# Investigating the Effects of 2-Aminoadipic Acid on Beta-Cell and Human Islet Function

by

Matthew Hubert

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

Master of Science

Department of Pharmacology
University of Alberta

Abstract

2-aminoadipic acid (2-AAA) has been identified as an accurate long-term biomarker for the future development of diabetes. An investigation of over 2000 patients found that patients in the upper quartile of initial blood 2-AAA levels showed a 2-4x higher incidence of diabetes development over the next 12-13 years. Mouse models and static mouse and human islet cultures both suggest that 2-AAA increases insulin release at low glucose. Additionally, previous 2-AAA studies indicate 2-AAA's toxicity towards glial cells is the result of inhibited glutathione production. This toxicity inducing inhibition could also be taking place in β-cells of the pancreatic islet, as the affected transporters and enzymes are present and required in both cell types. Decreases in glutathione production in β-cells could lead to increased intracellular reactive oxygen species (ROS) levels. This could increase hydrogen peroxide ($H_2O_2$) mediated insulin secretion, accounting for the increased basal insulin secretion observed in mice. Additionally, bacterial salt stress pathways are linked with lysine breakdown pathways which can produce 2-AAA. 2-AAA production in the body may decrease if the population of such bacteria in the gut decreases.

To investigate if 2-AAA may be involved in the development of diabetes I performed the following experiments. The rat β-cell line INS-1 and human islets were treated with buffer containing 2-AAA along with $H_2O_2$ and/or 20mM glucose. Necrosis, $H_2O_2$ production, ROS and glutathione levels were measured in different experiments after treatment. Isolated human islets were cultured overnight, treated with 2.5mM glucose buffer containing 2-AAA for three hours, and then perifused at 2.5mM glucose and then 11.1mM glucose. Three-hours of static insulin secretion and one-hour of dynamic insulin secretion was then measured. Cystine and glutamate treatment was included in the buffer of many experiments to mirror previous 2-AAA studies in glial cells. Serum 2-AAA levels of germ-free mice were also measured and compared to wild-type mice.

In summary, treating INS-1 cells with 2-AAA shows significant increases in necrosis, $H_2O_2$ production, and ROS levels, as well as a decrease in total glutathione levels. Treating human islets with 2-AAA shows significant decreases in total glutathione levels with no significant change in $H_2O_2$ production. Our data also suggests that 2-AAA does not modify insulin secretion from human islets at low 2.5mM or high 11.1mM glucose. A significant negative correlation was found between 2-AAA induced three-hour low

glucose insulin secretion and the age of the male donors of those islets. No change in serum 2-AAA levels between germ-free and wild-type mice was observed.

Similar toxic effects of 2-AAA as previously seen in glial cells were observed in the INS-1 β-cell line. Only some of 2-AAA's toxic effects were observed in human islets. This may be due to the different cellular composition or oxidative resilience of human islets, or may be due to an insufficient treatment time of human islets due to limited culture viability of the explanted tissue. The lack of increased $H_2O_2$ production with 2-AAA treatment in human islets may account for a lack of insulin secretion increase with 2-AAA treatment. While germ-free mice did not have a lower 2-AAA level, it is still possible that modification of salt/lysine in diets may result in modifications of 2-AAA production from the gut microbiome. It is also possible that myeloperoxidase mediated 2-AAA production during inflammation may lead to modified blood 2-AAA levels.

This research provides a more complete understanding of 2-AAA's mechanism, suggesting a causative role for 2-AAA in the etiology of diabetes and provides support to utilize 2-AAA as a predictive biomarker to treat non-diabetic patients with high blood 2-AAA levels to prevent them from developing diabetes.

## Preface

This research was conducted at the University of Alberta under the supervision and guidance of Dr. Peter Light, Dr. Patrick MacDonald, and Dr. Jean Buteau. The research presented in this thesis received research ethics approval from the University of Alberta Research Ethics Board. De-identified human islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines (Pro00014814). Serum samples from germ-free and wild-type mice were a serum gift from Dr. Ramer-Tait, Food Science and Technology Department, Institute of Agriculture and Natural Resources, University of Nebraska. My role in this project includes cell culturing, all fluorescence experiments on tissue, human islet secretion and perifusion experiments, insulin and glutathione assays, data collection and analysis, creating and finalizing figures, and manuscript preparation. Human insulin assays were completed with the help of Dr. Scott Campbell, Katarina Ondrusova, and Janyne Johnson of the Light lab. Western blot analysis of islet and INS-1 insulin content were performed by Dr. Wentong Long of the Light lab. This thesis is original work written by myself, the author, with edits suggested by my supervisory committee. This research received funding support from the Canadian Institutes for Health Research and the Alberta Diabetes Foundation.

Acknowledgements

Thank you to my supervisor Dr. Peter Light who gave me the opportunity to explore research and allowed me the lenience to explore a multitude of other opportunities such as teaching during my graduate studies. I have learned a great deal from you and all the experiences you've both encouraged me to and let me pursue. Thank you to my supervisory committee Dr. Patrick MacDonald and Dr. Jean Buteau for answering all my questions, no matter how vague, and providing helpful feedback on my research. A ridiculously large thank you to my lab manager Amy Barr who guided my mind and hands through the day to day of laboratory life with abundant grace and appropriate sternness. Thank you to all my fellow lab members throughout the years – Dr. Scott Campbell, Katarina Ondrusova, Janyne Johnson, Dr. Wentong Long, Dr. Koenraad Philippaert, Dr. Mohammad Fatehi, Dr. Jyoti Singh and Dr. Dominic Golec – who offered guidance, support, criticism, and an ear to listen during my time in the lab.

Thank you to the manager of the 6$^{th}$ floor LKS core Dr. Kunimasa Suzuki for his practical help with using equipment of the floor. Thank you to my peers in pharmacology through the years, offering advice when asked, laughs when not asked, and help with all aspects of science and teaching always. Thank you to my all my students throughout the years, I genuinely would not have made it through this program without getting to take intermittent breaks from the science to spend time teaching and talking with you folks. Thank you to the Professors in the Department of Pharmacology – Dr. Frances Plane, Dr. Martin Davies, and Dr. James Hammond – that offered guidance and opportunities for me to explore being an educator within the department.

Finally, thank you to my friends and family that supported me outside the lab and classroom over the years. Your support and love, care and steadfastness, kindness and companionship, kept me going. I will leave you with the full verse from Philippians that the University of Alberta takes its motto *Quaecumque vera* from. This verse is a fine summation of what I have endeavored to explore and think about through this program. I will endeavor to keep on exploring and thinking about these things the rest of my life.

"Finally, brothers and sisters, whatever is true, whatever is noble, whatever is right, whatever is pure, whatever is lovely, whatever is admirable--if anything is excellent or praiseworthy--think about such things." (Philippians 4:8, NIV)

Table of Contents

List of Figures

Glossary of Terms & Abbreviations

2-Aminoadipic Acid: 2-AAA

Alpha-cell: α-cell

Beta-cell: β-cell

Bovine Serum Albumin: BSA

Cystine and Glutamate: Cys & Glu

Cystine/Glutamate Transporter: Xc-

Delta-cell: δ-cell

Dulbecco's Modified Eagle Medium: DMEM

Glutamate Transporter 1: GLT-1

Glutamate-Cysteine Ligase: GCL

Glutathione Disulphide: GSSG

Glutathione: GSH

Hemoglobin A1c: HbA1c

Hydrogen Peroxide: $H_2O_2$

Islets of Langerhans of the Pancreas: Islets

Myeloperoxidase: MPO

Oxidized form of two cysteine molecules: Cystine

Phosphate Buffered Saline: PBS

Reactive Oxygen Species: ROS

## 1. Introduction

Diabetes is a condition that affects up to 8.9% of men and 6.0% of women worldwide by the age of 70 (Gnatiuc et al., 2018). Diabetic patients lose their ability to regulate blood glucose levels. Drug based medical intervention and/or lifestyle changes such as dieting and exercise are initiated to properly regulate blood glucose. The disruption of blood glucose regulation is due to the combined loss of insulin action and production in the body (DeFronzo, 2004; Guthrie & Guthrie, 2004).

High blood glucose levels can lead to both vascular and organ damage resulting in disease states such as atherosclerosis, chronic kidney disease and several types of heart disease (de Ritter et al., 2020; Gnatiuc et al., 2018; Thomas, Cooper, & Zimmet, 2016). High blood glucose levels can also damage specific tissues such as peripheral nerves and the retina (Bourne et al., 2013). These diabetic complications lead to reduced quality of life, with the duration of diabetes correlating with reductions in quality of life measurements (Cannon, Handelsman, Heile, & Shannon, 2018; Jing et al., 2018).

Two major types of diabetes exist, type 1 and type 2. Type 1 diabetes involves autoimmune destruction of β-cells within the islets of Langerhans (islets) of the pancreas (Atkinson, Eisenbarth, & Michels, 2014; Guthrie & Guthrie, 2004). As β-cells are the sole source of insulin secretion in the body, this leads first to lowered insulin production in response to changes in glucose and finally to effectively little to no insulin production. Thus, type 1 diabetes ultimately requires exogenous insulin treatment to maintain blood glucose levels – though islet and pancreatic transplant options are available to treat certain cases (Atkinson et al., 2014; Gruessner & Sutherland, 1996; Shapiro et al., 2006).

Type 2 diabetes development involves a complex interplay of increasing insulin resistance in peripheral tissues such as the liver, skeletal muscles, and fat, along with increasing β-cell growth and/or insulin production from β-cells in the pancreas to meet the demand of glucose storage (DeFronzo, 2004; Guthrie & Guthrie, 2004). While the causal direction remains unclear, raised blood glucose levels and subsequent type 2 diabetes results when β-cells can no longer grow in insulin production capacity to meet the demands of the increasing glucose levels and insulin resistance (Malone & Hansen, 2019; Sun & Han, 2019). Treatment of type 2 diabetes involves lifestyle changes in exercise and diet, as well as drug treatment to decrease insulin resistance and increase β-cell health, growth, and insulin secretion. (Pfeiffer & Klein, 2014). My focus in this study is type 2 diabetes.

β-cell damage and insulin resistance are progressive in type 2 diabetes. However, type 2 diabetes progression can be halted and sometimes reversed with both lifestyle and medical therapies (Stevens et al., 2015). Due to this, a potentially helpful diagnostic tool that has not yet been fully developed for type 2 diabetes would be the discovery of one or several early biomarkers for the long-term prediction of diabetes development risk.

Some biomarkers for diabetes have been identified and developed into clinical tests. For example, the biomarker hemoglobin A1c (HbA1c) is currently used to determine the relative average glucose levels of an individual over the last 3 months (Nathan et al., 1993; Turner, 1998). This marker is useful for determining the risk of complications in diabetic patients and for determining the effectiveness of diabetes treatment in lowering average glucose levels. It is also used to determine those people who are not yet fully diabetic but do have moderate increases in blood glucose levels. These pre-diabetic patients are at risk of developing diabetes, and HbA1c is a biomarker for this risk.

However, β-cell dysfunction leading to reduced maximum insulin secretion and the development of insulin resistance precedes the rise in blood glucose and HbA1c levels. Initial insulin resistance, increased glucose intake from the diet, and reduced glucose breakdown due to a sedentary lifestyle leads to increased β-cell insulin production. As long as this increase in insulin production can be maintained and can sequester blood glucose, no effect on glucose levels will be observed (Cox et al., 2016; Linnemann, Baan, & Davis, 2014). During this stage increased insulin production leads to both more insulin resistance and increased β-cell metabolic stress followed by eventual dysfunction. This insulin resistance and β-cell dysfunction can all begin to occur well before type 2 diabetes and pre-diabetes, as these conditions are clinically determined by blood glucose and HbA1c measures. Once the changes in blood glucose have been observed, significant tissue dysfunction and damage has already occurred. If an earlier marker could be determined, therapies could be initiated at an earlier stage before tissue dysfunction and damage. This allows for the possibility of significantly delaying or even preventing β-cell damage and/or the development of insulin resistance.

An additional reason to investigate biomarkers is the possibility of finding a biomarker that is not only predictive of but also causative in the disease progression. If the high levels of a biomarker are causing damage by a specific mechanism, then lowering levels of the biomarker and/or preventing its effect

would likely lead to a lower incidence of the predicted outcome. If a biomarker did not appear to be the causative agent, metabolic and catabolic systems could be examined to determine if it is a by-product of a different system or compound that is causative, which may then be a new target for diabetes prevention for patients flagged to be at high risk of diabetes due to this biomarker.

### *1.1 2-aminoadipic acid as a biomarker for diabetes*

2-aminoadipic acid (2-AAA) has been identified as a long-term predictive biomarker for type 2 diabetes risk (T. J. Wang et al., 2013). 2-AAA is a metabolite of the amino acid lysine normally found in the human body at low levels (approximately 0.5-1μM) in the blood (Fig 1). Structurally, 2-AAA is similar to glutamate with the R-chain ending with a carboxylic acid group. Unlike glutamate, 2-AAA's R-chain contains four rather than three carbons.

Wang *et al.*, 2013, found 2-AAA to be a type 2 diabetes biomarker by examining two large cohorts of patients in their 50s: 1937 patients from the Framingham Heart Study and 162 patients from the Malmö Diet Cancer Study. These patients had blood samples taken at the beginning of the study and these samples were screened for a large number of potential biomarkers such as amino acids and their metabolites. The study was expected to find that one or more of the branched-chain amino acids would be a biomarker for the development of diabetes based on previously published studies (T. J. Wang et al., 2011). However, this study found that 2-AAA was consistently the most promising candidate for a long-term biomarker. Measuring the 2-AAA levels of blood samples from patients and placing them into quartiles based on their initial blood 2-AAA levels, it was found that patients in the fourth quartile of highest blood 2-AAA levels (approximately 2-8μM) had a 2-4 fold elevated risk of developing type 2 diabetes over the next 12-13 years. Additionally, it was found that the second-best biomarker in the study was lysine, of which 2-AAA is a major metabolite (Fig 1).

