EDC REAGENT IN ADVANCE**
6. Activate beads - Part II	6.1. Add 10 µL EDC solution to each tube 6.2. Vortex the reaction tube for 20 seconds 6.3. Wrap each tube in foil	
7. Incubate beads on rotator for 20 minutes	7.1. Place each foil-wrapped bead vial into a slot on the rotator 7.2. Incubate for 20 minutes at room temperature while rotating	

Step	Detail	Information
8. Wash microspheres 3 times	8.1. Centrifuge all tubes at 8000g for 2 minutes 8.2. Using a P1000, remove 480-490 µL and discard supernatant 8.3. Add 500 µL of Activation Buffer to each tube 8.4. Vortex each tube for 30 seconds 8.5. Centrifuge all tubes at 8000g for 2 minutes 8.6. Using a P1000, remove 480-490 µL and discard supernatant 8.7. Add 500 µL of Activation Buffer to each tube 8.8. Vortex each tube for 30 seconds 8.9. Centrifuge all tubes at 8000g for 2 minutes 8.10. Using a thin tipped transfer pipette, remove and discard supernatant	
9. Add BSA-antigen to the solution	9.1. Calculate the required volume of each ABO antigen and document on the Excel worksheet 9.2. Calculate the required volume of Activation Buffer to bring volume up to a total of 500 µL and document on the worksheet 9.3. Add the Activation Buffer volume determined in step 9.2 9.4. Add the volume of each ABO antigen as determined in step 9.1 9.5. Vortex each tube for 20 seconds 9.6. Wrap each tube in aluminum foil	Total 5ug of antigen are used for each 1.0 x 10^6 beads Calculate the required volume of Activation Buffer by subtracting volume of ABO antigen to be added from 500 µL Eg.: 12.5 µL antigen + 487.5 µL Activation Buffer

Step	Detail	Information
10. Incubate beads on rotator for 2 hours	10.1. Place each foil-wrapped bead vial into a slot on the rotator 10.2. Incubate for 2 hours at room temperature	
Wash Buffer is used for remaining steps: DO NOT continue to use Activation Buffer		
11. Wash microspheres 3 times	11.1. Centrifuge all tubes at 8000g for 2 minutes 11.2. Using a P1000, remove 480-490 µL and discard supernatant 11.3. Add 500 µL of Wash Buffer to each tube 11.4. Vortex each tube for 30 seconds 11.5. Centrifuge all tubes at 8000g for 2 minutes 11.6. Using a P1000, remove 480-490 µL and discard supernatant 11.7. Add 500 µL of Wash Buffer to each tube 11.8. Vortex each tube for 30 seconds 11.9. Centrifuge all tubes at 8000g for 2 minutes 11.10. Using a thin tipped transfer pipette, remove and discard supernatant 11.11. Add 500 µL Wash Buffer to the reaction tube, vortex 30 seconds	This final bead volume can be increased or decreased depending on the desired concentration of beads
12. Count the microspheres on a hemocytometer and store	12.1. Count the number of microspheres recovered and document the percentage of doublet beads 12.2. Record bead counts on the worksheets 12.3. Store coupled beads at 2-8°C in the dark	See Additional Information section of SOP for counting details

Procedural Notes

Sterile conditions are not required for the coupling confirmation
Not for use same day.
Refer to Luminex xMAP® Antibody Coupling Kit for more information.

Instruction for magnet wash steps if swing out microcentrifuge is not available:

If a swing out microcentrifuge is not available, all wash steps can be performed in a magnetic tube separator. *If coupling many beads, this will take much longer to perform and is not recommended*.

1. Place the reaction tube in a magnetic separator for 1-2 minutes, *90 seconds* w/ caps open
2. With tube still in magnetic separator, remove the supernatant with a pipette
3. Remove the tube from the magnetic separator and add 500 µL of Activation Buffer into the reaction tube
4. Vortex the reaction tube for 30 seconds
5. Repeat steps until required number of washes is achieved

Additional Information

Coupling Chemistry:

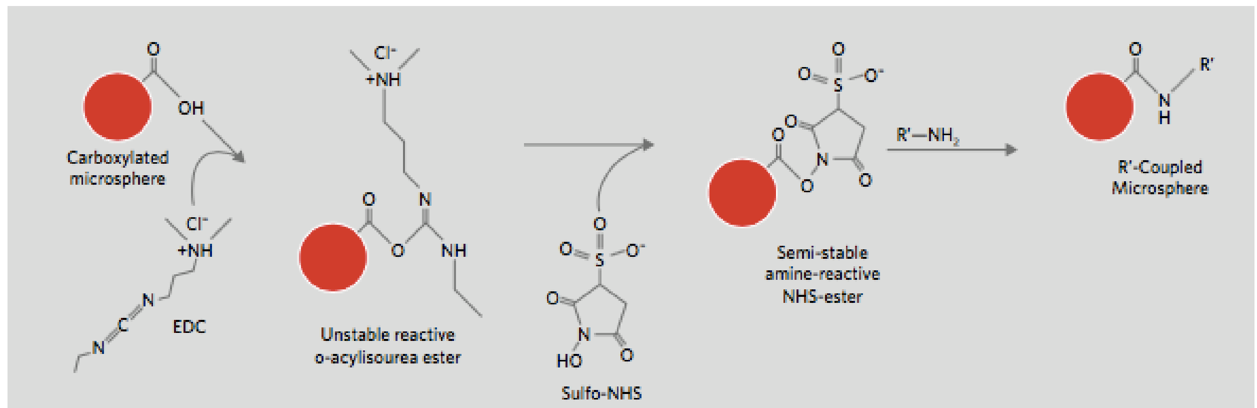


Figure 1: Coupling Chemistry from Angeloni et al. Luminex xMAP Cookbook. Luminex Corporation; 2016. p. 14

Coupling Kit:

The coupling kit is preferred to making each reagent for consistency of concentration and pH. Refer to the Luminex Reagent Job Aid for instructions on reagent preparation without the kit.

The reagents included in the coupling kit:

- EDC
- Sulfo-NHS
- Activation buffer
- Wash Buffer

ABO Antigens Detail:

Antigen Source: A and B tetrasaccharide antigens (A-VI) were created and BSA-linked and procured from Lowary Laboratory at the University of Alberta.[1–3] Detailed summary of these glycans is provided by Jeyakanthan et al. [4]

All antigens are reconstituted 2 mg/mL in 14 Ω water and stored at -80°C.

Antigen	**Source**	**Coupled to MagPlex Bead ID**
A-I tetrasaccharide	Lowary Laboratory	12
A-II tetrasaccharide	Lowary Laboratory	13
A-III tetrasaccharide	Lowary Laboratory	14
A-IV tetrasaccharide	Lowary Laboratory	15
A-V tetrasaccharide	Lowary Laboratory	18
A-VI tetrasaccharide	Lowary Laboratory	19
B-I tetrasaccharide	Lowary Laboratory	45
B-II tetrasaccharide	Lowary Laboratory	46
B-III tetrasaccharide	Lowary Laboratory	47
B-IV tetrasaccharide	Lowary Laboratory	48
B-V tetrasaccharide	Lowary Laboratory	51
B-VI tetrasaccharide	Lowary Laboratory	53
BSA	Sigma Aldrich, Cat# A3059	20
α-Gal (Gala1-3Galb1-4GlcNAc-BSA(3 atom spacer	Dextra UK, Cat# NGP0334	21
No antigen (water)	Li Ka Shing Core Ultra Pure water	22
A-trisaccharide	Lowary Laboratory	61
B-trisaccharide	Lowary Laboratory	62

Selection of Optimum Antigen Coupling Concentration

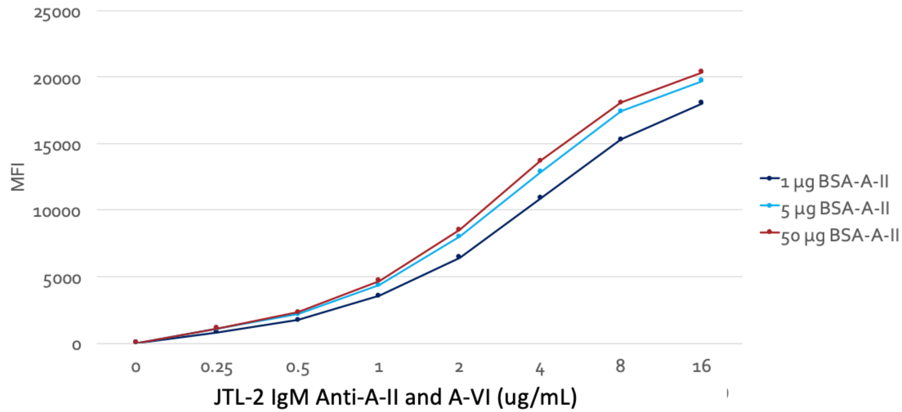


Figure 2: 5ug/mL concentration of BSA-conjugated glycan is was selected as optimum bead coupling concentration.

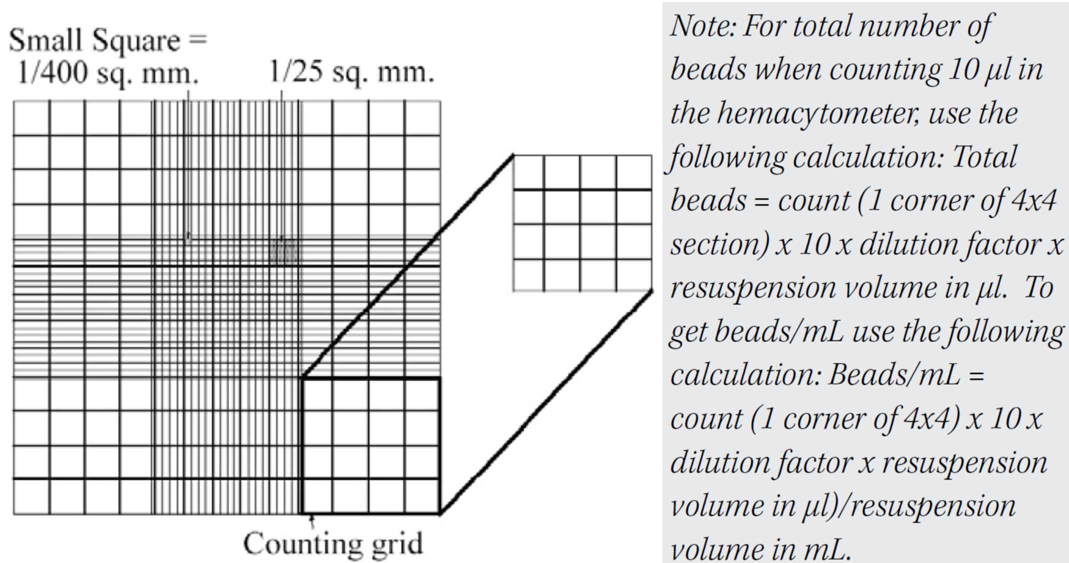


Figure 3: This diagram adapted from the Angeloni et al. Luminex xMAP Cookbook. Luminex Corporation; 2016.[5]

Add 2 µL to 18 µL PBS-TBN to create a 1/10 dilution for counting and load 10 µL into the hemocytometer.

General References

Product Insert: Luminex xMAP® Antibody Coupling Kit User Manual 89-00002-00-319

Publication References

1. Meloncelli PJ, Lowary TL. Synthesis of ABO histo-blood group type I and II antigens. *Carbohydr Res*. 2010;345(16):2305-2322. doi:https://doi.org/10.1016/j.carres.2010.08.012
2. Meloncelli PJ, West LJ, Lowary TL. Synthesis and NMR studies on the ABO histo-blood group antigens: synthesis of type III and IV structures and NMR characterization of type I–VI antigens. *Carbohydr Res*. 2011;346(12):1406-1426. doi:https://doi.org/10.1016/j.carres.2011.03.008
3. Meloncelli PJ, Lowary TL. Synthesis of ABO Histo-Blood Group Type V and VI Antigens*. *Aust J Chem*. 2009;62(6):558-574. https://doi.org/10.1071/CH09058.
4. Jeyakanthan M, Meloncelli PJ, Zou L, et al. ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation. *Am J Transplant*. 2016;16(5):1548-1558. doi:10.1111/ajt.13625
5. Angeloni S, Cordes R, Dunbar S, et al. *XMAP Cookbook: A Collection of Methods and Protocols for Developing Multiplex Assays with XMAP Technology*. 3rd ed. Austin, TX: Luminex Corporation; 2016.

Coupling Confirmation Luminex Beads

Purpose This SOP outlines the procedure for the assessment of coupling success/efficiency of the A or B glycan subtypes, and controls (BSA and ∝Gal) to Luminex beads.[1] This step follows the coupling and must be performed before the beads are used. It is to be run in parallel with the most recent lot/batch of beads. This coupling confirmation must be followed with another QC run of human negative and positive control as well as other recently tested sera as further QC for the new beads.

This protocol can also be used for quality control of beads that have been in storage or perform shipment/lot QC on new Novoclone and secondary antibody shipments. Note that the specific concentration of Novoclone reagents is not provided by the vendor and that the vendor QC for this reagent is performed by hemagglutination. As such, there may be some variation from lot to lot on this Luminex platform which makes lot QC on the Luminex beads very important for the Novoclone reagents.

Materials

Reagents	Supplies	Equipment
• A/B glycan-coupled beads • BSA-coupled beads • ∝Gal glycan-coupled beads • PBS-TBN buffer • Novaclone anti-A Immucor (cat# 5170022) • Novaclone anti-B Immucor (cat# 517502) • JTL-2 [2] • JTL-4 [2] • PE-labeled goat anti-mouse IgM secondary antibody Southern Biotech (cat# 1020-09)	• 96-well plate Corning 3605 (round bottom non-binding white polystyrene) • Adhesive plastic plate seal • 1.5 mL microcentrifuge tubes • Disposable pipette tips • Aluminum foil	• Luminex cytometer • Vortex • Plate shaker (Barnstead Labline) • Luminex Magnetic Plate Separator (Part# CN-0269-01) • Pipettes -P10 -P200 -P1000 -P200 multi

Specimen Specimens are not used at this stage of the Luminex bead-based ABH antigen assay, as this Standard Operating Procedure provides an outline for the coupling confirmation of ABO-BSA-Antigen coupled Luminex beads to be used later to test specimens.
Only monoclonal antibodies are required.

Safety See specific SDS for detailed information.

Quality Control Previously characterized antibodies are used for this purpose. New lot # or new antibodies ***must*** be tested in parallel with previously confirmed coupled bead lot/batch (or in parallel with new reagent). A PBS-PBN well is include to ensure that there is not non-specific fluorescence to any specific bead.

Precautions NA

Procedure

<table>
<tr><th>Step</th><th>Detail</th><th>Information</th></tr>
<tr><td colspan="3">Confirm with laboratory personnel that the Luminex instrument will be available; request that it is left on if it is already in use
Assemble required reagents as listed above, selecting the appropriate antibody-coupled microsphere set(s) and ensure that plate shaker is available
Warm PBS-TBN to room temperature</td></tr>
<tr><td>1. Design tray layout and complete Excel Coupling Confirmation Worksheet</td><td>1.1. Retrieve an Excel Coupling Confirmation Worksheet from the server drive

1.2. Prepare tray layout to include all monoclonal antibodies tested. For a full A/B panel this includes:
• Novoclone anti-A
• Novoclone anti-B
• JTL-2
• JTL-2
• PBS-TBN (background control)

1.3. Fill in information, such as:
• Bead Counts
• Date of Run
• Lot information of Novaclones
• Preparation date of the JTL reagents
• Lot information of anti-mouse IgM secondary
• Required bead volume per well</td><td>Excel worksheet will calculate required bead volumes to achieve 2500 beads per well

Measure extra bead volume for 2 extra wells

Details on antibody specificity and dilutions are provided in the Additional Information Section of this SOP

Refer to last worksheet to confirm the required dilutions to test.</td></tr>
</table>

Step	Detail	Information
2. Prepare beads	2.1. Label a 1.5mL microcentrifuge tube as pooled beads and with the date 2.2. Vortex each individual bead preparation for 30 seconds 2.3. Add the pre-determined bead volume for each panel (A and B) into a microcentrifuge tube as was calculated in step 1 2.4. Spin the tube in a swing-out microcentrifuge at 8000g for 2 minutes 2.5. Remove the supernatant using a pipette 2.6. Add the determined volume of PBS-TBN from Excel worksheet	
3. Prepare Novaclone dilutions	3.1. Prepare dilutions of each of Novaclone anti-A and Novaclone anti-B dilutions using PBS-TBN as the diluent and document the preparation of the dilutions in the plate lay out tab of the worksheet • 1/100 • 1/200 • 1/400 • 1/800 • 1/1600 3.2. Document the preparation of the dilutions in the plate lay out tab of the worksheet	Anti-A is blue Anti-B is yellow The 1/100 dilution will provide saturated MFI values Note that the 1/1600 dilution will not result in a negative (low MFI) result and if titration to negative is required, dilution up to approximately 1/5000

Step	Detail	Information
4. Prepare the JLT 2 and 4 dilutions	4.1. Prepare JTL-2 and -4 dilutions using PBS-TBN as the diluent • 1250 ng/mL • 625 ng/mL • 312.5 ng/mL • 156.25 ng/mL • 78.13 ng/mL • 39.06 ng/mL • 19.53 ng/mL 4.2. Document the preparation of the dilutions in the plate lay out tab of the worksheet	These dilutions will titrate to negative. If saturated bead dilutions are required, higher concentrations can be made from the stock solution
5. Add beads and monoclonal antibody dilutions	5.1. Refer to plate layout and mark the columns and rows to be used using a black sharpie 5.2. Add 5 µL of the multiplexed (pooled) bead suspension to each well as per tray layout 5.3. Add 50 µL of each Novaclone anti-A and B dilution as per tray layout 5.4. Add 50 µL of each JLT 2 and 4 dilution as per tray layout 5.5. Tap plate to mix	Some coloured sharpie pens may affect fluorochromes and black is preferable
6. Incubate the plate for 30 minutes at room temperature	6.1. Apply a fresh adhesive plate seal to the plate ensuring the edges of the outside wells are sealed 6.2. Wrap the plate in aluminum foil 6.3. Incubate the plate for 30 minutes at room temperature on a plate shaker at setting ‘2’	This precaution is taken to protect the bead fluorochromes from light
Perform step 7 during the step 6 incubation		

Step	Detail	Information
7. Prepare secondary antibody	7.1. Prepare the PE-labeled anti-mouse IgM secondary antibody at a 1/100 dilution 7.2. Document the preparation of the secondary antibody in the plate lay out tab of the worksheet 7.3. Protect the secondary antibody from light by wrapping in foil or placing in a drawer	
8. Wash 3 times with PBS-TBN buffer	8.1. Carefully remove adhesive plate seal. 8.2. Add 150 uL of PBS-TBN buffer to each well using an 8 channel multipipette 8.3. WITHOUT REMOVING THE PLATE FROM THE MAGNET, flick the plate with one firm motion Do this over a non-handwashing sink or biohazard reservoir. 8.4. WITHOUT FLIPPING THE PLATE BACK OVER, dab the plate onto a paper towel to capture any residual droplets 8.5. Perform the second and third washes by adding 200uL of wash buffer and repeating steps 8.3 and 8.4 twice	Avoid splashes or liquid transfer from well to well
9. Add PE-labeled anti-mouse IgM secondary antibody	9.1. Add 100 µL of the prepared 1/100 diluted PE-labeled anti-mouse IgM secondary antibody to each well as per tray layout 9.2. Re-seal the plate with a fresh adhesive seal 9.3. Tap plate to mix	
10. Incubate the plate for 30 minutes at room temperature	10.1. Wrap plate in aluminum foil 10.2. Incubate for 30 minutes at room temperature on a plate shaker at setting '2'	This precaution is taken to protect the plate from light

Step	Detail	Information
11. Wash microspheres 3 times and resuspend in PBS-TBN buffer	11.1. Remove adhesive plate seal. 11.2. Add 100 uL of PBS-TBN buffer to each well 11.3. Place plate on a magnetic separator for 2 minutes 11.4. WITHOUT REMOVING THE PLATE FROM THE MAGNET, flick the plate with one firm motion Do this over a non-handwashing sink or biohazard reservoir. 11.5. WITHOUT FLIPPING THE PLATE BACK OVER, dab the plate onto a paper towel to capture any residual droplets 11.6. Perform the second and third washes by adding 200uL of wash buffer and repeating steps 12.3 to 12.5 twice 11.7. Resuspend in 80 uL of TBN-PBS wash buffer	
12. Acquire the beads	12.1. Acquire beads on the Luminex flow cytometer	This step is to be done as soon as possible and within two hours

Procedural Notes Sterile conditions are not required for these steps.

Calculating Beads Required

The Excel worksheet will calculate the beads as per the following calculations.
2500 beads are needed per well (with 2 extra wells included for pipetting loss of reagent)

Beads Calculation
C_1V_1 = 2500 beads
Where: C_1 = concentration of beads in original stock solution of coupled beads (beads/µL);
V_1 = volume of beads to add to each well from the original stock solution (µL);

Example Calculation:
C_1V_1 = 2500 beads where: C_1 = concentration of beads /mL (ex 3.75×10^5);

$V_1 = x$ (# μL to be determined per well);

$(3.75 \times 10^5$ beads/mL)(x) = 2500 beads

x = 6.7 μL beads per well, then bring up to 50 μL

Secondary Antibody Preparation of 1/100 – prepare in 5 mL Falcon tube
(# wells + 1) × 100 μL = total volume required

Example calculation: (14+1) × 100 μL = 1500 μL

1/1000

1/1000 = x /1500 μL
x = 1.5 μL
1.5 μL + 1500 μL PBS-TBN

Additional Information

Typical reactivity patterns for each monoclonal antibody are shown below in Figures 1 and 2. These results are from Feb 2021 coupling experiments. New lot A and B antigens were used. Data were acquired on a Luminex 200 instrument; multiply all MFI values by 1.67 to approximate values on the FlexMap 3D.

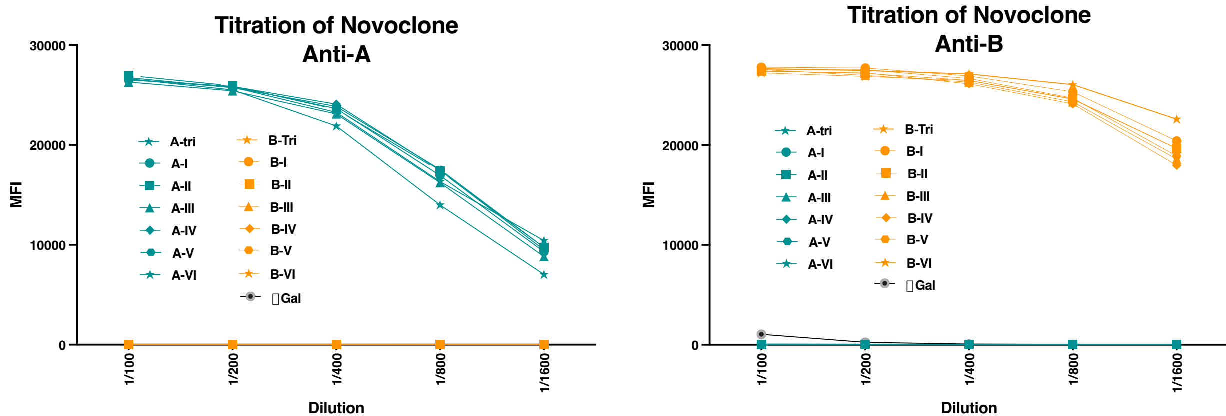


Figure 1: Novoclone monoclonal antibodies will react with all A and B subtype glycans as well as A-Tri and B-Tri. The F