Since this initial study indicating the importance of 2-AAA as a potential long-term biomarker for the development of diabetes, subsequent studies have found corroborating evidence in different patient groups and cohorts.

One 2018 study examining 30 and 40-year-old patients found that 2-AAA levels were higher in obese and type 2 diabetic obese patients compared to lean controls (Libert, Nowacki, & Natowicz, 2018). While patient numbers were lower in this study, 20-25 per group, it was found that the metabolic health of

the obese patients did not significantly affect the observed 2-AAA levels of the patients. Unfortunately, this study did not examine non-obese type 2 diabetic patients, so it is unclear if 2-AAA levels are increased both in diabetic and obese patients, or simply just in obese patients, with the diabetes status being incidental.

A more recent study investigated a group of younger subjects aged from 10-14 (Lee et al., 2019). In these children/youth, blood levels of 2-AAA were found to correlate with several markers of metabolic syndrome, obesity, and pre-diabetes. These markers included increased fat mass, fat percentage, waist circumference, BMI, triglycerides, fasting glucose, fasting insulin, and the homeostasis model assessment of insulin resistance. More interestingly, all of these parameters except fasting glucose remained significantly predicted by 2-AAA, even when the parameters were measured 2 years after the initial 2-AAA measurement. As the title of the paper suggests, "2-AAA is a potential biomarker for childhood insulin resistance and metabolic disorder", and therefore, may be a biomarker that is useful for predicting type 2 diabetes not just in middle-aged or elderly patients as previously suggested, but in children and youth as well.

Another recent study examined two cohorts of patients in their 60s from the PREDIMED cohort (Razquin et al., 2019). This study works with a similar age of patients to the original 2-AAA biomarker paper and its results do "provide an independent prospective replication of the association of 2-AAA with future risk of type 2 diabetes", though this paper only studied diabetes incidence up to 1 year after follow-up. Interestingly they also found that lysine itself, while not a biomarker for diabetes risk, is associated with a diabetes dependent increase in cardiovascular disease risk.

These studies provide support for the initial biomarker conclusions of Wang et al., 2013. Now that it is clear 2-AAA is a biomarker for future diabetes risk further questions can be raised. Is 2-AAA a causative factor in the development of diabetes? Or is 2-AAA merely an associative factor that is increased in the blood due to a separate cause or mechanism that is the actual factor involved in the development of diabetes? To explore this, Wang et al., 2013 investigated the insulin secretion and sensitivity effects of 2-AAA treatment in-vivo in mice and ex-vivo on islets.

### *1.2 In-vivo effect of 2-AAA on insulin secretion in mice*

To examine the effects of 2-AAA on insulin secretion and sensitivity in mice, 2-AAA was added to the drinking water of the mice, leading to a 33% increase in blood 2-AAA levels (T. J. Wang et al., 2013).

Mice treated with 2-AAA for 5 weeks showed increased fasting plasma insulin and a corresponding decrease in fasting plasma glucose. The increased fasting plasma insulin may have indicated increased insulin secretion in response to insulin resistance. However, the corresponding decrease in fasting plasma glucose indicates insulin resistance is either not present or is at least highly limited. The increased low glucose insulin secretion is therefore likely a direct effect of treatment. These mice treated with 2-AAA also showed increased glucose tolerance in response to a glucose tolerance test, which also suggests that increased insulin resistance is unlikely. Other evidence in the paper suggested that in high-fat diet treated mice, 2-AAA treatment may have increased insulin sensitivity to a small degree.

Recently a 2018 paper has repeated several of these experiments over a longer time frame of 8 and 12 weeks, and found the same increased fasting plasma insulin and decreased fasting plasma glucose results (Xu et al., 2018).

*1.3 2-AAA levels in the mouse pancreas*

Interestingly when tissue samples from mice were taken and homogenized, nanomolar levels of 2-AAA per gram of tissue were higher in the pancreas compared to other tissues and these levels increased significantly with 2-AAA treatment (T. J. Wang et al., 2013). While 2-AAA levels in adipose tissue did increase significantly with 2-AAA treatment, these elevated 2-AAA levels were still lower than pancreatic 2-AAA levels of non-treated animals. Liver levels of 2-AAA did not correlate with increased blood 2-AAA levels, which may be due to a high level of lysine breakdown enzymes in liver tissue (Higashino, Fujioka, & Yamamura, 1971). While not conclusive this information does suggest that if blood 2-AAA levels are increased, good candidates for investigation would include the pancreas and to a lesser extent adipose tissue, rather than the liver.

*1.4 Ex-vivo effect of 2-AAA on the insulin secretion of islets*

To examine any potential effect of 2-AAA in the pancreas, mouse and human islets were isolated and 2-AAA was added to the buffer of islets undergoing a glucose-stimulated insulin secretion experiment (T. J. Wang et al., 2013). Both mouse and human islets treated with 2-AAA had increased insulin secretion at low 2.5mM glucose levels compared to buffer treated controls. No increase or decrease in insulin secretion was observed at high glucose. Though only an n of 3 for each type of islet was used,

this data does match the low glucose insulin secretion effect seen in the whole mice. Interestingly, a pre-treatment with 2-AAA greater than two hours was required to see this effect of increased insulin secretion.

The same 2018 paper which repeated 2-AAA treatment experiments in mice also completed low glucose insulin secretion experiments on mouse islets. While no high glucose insulin secretion data was presented in the paper, the low glucose data is consistent with the original biomarker paper – low glucose insulin secretion from mouse islets is increased with 2-AAA treatment (Xu et al., 2018).

### *1.5 2-AAA and basal hyperinsulinemia*

The data from T. J. Wang et al., 2013 and Xu et al., 2018 suggests that in mice, isolated mouse islets, and isolated human islets increased insulin secretion at low glucose seems to be an important factor in 2-AAA's effect. At first glance this seems counter-intuitive. Often the objective of small molecule diabetes intervention, such as sulphonylureas or GLP-1 mimetics, is the increase of insulin secretion (Pfeiffer & Klein, 2014). However, at low glucose additional insulin secretion is not required as insulin decreases glucose levels, which is unnecessary when blood glucose levels are already low. Increased low glucose insulin secretion is observed in pre-diabetic and diabetic patients (Del Prato, 1992; Shanik et al., 2008). Labeled basal hyperinsulinemia, this condition is part of the complex increase in both insulin secretion and insulin resistance leading up to and during the early stages of diabetes. It has even been shown that detection of basal hyperinsulinemia in patients without diabetes can itself be a biomarker for diabetes development (Dankner, Chetrit, Shanik, Raz, & Roth, 2012). If 2-AAA can cause basal hyperinsulinemia in humans, as it seems to be able to in mice, the basal hyperinsulinemia itself may be why, or part of why, 2-AAA is an effective biomarker for future development of diabetes.

Similar small molecule induced basal hyperinsulinemia caused by drugs does have a history of being detrimental to diabetes severity in the long run. It is known that the sulphonylurea class of anti-diabetic drugs increases insulin secretion regardless of glucose, as it bypasses the glucose sensing mechanism of β-cells (Pfeiffer & Klein, 2014). Constantly increased insulin secretion at low glucose can lead to β-cells having little to no slow down in metabolism and time to undergo repair. This can lead to a "secondary failure", where β-cells have increased insulin secretion in the short term but have a decreased maximum secretion in the long term due to overwork causing β-cell dysfunction and loss of β-cell mass (Matthews, Cull, Stratton, Holman, & Turner, 1998; H. Wang et al., 2017).

Considering this observed increase in low glucose insulin secretion in mice and from islets and the link between hyperinsulinemia and type 2 diabetes, the next question to answer is: what is a possible mechanism for 2-AAA's actions on insulin secretion from β-cells?

*1.6 2-AAA transporter and enzyme targets*

2-AAA has been known in the neuroscience literature as a glial toxin for many years. During the 1990s, several papers examined 2-AAA's effects on several transporters and enzymes. Recent literature has corroborated this evidence. The main transporters affected are glutamate transporter 1 (GLT-1) which transports glutamate into the cytosol and the antiporter cystine/glutamate transporter (Xc-) which transports glutamate out of the cytosol in exchange for cystine moving into the cytosol (Fig 2). The main enzyme affected by 2-AAA is the glutamate-cysteine ligase (GCL) which combines cysteine and glutamate to produce γ-glutamyl cysteine (Fig 2). This precursor for glutathione is needed for proper cell antioxidant production with GCL being the rate-limiting enzyme.

First, it was determined that 2-AAA uptake into cells was both sodium and chloride dependent (Tsai, Chang, Schwarcz, & Brookes, 1996). This, along with 2-AAA's similarity to glutamate in structure, implied that the transporters involved also cotransported these two ions. GLT-1 does co-transport sodium into the cell along with glutamate, and Xc- does co-transport chloride ions along with cystine (Pines et al., 1992). Furthermore, the chloride dependent uptake was not found to be also sodium dependent, which is also consistent with the ionic transport profile of the two transporters (Tsai et al., 1996).

Further studies also indicate that 2-AAA inhibits the uptake of cystine as measured by radiolabeled [$^{35}$S]cystine (Kato, Ishita, Sugawara, & Mawatari, 1993; Kato, Negishi, Mawatari, & Kuo, 1992). Significant but incomplete inhibition of glutamate influx through GLT-1 by 2-AAA into liposomes containing GLT-1 was also shown (Pines et al., 1992). Later 2-AAA was also shown to inhibit the inward flux of glutamate measured by inward sodium current, though again 2-AAA did not completely inhibit glutamate influx (Reichelt, Stabel-Burow, Pannicke, Weichert, & Heinemann, 1997).

Both glutamate and cysteine are components of glutathione, the major antioxidant for most cells, and 2-AAA limits the influx of these amino acids. 2-AAA inhibition of GLT-1 and Xc- was linked to reduced cellular glutathione levels, which then lead to glial cell toxicity (da Silva et al., 2017; Kato et al., 1993; Reichelt et al., 1997). Xc- inhibition by 2-AAA did result in lowered glutathione levels in retinal cells

and this reduction in glutathione was shown to be a reduction in newly produced glutathione with the radiolabeled [$^{35}$S]cystine incorporated (Kato et al., 1993). This reduction in glutathione was repeated in several other studies and was at least partially dependent on glutamate and cystine being present in the buffer (Reichelt et al., 1997). By examining inhibitors that block GLT-1 more completely than 2-AAA, it was found that 2-AAA's effect on lowering glutathione levels was more considerable than would be suggested by 2-AAA's partial block of GLT-1 alone (Reichelt et al., 1997). This indicates that an additional mechanism such as Xc- or GCL was likely involved. The GCL inhibitor buthionine sulfoximine was examined and showed a similar decrease in glutathione levels that was dependent on glutamate and cystine being present in the buffer (Reichelt et al., 1997).

More recently, it was found that 2-AAA's lowered glutamate uptake was not affected by antioxidants, implying that the reduction in GLT-1 activity is not a secondary feature of glutathione reduction (da Silva et al., 2017). 2-AAA also did not influence any mitochondrial kinetics measured in this study, implying the glutathione reduction is not a product of reduced energy reserves for anabolism or increased reactive oxygen species (ROS) production from the mitochondria. Importantly carboxylic acids similar to 2-AAA such as adipic acid or methylsuccinic acid did not show any glutathione reducing effects, further implying that the amino acid structure and inhibition of amino acid utilizing transporters and enzymes are the likely target of action. Reductions in glutathione would be expected to increase markers of oxidative damage from ROS, and levels of both protein and lipid oxidation were seen to increase in 2-AAA treated cerebral cortex cells of rats.

### *1.7 Detrimental effects of 2-AAA effects on glial cells*

The cumulative effect of 2-AAA's action on glial cells can be summarized in a model proposed by Reichelt W. et al 1997. GLT-1 inhibition by 2-AAA lowers intracellular glutamate levels and raises extracellular glutamate levels. Xc- function is compromised by low intracellular glutamate and high extracellular glutamate levels. Xc- is also directly inhibited by 2-AAA. GCL requires both glutamate and cysteine to function, which have now been depleted in the cell. GCL is also directly inhibited by 2-AAA. This cumulative effect lowers or prevents the production of additional cellular glutathione. This lowers the cell's antioxidant capacity, increasing the cell's susceptibility to ROS mediated damage and death. This susceptibility in glial cells is sufficient to lead to glial cell death in-vitro and in-vivo.

*1.8 Blocking GLT-1, Xc-, glutamate-cysteine ligase in β-cells and islets*

Interestingly, β-cell lines and isolated islets contain GLT-1, Xc-, and GCL. Additionally, disrupting this system disrupts glutathione production. The rat β-cell line INS-1 shows sodium independent Xc- transporter activity by measuring uptake of radiolabeled [$^{35}$S]cystine (Janjic & Wollheim, 1992). This uptake was reduced after long term exposure to the cell permeable antioxidant 2-mercaptoethanol, which artificially maintains a high cellular glutathione level and glutathione/glutathione disulphide (glutathione/GSSG) ratio. GLT-1 is also expressed in the INS-1 β-cell line and was also found to be expressed in the β-cells of the human islet (Di Cairano et al., 2011). In the same study an Xc- subunit was found to be expressed in human islets as well. Increased islet oxidative stress markers and increased apoptosis were observed when Xc- was blocked by excess extracellular glutamate and when GLT-1 was blocked by dihydrokainate. Interestingly this effect was not prevented by cotreating with antagonists of the AMPA and Kinate glutamate receptors. When the apoptosis was examined on a cell by cell basis it was only β-cells and not α-cells which showed increased apoptosis. The excitatory AMPA and Kinate receptors are found on α-cells not β-cells, and the lack of inhibitor effect and lack of α-cell apoptosis implies that any glutathione lowering effect in islets via GLT-1 or Xc- inhibition is likely happening in β-cells not α-cells. Additionally, a recent study treated a β-cell line with the same GCL inhibitor buthionine sulfoximine used in the glial cell studies and found a similar result of increased ROS production (Melo et al., 2017).