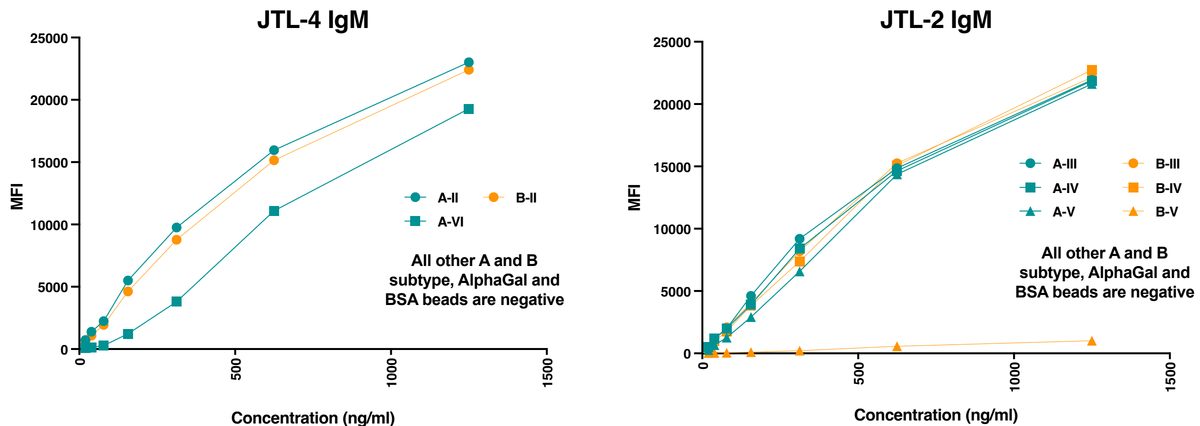


Figure 2: The in-house JTL monoclonal antibodies react as follows: JLT-4 with A-II, B-II, and A-VI (A-IV more weakly than the II glycans). The JTL-2 reacts with A-III, A-IV, A-V, B-III, and B-IV

References

1. Angeloni, S. *et al. xMAP Cookbook: A Collection of Methods and Protocols for Developing Multiplex Assays with xMAP Technology*. (Luminex Corporation, 2016).
2. Jeyakanthan, M. *et al.* Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation. *Am. J. Transplant.* 2602 (2015) doi:10.1111/ajt.13328.

Also refer to product inserts for Novaclone and secondary antibody reagents

Luminex Human Sample ABO Antibody Detection

Purpose This SOP outlines the procedure for the measurement of IgG and IgM ABO subtype-specific antibodies using Luminex single antigen beads. This assay was adapted for Luminex from the microarray method published by Jeyakantham et al[1]

This assay uses a Luminex bead-based panel of ABO-A and ABO-B subtype I-VI glycans which have been coupled to individual Luminex beads. Additional beads to bovine serum albumin (BSA), galactose-α-1,3-galactose (α-Gal) glycan, and A- and B-trisaccharides are also included in this panel.

Phycoerythrin (PE) secondary antibodies specific to human IgG and IgM are used to detect ABO antibodies bound to individual beads. Beads can be acquired on a Luminex 200™ or FlexMap 3D® Luminex instrument.

Materials

Reagents	Supplies	Equipment
• ABO bead panel • Negative control serum (AB-1) • Positive control serum (VP-5) • TBN-PBS wash buffer • IgM secondary antibody: PE-Anti-Human IgM, Cat# IGM-PEC1, One Lambda Thermo Fisher • IgG secondary antibody: PE-Anti-Human IgG, Cat# LS-AB2, One Lambda Thermo Fisher	• 96-well plate, Corning #3605 round bottom non-binding white polystyrene, non-binding surface, 330 µL capacity, 75-200 µL working volume • Plate seal, Excel Scientific Sealing Film 100SEALPLT) • 1.5 mL clear microfuge tubes, MCT-150-C Axygen 311-08-051 • Disposable pipette tips	• Microcentrifuge • Luminex Magnetic Plate Separator (Part# CN-0269-01) • Vortex • Plate shaker • Pipettes (P10, P200, P1000) and tips • 300uL volume multi-channel pipette (8 well)

Specimen This method has been validated for use with serum, heparin, and EDTA plasma. Samples do not require EDTA treatment.

Safety Individual SDS for the beads and wash buffer are stored in the West laboratory. There are no safety concerns with these reagents.

Quality Control There are negative and positive control sera included in this assay.
AB-1: Plasma unit from an ABO-AB individual sourced from the University of Alberta Transfusion Medicine Laboratory
VP-5: Pool of two ABO-O plasma units sourced from the University of Alberta Transfusion Medicine Laboratory

Control beads include:
BSA coupled bead (negative control/background bead)
No antigen bead (negative control/background bead)
α-Gal as a (positive control bead)

Precautions Universal precautions must be taken when handling human specimens

Procedure

Step	Detail	Information
Turn on Luminex instrument and perform standard daily start up and calibration **Bring the wash buffer to room temperature**		
1. Initiate and document samples in the excel ABO plate worksheet	1.1. Open the 'in use' Excel template for this bead batch 1.2. Fill in the number of samples run to calculate the bead volumes required in the 'Beadcalc' tab 1.3. Fill in the plate map details for each sample to be tested as well as the negative and positive control and assign locations on the plate in the 'LAYOUT tab 1.4. Document the lot# of the secondary antibody reagents in the 'Reagent' tab of the worksheet	Bead numbers vary from batch to batch of beads. Ensure the correct version for this bead batch date is used The Excel worksheet will automatically calculate the required bead volumes for each bead address
2. Prepare the bead mix	2.1. Using the calculations in the Excel sheet from Step 1, prepare the bead mix to be used for all beads and controls	

Step	Detail	Information
3. Prepare samples/controls and perform dilutions	3.1. Centrifuge samples in microfuge at 10,000 rpm for 5 minutes 3.2. Using the TBN wash buffer, prepare 1/25 dilutions of all samples and the controls	EDTA treatment is not required *Sample calculation:* 1/25 dilution for each sample: 8uL + 192uL wash buffer 1/50 dilution for pos control: 4uL + 196uL wash buffer
4. Prepare plate adding beads and samples	4.1. Add 5 µL ABO A and B beads to every well as indicated on plate worksheet 4.2. Add 50uL of each diluted sample and control to the IgG as well as IgM wells on the plate worksheet	As per plate layout tab of Excel worksheet
5. Seal and place on plate shaker for 30 minutes	5.1. Apply plate/tray seal 5.2. Wrap plate in aluminum foil 5.3. Incubate for 30 minutes at room temperature on the plate shaker at setting '2'	This precaution is taken to protect the plate from light
6. Prepare secondary antibodies	6.1. Prepare the IgG secondary antibody at a 1/50 dilution 6.2. Prepare the IgM secondary antibody at a 1/50 dilution	100uL are needed for each well. Prepare enough for one extra well *Example of calculations for a run of 12 IgG wells:* • 28uL of IgG secondary Ab • 1372uL of wash buffer

Step	Detail	Information
These beads are the MagPlex beads so they can be 'pelleted' using a magnet, centrifuge or vacuum apparatus. The following steps are using a magnet wash method.		
7. Wash plate 3 times	7.1. Remove plate/tray seal 7.2. Using the 8 channel multi-pipette, add 150uL of wash buffer to each well 7.3. Place the plate on the Luminex Magnetic Plate separator and fold up the side clips to secure the plate 7.4. Leave the plate on the magnet for 2 minutes 7.5. WITHOUT REMOVING THE PLATE FROM THE MAGNET, flick the plate with one firm motion Do this over a non-handwashing sink or biohazard reservoir. 7.6. WITHOUT FLIPPING THE PLATE BACK OVER, dab the plate onto a paper towel to capture any residual droplets 7.7. Perform the second and third washes by adding 200uL of wash buffer and repeating steps 7.3 to 7.5 twice	If an alternate wash method is used, document on the plate layout worksheet Ensure that face cover is used to prevent exposure to aerosols
8. Add the IgG and IgM secondary antibodies	8.1. To the designated IgG wells , add 100 µL of the prepared 1/50 PE labeled IgG secondary antibody 8.2. To the designated IgM wells, add 100 µL of the prepared 1/50 PE labeled IgG secondary antibody 8.3. Apply a plate/tray seal 8.4. Tap plate to gently mix	Refer to plate map in worksheet for well locations
9. Place on plate shaker for 30 minutes	9.1. Wrap plate in aluminum foil 9.2. Incubate for 30 minutes at room temperature on the plate shaker at setting '2'	This precaution is taken to protect the plate from light

Step	Detail	Information
10. Wash plate 3 times	10.1. Remove plate/tray seal 10.2. Place the plate on the Luminex Magnetic Plate separator and fold up the side clips to secure the plate 10.3. Leave the plate on the magnet for 2 minutes 10.4. WITHOUT REMOVING THE PLATE FROM THE MAGNET, flick the plate with one firm motion Do this over a non-handwashing sink or biohazard reservoir. 10.5. WITHOUT FLIPPING THE PLATE BACK OVER, dab the plate onto a paper towel to capture any residual droplets 10.6. Perform the second and third washes by adding 200uL of wash buffer and repeating steps 10.3 to 10.5 twice	
11. Resuspend beads for acquisition	11.1. Add 80 µL of wash buffer to each well and gently mix	Please acquire beads within 2 hours. If a delay is necessary, please document on the Tray Worksheet
12. Acquire the beads	12.1. Read/ acquire on the Luminex cytometer	This step is to be done as soon as possible and within two hours

Procedural Notes

Sterile conditions are not required for this SOP.

The optimal dilution for serum/plasma samples as well as PE-labelled anti-human IgG and IgM was determined by performing a checkerboard experiment

Additional Information

Alternate wash steps may be used such as filter plate, centrifuge and flick wash, etc but these wash steps must be validated for use prior to implementation.

References

1. Angeloni S, Cordes R, Dunbar S, Garcia C, Gibson G, Martin C, et al. Luminex xMAP Cookbook. Luminex Corporation; 2016. p. 1–116
2. Jeyakanthan M, Meloncelli PJ, Zou L, et al. ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation. *Am J Transplant*. 2016:n/a. doi:10.1111/ajt.13625

Luminex Reagent Job Aid

Purpose This job aid provides instructions for making Luminex-related reagents. The TBN buffer is required for all assays and is the wash buffer used in every run. The remaining reagents are now replaced by the Luminex coupling kit xMAP 40-50016 Antibody Coupling Kit but can be used as a back up in the event that the coupling kit is unavailable. The kit is preferred for consistency and ease of use.

Reagent	Materials	Preparation	Stability/ Expiry	Note
Activation Buffer 100 mL **1.1 M NaH_2PO_4**	• NaH_2PO_4 (MW=119.98) stored near Jean's bench in MLS box Vendor: Sigma Cat # S3139 • ddH_2O at handwashing sink	1. Label a 100mL glass bottle with reagent name, date made, expiry date, and initials 2. Tare scale 3. Weigh out 1.1998 grams NaH_2PO_4 and add to the labelled bottle 4. Measure 100 mL ddH_2O in a graduated cylinder and add to the labelled bottle 5. pH to 6.2 6. Store (Anne's shelf) at 2-8 ° C	2 weeks	This reagent is to be used at a pH of 6.2

Reagent	Materials	Preparation	Stability/ Expiry	Note
Coupling Buffer 100 mL MES **50 mM (0.05M) (2[n-Morpholino] ehtanesulfonic acid)**	●(2[n-Morpholino] ehtanesulfonic acid) MES (MW=195.246) stored in bottommost left pull-out drawer of bottom half of -30 ° C fridge Vendor: Sigma Cat # M2933 ●ddH_2O at handwashing sink	1. Label a 100mL glass bottle with reagent name, date made, expiry date, and initials 2. Tare scale 3. Weight out 0.9762 grams MES and add to the labelled bottle 4. Measure out 100mL of ddH_2O and add to the labelled bottle 5. pH to 5.0 6. Store at 2-8 ° C	2 weeks	This reagent is to be used at a pH of 5.0
EDC 100µL **1-Ethyl-3-[3-dimethylaminoprop yl]carbodiimide hydrochloride** **50 mg/mL**	●1-Ethyl-3-[3-dimethylaminopropy l] carbodiimide hydrochloride (EDC) stored in bottommost left pull-out drawer of bottom half of -30 ° C fridge Vendor: Pierce Cat #: 77149 ●ddH_2O at handwashing sink	1. Label 1.5mL eppendorf tube with reagent name and date made 2. Tare scale 3. Weigh out 50 mg EDC (equivalent to 0.05 grams) 4. Add 1mL ddH2O, using a rinsing technique to wash the weighboat and fully dissolve the solid 5. Decant into labelled eppendorf 6. Dispose of immediately after experiement	**Must be made fresh**	10 µL require d per bead couplin g

Reagent	Materials	Preparation	Stability/ Expiry	Note
5% NaAzide	●NaAzide stored on shelf near filed MSDS sheets Vendor: Cat #: ●ddH_2O at handwashing sink	1. Label a 50 or 100mL glass bottle with reagent name, date made, and initials 2. Put on and adjust mask to fit 3. Double glove for safety before handling this reagent 4. Select a spatula or appropriate scoopula that is ***not metal*** 5. Tare scale and weigh out 2.5g of NaAzide 6. Add the 2.5 g NaAzide to the labelled container 7. Add 50mL of ddH_2O 8. Mix to dissolve completely	Stable for 3 years	**Toxic** Added to prevent bacteri al growth

PBS-TBN Buffer	Vendor: Sigma • PBS Cat #: P3813 stored near Jean's bench in MLS box in little individual packets • 1% BSA Cat #: A7888 stored in the bottom left shelf of the deli fridge at 2-8 ° C • 0.05% Tween Cat #: P9416 • NaAzide 0.1% will have been made up previously, but the powder reagent is stored on shelf near filed MSDS sheets • ddH_2O at handwashing sink	1. Ensure 5% NaAzide is available 2. Add 1000 mL ddH_2O to a 1000 mL Erlenmeyer flask 3. Place a stir bar in the bottom of the flask and place the flask on the magnetic stir plate; DO NOT HEAT 4. Add approximately 700-800 mL of the total volume of ddH_2O to the flask and add PBS packet and cover with foil 5. Turn the magnetic stir plate on to a low/moderate speed 6. Stir until solution is completely mixed 7. Tare scale 8. Weigh 10 g BSA 9. Add all BSA into the flask, replace the foil cover and allow solution to mix 10. Stir until solution is completely mixed 11. Using a pipette, add 500 µL Tween to flask and let dissolve		

Reagent	Materials	Preparation	Stability/ Expiry	Note
		12. Stir until solution is completely mixed 13. Remove flask from stir plate, add 200 µL 5% Sodium Azide 14. Mix thoroughly 15. Add remaining ddH_2O in the graduated cylinder while the solution mixes; final volume is 1000mL 16. Label with a 1000mL glass bottle with reagent name, date made, expiry date, and initials 17. Pour buffer from the flask into the upper reservoir of 0.22 um filter unit and filter. 18. pH to ~7.4 (omitted in 2021) 19. Store at 2-8 ° C		

Reagent	Materials	Preparation	Stability/ Expiry	Note
Sulfo-NHS 100 µL **N-Hydroxysulfosuccinimide** **50 mg/mL**	●N-Hydroxysulfosuccinimide (Sulfo-NHS) stored in bottommost left pull-out drawer of bottom half of -30 ° C fridge Vendor: Pierce Cat #: 24510 ●ddH_2O at handwashing sink	1. Label 1.5mL eppendorf tube with reagent name and date made 2. Weigh out Sulfo-NHS appropriate for making up a concentration of 50 mg/mL – equal to 5 mg in 0.100 mL 3. Add 5 mg Sulfo-NHS to 0.100 mL ddH_2O 4. Dissolve completely 5. Decant into labelled eppendorf 6. Discard immediately when experiment is finished	**Must be made fresh**	10 µL required per bead coupling

References:

Angeloni S, Cordes R, Dunbar S, Garcia C, Gibson G, Martin C, et al. Luminex xMAP Cookbook. Luminex Corporation; 2016. p. 1–116.

Complete List of References

Adachi, Iki, Muhammad S. Khan, Francisco A. Guzmán-Pruneda, Charles D. Fraser, Carlos M. Mery, Susan W. Denfield, William J. Dreyer, David L. S. Morales, E. Dean McKenzie, Jeffrey S. Heinle, and Charles D. Fraser. 2015. "Evolution and Impact of Ventricular Assist Device Program on Children Awaiting Heart Transplantation." *The Annals of Thoracic Surgery* 99(2):635–40.

Alba, Ana C., Kathryn Tinckam, Farid Foroutan, Laerke M. Nelson, Finn Gustafsson, Kam Sander, Hellen Bruunsgaard, Sharon Chih, Helen Hayes, Vivek Rao, Diego Delgado, and Heather J. Ross. 2015. "Factors Associated with Anti-Human Leukocyte Antigen Antibodies in Patients Supported with Continuous-Flow Devices and Effect on Probability of Transplant and Post-Transplant Outcomes." *Journal of Heart and Lung Transplantation* 34:685–92.

Almond, C. S., R. R. Thiagarajan, G. E. Piercey, K. Gauvreau, E. D. Blume, H. J. Bastardi, F. Fynn-Thompson, and T. P. Singh. 2009. "Waiting List Mortality among Children Listed for Heart Transplantation in the United States." *Circulation* 119(5):717–27.

Almond, Christopher S., Helena Hoen, Joseph W. Rossano, Chesney Castleberry, Scott R. Auerbach, Lingyao Yang, Ashwin K. Lal, Melanie D. Everitt, Matthew Fenton, Seth A. Hollander, Elfriede Pahl, Elizabeth Pruitt, David N. Rosenthal, Doff B. McElhinney, Kevin P. Daly, and Manisha Desai. 2018. "Development and Validation of a Major Adverse Transplant Event (MATE) Score to Predict Late Graft Loss in Pediatric Heart Transplantation." *The Journal of Heart and Lung Transplantation* 37(4):441–50.

Ameduri, Rebecca K., Jie Zheng, Kenneth B. Schechtman, Timothy M. Hoffman, Robert J. Gajarski, Richard Chinnock, David C. Naftel, James K. Kirklin, Anne I. Dipchand, and Charles E. Canter. 2012. "Has Late Rejection Decreased in Pediatric Heart Transplantation in the Current Era? A Multi-Institutional Study." *The Journal of Heart and Lung Transplantation* 31(9):980–86.

Andersen, Ditte C., Claus Koch, Charlotte H. Jensen, Karsten Skjødt, Jette Brandt, and Børge Teisner. 2004. "High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies." *Journal of Immunoassay and Immunochemistry* 25(1):17–30.

Angeloni, Stephan, Robert Cordes, Sherry Dunbar, Carlos Garcia, Grant Gibson, Charles Martin, and Valerie Stone. 2016. *XMAP Cookbook: A Collection of Methods and Protocols for Developing Multiplex Assays with XMAP Technology*. 3rd ed. Austin, TX: Luminex Corporation.

Askar, M., D. Bhakta, H. Zafar, A. Alam, L. Pittmon, S. Kang, L. Kllingman, J. Felius, SM Joseph, D. Wencker, DM Meyer, and SA Hall. 2020. "Multiplicity of Non-HLA Targets for Antibodies Developed in Association with Ventricular Assist Device Implantation." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 20 Suppl 3:5–1166.

Askar, Medhat, Eileen Hsich, Patrick Reville, Amy S. Nowacki, William Baldwin, Suzanne Bakdash, Jenna Daghstani, Aiwen Zhang, Lynne Klingman, Nicholas Smedira, Nader Moazami, David O. Taylor, Randall C. Starling, and Gonzalo Gonzalez-Stawinski. 2013. "HLA and MICA Allosensitization Patterns among Patients Supported by Ventricular Assist Devices." *The Journal of Heart and Lung Transplantation* 32(12):1241–48.

AuBuchon, JP, J. de Wildt-Eggen, and LJ Dumont. 2008. "Reducing the Variation in Performance of Antibody Titrations." *Archives of Pathology & Laboratory Medicine* 132(7):1194–1201.

Aurora, Paul, Leah B. Edwards, Anna Y. Kucheryavaya, Jason D. Christie, Fabienne Dobbels, Richard Kirk, Axel O. Rahmel, Josef Stehlik, and Marshall I. Hertz. 2010. "Registry Series: The Registry of the International Society for Heart and Lung Transplantation: Thirteenth Official Pediatric Lung and Heart-Lung Transplantation Report—2010." *Journal of Heart and Lung Transplantation* 29:1129–41.

Bailey, L. L., S. R. Gundry, A. J. Razzouk, N. Wang, C. M. Sciolaro, and M. Chiavarelli. 1993. "Bless the Babies: One Hundred Fifteen Late Survivors of Heart Transplantation during the First Year of Life. The Loma Linda University Pediatric Heart Transplant Group." *The Journal of Thoracic and Cardiovascular Surgery* 105(5):805.

Bentall, Andrew, Mylvaganam Jeyakanthan, Manjit Braitch, Christopher W. Cairo, Todd L. Lowary, Stephanie Maier, Anne Halpin, Bruce Motyka, Lu Zou, Lori J. West, and Simon Ball. 2021. "Characterization of ABH-Subtype Donor-Specific Antibodies in ABO-A-Incompatible Kidney Transplantation." *American Journal of Transplantation* 21(11):3649–62.

Bentall, Andrew, A. Nicholas R. Barnett, Manjit Braitch, Nicos Kessaris, Will McKane, Chas Newstead, Gavin McHaffie, Alison Brown, Sian Griffin, Nizam Mamode, David Briggs, and Simon Ball. 2016. "Clinical Outcomes with ABO Antibody Titer Variability in a Multicenter Study of ABO-Incompatible Kidney Transplantation in the United Kingdom." *Transfusion* 56(11):2668–79.

Berry, Gerald J., Margaret M. Burke, Claus Andersen, Patrick Bruneval, Marny Fedrigo, Michael C. Fishbein, Martin Goddard, Elizabeth H. Hammond, Ornella Leone, Charles Marboe, Dylan Miller, Desley Neil, Doris Rassl, Monica P. Revelo, Alexandra Rice, E. Rene Rodriguez, Susan Stewart, Carmela D. Tan, Gayle L. Winters, Lori West, Mandeep R. Mehra, and Annalisa Angelini. 2013. "ISHLT CONSENSUS: The 2013 International Society for Heart and Lung Transplantation Working Formulation for the Standardization of Nomenclature in the Pathologic Diagnosis of Antibody-Mediated Rejection in Heart Transplantation." *Journal of Heart and Lung Transplantation* 32:1147–62.

Bianchi, Maria, Stefania Vaglio, Simonetta Pupella, Giuseppe Marano, Giuseppina Facco, Giancarlo M. Liumbruno, and Giuliano Grazzini. 2016. "Leucoreduction of Blood Components: An Effective Way to Increase Blood Safety?" *Blood Transfusion = Trasfusione Del Sangue* 14(2):214–27.

Brian, Duncan Tait. 2016. "Detection of HLA Antibodies in Organ Transplant Recipients-Triumphs and Challenges of the Solid Phase Bead Assay." *Frontiers in Immunology, Vol 7 (2016)*.

Bruneval, P., A. Angelini, D. Miller, L. Potena, A. Loupy, A. Zeevi, E. F. Reed, D. Dragun, N. Reinsmoen, R. N. Smith, L. West, S. Tebutt, T. Thum, M. Haas, M. Mengel, P. Revelo, M. Fedrigo, J. P. Duong Van Huyen, and G. J. Berry. 2017. "The XIIIth Banff Conference on Allograft Pathology: The Banff 2015 Heart Meeting Report: Improving Antibody-Mediated Rejection Diagnostics: Strengths, Unmet Needs, and Future Directions." *American Journal of Transplantation* 17.