*1.9 Applying the glial cell model to β-cells*

The model proposed by Reichelt W. et al 1997 stated above to work in glial cells may be working similarly in β-cells of the islets. If β-cells contain the same transporters and enzymes, 2-AAA blocks these transporters and enzymes in a similar manner, and inhibition of these transporters and enzymes is detrimental to β-cell health, then the mechanism of action of 2-AAA observed in glial cells may also be occurring in β-cells (Fig 2).

Considering the previous studies indicating that 2-AAA is a biomarker for the development of future diabetes, the proposal of 2-AAA decreasing β-cell antioxidant capacity leading to an increase in ROS mediated β-cell dysfunction and death, seems plausible. ROS mediated β-cell dysfunction and death leading to increased diabetes incidence and severity is known in the literature, and general β-cell dysfunction and death are part of the etiology of both type 1 and type 2 diabetes (Atkinson et al., 2014;

DeFronzo, 2004; Guthrie & Guthrie, 2004). Therefore, of importance is precisely how might this 2-AAA mechanism of decreased glutathione link to the previous data concerning insulin secretion?

*1.10 Increased hydrogen peroxide levels can increase insulin secretion from β-cells*

Surprisingly, treating β-cell lines and islets with the reducing agent 2-mercaptoethanol, which may be expected to prevent ROS mediated β-cell dysfunction and increase insulin secretion, actually decreased high glucose insulin secretion (Janjic & Wollheim, 1992). In islets, even low glucose basal insulin secretion was decreased with 2-mercaptoethanol treatment (Janjic & Wollheim, 1992). Later research subsequently discovered that short term hydrogen peroxide ($H_2O_2$) treatment or increasing the $H_2O_2$ production of β-cells can lead to increased insulin secretion, even at low glucose (Leloup et al., 2009; Pi et al., 2007). This $H_2O_2$ mediated increase in insulin secretion can be prevented with pegylated catalase treatment to break down the $H_2O_2$ and N-acetylcysteine treatment to reduce the $H_2O_2$ level, similar to what was previously seen with 2-mercaptoethanol treatment (Pi et al., 2007). A recent study suggests that cytosolic $H_2O_2$ may mediate some insulin secretion by contributing to the inhibition of adenosine-triphosphate sensitive potassium channels, though was only shown to occur at high glucose levels (Plecitá-Hlavatá et al., 2020). Another recent study suggests that low glucose insulin secretion may instead be mediated through mitochondrial proton leak, though does not directly link this to increased $H_2O_2$ levels (Taddeo et al., 2020). The exact mechanism by which $H_2O_2$ mediates insulin secretion at low glucose has not been fully elucidated.

While long-term and extreme ROS exposure can lead to β-cell dysfunction and death, $H_2O_2$ signaling, particularly normal production from the mitochondria via cellular respiration, is important for intracellular β-cell signaling and insulin secretion.

*1.11 Blocking GLT-1 and Xc- increases insulin secretion from human islets*

Interestingly, blocking glutamate transport through GLT-1 into human islets using dihydrokainate can lead to increased basal insulin secretion (Di Cairano et al., 2011). Xc- block by introducing excess extracellular glutamate to lower cystine uptake also increased basal insulin secretion from human islets. Even more interestingly these effects were additive, implying that a substance capable of inhibiting both transporters at once may be able to achieve a combined effect greater than an inhibitor of only one of these transporters.

### *1.12 A model for 2-AAA action in β-cells and islets*

Combining the 2-AAA glutathione depletion model with this data we can start to see the potential connection between the biomarker data, the basal insulin secretion data, the β-cell transporter inhibition data, and the $H_2O_2$ mediated insulin secretion data in the literature.

2-AAA leads to a decrease in glutathione production via GLT-1, Xc-, and GCL. This decrease in glutathione production leads to a long-term reduction in cellular antioxidant capacity. This leads to a long-term increase in effective ROS production, as fewer ROS molecules that are produced are subsequently reduced. This increase in ROS, particularly $H_2O_2$, leads to increased basal insulin secretion. Constant basal insulin secretion, which may be observed as the diabetic condition basal hyperinsulinemia, may itself lead to further increases in $H_2O_2$ production due to increased protein production without rest periods. Long-term increased ROS production along with lower glutathione production results in increased β-cell susceptibility to dysfunction and death, leading to increased risk of diabetes development. In addition, the basal hyperinsulinemia itself may lead to long term increases in insulin resistance, also increasing the patient's risk for diabetes development.

Considering this potential model, the first steps in examining it will involve investigating 2-AAA's effect on β-cell and islet survival, ROS production, and total glutathione levels. The rat β-cell line INS-1 will be our model for β-cell health, as it is a long-used and well-characterized β-cell model. Normally, the next step would be investigations into the health of mouse islets; however, the University of Alberta with both its clinical and research core human islet isolation teams, is uniquely situated to begin islet work directly on human islets.

Along with investigations into human islet health with 2-AAA treatment, further work will be completed on human islet insulin secretion with 2-AAA treatment. While several papers have investigated mouse islets, our lab's n=3 contribution to the original biomarker paper constitutes the only measurement of insulin secretion from human islets treated with 2-AAA I have found in the literature (T. J. Wang et al., 2013; Xu et al., 2018). While a good first step, I wish to repeat, with human islets, the static low glucose insulin secretion experiments completed in previous papers on mouse islets. In addition, I would like to go further and examine human islet insulin secretion with 2-AAA treatment in a perifusion system. This will allow us to examine the first and second phases of insulin secretion separately, which may help to elucidate

the underexamined observation of increased glucose tolerance in mice without a matching increase in high glucose insulin secretion in islet studies (T. J. Wang et al., 2013; Xu et al., 2018).

### *1.13 Cell population of human islets and rodent islets*

When comparing human islets to mouse islets it is important to consider the differences that exist which may modify the effect of 2-AAA treatment. One of the most defined differences between mouse and human islets is the percentage of β-cells, ~75% and 55% respectively, and α-cells, 15% and 35% respectively (Brissova et al., 2005; Cabrera et al., 2006).

Human islets are also more heterogenous with α-cells, β-cells, and other cells being distributed throughout the islet (Brissova et al., 2005; Cabrera et al., 2006). Mouse islets instead have α-cells and other cells in a roughly 1 or 2 cell thick layer on the outside surface of the islet, with the rest of the center of the islet filled almost exclusively by β-cells (Brissova et al., 2005; Cabrera et al., 2006).

Also, while islets are known to have a lower expression of antioxidant enzymes compared to other tissues, it has been found that human islets are significantly more resilient to oxidative stress than rat or mouse islets and this resilience is consistent with a relatively higher level of antioxidant enzymes (Lenzen, Drinkgern, & Tiedge, 1996; Welsh et al., 1995).

This may mean that human islets are slightly more protected from β-cell specific 2-AAA toxicity than rodent islets or pure β-cell lines.

### *1.14 Potential for 2-AAA production by the gut microbiome*

While determination of 2-AAA's mechanism is the main thrust of this investigation, one preliminary experiment will be completed to examine the potential source of 2-AAA in the body. If 2-AAA is a causative agent in the development of diabetes, then prevention of its production and accumulation in the body becomes an important next step.

It has been found that in bacteria, lysine breakdown pathways and cellular pathways that prevent damage from salt stress have a linked regulation (Neshich, Kiyota, & Arruda, 2013). Bacteria containing lysine breakdown pathways were more resilient to salt stress than bacteria that did not contain these linked pathways. Furthermore, bacteria with the lysine breakdown pathways were most resilient to salt stress when co-treated with lysine to induce the upregulation of the lysine breakdown pathway. If gut bacteria of the intestinal microbiome share this upregulation link between the two pathways, it is possible

that a high salt diet, similar to that seen in modern western societies, may lead to a higher survival rate and prevalence of bacteria with lysine breakdown pathways. If the diet contained lysine, which is likely as lysine is an essential amino acid that can-not be produced by the human body, then we may expect to see a significant increase in 2-AAA production from the gut bacteria.

To investigate possible 2-AAA production from the gut bacteria I will examine serum samples previously retrieved from wild-type mice and germ-free mice. If 2-AAA is being produced from the microbiome, it is hypothesized that germ-free mice will have reduced serum 2-AAA levels, as no bacterial 2-AAA production will be present. If this hypothesis is confirmed it is likely due to this lysine breakdown pathway effect, though further work investigating this must be completed. If this hypothesis is not confirmed, further long-term high salt diet investigations can be completed and other hypotheses for the origin of 2-AAA production should be investigated.

### *1.15 Hypothesis and Aims*

#### *1.15.1 Main Hypothesis and Aims – The Mechanism of 2-AAA*

2-AAA treatment reduces glutathione production in β-cells leading to an increase in $H_2O_2$ production and subsequent increase in insulin secretion.

Aim 1: Investigate 2-AAA treatment in the rat INS-1 β-cell line to observe changes in β-cell glutathione levels and the resulting alterations in $H_2O_2$ production and cell death.

Aim 2: Investigate 2-AAA treatment in human islets to observe changes in insulin secretion at low and high glucose levels.

Aim 3: Investigate 2-AAA treatment in human islets to observe changes in human islet glutathione levels and $H_2O_2$ production.

#### *1.15.2 Secondary Hypothesis and Aims – The Source of 2-AAA*

Increased serum 2-AAA levels are linked to increased 2-AAA production by gut bacteria through salt stress pathways linked to lysine breakdown pathways.

Aim 1: Investigate the effect of a lack of microbiome of 2-AAA production in mice.

Aim 2: Propose future experiments to further investigate this hypothesis more fully.

Aim 3: Investigate the literature to determine other possible sources of increased 2-AAA production.

## 2. Methods

### *2.1 Materials*

Amplex Red (Cayman 10010469), Bovine Serum Albumin (BSA) Fatty Acid Free (Equitech-Bio BAH66), CellROX Deep Red (Invitrogen C10422), Cysteine (Sigma-Aldrich C4022), dimethyl sulfoxide (Fisher BP231), Dipotassium hydrogen phosphate (Fisher P288), ECL plus (Bio-Rad, 1705060), Glucose (Sigma-Aldrich G8270), Glutamic Acid (Sigma-Aldrich G1501), HEPES (Fisher BP310), Hydrogen peroxide (Sigma H1009), Instant skim milk powder (Nestle 011), L-2-aminoadipic acid (Fisher AAL1392403), Magnesium Chloride Hexahydrate (MilliporeSigma MX0045-1), 2-Mercaptoethanol (MP biomedicals 194705), 4-20% Mini-Protein TGX Stain-Free Protein gels (Bio-Rad 4568094), Nitrocellulose membrane 0.2 µM (Bio-Rad 162-0012), Peroxidase Type II From Horseradish (Sigma-Aldrich P8250), Potassium Chloride (Fisher P2173), Potassium phosphate monobasic (Sigma P5655), primary insulin antibody (Cell signaling, L6B10), protease inhibitor (Sigma P-8340), secondary anti-mouse antibody (Santa Cruz SC-2055), Sodium Bicarbonate (Sigma-Aldrich S5761), Sodium Chloride (Sigma-Aldrich 746398), Sodium deoxycholate (Thermo-Fisher 89905), 20% sodium dodecyl sulfate (Thermo-Fish BP1311 1), Sytox Green (Invitrogen S7020), Tris-HCL (USB 22638), Triton X-100 (VWR VW3929-2), Trypsin EDTA 0.25% (Sigma-Aldrich T4049),

*2.1.1 Media.* INS-1 cells cultured in RPMI complete media: RPMI 1640 Medium (Gibco 11875-119) - Including additional: 0.01M HEPES (Gibco 15630-080), 10% fetal bovine serum (Sigma F1051), 100U/0.1mg Penicillin-Streptomycin (Sigma-Aldrich P0781), 1mM sodium pyruvate (Gibco 11360-070), 2mM L-glutamine (Gibco 25030-081)

Islets cultured in DMEM complete media: DMEM – low glucose with 100mg/L L-glutamine, and sodium bicarbonate (Sigma D6046-500mL) – Including additional: 10% fetal bovine serum (Sigma F1051), 1% 100U/1mg Penicillin-Streptomycin (Sigma-Aldrich P0781)

*2.1.2 Phosphate buffered saline.* PBS. 3.7M NaCl, 0.27M KCl, 0.8M $Na_2HPO_4$, 0.2M $KH_2PO_4$, in deionized double distilled deionized $H_2O$ pH 7.4.

*2.1.3 Acid Ethanol.* 13.1M ethanol (Commercial Alcohols P016EA95), 4.1M Acetic Acid (Fisher 351270212), 0.18M HCl (Fisher A144-212).

*2.1.4* *Buffer.* Using double distilled deionized $H_2O$, prepare a solution including: 115mM NaCl, 5mM KCl, 24mM $NaHCO_3$, 2.5mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 0.1% BSA (1g/L). Add glucose to the required level: 2.5mM glucose, 11.1mM glucose, 20mM glucose. Add 2-AAA, Cystine, and glutamate to the required level: 30μM or 100μM 2-AAA, 25μM Cystine, 40μM Glutamate**.** Stocks of 2-AAA, cystine, and glutamate were made up initially in 0.1mM HCl. These stocks were frozen at -20°C and drawn from to add amino acids to the appropriate level before the buffer was pH'd to 7.4. For perifusion experiments new buffer was made each time. For each of the other experiments one batch of buffer was made, this was filter sterilized, and used for that all runs of that experiment, refrigerating between uses.