Bux, Jürgen and Ulrich J. H. Sachs. 2007. "The Pathogenesis of Transfusion-Related Acute Lung Injury (TRALI)." *British Journal of Haematology* 136(6):788–99.

Cardinal, H., M. Dieudé, N. Brassard, S. Qi, N. Patey, M. Soulez, D. Beillevaire, F. Echeverry, C. Daniel, Y. Durocher, F. Madore, and M. J. Hébert. 2013. "Antiperlecan Antibodies Are Novel Accelerators of Immune-Mediated Vascular Injury." *American Journal of Transplantation* 13(4):861–74.

Carroll, Robert P., Sue Deayton, Timothy Emery, Watsala Munasinghe, Eleni Tsiopelas, Adrian Fleet, Meg Lake, Ian Humphreys, Maisarah Jalalonmuhali, and Patrick Coates. 2019. "Proactive Treatment of Angiotensin Receptor Antibodies in Kidney Transplantation with Plasma Exchange and/or Candesartan Is Safe and Associated with Excellent Graft Survival at 4 years: A Single Centre Australian Experience." *Human Immunology* 80(8):573–78.

Carroll, Robert P., Michael Riceman, Christopher M. Hope, Anna Zeng, Sue Deayton, Greg D. Bennett, and P. T. Coates. 2016. "Angiotensin II Type-1 Receptor Antibody (AT1Rab) Associated Humoral Rejection and the Effect of Peri Operative Plasma Exchange and Candesartan." *Human Immunology* 77(12):1154–58.

Castleberry, Chesney, Farhan Zafar, Tamara Thomas, Muhammad S. Khan, Roosevelt Bryant, Clifford Chin, David L. S. Morales, and Angela Lorts. 2016. "Allosensitization Does Not Alter Post-Transplant Outcomes in Pediatric Patients Bridged to Transplant with a Ventricular Assist Device." *Pediatric Transplantation* 20(4):559–64.

Clausen, H. and S. Hakomori. 1989. "ABH and Related Histo-Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution." *Vox Sanguinis* 56(1):1–20.

Clausen, H., S. B. Levery, E. Nudelman, S. Tsuchiya, and S. Hakomori. 1985. "Repetitive A Epitope (Type 3 Chain A) Defined by Blood Group A1-Specific Monoclonal Antibody TH-1: Chemical Basis of Qualitative A1 and A2 Distinction." *Proceedings of the National Academy of Sciences of the United States of America* 82(4):1199–1203.

Coppage, Myra. 2013. "Allosensitization and the Ventricular Assist Device: Dual Evolution of Technology." Pp. 67–79 in *Recent Advances in the Field of Ventricular Assist Devices*, edited by K. Komamura. InTech.

Coppage, Myra, Marc Baker, Lawrence Fialkow, Danielle Meehan, Kelly Gettings, Leway Chen, H. Todd Massey, and Neil Blumberg. 2009. "Lack of Significant de Novo HLA Allosensitization in Ventricular Assist Device Recipients Transfused with Leukoreduced, ABO Identical Blood Products." *Human Immunology* 70(6):413–16.

Cowan, P. J. and A. J. Tector. 2017. "The Resurgence of Xenotransplantation." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 17(10):2531–36.

D'alessandro, Angelo. 2016. "Leucoreduction of Blood Components: Clinical and Molecular Evidence." *Blood Transfusion = Trasfusione Del Sangue* 14(2):212–13.

Daga, Sunil, Shimon Hussain, Nithya Krishnan, David Lowe, Manjit Braitch, Prashanth Patel, Andrew Bentall, Simon Ball, Daniel A. Mitchell, Robert Higgins, Ian Skidmore, Daniel Zehnder, and David Briggs. 2021. "Immunoglobulin Isotype Compositions of ABO Specific Antibodies Are Dependent on the Individual Patient Blood Group and Blood Group Specificity: Results from a Healthy Donor Cohort." *Journal of Immunological Methods* 494:113053.

Datta, Pradip. 2013. *Immunoassay Design and Mechanisms of Interferences*. First Edit. Elsevier Inc.

Datta, S. S., S. Basu, M. Reddy, K. Gupta, and S. Sinha. 2021. "Comparative Evaluation of the Conventional Tube Test and Column Agglutination Technology for ABO Antibody Titration in Healthy Individuals: A Report from India." *Immunohematology* 37(1):25–32.

Deltombe, Clement, Florence Gillaizeau, Daniel Anglicheau, Emmanuel Morelon, Katy Trébern-Launay, Florent Le Borgne, Marie Rimbert, Pierrick Guérif, Stéphanie Malard-Castagnet, Yohann Foucher, and Magali Giral. 2017. "Is Pre-Transplant Sensitization against Angiotensin II Type 1 Receptor Still a Risk Factor of Graft and Patient Outcome in Kidney Transplantation in the Anti-HLA Luminex Era? A Retrospective Study." *Transplant International* 30(11):1150–60.

Denomme, Gregory A. and Waseem Q. Anani. 2020. "ABO Titers: Harmonization and Identifying Clinically Relevant ABO Antibodies." *Transfusion* 60(3):441–43.

Dipchand, A. I., S. M. Pollock BarZiv, C. Manlhiot, L. J. West, M. VanderVliet, and B. W. McCrindle. 2010. "Equivalent Outcomes for Pediatric Heart Transplantation Recipients: ABO-Blood Group Incompatible versus ABO-Compatible." *American Journal of Transplantation* 10(2):389–97.

Dipchand, Anne I. 2018. "Current State of Pediatric Cardiac Transplantation." *Annals of Cardiothoracic Surgery; Vol 7, No 1 (January 2018): Heart Transplantation* 7(1):31–55.

Dipchand, Anne I. and Jessica A. Laks. 2020. "Pediatric Heart Transplantation: Long-Term Outcomes." *Indian Journal of Thoracic and Cardiovascular Surgery* 36(2):175–89.

Dragun, Duska and Aurélie Philippe. 2018. "Tempest in a Sugar-Coated Lab Vial." *American Journal of Transplantation* 18(11):2622–23.

El-Awar, N., J. Lee, and P. I. Terasaki. 2005. "HLA Antibody Identification with Single Antigen Beads Compared to Conventional Methods." *Human Immunology* 66(9):989–97.

El-Awar, Nadim, Vadim Jucaud, and Anh Nguyen. 2017. "HLA Epitopes: The Targets of Monoclonal and Alloantibodies Defined." *Journal of Immunology Research* 2017.

Elkind, Jae, Juliana Sobczyk, Oscar Ostberg-Braun, Jorge Silva Enciso, Eric Adler, and Gerald P. Morris. 2020. "Factors Influencing Transfusion-Associated HLA Sensitization in Patients Bridged to Heart Transplantation Using Ventricular Assist Device." *Clinical Transplantation* 34(1):e13772.

Fan, Xiaohu, Andrew Ang, Stacey Pollock-BarZiv, Anne I. Dipchand, Phillip Ruiz, Gregory Wilson, Jeffrey L. Platt, and Lori J. West. 2004. "Donor-Specific B-Cell Tolerance after ABO-Incompatible Infant Heart Transplantation." *Nature Medicine* 10(11):1227–33.

Fichtner, Alexander, Caner Süsal, Claudia Schröder, Britta Höcker, Susanne Rieger, Rüdiger Waldherr, Jens H. Westhoff, Anja Sander, Duska Dragun, and Burkhard Tönshoff. 2018. "Association of Angiotensin II Type 1 Receptor Antibodies with Graft Histology, Function and Survival in Paediatric Renal Transplant Recipients." *Nephrology Dialysis Transplantation* 33(6):1065–72.

Fontaine, Magali J., Jenny Kuo, Ge Chen, Susan A. Galel, Evelyn Miller, Flavia Sequeira, Maurene Viele, Lawrence T. Goodnough, Dolly B. Tyan, G. D. Wool, M. Moayeri, and S. Pandey. 2012. "Complement (C1q) Fixing Solid-Phase Screening for HLA Antibodies Increases the Availability of Compatible Platelet Components for Refractory Patients." *Transfusion* 51; 52(12):2618; 192A.

Foster, Bethany J., Xun Zhang, Alexia de Simone, Mourad Dahhou, Ruth Sapir-Pichhadze, Heloise Cardinal, and Lori West. 2021. "Differences in Heart Graft Survival by Recipient Sex." *Transplantation Direct* 1–10.

Gao, Bingsi, Cassandra Long, Whayoung Lee, Zhongqiang Zhang, Xiaotian Gao, Doug Landsittel, Mohamed Ezzelarab, David Ayares, Yuliang Huang, David K. C. Cooper, Yi Wang, and Hidetaka Hara. 2017. "Anti-Neu5Gc and Anti-Non-Neu5Gc Antibodies in Healthy Humans." *PLOS ONE* 12(7):e0180768.

Gebel, H., B. Bray, and R. Kerman. 2000. "FlowPRA(TM) Detects Clinically Relevant Pretransplant HLA Antibodies in Renal Allograft Recipients." *VISUALS OF THE CLINICAL HISTOCOMPATIBILITY WORKSHOP* 29–30.

Gebel, Howard M. and Robert A. Bray. 2010. "The Evolution and Clinical Impact of Human Leukocyte Antigen Technology." *Current Opinion in Nephrology and Hypertension* 19(6):598–602.

George, Isaac, Patrick Colley, Mark J. Russo, Timothy P. Martens, Elizabeth Burke, Mehmet C. Oz, Mario C. Deng, Donna M. Mancini, and Yoshifumi Naka. 2008. "Association of Device Surface and Biomaterials with Immunologic Sensitization after Mechanical Support." *The Journal of Thoracic and Cardiovascular Surgery* 135(6):1372-1379.e1.

Giral, M., Y. Foucher, A. Dufay, J. P. D. Van Huyen, K. Renaudin, A. Moreau, A. Philippe, B. Hegner, R. Dechend, H. Heidecke, S. Brouard, A. Cesbron, S. Castagnet, A. Devys, J. P. Soulillou, D. Dragun, D. Dragun, A. Philippe, and R. Catar. 2012. "Pretransplant Sensitization Against Angiotensin II Type 1 Receptor Is a Risk Factor for Acute Rejection and Graft Loss." *CURRENT OPINION IN ORGAN TRANSPLANTATION* 13; 17(10; 4):2576; 445.

Growe, G. H., T. R. Petraszko, and Mark Bigham. 2008. "The Approach Taken to Reducing the Risk of Transfusion Related Acute Lung Injury in Canada." *Asian Journal of Transfusion Science* 2(2):84–86.

Haas, M., A. Loupy, C. Lefaucheur, C. Roufosse, D. Glotz, D. Seron, B. J. Nankivell, P. F. Halloran, R. B. Colvin, N. Alachkar, S. Bagnasco, Y. Bouatou, J. U. Becker, L. Cornell, J. P. Duong van Huyen, I. Gibson, R. Mannon, M. Naesens, V. Nickeleit, P. Nickerson, D. L. Segev, H. K. Singh, M. Stegall, P. Randhawa, L. Racusen, K. Solez, and M. Mengel. 2017. "The Banff 2017 Kidney Meeting Report: Revised Diagnostic Criteria for Chronic Active T Cell-Mediated Rejection, Antibody-Mediated Rejection, and Prospects for Integrative Endpoints for Next-Generation Clinical Trials." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*.

Halpin, Anne M., Robert A. Bray, Chris W. Cairo, Patricia M. Campbell, Dongfeng\ Chen, Esme Djike, Tess Ellis, Howard M. Gebel, Anna Greenshields, Sebastiaan Heidt, Annette M\ Jackson, Gonca Karahan, Robert Liwksi, Todd L. Lowary, Stephanie Maier, Bruce Motyka, Cathi L. Murphey, Jean Pearcey, Harold C. Sullivan, Anat R. Tambur, Paul R. Warner, and Lori J. West. 2020. "ABO Antibody Detection: Comparing Apples to Apples in a Multisite Luminex Tool Evaluation." *Human Immunology* 81(Supplementao):36.