*2.1.5* *RIPA Buffer.* 1% Triton X, 150mM NaCl, 0.5% sodium deoxycholate, 0.1% % sodium dodecyl sulfate, 50mM Tris-HCl, 1x protease inhibitors, pH 8.0

*2.1.6* *Plates and dishes.* Fluorescence based INS-1 experiments in 96 well plates used black tissue-culture-treated plates (Greiner 655090). Fluorescence based INS-1 experiments in 35mm dishes used tissue-culture-treated dishes (Fisher FB012920). Fluorescence based human islet experiments in 96 well plates used black nontreated plates (Thermo 265301). Luminescence based INS-1 and Human islet experiments in 96 well plates used white tissue-culture-treated plates (Costar 3610). INS-1 cells cultured in tissue culture tissue-culture-treated 75$cm^2$ flask (Corning 353136). Human islets cultured in nontreated 35mm dish (Fisher FB0875711YZ)

*2.2* *INS-1 cells*

INS-1 cells grown in a 75$cm^2$ flask to ~100% confluency and split approximately weekly with one media change between splits. To begin splitting, cells were washed 3 times with 1x PBS. To begin liberating the cells ~1.5mL of 0.25% Trypsin EDTA solution was added and cells were placed in a 37°C 5% $CO_2$ incubator for 2 minutes. Once lifting is observed, the flask is tapped to fully liberate the cells. Trypsin is then neutralized with ~3.5mL of RPMI complete media. Cells are triturated to single cells and seeded either back into the growth flask or into the experimental vessel. Split ratio was approximately 1/10. Passage numbers were maintained between 50-70. Thank you to the McDonald Lab, Department of Pharmacology, University of Alberta, for providing our lab with this cell line. Cell line confirmed to produce insulin by western blot. (Fig 17)

*2.2.1 96 well plate Sytox Green necrosis – 2 hour buffer.* 200µL of RPMI complete media with 2-mercaptoethanol and 20µL of 5-6mL of INS-1 cells (~1.5mL of trypsin and ~3.5mL of media from the split) was placed into each well. The plate was then placed in incubator at 37°C and 5% CO2 for 48hr. Cells were allowed to grow to ~90-100% confluency in a 96 well black plate. Cell growth was checked well by well visually using a 10x magnification microscope. Any cell shearing during the experiment led to that well being excluded from the analysis. To begin the experiment media in each well was incrementally diluted and replaced with 2.5mM glucose buffer: Remove 170µL, Add 250µL of buffer, remove 250µL, add 250µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL. ~1/200 dilution, final volume 100µL. Gradual dilution to limit damage to cells, both physical perturbation and change in chemical environment. Buffer in experimental wells included 100µM 2-AAA. Buffer in cystine and glutamate wells contained 25µM cystine and 40µM glutamate. Dilute 1µL of 9.8M $H_2O_2$ in 1633µL of buffer (6mM final). Add 0.5µL of the 6mM $H_2O_2$ solution into each well (30µM final). Dilute 5mM Sytox Green 1µL in 199µL of 0µM 2-AAA buffer (25µM final). Add 0.8µL of the 25µM to each well (200nM final). Keep the plate out of the light from this point onward. Place in incubator at 37°C and 5% CO2 for 2hr. Read on the Synergy H4 plate reader. Set to read “Costar 96 clear bottom black side” at excitation 485/9, emission 528/9. Optic position bottom, gain 100, read speed normal, read height 8mm.

*2.2.2 96 well plate Sytox Green necrosis – 24 hour media.* 200µL of RPMI complete media without 2-mercaptoethanol + 10µL of 5-6mL of INS-1 cells (~1.5mL of trypsin and ~3.5mL of media from the split) was placed into each well. RPMI complete media in experimental wells included 30µM 2-AAA. The plate was then placed in incubator at 37°C and 5% CO2 for 24hr. Cells grown to ~90% confluency in a 96 well black plate. Cell growth was checked well by well visually using a 10x magnification microscope. Due to the length of treatment in media some cell growth to ~100% confluency was expected and seen. Any cell shearing during the experiment led to that well being excluded from the analysis. To begin the experiment, dilute 1µL of 9.8M $H_2O_2$ in 1633µL of buffer (6mM final). Add 1.05µL of the 6mM $H_2O_2$ solution into each well (30µM final). Place in incubator at 37°C and 5% CO2 for 24hr. Dilute 5mM Sytox Green 1µL in 199µL of 0µM 2-AAA buffer (25µM final). Add 1.68µL of the 25µM to each well (200nM final). Read on the Synergy H4 plate reader. Set to read “Costar 96 clear bottom black side” at excitation 485/9, emission 528/9. Optic position bottom, gain 100, read speed normal, read height 8mm.

2.2.3 *96 well plate Amplex Red hydrogen peroxide production rate.* 100µL of RPMI complete media with 2-mercaptoethanol + 20µL of 5-6mL of INS-1 cells (~1.5mL of trypsin and ~3.5mL of media from the split) was placed into each well. The plate was then placed in incubator at 37°C and 5% CO2 for 48hr. Cells were allowed to grow to ~90-100% confluency in a 96 well black plate. Cell growth was checked well by well visually using a 10x magnification microscope. Any cell shearing during the experiment led to that well being excluded from the analysis. To begin the experiment media in each well was incrementally diluted and replaced with 20mM glucose buffer: Remove 55µL, Add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 165µL. ~1/200 dilution, final concentration 100µL. Gradual dilution to limit damage to cells, both physical perturbation and change in chemical environment. Buffer in experimental wells included 30µM 2-AAA. Buffer in cystine and glutamate wells contained 25µM cystine and 40µM glutamate. Place in incubator at 37°C and 5% CO2 for 48hr. Create the Amplex Red/HRP Solution: Dissolve 0.005g of Amplex Red in 7.406mL dimethyl sulfoxide. Add 0.38mL of this solution to 1.56mL of a 26.25U/mL PBS solution of HRP. Keep lights off during all Amplex Red dilutions. Cover tubes and plates with tinfoil when possible. Add 5µL of the Amplex Red/HRP Solution to each well. Read on the Synergy H4 plate reader. Set temperature setpoint to 37°C. Set to read "Nuc 96 flat bottom generic" at excitation 565/9, emission 585/9. Optics position Top, gain 70, read speed normal, read height 8mm. Kinetic measurements for 2hr at 37°C, 2 minute intervals.

*2.2.4 Microscope CellROX Deep Red intracellular reactive oxygen species.* 1mL of RPMI complete media with 2-mercaptoethanol + 50µL of 5-6mL of INS-1 cells (~1.5mL of trypsin and ~3.5mL of media from the split) was placed into 35mm petri dishes. INS-1 cells were then cultured in RPMI complete media overnight at 37°C and 5% CO2. Cells were grown to <50% confluency to allow visualization of individual cells. The media is then replaced with 20mM glucose buffer: Remove all solution and replace with 1mL of buffer x3. Buffer in experimental dishes included 30µM 2-AAA. Buffer in cystine and glutamate dishes contained 25µM cystine and 40µM glutamate. Dilute 1µL of 9.8M $H_2O_2$ in 1633µL of buffer (6mM final). Add 5µL of the 6mM $H_2O_2$ solution into each well (30µM final). Incubate at 37°C and 5% $CO_2$ for 30 minutes and replace the buffer with fresh buffer: Remove all solution and replace with 1mL of buffer x3. Add 1µL of 5mM CellROX Deep Red to the dish (5µM final). Keep the dish out of the light from this point onward. Place in incubator at 37°C and 5% CO2 for 30min. Wash three times with PBS: remove all solution and replace

with 1mL PBS x3. CellROX Deep Red Fluorescence was then measured using an Olympus fluorescent microscope. 20x objective lens and ANDOR iXon Ultra camera were used for image capture. Two images were obtained per dish. Cells were found and focused on at lowest fluorescence setting before a snapshots were taken to reduce possible bleaching. Fluorescence cube used was a 49006-BX3; ET-CY5 620/60X, BS660, 700/75M, using the X-Cite 120 LED Boost fluorescence light system. Individual cell regions of interest were examined using the Count and Measure Tool in the Olympus cellSens Dimension software. Pictures were manually thresholded, segmented, and then "count and measured". Individual non-overlapping cells with no overlapping ROIs and with no cell portions possibly missing in the segmentation were then highlighted and the ROI fluorescence and area values were obtained cell by cell to calculate individual Fluorescence/Area for each cell.

*2.2.5 Glutathione/GSSG Glo Assay.* 100µL of RPMI complete media with 2-mercaptoethanol + 20µL of 5-6mL of INS-1 cells (~1.5mL of trypsin and ~3.5mL of media from the split) was placed into each well. The plate was then placed in incubator at 37°C and 5% CO2 for 48hr. Cells were allowed to grow to ~90-100% confluency in a 96 well white plate. Cell growth was checked well by well visually using a 10x magnification microscope. Any cell shearing during the experiment led to that well being excluded from the analysis. To begin the experiment media in each well was incrementally diluted and replaced with 20mM glucose buffer: Remove 55µL, Add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 200µL, add 200µL of buffer, remove 165µL. ~1/200 dilution. Gradual dilution to limit damage to cells, both physical perturbation and change in chemical environment. Buffer in experimental wells included 30µM 2-AAA. Buffer in cystine and glutamate wells contained 25µM cystine and 40µM glutamate. The plate was then cultured at 37°C and 5% $CO_2$ for 48hr. Glutathione and glutathione disulphide levels are then measured using the GSH/GSSG Glo assay (Promega V6611). Assay completed as per manufacturer instructions. Read on the Synergy H4 plate reader. Set to red "Corning 96 well flat bottom", at emission Luminescence Hole setting. Optics position Top, Gain 150, Integration Time 1s, read height 1mm.

*2.3 Human islets*

Human islets are picked twice by hand after being received from either the Alberta Diabetes Institute Islet Core or the clinical islet teams. After being picked, the human islets are cultured in DMEM

complete media at 37°C and 5% $CO_2$. Thank you to the Clinical Islet Team, University of Alberta Hospital and the Alberta Diabetes Institute Islet Core Team, Alberta Diabetes Institute, University of Alberta, for their work in receiving donations and preforming world class human pancreatic islet isolations. Thank you to the donors and their families, without their support and trust this work would not be possible.

*2.3.1 Perifusion and static insulin secretion.* After culturing islets overnight in DMEM complete media at 37°C and 5% $CO_2$. 25 islets per perifusion lane are placed into petri-dishes containing 2.5mM low glucose buffer. Buffer in experimental dishes included 30μM 2-AAA. Buffer in cystine and glutamate dishes contained 25μM cystine and 40μM glutamate. The six petri-dishes containing 25 islets in buffer each are placed in the 37°C and 5% $CO_2$ incubator for 3 hours. After the three hours islets are transferred to the perifusion system, 25 islets are placed into the heated reaction chamber of each lane. The system runs heated 2.5mM low glucose buffer containing the treatment compounds for that lane at a rate of 250μL/min over these islets. A 20 minute equilibration wait step with flow is then followed before any measurements are taken. During this wait step the remaining buffer from the petri-dishes is collected and frozen at -20°C to later determine the 3hr low glucose insulin secretion of these islets. After the wait step buffer is collected into sets of Eppendorf tubes for each lane over different intervals for ~58 minutes. First 20 minutes of 2.5mM glucose insulin secretion is measured. The buffer is then switched to a 11.1mM high glucose buffer, also containing any treatment compounds for that lane, and the first and second phases of high glucose insulin secretion are measured. All tubes containing buffer are frozen at -20°C to later determine the dynamic insulin secretion of these islets. After all buffer is collected, islets are removed from the reaction chambers and placed in Eppendorf tubes. Acid ethanol is added to these Eppendorf tubes containing islets to dissolve them. After 30 seconds of vortexing these Eppendorf tubes are also placed in a -20°C freezer for later determination of total insulin levels of these islets.

*2.3.2 Human insulin assay.* The Stellux Chemi Human Insulin ELISA kit by ALPCO was used to measured insulin levels from our samples as per manufacturer instructions. -20°C samples were thawed in the fridge overnight and kept on ice until the samples were fully thawed. Samples were then transferred to the plate after vortexing. Acid ethanol containing total insulin samples were diluted 600 times in the zero standard. Plates were measured on the APLCO Stellux Chemiluminescence Plate Reader (STX-4400) using the SoftMax Pro GxP v5.4.4 software.

*2.3.3 96 well plate Amplex Red hydrogen peroxide production rate.* After handpicking human islets and culturing them overnight in DMEM complete media at 37°C and 5% $CO_2$. Islets were moved into 35mm dishes with 1 mL of buffer containing 20mM glucose and cultured at 37°C and 5% $CO_2$ for 8 hours. Buffer in experimental dishes included 30μM 2-AAA. Buffer in cystine and glutamate dishes contained 25μM cystine and 40μM glutamate. Create the Amplex Red/HRP Solution: Dissolve 0.005g of Amplex Red in 7.406mL dimethyl sulfoxide. Add 0.38mL of this solution to 1.56mL of a 26.25U/mL PBS solution of HRP. Keep lights off during all Amplex Red dilutions. Cover tubes and plates with tinfoil when possible. Move one islet per 96 black plate well to wells containing 100μL of the appropriate buffer. Add 5μL of the Amplex Red/HRP Solution to each well. Read on the Synergy H4 plate reader, excitation 565/9, emission 585/9. Measurements for 2hr at 37°C. Read on the Synergy H4 plate reader. Set temperature setpoint to 37°C. Set to read "Nuc 96 flat bottom generic" at excitation 565/9, emission 585/9. Optics position Top, gain 70, read speed normal, read height 8mm. Measurements for 2hr at 37°C, intervals variable due to practical limitations, interval times consistent between experiments.

*2.3.4 Glutathione/GSSG Glo Assay.* After handpicking human islets and culturing them overnight in DMEM complete media at 37°C and 5% $CO_2$. Islets were moved into 35mm dishes with 1 mL of buffer containing 20mM glucose and cultured at 37°C and 5% $CO_2$ for 8 hours. Buffer in experimental dishes included 30μM 2-AAA. Buffer in cystine and glutamate dishes contained 25μM cystine and 40μM glutamate. After all treatments islets were washed with 4x ice cold PBS in Eppendorf tubes and frozen with liquid nitrogen. At the time of measurement ~2μL of ice cold PBS is added per islet (50μL of PBS minimum) and then the tubes are sonicated. Sonicator used is Qsonica's S-4000MPX. Eppendorf tubes were placed in a foam holder and floated in ice cold water in the sonicator. The portion of the Eppendorf tube which contains PBS must be fully submerged. To fully sonicate an amplitude of 30 for ~10min was used – approximately 3000J of energy total. Glutathione and glutathione disulphide levels are then measured using the GSH/GSSG Glo assay (Promega V6611). Assay completed as per manufacturer instructions. Read on the Synergy H4 plate reader. Set to red "Corning 96 well flat bottom", at emission Luminescence Hole setting. Optics position Top, Gain 150, Integration Time 1s, read height 1mm. The characterization experiment shown in figure 18 suggests that control buffer treatment provides similar total glutathione levels to media treatment alone, and a glutathione/GSSG ratio between media treatment and a highly oxidative

environment, which is expected. Data in figure 18 also matches previous data in β-cell lines and mouse islets indicating that with 2-mercaptoethanol treatment both total glutathione and glutathione/GSSG ratios rise dramatically (Janjic & Wollheim, 1992).

*2.4 Serum samples*

Frozen serum samples from germ-free and wild-type C57BL/6 mice were a gift from Dr. Ramer-Tait, Food Science and Technology Department, Institute of Agriculture and Natural Resources, University of Nebraska. Mice were 5 weeks old when serum samples were retrieved.

*2.5 Western blot protocol*

INS-1 cells and frozen islets were lysed in RIPA buffer. Samples were prepared with 2 parts of cell lysate and 1 part of loading buffer containing 2-mecaptoethanol. Loading samples were heated at 55°C for 5 minutes followed by a 2-minute cool down on ice. All samples were loaded to a TGX stain-free precast gradient (4-20%) gel and subjected to electrophoresis. Proteins were transferred from the gel to a 0.2μM nitrocellulose membrane and membrane was dried. Membrane was blocked at 4°C overnight in a 5% milk protein PBS solution and then washed 5x with PBS. The membrane was incubated with primary insulin antibody (1:1000) at 4°C overnight and then washed 5x with PBS. This was followed by secondary antibody incubation for 1 hour at room temperature (1:1000). Membrane was washed 8x with PBS and then developed using ECL plus and images were taken by Bio-Rad's ChemiDoc XRS+ system. Thank you to Wentong Long, Light Lab, Department of Pharmacology, University of Alberta, for completing this western blot experiment to confirm the insulin producing character of the INS-1 cell line (Fig 15).

*2.6 Statistical Analysis.* All statistical analyses were preformed as described in each figure of *3.5 Figures*. Significance set at $P<0.05$ for all experiments. Statistical analysis included: two-way unpaired t-test, one-way ANOVA with Tukey's Multiple Comparisons, two-way ANOVA with Sidak's Multiple comparisons, and two factor correlation.

## 3. Results

### *3.1 INS-1 rat β-cell line health*

*3.1.1 Necrosis of INS-1 cells treated with 2-AAA.* To investigate whether acute treatment of 2-AAA had any effect on the health of β-cells, the rat INS-1 β-cell line was treated with buffer containing 30μM $H_2O_2$ and 100μM 2-AAA. After 2 hours Sytox Green fluorescence was measured. Sytox Green fluoresces when bound to DNA and is non-cell permeable, therefore it is an indicator of cellular necrosis. Cellular necrosis of INS-1 cells increased to 119% of control levels with 2-AAA treatment (two-tailed t-test, $p<0.05$; Fig 3A). It was suggested in previous studies examining 2-AAA treatment in glial cells that cystine and glutamate may be required to observe the effect of 2-AAA inhibition (Reichelt et al., 1997). With cystine and glutamate added to the buffer cellular necrosis increased to 117% of control levels with 2-AAA treatment; however, this increase was not significant (two-tailed t-test, $p=0.053$; Fig 3B). INS-1 cells were also treated with RPMI complete media containing 30μM $H_2O_2$ and 30μM 2-AAA for 24 hours. Necrosis increased to 105% of control levels with 2-AAA treatment, though this was also a non-significant increase (two-tailed t-test, $p=0.057$; Fig 4).

*3.1.2 Rate of $H_2O_2$ production from INS-1 cells treated with 2-AAA.* Observing that co-treatment of 100μM 2-AAA with $H_2O_2$ increased acute INS-1 cellular necrosis, I expected that the lower level of 30μM 2-AAA co-treated with the more limited metabolic stress of 20mM glucose should show an increase in $H_2O_2$ production. 30μM 2-AAA was the same concentration used in the original biomarker paper for islet treatment (T. J. Wang et al., 2013). A longer treatment time of 24hr was also used so the more limited metabolic stressor of 20mM glucose could have time to raise $H_2O_2$ production. To measure $H_2O_2$ production Amplex Red was included in the buffer to measure excess $H_2O_2$ produced and released by diffusion from the cells. Considering the necrosis experiments, I also expected that 2-AAA treatment without cystine and glutamate added into the buffer would result in higher level of $H_2O_2$ production, though we may still observe some increase in $H_2O_2$ production in cells treated with cystine and glutamate.

INS-1 cells treated with buffer containing 20mM glucose and 30μM 2-AAA for 24hr showed a 127% higher rate of $H_2O_2$ production compared to control (two-tailed t-test, $p<0.0001$; Fig 5A). This was without the addition of cystine and glutamate in the buffer. With the addition of cystine and glutamate,

2-AAA treatment showed a 110% higher rate of $H_2O_2$ production compared to control (two-tailed t-test, $p<0.05$; Fig 5B).

*3.1.3 ROS levels of INS-1 cells treated with cystine, glutamate, and 2-AAA.* Finding that there seems to potentially be a protective effect of the glutamate and cystine added to the buffer, I attempted to overcome this potential effect with 2-AAA treatment under a heavier chronic stress.

INS-1 cells were grown to <50% confluency and treated with 20mM glucose buffer containing 30μM 2-AAA for 48hr. After this treatment, cells were treated with $H_2O_2$ for 30min, washed with buffer and then loaded with the CellROX Deep Red dye. This cell permeable dye fluoresces when oxidized by ROS species. Measurement of individual cell fluorescence/area showed that cystine and glutamate treated cells showed a significantly lower ROS level than control (2351$\mu m^{-2}$ vs 3422$\mu m^{-2}$; One-way ANOVA with Tukey's Multiple Comparisons, $p<0.0001$; Fig 6). 30μM 2-AAA treatment did show significantly increased ROS levels compared to cystine and glutamate alone (2910$\mu m^{-2}$ vs 2351$\mu m^{-2}$; One-way ANOVA with Tukey's Multiple Comparisons, $p<0.0001$; Fig 6). 30μM 2-AAA treatment did not completely overcome the apparent protective effect of cystine and glutamate and reach control ROS levels (2910$\mu m^{-2}$ vs 3422$\mu m^{-2}$; One-way ANOVA with Tukey's Multiple Comparisons, $p<0.0001$; Fig 6).

*3.1.4 Glutathione levels and ratio of INS-1 cells treated with 2-AAA.* Finally, I investigated both the glutathione/GSSG ratio and the total glutathione levels in INS-1 cells. INS-1 cells were treated with a buffer containing 20mM glucose and 30μM 2-AAA for 48hr before glutathione levels were measured using a luminescence kit. No significant change in the glutathione/GSSG ratio was observed with 2-AAA treatment in either the buffer alone or cystine and glutamate added groups (two-tailed t-test, $p>0.1$; Fig 7A,B). With 2-AAA treatment total glutathione levels lowered to 84% of control levels in the buffer alone group and to 86% of control levels in the cystine and glutamate added buffer group (two-tailed t-test, $p<0.05$; Fig 7C,D).

*3.2 Human islet insulin secretion*

*3.2.1 Dynamic insulin secretion during human islet perifusion.* Human islets were isolated and pre-treated with 2.5mM low glucose buffer for 3 hours before undergoing a perifusion treatment over approximately 100 minutes to measure insulin secretion over time. Islets were treated with 30μM 2-AAA or 30μM 2-AAA along with cystine and glutamate over the whole period. No significant changes in insulin secretion were observed at any point measured with either treatment (two-way ANOVA with Sidak's multiple

comparisons, p>0.1; Fig 8A,C). When area under the curve was determined for the low glucose and high glucose portions of the curves, no significant change was observed (two-way ANOVA with Sidak's multiple comparisons, p>0.1; Fig 8B,D). No significant change was observed when the first and second phases of insulin secretion, the first ten minutes and the rest of the high glucose secretion period respectively, had their areas under the curve examined separately (two-way ANOVA with Sidak's multiple comparisons, p>0.1; Fig 8B,D).

Additional analyses were preformed to confirm that the unexpected lack of a significant change in insulin secretion is not a false negative. The same data was analyzed as a percent of the maximum control insulin secretion for each islet set. Though error bars do appear to be superficially reduced, no significant change with 2-AAA treatment was observed (two-way ANOVA with Sidak's multiple comparisons, p>0.1; Fig 9A,B). Data was also analyzed as absolute insulin secretion values not compared to the total insulin levels of the respective islets. Again, no significant change in insulin secretion with 2-AAA treatment was observed (two-way ANOVA with Sidak's multiple comparisons, p>0.1; Fig 9C,D) and no qualitative change in curve shape was observed (Fig 9C,D). To confirm that total insulin levels were not considerably different between different islet isolations, total insulin levels were assayed additional times for accuracy and compared. No change in total insulin levels with 2-AAA treatment was observed, either with or without cystine and glutamate in the treatment buffer (two-tailed t-test, p>0.1; Fig 10).

*3.2.2 Static insulin secretion before human islet perifusion.* To compare with the static insulin secretion data found in other studies on mouse islets, the 3hr low glucose pre-treatment was examined as a static insulin secretion experiment (T. J. Wang et al., 2013; Xu et al., 2018). Low glucose (2.5mM) insulin secretion does not appear to increase with 2-AAA treatment in the absence of cystine and glutamate, being only 97% of the control values (two-tailed t-test, p>0.1; Fig 11A,B). While 2-AAA treatment with cystine and glutamate treatment does increase insulin secretion to 164% of control values, this increase is not significant (two-tailed t-test, p>0.1; Fig 11C,D).

*3.2.3 Insulin secretion donor demographic correlations.* Contrary to what has been observed in mouse islets no significant change in insulin secretion was seen. To investigate potential reasons for this difference I used the available demographic data to investigate any possible correlations with our insulin data. The BMI, age, and sex demographic information were available. This demographic information was

correlated with the 3hr low glucose insulin secretion from islets treated with 2-AAA over control secretion, expressed as a percent of control (Fig 12A-C,13A-C). Similarly, demographic information was correlated with total insulin levels of 2-AAA treated islets compared to control islets, expressed as a percent of control (Fig 12D-F,13D-F). Data was analyzed both with and without sexes separated.

The only significant correlation found is a negative correlation of age with low glucose insulin secretion, in the islets from male donors treated with a cystine and glutamate buffer (two factor correlation, $r^2$=0.9, p<0.05; Fig 13A). It appears that with increasing age the islets of these male donors show a subsequent decrease in the ability of 2-AAA to increase insulin secretion at low glucose.

*3.3 Human islet health*

*3.3.1 Rate of $H_2O_2$ production from human islets treated with 2-AAA.* Isolated human islets were treated with 8hrs of 20mM glucose buffer to induce a high metabolic stress environment within islets and increase $H_2O_2$ production. Along with this treatment, 30μM 2-AAA was added to experimental groups. No significant change in $H_2O_2$ production rate was observed with 2-AAA treatment when measured by Amplex Red, both with or without cystine and glutamate added (two-way t-test, p>0.1; Fig 14).

*3.3.2 Glutathione levels and ratio of human islets treated with 2-AAA.* The glutathione/GSSG ratios and total glutathione levels were also measured per islet in islets receiving the same 8hr 20mM glucose treatment. As observed in the INS-1 cells, no significant change in the glutathione/GSSG ratio was seen. Total glutathione levels of 2-AAA treated islets were 90% of control islet levels when the buffer did not contain cystine and glutamate (two-way t-test, p<0.01; Fig 15C). With cystine and glutamate present no significant change in total islet glutathione levels were observed with 2-AAA treatment, perhaps reflecting the apparent protective effect observed in the INS-1 cells (two-way t-test, p=0.055; Fig 15D).

*3.4 Serum 2-AAA levels of germ-free mice*

Serum samples retrieved from 5-week-old wild-type mice and germ-free mice. Serum 2-AAA levels were determined and no significant difference in 2-AAA levels was observed between the two groups (two-way t-test, p>0.1; Fig 16).