Halpin, Anne, Susan Nahirniak, Patricia Campbell, Simon Urschel, Daniel Kim, Lori West, Tara Pidorochynski, Holger Buchholz, and Jennifer Conway. 2019. "HLA Allo-Immunization Following Ventricular Assist Device Support Across the Age Spectrum." *Transplantation*.

Halpin, Anne, Janet Zhou, Jean Pearcey, Todd L. Lowary, Christopher W. Cairo, Gour Daskhan, Bruce Motyka, Stephanie Maier, and Lori J. West. 2018. "OR34 Modernizing ABO Antibody Detection: Development of a Bead-Based ABO Antibody Detection Assay." *Human Immunology*.

Henderson, Heather T., Charles E. Canter, William T. Mahle, Anne I. Dipchand, Kelci LaPorte, Kenneth B. Schechtman, Jie Zheng, Alfred Asante-Korang, Rakesh K. Singh, and Kirk R. Kanter. 2012. "ABO-Incompatible Heart Transplantation: Analysis of the Pediatric Heart Transplant Study (PHTS) Database." *The Journal of Heart and Lung Transplantation* 31(2):173–79.

Herrera, Marcela, Matthew A. Sparks, Adolfo R. Alfonso-Pecchio, Lisa M. Harrison-Bernard, and Thomas M. Coffman. 2013. "Lack of Specificity of Commercial Antibodies Leads to Misidentification of Angiotensin Type 1 Receptor Protein." *Hypertension* 61(1):253–58.

Honger, G., H. Cardinal, M. Dieude, A. Buser, I. Hosli, D. Dragun, M. J. Hebert, and S. Schaub. 2014. "Human Pregnancy and Generation of Anti-Angiotensin Receptor and Anti-Perlecan Antibodies." *Transplant International* 27(5):467–74.

Hussey, J. C., J. Parameshwar, and N. R. Banner. 2007. "Influence of Blood Group on Mortality and Waiting Time before Heart Transplantation in the United Kingdom: Implications for Equity of Access." *The Journal of Heart and Lung Transplantation : The Official Publication of the International Society for Heart Transplantation* 26(1):30–33.

Intermacs. n.d. "Interagency Registry for Mechanically Assisted Cirulatory Support." Retrieved (https://www.uab.edu/medicine/intermacs/).

Irure, Juan, Esther Asensio, Emilio Rodrigo, igo Romn, Javier Gmez, Manuel Arias, Marcos Lpez-Hoyos, and David San Segundo. 2017. "Improvement in the Definition of Anti-HLA Antibody Profile in Highly Sensitized Patients." *PLoS ONE* 12(2):1–13.

Itescu, Silviu and Ranjit John. 2003. "Interactions between the Recipient Immune System and the Left Ventricular Assist Device Surface: Immunological and Clinical Implications." *The Annals of Thoracic Surgery* 75:S65.

Jackson, Annette M., Sai Kanaparthi, Bryna E. Burrell, Donna P. Lucas, Renato M. Vega, Anthony J. Demetris, and Sandy Feng. 2020. "IgG4 Donor-Specific HLA Antibody Profile Is Associated with Subclinical Rejection in Stable Pediatric Liver Recipients." *American Journal of Transplantation* 20(2):513–24.

Javier Delmo, Eva Maria, Mariano Francisco del Maria Javier, and Roland Hetzer. 2020. "The Role of Ventricular Assist Device in Children." *Cardiovascular Diagnosis and Therapy; Vol 11, No 1 (February 2021): Cardiovascular Diagnosis and Therapy*.

Jeyakanthan, M., P. J. Meloncelli, L. Zou, T. L. Lowary, I. Larsen, S. Maier, K. Tao, J. Rusch, R. Chinnock, N. Shaw, M. Burch, K. Beddows, L. Addonizio, W. Zuckerman, E. Pahl, J. Rutledge, K. R. Kanter, C. W. Cairo, J. M. Buriak, D. Ross, I. Rebeyka, and L. J. West. 2016. "ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation." *American Journal of Transplantation* 16(5):1548–58.

Jeyakanthan, M., K. Tao, L. Zou, P. J. Meloncelli, T. L. Lowary, K. Suzuki, D. Boland, I. Larsen, M. Burch, N. Shaw, K. Beddows, L. Addonizio, W. Zuckerman, B. Afzali, D. H. Kim, M. Mengel, A. M. J. Shapiro, and L. J. West. 2015. "Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation." *American Journal of Transplantation* (10):2602.

Jeyakanthan, M. and L. J. West. 2012. "Donor-Specific Isohemagglutinins: Measuring the Unknown." *American Journal of Transplantation* 12(4):803–5.

Kahlyar, Htar, David Roxby, Tony Badrick, and Thiru Vanniasinkam. 2022. "Challenges in Antibody Titration for ABO-Incompatible Renal Transplantation." *Vox Sanguinis* 117(1):109–18.

Kang, Seon Joo, Young Ae Lim, and Sae Yun Baik. 2014. “Comparison of ABO Antibody Titers on the Basis of the Antibody Detection Method Used.” *Annals Of Laboratory Medicine* 34(4):300–306.

Karahan, Gonca E., Frans H. J. Claas, and Sebastiaan Heidt. 2015. “Detecting the Humoral Alloimmune Response: We Need More Than Serum Antibody Screening.” *TRANSPLANTATION* 99(5):908–15.

Karahan, Gonca E., Juliette Krop, Caroline Wehmeier, Yvonne de Vaal, Janneke Langerak-Langerak, Dave L. Roelen, Neubury M. Lardy, Frederike J. Bemelman, Ineke ten Berge, Marlies E. J. Reinders, Cees van Kooten, Frans H. J. Claas, and Sebastiaan Heidt. 2018. “An Easy and Sensitive Method to Profile the Antibody Specificities of HLA-Specific Memory B Cells.” *Transplantation* (Publish Ahead of Print, 9 November 2018).

Kelsch, Reinhard, Anne Schulze Everding, Eberhard Kuwertz-Bröking, Eva Brand, Bernd M. Spriewald, Walter Sibrowski, Martin Konrad, and Duska Dragun. 2011. “Accelerated Kidney Transplant Rejection and Hypertensive Encephalopathy in a Pediatric Patient Associated With Antibodies Against Angiotensin Type 1 Receptor and HLA Class II.” *Transplantation* 92(10).

Khalili, Iman, Martina Koch, Friedrich Thaiss, Rosemarie Plaetke, Jan Bruegger, and Sven Peine. 2017. “Systematic Comparison of IgM and IgG ABO Antibody Titers by Using Tube and Gel Card Techniques and Its Relevance for ABO-Incompatible Kidney Transplantation.” *Clinical Laboratory* 63(09/2017).

Khoury, Michael, Tara Pidborochynski, Anne Halpin, Patricia Campbell, Simon Urschel, Daniel Kim, Lori West, Holger Buchholz, and Jennifer Conway. 2021. “Human Leukocyte Antigen Antibody Sampling in Ventricular Assist Device Recipients: Are We Talking?” *Transplantation Proceedings* 53(7):2377–81.

Kiernan, Jeffrey J., Cynthia A. Ellison, and Kathryn J. Tinckam. 2018. “Measuring Alloantibodies.” *Current Opinion in Organ Transplantation* 1.

Kim, D., I. Gimferrer, P. Warner, K. Nelson, L. Sibulesky, R. Bakthavatsalam, and N. Leca. 2018. “Preformed Angiotensin II Type-1 Receptor Antibodies Are Associated With Rejection After Kidney Transplantation: A Single-Center, Cohort Study.” *Transplantation Proceedings* 50(10):3467–72.

Klein, Sabra L. and Katie L. Flanagan. 2016. “Sex Differences in Immune Responses.” *Nature Reviews Immunology* 16(10):626–38.

Kobashigawa, Jon, Monica Colvin, Luciano Potena, Duska Dragun, Maria G. Crespo-Leiro, Juan F. Delgado, Michael Olymbios, Jayan Parameshwar, Jignesh Patel, Elaine Reed, Nancy Reinsmoen, E. Rene Rodriguez, Heather Ross, Randall C. Starling, Dolly Tyan, Simon Urschel, and Andreas Zuckermann. 2018. “The Management of Antibodies in Heart Transplantation: An ISHLT Consensus Document.” *Journal of Heart and Lung Transplantation* 1–11.

Kwon, Murray H., Jennifer Q. Zhang, Joanna M. Schaenman, Martin Cadeiras, David W. Gjertson, Carolyn A. Krystal, Hillel Laks, Abbas Ardehali, Mario C. Deng, Richard J. Shemin, and Elaine F. Reed. 2015. “Characterization of Ventricular Assist Device-Mediated Sensitization in the Bridge-to-Heart-Transplantation Patient.” *The Journal of Thoracic and Cardiovascular Surgery* 149(4):1161–66.

Laing, Bryce J., David B. Ross, Steven R. Meyer, Patricia Campbell, Anne M. Halpin, Lori J. West, and Ivan M. Rebeyka. 2010. "Congenital Heart Disease: Glutaraldehyde Treatment of Allograft Tissue Decreases Allosensitization after the Norwood Procedure." *The Journal of Thoracic and Cardiovascular Surgery* 139:1402–8.

Landsteiner, K. 2001. "[Agglutination phenomena of normal human blood]." *Wiener klinische Wochenschrift* 113(20–21):768–69.

Lane, William. 2016. "Next Generation Sequencing for Blood Group Antigen Profiling." *Blood* 128(22):SCI-24 LP-SCI-24.

Lefaucheur, Carmen, Denis Viglietti, Yassine Bouatou, Aurélie Philippe, Daniele Pievani, Olivier Aubert, Jean-Paul Duong Van Huyen, Jean-Luc Taupin, Denis Glotz, Christophe Legendre, Alexandre Loupy, Philip F. Halloran, and Duska Dragun. 2019. "Non-HLA Agonistic Anti-Angiotensin II Type 1 Receptor Antibodies Induce a Distinctive Phenotype of Antibody-Mediated Rejection in Kidney Transplant Recipients." *Kidney International* 96(1):189–201.

Lepeytre, Fanny, Mourad Dahhou, Xun Zhang, Julie Boucquemont, Ruth Sapir-Pichhadze, Heloise Cardinal, and Bethany J. Foster. 2017. "Association of Sex with Risk of Kidney Graft Failure Differs by Age." *Journal of the American Society of Nephrology* 28(10):3014 LP – 3023.

Lindberg, Linda, Sandra M. Johansson, Jining Liu, Per Grufman, and Jan Holgersson. 2011. "Is There a Clinical Need for a Diagnostic Test Allowing Detection of Chain Type-Specific Anti-A and Anti-B?" *Transfusion* 51(3):494–503.

Luft, Friedrich C. 2013. "Activating Autoantibodies and Cardiovascular Disease." *Physiology (Bethesda, Md.)* 28(4):254–61.

Magdo, H. Sonali, Kurt R. Schumacher, Sunkyung Yu, Robert J. Gajarski, and Joshua M. Friedland-Little. 2017. "Clinical Significance of Anti-HLA Antibodies Associated with Ventricular Assist Device Use in Pediatric Patients: A United Network for Organ Sharing Database Analysis." *Pediatric Transplantation* 21(5):e12938.

Mahle, William T., Margaret A. Tresler, R. E. Edens, Paolo Rusconi, James F. George, David C. Naftel, and Robert E. Shaddy. 2011. "Allosensitization and Outcomes in Pediatric Heart Transplantation." *The Journal Of Heart And Lung Transplantation: The Official Publication Of The International Society For Heart Transplantation* 30(11):1221–27.

Mangiola, Massimo, Marilyn Marrari, Brian Feingold, and Adriana Zeevi. 2017. "Significance of Anti-HLA Antibodies on Adult and Pediatric Heart Allograft Outcomes." *Frontiers in Immunology* 8(JAN).

Massie, Allan B., Babak J. Orandi, Madeleine M. Waldram, Xun Luo, Anh Q. Nguyen, Robert A. Montgomery, Krista L. Lentine, and Dorry L. Segev. 2020. "Impact of ABO-Incompatible Living Donor Kidney Transplantation on Patient Survival." *American Journal of Kidney Diseases* 76(5):616–23.

de Mattos, Luiz Carlos. 2016. "Structural Diversity and Biological Importance of ABO, H, Lewis and Secretor Histo-Blood Group Carbohydrates." *Revista Brasileira de Hematologia e Hemoterapia* 38(4):331–40.