*3.5 Figures*

**Figure 1**

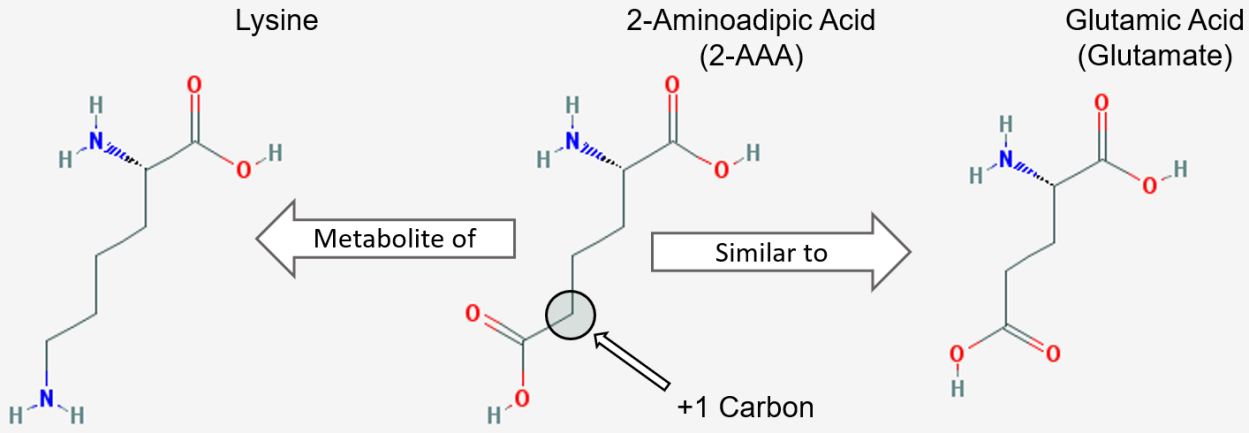


**Figure 1. Structure of 2-aminoadipic acid.** 2-aminoadipic acid in a metabolite of lysine. It is similar in structure to glutamate, with its R-group being only one carbon longer. 2D chemical structures obtained from "pubchem.ncbi.nlm.nih.gov". PubChem Identifiers: Lysine, CID 5962; 2-Aminoadipic Acid, CID 469; Glutamic Acid, CID 33032. (S. Kim et al., 2019) URL:

https://pubchem.ncbi.nlm.nih.gov/compound/5962#section=2D-Structure

https://pubchem.ncbi.nlm.nih.gov/compound/469#section=2D-Structure

https://pubchem.ncbi.nlm.nih.gov/compound/33032#section=2D-Structure

**Figure 2**

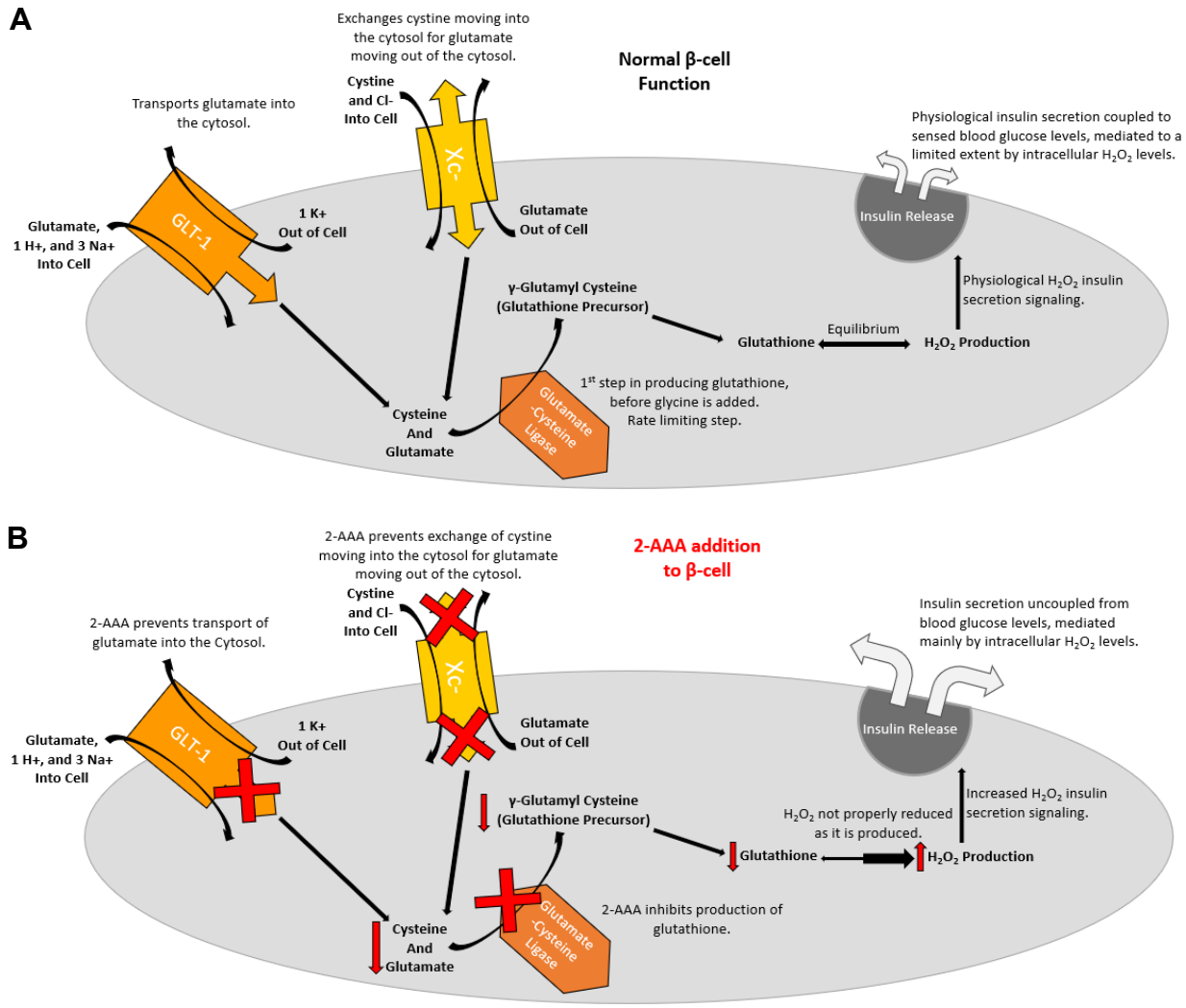


**Figure 2. β-cell model of 2-AAA toxicity.** A) Glutamate enters a β-cell via GLT-1 and some of this glutamate is exchanged for cystine via Xc-. Cysteine and glutamate form the glutathione precursor γ-glutamyl cysteine via GCL. Glutathione produced, reduces $H_2O_2$ produced by metabolic processes in the cell. Insulin secretion is coupled to sensed blood glucose levels, with limited $H_2O_2$ signaling. B) 2-AAA inhibits GLT-1 lowering intracellular glutamate. 2-AAA inhibits Xc- lowering intracellular cysteine levels. GCL requires glutamate and cysteine to produce γ-glutamyl cysteine. GCL is also directly inhibited by 2-AAA. Decreased glutathione levels do not properly reduce $H_2O_2$ produced by metabolic processes. Increased $H_2O_2$ signals for increased insulin secretion, uncoupling insulin secretion from blood glucose levels. 2-AAA, 2-Aminoadipic Acid GLT-1, Glutamate Transporter 1; Xc-, Cystine/Glutamate Transporter; GCL, Glutamate-Cysteine Ligase $H_2O_2$, Hydrogen Peroxide. Adapted from model by Reichelt et al., 1997.

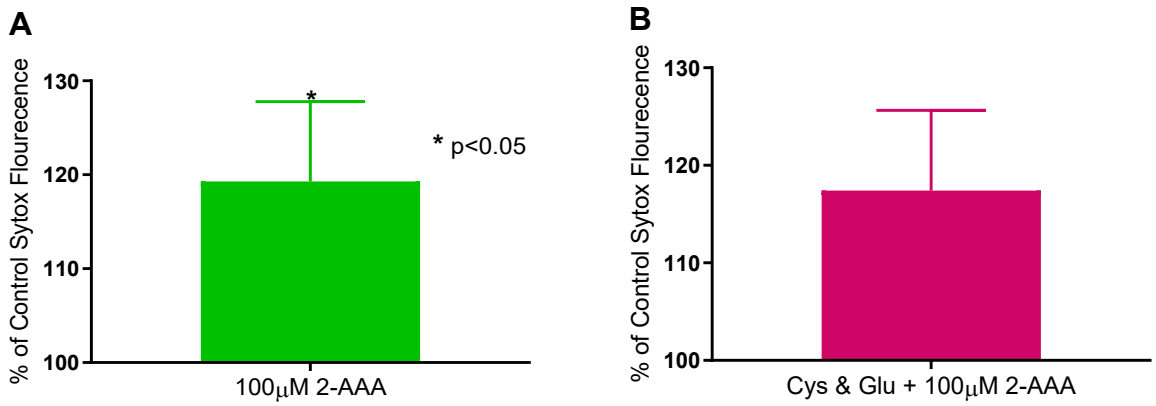


**Figure 3. Necrosis of INS-1 cells treated with $H_2O_2$ in buffer.** A) 2-hour 100μM 2-AAA treatment significantly increased necrosis of INS-1 cells concurrently treated with 30μM $H_2O_2$. B) 2-hour 100μM 2-AAA treatment in the presence of cystine and glutamate does not significantly increase necrosis of INS-1 cells concurrently treated with 30μM $H_2O_2$. INS-1 cells cultured in RPMI complete media overnight. The media is then replaced with buffer containing 30μM $H_2O_2$. The buffer of experimental groups also contained 100μM 2-AAA. A) Buffer contained no added cystine or glutamate. B) Buffer also contained 25μM cystine and 40μM glutamate. After 2 hours of treatment, Sytox Green fluorescence was measured on a plate reader. Increased fluorescence indicates increased cellular necrosis. All graphs are mean ± SEM. n=8. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate.

**Figure 4**

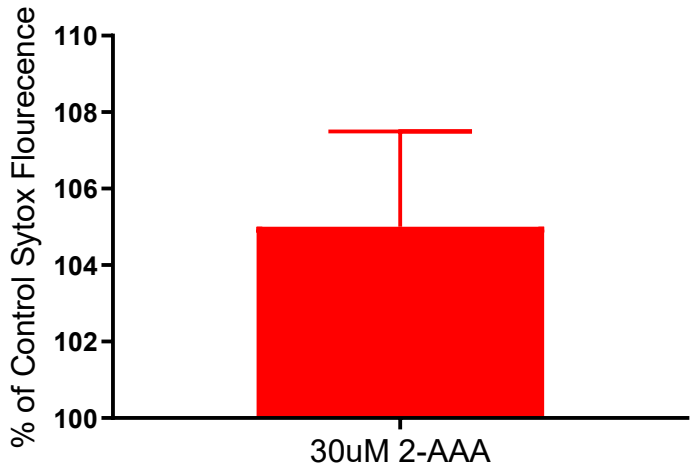


**Figure 4. Necrosis of INS-1 cells treated with $H_2O_2$ in media.** 24-hour 30μM 2-AAA treatment does not significantly increase necrosis of INS-1 cells concurrently treated with 30μM $H_2O_2$ in RPMI complete media. INS-1 cells cultured in RPMI complete media overnight. The media is then replaced with media containing 30μM $H_2O_2$. The media of experimental groups also contained 30μM 2-AAA. After 24 hours of treatment Sytox Green fluorescence was measured on a plate reader. Increased fluorescence indicates increased cellular necrosis. Graph presented as mean ± SEM. n=13. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide.

**Figure 5**

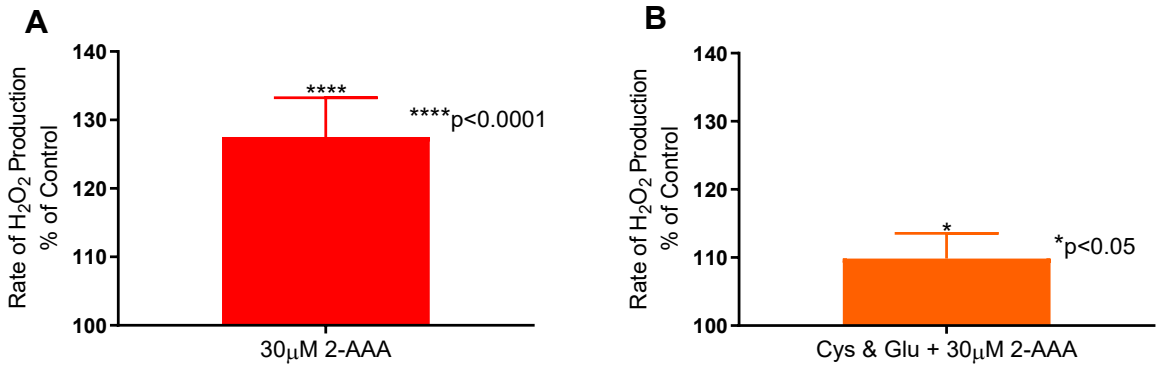


**Figure 5. Rate of $H_2O_2$ production from INS-1 cells treated with high glucose in buffer.** INS-1 cells in buffer both A) without and B) with cystine and glutamate showed significantly increased $H_2O_2$ production with 30μM 2-AAA treatment compared to control INS-1 cells. INS-1 cells cultured in RPMI complete media overnight. The media is then replaced with buffer containing 20mM glucose. The buffer of experimental groups also contained 100μM 2-AAA. A) Buffer contained no added cystine or glutamate. B) Buffer also contained 25μM cystine and 40μM glutamate. Cells were then cultured at 37°C and 5% $CO_2$ for 24hr and then 100 minutes of Amplex Red fluorescence over time was then measured on a plate reader. Increased fluorescence indicates increased ROS production and release from the cells. Percent changes in fluorescence over time were compared to control and expressed as a percentage, this indicates the rate of $H_2O_2$ production compared to control. All graphs are mean ± SEM. n=14. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate.

**Figure 6**

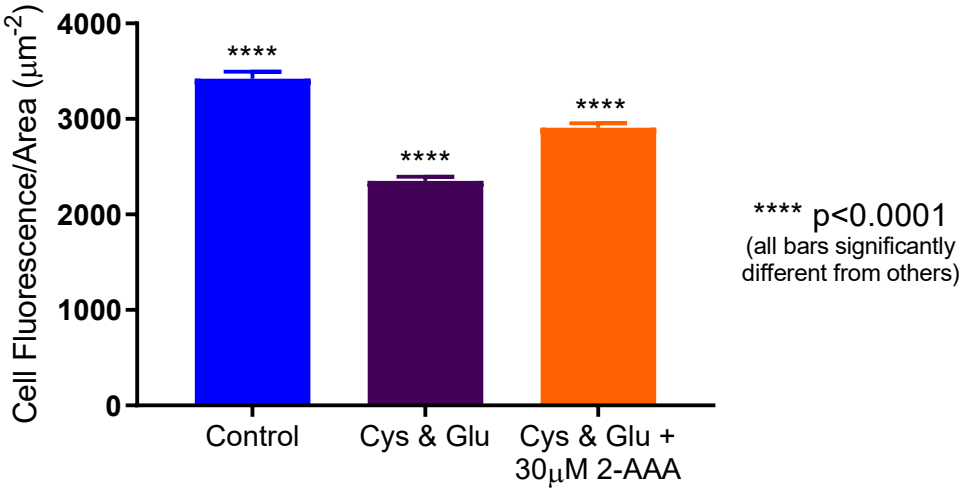


**Figure 6. ROS levels of INS-1 cells treated with high glucose and $H_2O_2$.** INS-1 cells treated in 20mM glucose and 30μM $H_2O_2$ buffer without cystine and glutamate, with cystine and glutamate, and with cystine, glutamate, and 30μM 2-AAA, all showed significantly different ROS levels from each other. INS-1 cells cultured in RPMI complete media overnight. The media is then replaced with buffer containing 20mM glucose. The buffer of the indicated group also contained 30μM 2-AAA. Control buffer contained no added cystine or glutamate. Both experimental buffers also contained 25μM cystine and 40μM glutamate. Cells were cultured at 37°C and 5% $CO_2$ for 48hr. 30μM $H_2O_2$ was added to the buffer for 30 minutes and then the buffer was replaced with fresh buffer and cells were loaded for 30min with CellROX Deep Red. CellROX Deep Red Fluorescence was then measured on a fluorescent microscope at 10x. Individual cell regions of interest were thresholded and cell fluorescence and area was measured. Increased fluorescence indicates increased cellular ROS levels. Graph presented as mean ± SEM. Control n=748 cells, Cys & Glu n=1216 cells, Cys & Glu + 30μM 2-AAA n=1202 cells. Cell images acquired from 13 cell passages; all passages received all treatments. Significance set at $P<0.05$, One-Way ANOVA with Tukey's Multiple Comparisons. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate.

**Figure 7**

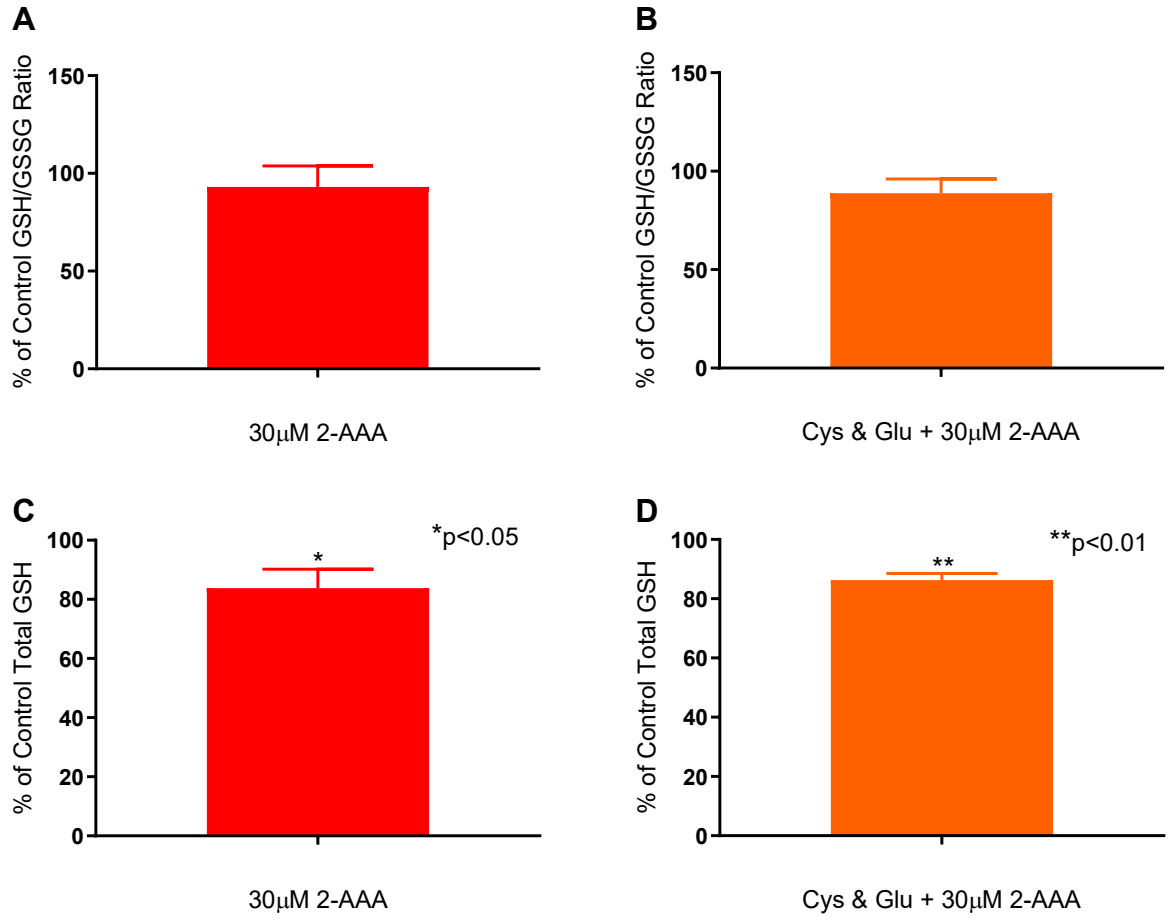


**Figure 7. Glutathione/GSSG and total glutathione levels of INS-1 cells treated with high glucose.** A,B) No significant change in glutathione/GSSG ratio. INS-1 cells in buffer both C) without and D) with cystine and glutamate showed significantly decreased total glutathione levels with 30μM 2-AAA treatment compared to INS-1 cells treated only with 20mM glucose. INS-1 cells cultured in RPMI complete media overnight. The media is then replaced with buffer containing 20mM glucose. The buffer of the experimental group also contained 30μM 2-AAA. A,C) Buffer contained no added cystine or glutamate. B,D) Buffer also contained 25μM cystine and 40μM glutamate. Cells were then cultured at 37°C and 5% $CO_2$ for 48hr. Glutathione and GSSG levels are then measured using a GSH/GSSG Glo assay. All graphs are mean ± SEM. n=4. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate; GSH, Glutathione; GSSG, Glutathione Disulphide.

**Figure 8**

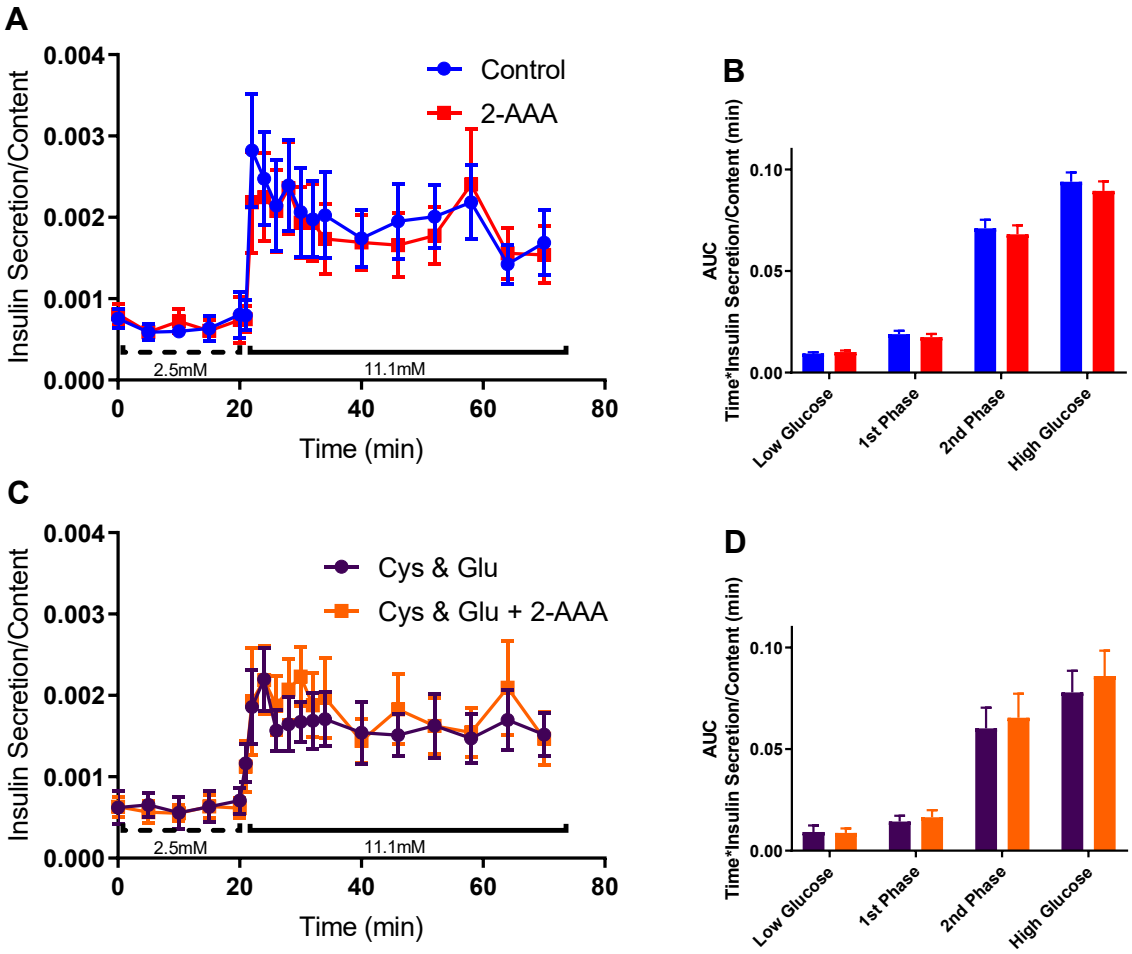


**Figure 8. Perifusion of human islets to determine dynamic insulin secretion.** A-D) No significant difference observed. Islets are picked and then cultured overnight in DMEM complete media at 37°C and 5% $CO_2$. Islets are then separated into dishes containing 2.5mM glucose buffer. The buffer of the experimental group also contained 30μM 2-AAA. A,B) Buffer contained no added cystine or glutamate. C,D) Buffer also contained 25μM cystine and 40μM glutamate. These islets are then cultured at 37°C and 5% $CO_2$ for 3 hours. 25 islets per lane are then placed into each perifusion reaction vessel and then perifused at a flow rate of 250μL/min and allowed to equilibrate for 20 minutes before measurements. The buffer concentration of glucose is increased from 2.5mM to 11.1mM at 20 minutes. All graphs are mean ± SEM. A,B) n=12, C,D) n=9. Significance set at $P<0.05$, Statistics: two-way ANOVA with Sidak's Multiple comparisons. 2-AAA, 2-Aminoadipic Acid; Cys & Glu, Cystine and Glutamate; 1st phase, first 10 minutes of high glucose insulin secretion; 2nd phase, remaining high glucose insulin secretion period.

**Figure 9**

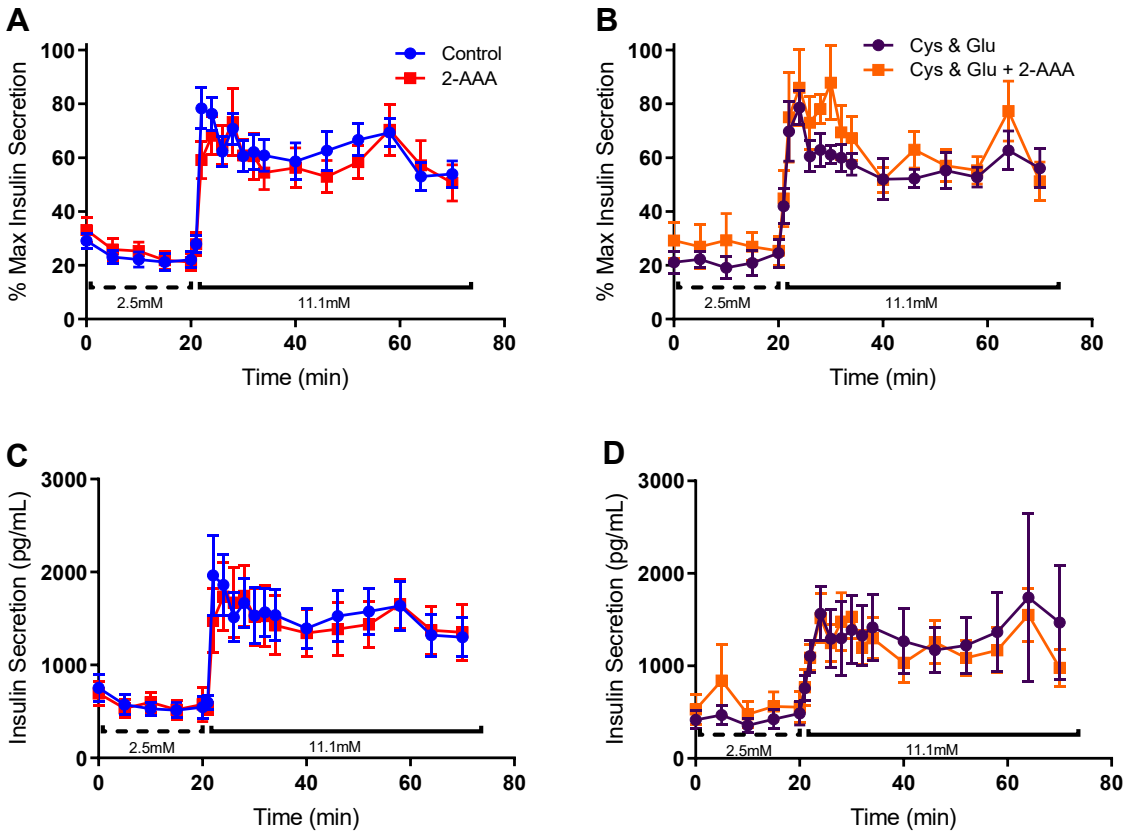


**Figure 9. Modified analysis of human islet perifusion to determine dynamic insulin secretion.** A-D) No significant difference observed. Instead of plotting insulin secretion over total insulin levels as was done in Figure 6, A,B) insulin secretion over total insulin levels as a percentage of the highest control insulin secretion level and (C,D) insulin secretion alone were plotted. This was done to observe if any significant changes may have been overlooked by presenting the data as insulin secretion over total insulin levels. All graphs are mean ± SEM. A,B) n=12, C,D) n=9. Significance set at $P<0.05$, Statistics: two-way ANOVA with Sidak's Multiple comparisons. 2-AAA, 2-Aminoadipic Acid; Cys & Glu, Cystine and Glutamate.