McKenna David H, Jr., Ted Eastlund, Miriam Segall, Harriet J. Noreen, Soon Park, Jr. McKenna David H, Ted Eastlund, Miriam Segall, Harriet J. Noreen, and Soon Park. 2002. "Original Article: HLA Alloimmunization in Patients Requiring Ventricular Assist Device Support." *Journal of Heart and Lung Transplantation* 21:1218–24.

Mehra, Mandeep R., Maria G. Crespo-Leiro, Anne Dipchand, Stephan M. Ensminger, Nicola E. Hiemann, Jon A. Kobashigawa, Joren Madsen, Jayan Parameshwar, Randall C. Starling, and Patricia A. Uber. 2010. “International Society for Heart and Lung Transplantation Working Formulation of a Standardized Nomenclature for Cardiac Allograft Vasculopathy—2010.” *The Journal of Heart and Lung Transplantation* 29(7):717–27.

Meloncelli, Peter J. and Todd L. Lowary. 2009. “Synthesis of ABO Histo-Blood Group Type V and VI Antigens*.” *Australian Journal of Chemistry* 62(6):558–74.

Meloncelli, Peter J. and Todd L. Lowary. 2010. “Synthesis of ABO Histo-Blood Group Type I and II Antigens.” *Carbohydrate Research* 345(16):2305–22.

Meloncelli, Peter J., Lori J. West, and Todd L. Lowary. 2011. “Synthesis and NMR Studies on the ABO Histo-Blood Group Antigens: Synthesis of Type III and IV Structures and NMR Characterization of Type I–VI Antigens.” *Carbohydrate Research* 346(12):1406–26.

Meyer, Steven R., Patricia M. Campbell, Jennifer M. Rutledge, Anne M. Halpin, Lois E. Hawkins, Jonathan R. T. Lakey, Ivan M. Rebeyka, and David B. Ross. 2005. “Use of an Allograft Patch in Repair of Hypoplastic Left Heart Syndrome May Complicate Future Transplantation.” *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery* 27(4):554–60.

Michielsen, Laura A., Arjan D. van Zuilen, Merle M. Krebber, Marianne C. Verhaar, and Henny G. Otten. 2016. “Clinical Value of Non-HLA Antibodies in Kidney Transplantation: Still an Enigma?” *Transplantation Reviews (Orlando, Fla.)* 30(4):195–202.

Middleton, D., J. Jones, and D. Lowe. 2014. “Nothing’s Perfect: The Art of Defining HLA-Specific Antibodies.” *Transplant Immunology* 30(4):115–21.

Milland, J. and M. S. Sandrin. 2006. “ABO Blood Group and Related Antigens, Natural Antibodies and Transplantation.” *Tissue Antigens* 68(6):459–66.

Moazami, N., S. Itescu, M. R. Williams, M. Argenziano, A. Weinberg, and M. C. Oz. 1998. “Platelet Transfusions Are Associated with the Development of Anti-Major Histocompatibility Complex Class I Antibodies in Patients with Left Ventricular Assist Support.” *Journal of Heart and Lung Transplantation* 17(9):876–80.

Montgomery, Robert A., Jeffrey M. Stern, Bonnie E. Lonze, Vasishta S. Tatapudi, Massimo Mangiola, Ming Wu, Elaina Weldon, Nikki Lawson, Cecilia Deterville, Rebecca A. Dieter, Brigitte Sullivan, Gabriella Boulton, Brendan Parent, Greta Piper, Philip Sommer, Samantha Cawthon, Erin Duggan, David Ayares, Amy Dandro, Ana Fazio-Kroll, Maria Kokkinaki, Lars Burdorf, Marc Lorber, Jef D. Boeke, Harvey Pass, Brendan Keating, Adam Griesemer, Nicole M. Ali, Sapna A. Mehta, and Zoe A. Stewart. 2022. “Results of Two Cases of Pig-to-Human Kidney Xenotransplantation.” *New England Journal of Medicine* 386(20):1889–98.

Moore, S., A. Chirnside, L. R. Micklem, D. B. McClelland, and K. James. 1984. “A Mouse Monoclonal Antibody with Anti-A,(B) Specificity Which Agglutinates Ax Cells.” *Vox Sanguinis* 47(6):427–34.

Morales-Buenrostro, Luis E., Paul I. Terasaki, Lluvia A. Marino-Vazquez, Jar-How Lee, Nadim El-Awar, and Josefina Alberu. n.d. “‘Natural’ Human Leukocyte Antigen Antibodies Found in Nonalloimmunized Healthy Males.” *TRANSPLANTATION* 86(8):1111–15.

Murphy, M. F. 2013. “Managing the Platelet Refractory Patient.” *ISBT Science Series* 9(1):234–38.

Muthana, Saddam M. and Jeffrey C. Gildersleeve. 2016. “Factors Affecting Anti-Glycan IgG and IgM Repertoires in Human Serum.” *Scientific Reports* 6(1):19509.

Newell, H., J. D. Smith, P. Rogers, E. Birks, A. J. Danskine, R. E. Fawson, and M. L. Rose. 2006. “Sensitization Following LVAD Implantation Using Leucodepleted Blood Is Not Due to HLA Antibodies.” *American Journal of Transplantation* 6(7):1712–17.

Ngo, S. T., F. J. Steyn, and P. A. McCombe. 2014. “Gender Differences in Autoimmune Disease.” *Frontiers in Neuroendocrinology* 35(3):347–69.

Nikaein, Afzal, Nadim El-Awar, Judson Hunt, Edward J. Rosenthal, Eric Eichhorn, Shelly Hall, James Boehrer, Mitchell Magee, Marc Pieniek, William Brinkman, and Todd Dewey. 2012. “Clinically Irrelevant Circulating Human Leukocyte Antigen Antibodies in the Presence of Ventricular Assist Devices.” *The Journal of Heart and Lung Transplantation* 31(5):443–47.

O’Connor, Matthew J., Terry O. Harville, Bobbie Rhodes-Clark, Sherry E. Pye, Kenneth R. Knecht, Michiaki Imamura, Elizabeth A. Frazier, and W. Robert Morrow. 2013. “Quantification, Identification, and Relevance of Anti-Human Leukocyte Antigen Antibodies Formed in Association with the Berlin Heart Ventricular Assist Device in Children.” *Transplantation* 95(12):1542–47.

O’Connor, Matthew J., JonDavid Menteer, Maryanne R. K. Chrisant, Dimitrios Monos, Curt Lind, Selena Levine, J. William Gaynor, Brian D. Hanna, Stephen M. Paridon, Chitra Ravishankar, and Beth D. Kaufman. 2010. “Ventricular Assist Device-Associated Anti-Human Leukocyte Antigen Antibody Sensitization in Pediatric Patients Bridged to Heart Transplantation.” *The Journal of Heart and Lung Transplantation* 29(1):109–16.

Oaks, Martin, Karen Michel, Francis X. Downey, and Vinay Thohan. 2018. “Xenoreactive Antibodies and Latent Fibrin Formation in VAD and Cardiac Transplant Recipients Can Confound the Detection and Measurement of Anti-AT1R Antibodies.” *American Journal of Transplantation* 18(11):2763–71.

Oriol, R., R. Mollicone, P. Coullin, A. M. Dalix, and J. J. Candelier. 1992. “Genetic Regulation of the Expression of ABH and Lewis Antigens in Tissues.” *APMIS. Supplementum* 27:28–38.

Patel, R. and P. I. Terasaki. 1969. “Significance of the Positive Crossmatch Test in Kidney Transplantation.” *The New England Journal of Medicine* 280(14):735–39.

Pendu, J. Le, A. M. Dalix, R. Mollicone, K. Crainic, and R. Oriol. 1989. “EXPRESSION OF ABH, LEWIS AND RELATED TISSUE ANTIGENS IN THE HUMAN THYMUS.” *International Journal of Immunogenetics* 16(1):19–32.

Philogene, Mary Carmelle, Serena Bagnasco, Edward S. Kraus, Robert A. Montgomery, Duska Dragun, Mary S. Leffell, Andrea A. Zachary, and Annette M. Jackson. 2017. “Anti-Angiotensin II Type 1 Receptor and Anti-Endothelial Cell Antibodies: A Cross-Sectional Analysis of Pathological Findings in Allograft Biopsies.” *Transplantation* 101(3):608–15.

Picascia, Antonietta, Chiara Sabia, Vincenzo Grimaldi, Maria Lourdes Montesano, Linda Sommese, Concetta Schiano, and Claudio Napoli. 2014. “Lights and Shadows of Anti-HLA Antibodies Detected by Solid-Phase Assay.” *Immunology Letters* 162(1, Part A):181–87.

Pinelli, David F., John J. Friedewald, Kelley M. K. Haarberg, Shree L. Radhakrishnan, Jennifer R. Zitzner, Wendy E. Hanshew, and Anat R. Tambur. 2017. “Assessing the Potential of Angiotensin II Type 1 Receptor and Donor Specific Anti-Endothelial Cell Antibodies to Predict Long-Term Kidney Graft Outcome.” *Human Immunology* 78(5–6):421–27.

Pochechueva, Tatiana, Alexander Chinarev, Marianne Spengler, Elena Korchagina, Viola Heinzelmann-Schwarz, Nicolai Bovin, and Robert Rieben. 2011. "Multiplex Suspension Array for Human Anti-Carbohydrate Antibody Profiling." *Analyst* 136(3):560–69.

Porrett, Paige M., Babak J. Orandi, Vineeta Kumar, Julie Houp, Douglas Anderson, A. Cozette Killian, Vera Hauptfeld-Dolejsek, Dominique E. Martin, Sara Macedon, Natalie Budd, Katherine L. Stegner, Amy Dandro, Maria Kokkinaki, Kasinath V Kuravi, Rhiannon D. Reed, Huma Fatima, John T. Killian Jr., Gavin Baker, Jackson Perry, Emma D. Wright, Matthew D. Cheung, Elise N. Erman, Karl Kraebber, Tracy Gamblin, Linda Guy, James F. George, David Ayares, and Jayme E. Locke. 2022. "First Clinical-Grade Porcine Kidney Xenotransplant Using a Human Decedent Model." *American Journal of Transplantation* 22(4):1037–53.

Ravn, V. and E. Dabelsteen. 2000. "Tissue Distribution of Histo-Blood Group Antigens." *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 108(1):1–28.

Reed, E. F., P. Rao, Z. Zhang, H. Gebel, R. A. Bray, I. Guleria, J. Lunz, T. Mohanakumar, P. Nickerson, A. R. Tambur, A. Zeevi, P. S. Heeger, and D. Gjertson. 2013. "Comprehensive Assessment and Standardization of Solid Phase Multiplex-Bead Arrays for the Detection of Antibodies to HLA." *American Journal of Transplantation* 13(7).

Reinsmoen, N. L., C. H. Lai, H. Heidecke, M. Haas, K. Cao, G. Ong, M. Naim, Q. Wang, J. Mirocha, J. Kahwaji, A. A. Vo, S. C. Jordan, and D. Dragun. 2010. "Anti-Angiotensin Type 1 Receptor Antibodies Associated With Antibody Mediated Rejection in Donor HLA Antibody Negative Patients." *Transplantation* 90(12):1473–77.

Reinsmoen, N. L., C. H. Lai, J. Mirocha, K. Cao, G. Ong, M. Naim, Q. Wang, M. Haas, M. Rafiei, L. Czer, J. Patel, and J. Kobashigawa. 2014. "Prediction of Organ Transplant Rejection by HLA-Specific and Non-HLA Antibodies - Brief Literature Review." *Transplantation* 97(2; 5):601.

Reynolds, Ben C. and Kathryn J. Tinckam. 2016. "Sensitization Assessment before Kidney Transplantation." *Transplantation Reviews (Orlando, Fla.)*.

Rossano, Joseph W., David L. S. Morales, Farhan Zafar, Susan W. Denfield, Jeffrey J. Kim, John L. Jefferies, and William J. Dreyer. 2010. "Impact of Antibodies against Human Leukocyte Antigens on Long-Term Outcome in Pediatric Heart Transplant Patients: An Analysis of the United Network for Organ Sharing Database." *The Journal of Thoracic and Cardiovascular Surgery* 140(3):694-699.e2.

Rothberg, Richard M. and Richard S. Farr. 1965. "ANTI-BOVINE SERUM ALBUMIN AND ANTI-ALPHA LACTALBUMIN IN THE SERUM OF CHILDREN AND ADULTS." *Pediatrics* 35(4):571 LP – 588.

See, Sarah B., Kevin J. Clerkin, Peter J. Kennel, Feifan Zhang, Matthew P. Weber, Kortney J. Rogers, Debanjana Chatterjee, Elena R. Vasilescu, George Vlad, Yoshifumi Naka, Susan W. Restaino, Maryjane A. Farr, Veli K. Topkara, Paolo C. Colombo, Donna M. Mancini, P. Christian Schulze, Bruce Levin, and Emmanuel Zorn. 2017. "Ventricular Assist Device Elicits Serum Natural IgG That Correlates with the Development of Primary Graft Dysfunction Following Heart Transplantation." *The Journal of Heart and Lung Transplantation* 36(8):862–70.

Seftel, Matthew D., Gershon H. Growe, Tanya Petraszko, W. Barrett Benny, Alan Le, Chao-Yong Lee, John J. Spinelli, Heather J. Sutherland, Peter Tsang, and Donna E. Hogge. 2004. "Universal Prestorage Leukoreduction in Canada Decreases Platelet Alloimmunization and Refractoriness." *Blood* 103(1):333 LP – 339.

Services, Canadian Blood. n.d. “Canadian CPRA Calculator.” *CPRA Calculator Online Tool*. Retrieved (https://ctr2.transplantregistry.ca/otd-cpra-client/ctr2.jsp).

Singh, Tajinder P., Christopher S. Almond, Gary Piercey, and Kimberlee Gauvreau. 2011. “Trends in Wait-List Mortality in Children Listed for Heart Transplantation in the United States: Era Effect Across Racial/Ethnic Groups.” *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11(12):2692–99.

Steiner, Jill M., Eric V Krieger, Karen K. Stout, April Stempien-Otero, Claudius Mahr, Nahush A. Mokadam, and Joshua L. Hermsen. 2017. “Durable Mechanical Circulatory Support in Teenagers and Adults with Congenital Heart Disease: A Systematic Review.” *International Journal of Cardiology* 245:135–40.

Stewart, Susan, Gayle L. Winters, Michael C. Fishbein, Henry D. Tazelaar, Jon Kobashigawa, Jacki Abrams, Claus B. Andersen, Annalisa Angelini, Gerald J. Berry, Margaret M. Burke, Anthony J. Demetris, Elizabeth Hammond, Silviu Itescu, Charles C. Marboe, Bruce McManus, Elaine F. Reed, Nancy L. Reinsmoen, E. Rene Rodriguez, Alan G. Rose, Marlene Rose, Nicole Suciu-Focia, Adriana Zeevi, and Margaret E. Billingham. 2005. “Revision of the 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection.” *The Journal of Heart and Lung Transplantation* 24(11):1710–20.

Stussi, G., K. Huggel, H. U. Lutz, U. Schanz, R. Rieben, and J. D. Seebach. 2005. “Isotype-Specific Detection of ABO Blood Group Antibodies Using a Novel Flow Cytometric Method.” *British Journal of Haematology* 130(6):954–63.

Sullivan, Harold C., Howard M. Gebel, and Robert A. Bray. 2017. “Understanding Solid-Phase HLA Antibody Assays and the Value of MFI.” *Human Immunology*.

Tait, Brian D., Caner Süsal, Howard M. Gebel, Peter W. Nickerson, Andrea A. Zachary, Frans H. J. Claas, Elaine F. Reed, Robert A. Bray, Patricia Campbell, Jeremy R. Chapman, P. Toby Coates, Robert B. Colvin, Emanuele Cozzi, Ilias I. N. Doxiadis, Susan V Fuggle, John Gill, Denis Glotz, Nils Lachmann, Thalachallour Mohanakumar, Nicole Suciu-Foca, Suchitra Sumitran-Holgersson, Kazunari Tanabe, Craig J. Taylor, Dolly B. Tyan, Angela Webster, Adriana Zeevi, and Gerhard Opelz. 2013. “Consensus Guidelines on the Testing and Clinical Management Issues Associated with HLA and Non-HLA Antibodies in Transplantation.” *Transplantation* 95(1):19–47.

Tambur, A. R., N. D. Herrera, K. M. K. Haarberg, M. F. Cusick, R. A. Gordon, J. R. Leventhal, J. J. Friedewald, and D. Glotz. 2015. “Assessing Antibody Strength: Comparison of MFI, C1q, and Titer Information.” *American Journal of Transplantation* 15(9).

Tambur, Anat, PhD DMD, and Chris Wiebe. 2018. “HLA Diagnostics: Evaluating DSA Strength by Titration.” *Transplantation* 102(1S):S23–30.

Tambur, Anat R., Patricia Campbell, Frans H. Claas, Sandy Feng, Howard M. Gebel, Annette M. Jackson, Roslyn B. Mannon, Elaine F. Reed, Kathryn Tinckam, Medhat Askar, Anil Chandraker, Patricia P. Chang, Monica Colvin, Anthony-Jake Demetris, Joshua M. Diamond, Anne I. Dipchand, Robert L. Fairchild, Mandy L. Ford, John Friedewald, Ronald G. Gill, Denis Glotz, Hilary Goldberg, Ramsey Hachem, Stuart Knechtle, Jon Kobashigawa, Deborah J. Levine, Joshua Levitsky, Michael Mengel, Edgar Milford, Kenneth A. Newell, Jacqueline G. O'Leary, Scott Palmer, Parmjeet Randhawa, John Smith, Laurie Snyder, Randall C. Starling, Stuart Sweet, Timucin Taner, Craig J. Taylor, Steve Woodle, Adriana Zeevi, and Peter Nickerson. 2018. "Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group Meeting Report." *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons*.

Terato, Kuniaki, Christopher T. Do, Dawn Cutler, Takaki Waritani, and Hiroshi Shionoya. 2014. "Preventing Intense False Positive and Negative Reactions Attributed to the Principle of ELISA to Re-Investigate Antibody Studies in Autoimmune Diseases." *Journal of Immunological Methods* 407:15–25.

Tinckam, K. and P. Campbell. 2013. "Angiotensin II Type 1 Receptor Antibodies: Great Expectations?" *American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons* 13(10):2515–16.

Tinckam, Kathryn. 2009. "Histocompatibility Methods." *Transplantation Reviews* 23:80–93.

Triulzi, Darrell J., Steven Kleinman, Ram M. Kakaiya, Michael. P. Busch, Philip J. Norris, Whitney R. Steele, Simone A. Glynn, Christopher D. Hillyer, Patricia Carey, Jerome L. Gottschall, Edward L. Murphy, Jorge A. Rios, Paul M. Ness, David J. Wright, Danielle Carrick, and George B. Schreiber. 2009. "The Effect of Previous Pregnancy and Transfusion on HLA Alloimmunization in Blood Donors: Implications for a Transfusion-Related Acute Lung Injury Risk Reduction Strategy." *Transfusion* 49(9):1825–35.

Urban, Marian, Antonij Slavcev, Tomas Gazdic, Peter Ivak, Josef Besik, and Ivan Netuka. 2016. "The Impact of Angiotensin II Type 1 Receptor Antibodies on Post-Heart Transplantation Outcome in Heart Mate II Bridged Recipients." *Interactive Cardiovascular And Thoracic Surgery* 22(3):292–97.

Urschel, S., P. M. Campbell, S. R. Meyer, I. M. Larsen, J. Nuebel, J. Birnbaum, H. Netz, K. Tinckam, T. Kauke, K. Derkatz, J. Y. Coe, J. L. Platt, and L. J. West. 2010. "Absence of Donor-Specific Anti-HLA Antibodies After ABO-Incompatible Heart Transplantation in Infancy: Altered Immunity or Age?" *American Journal of Transplantation* 10(1):149–56.

Urschel, Simon, Ingrid M. Larsen, Richard Kirk, Julie Flett, Michael Burch, Nadine Shaw, Julia Birnbaum, Heinrich Netz, Elfriede Pahl, Kathleen L. Matthews, Richard Chinnock, Joyce K. Johnston, Kim Derkatz, and Lori J. West. 2013. "ABO-Incompatible Heart Transplantation in Early Childhood: An International Multicenter Study of Clinical Experiences and Limits." *The Journal of Heart and Lung Transplantation* 32(3):285–92.

Urschel, Simon, Marie McCoy, Ryan S. Cantor, Devin A. Koehl, Warren A. Zuckerman, Anne I. Dipchand, Zdenka Reinhardt, Kirk R. Kanter, Jean A. Ballweg, Joshua Sparks, James K. Kirklin, and Waldemar F. Carlo. 2020. "A Current Era Analysis of ABO Incompatible Listing Practice and Impact on Outcomes in Young Children Requiring Heart Transplantation." *Journal of Heart and Lung Transplantation* 39(7):627–35.

de Weerd, Annelies E. and Michiel G. H. Betjes. 2018. “ABO-Incompatible Kidney Transplant Outcomes.” *Clinical Journal of the American Society of Nephrology* 13(8):1234 LP – 1243.
West, Lori J., Stacey M. Pollock-Barziv, Anne I. Dipchand, K. Jin Lee, Carl J. Cardella, Leland N. Benson, Ivan M. Rebeyka, and John G. Coles. 2001. “ABO-Incompatible Heart Transplantation in Infants.” *New England Journal of Medicine* 344(11):793–800.
Wright, Eric J., William P. Fiser, R. Erik Edens, Elizabeth A. Frazier, W. Robert Morrow, Michiaki Imamura, and Robert D. B. Jaquiss. 2007. “Cardiac Transplant Outcomes in Pediatric Patients with Pre-Formed Anti-Human Leukocyte Antigen Antibodies and/or Positive Retrospective Crossmatch.” *The Journal of Heart and Lung Transplantation* 26(11):1163–69.
Yang, B., M. Dieudé, K. Hamelin, M. Hénault-Rondeau, N. Patey, J. Turgeon, S. Lan, L. Pomerleau, M. Quesnel, J. Peng, J. Tremblay, Y. Shi, J. S. Chan, M. J. Hébert, and H. Cardinal. 2016. “Anti-LG3 Antibodies Aggravate Renal Ischemia–Reperfusion Injury and Long-Term Renal Allograft Dysfunction.” *American Journal of Transplantation* 16(12):3416–29.
Yang, J. A., Y. Naka, T. Ota, R. C. Neely, N. Uriel, P. C. Colombo, U. P. Jorde, D. M. Mancini, and H. Takayama. 2013. “Predictors of Prolonged Status 1A Wait Time Prior to Heart Transplant.” *The Journal of Heart and Lung Transplantation* 32(4):S143.
Yang, Jeremy, Cynthia Schall, Doug Smith, Lisa Kreuser, Mary Zamberlan, Karen King, and Robert Gajarski. 2009. “Mechanical Circulatory Support: HLA Sensitization in Pediatric Pre-Transplant Cardiac Patients Supported by Mechanical Assist Devices: The Utility of Luminex.” *Journal of Heart and Lung Transplantation* 28:123–29.
Yazer, Mark H. and Philip C. Spinella. 2018. “The Use of Low-Titer Group O Whole Blood for the Resuscitation of Civilian Trauma Patients in 2018.” *Transfusion* 58(11):2744–46.
Zhang, Qiuheng and Elaine F. Reed. 2016. “The Importance of Non-HLA Antibodies in Transplantation.” *Nature Reviews Nephrology* 12(June):484–95.