**Figure 10**

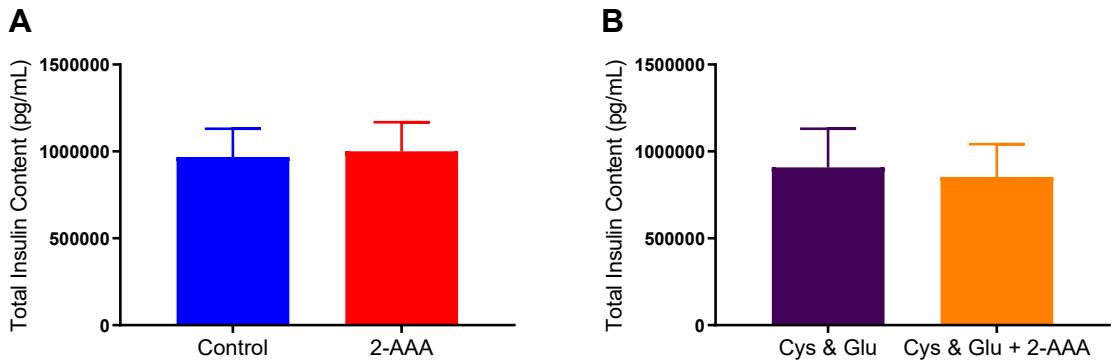


**Figure 10. Total insulin levels.** A,B) No significant difference observed. Following perifusions, islets were collected into Eppendorf tubes and dissolved in acid ethanol. After vortexing for 30 seconds these Eppendorf tubes were placed in -20°C freezer until they were analyzed. All total insulin content samples were measured at least twice on separate insulin assay kits. This was to ensure accurate total insulin values, as all other insulin values are normally displayed as a percent of total insulin content. All graphs are mean ± SEM. A,B) n=12, C,D) n=9. Significance set at $P<0.05$, Statistics: unpaired t-test. 2-AAA, 2-Aminoadipic Acid; Cys & Glu, Cystine and Glutamate.

**Figure 11**

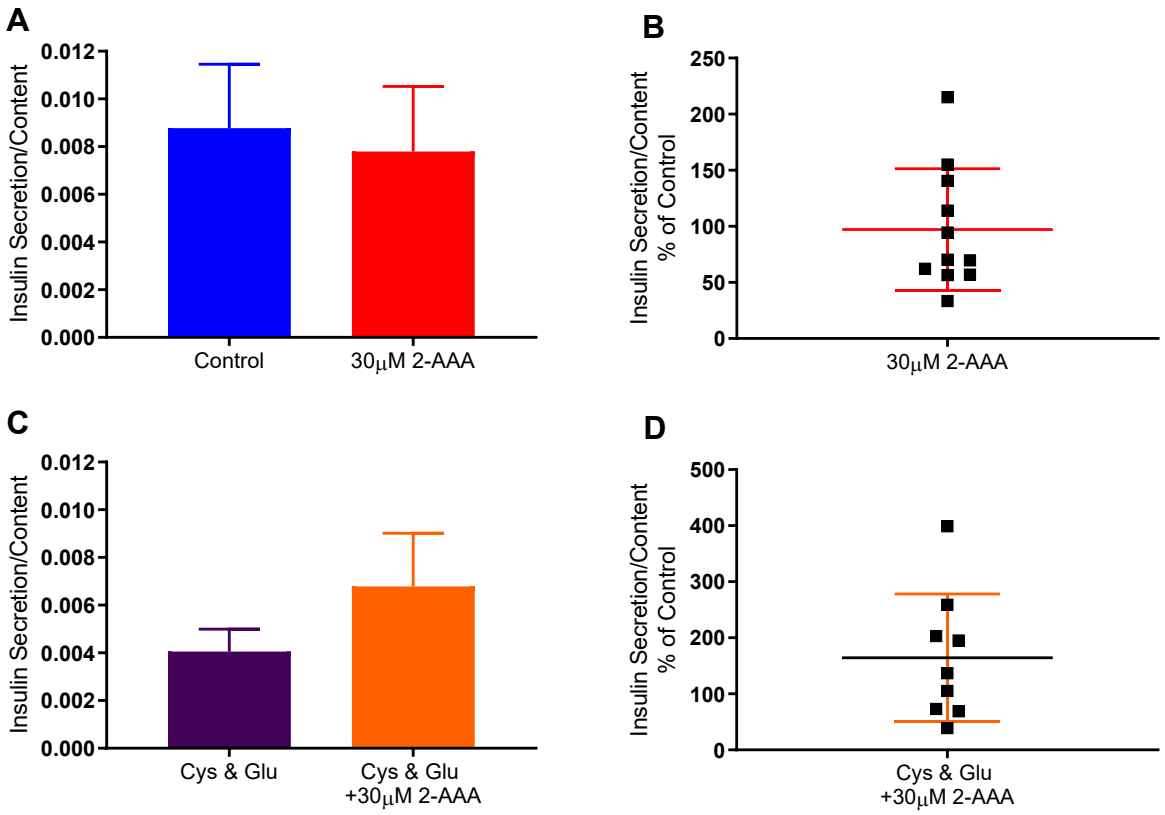


**Figure 11. Low glucose static insulin secretion from Human Islets.** A-D) No significant difference observed. Human islets are picked and then cultured overnight in DMEM complete media at 37°C and 5% $CO_2$. Islets are then separated into dishes containing 2.5mM glucose perifusion buffer. The buffer of the experimental group also contained 30μM 2-AAA. A,B) Buffer contained no added cystine or glutamate. C,D) Buffer also contained 25μM cystine and 40μM glutamate. These islets are then cultured at 37°C and 5% $CO_2$ for 3 hours. A buffer sample at the 3hr time was taken and measured to determine the low glucose insulin secretion over the 3hr period. All graphs are mean ± SEM. A,B) n=12, C,D) n=9. Significance set at $P<0.05$, Statistics: Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; Cys & Glu, Cystine and Glutamate.

**Figure 12**

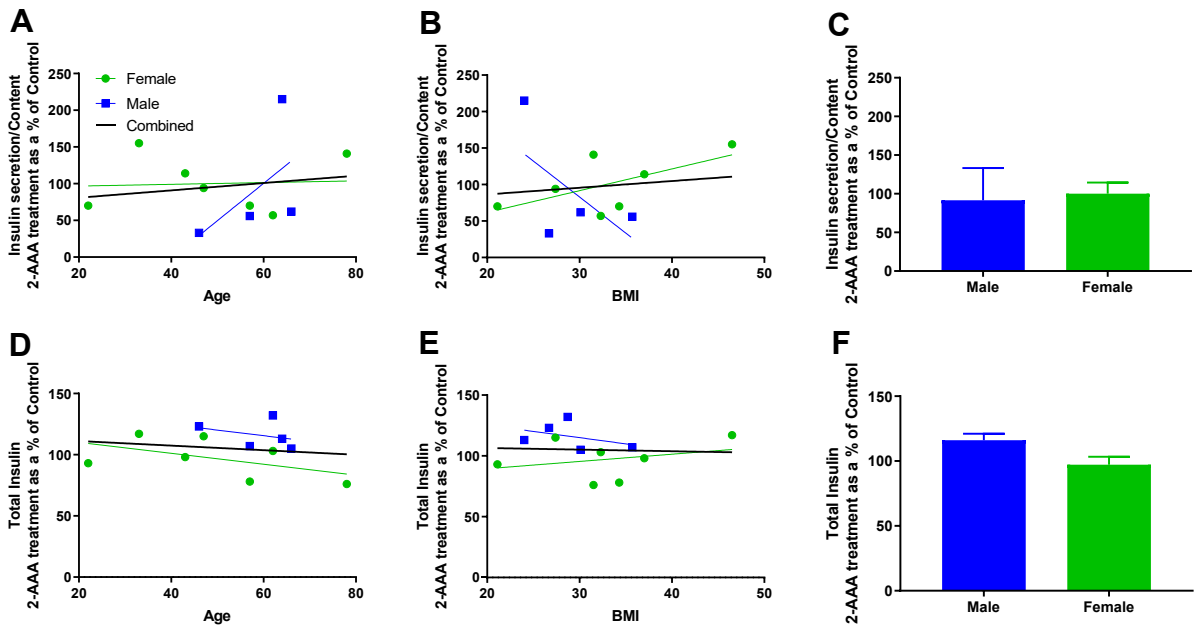


**Figure 12. Human islet data demographic correlation and comparison, no cystine and glutamate added.** A-F) No significant correlation or difference observed. A-C) 3 hour low glucose insulin secretion determined for each donor in figure 7. The 3 hour low glucose insulin secretion of islets treated with 2-AAA is expressed as a percent of the control islets' 3 hour low glucose insulin secretion in the same experiments. D-F) Total insulin secretion levels were determined for each donor from figures 6 and 7. The total insulin value of the islets treated with 2-AAA during the perifusion is expressed as a percent of the control islet total insulin values in the same experiments. Total insulin and 3hr low glucose insulin secretion percent control values were then compared or correlated with three demographic markers available for all patients, age, BMI, and sex. For each correlation graph, statistics were completed on the whole data set, females only, and males only. Females n=7, Males n=4/5 due to missing demographic data. Graphs A,B,D,E) Significance set at $P<0.05$, Statistics: Two factor correlation. Best fit lines in corresponding colours created using linear regression to aid in visualization only, black lines indicate best fit of all data regardless of sex. Graphs C,F) mean ± SEM. Significance set at $P<0.05$, Statistics: Unpaired t-test. 2-AAA, 2-Aminoadipic Acid

**Figure 13**

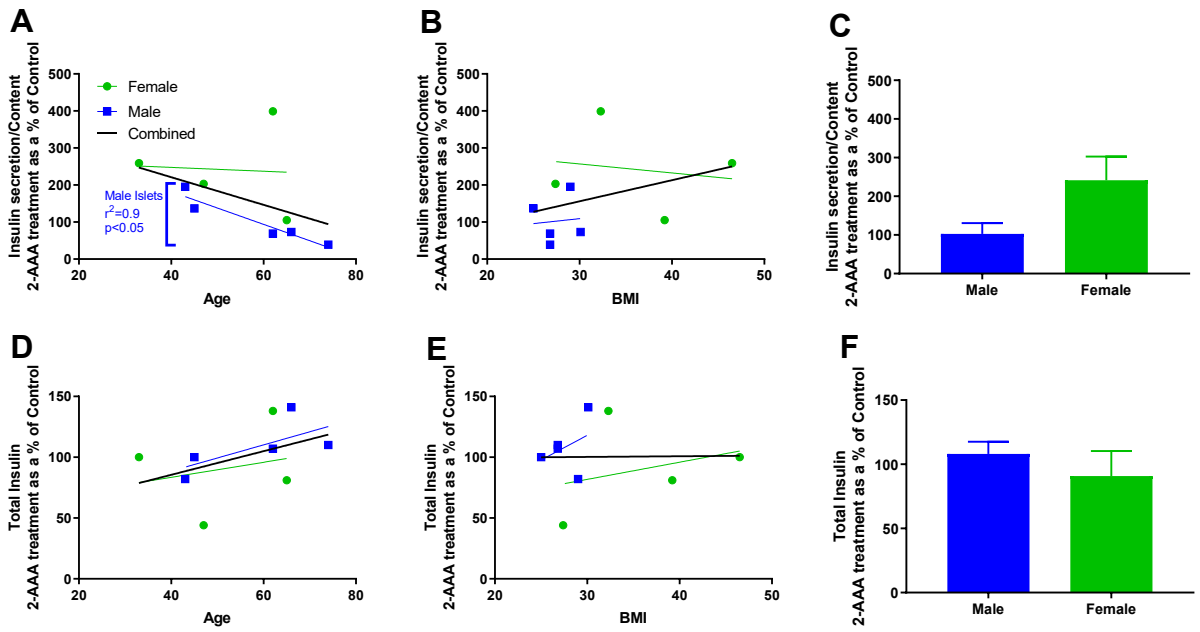


**Figure 13. Human islet data demographic correlation and comparison, with cystine and glutamate added.** A) Islets from males showed a significant negative correlation between percent control insulin secretion and age, $r^2$=0.9. B-F) No significant correlation or difference observed. A-C) 3 hour low glucose insulin secretion determined for each donor in figure 7. The 3 hour low glucose insulin secretion of islets treated with 2-AAA is expressed as a percent of the control islets' 3 hour low glucose insulin secretion in the same experiments. D-F) Total insulin secretion levels were determined for each donor from figures 6 and 7. The total insulin value of the islets treated with 2-AAA during the perifusion is expressed as a percent of the control islet total insulin values in the same experiments. Total insulin and 3hr low glucose insulin secretion percent control values were then compared or correlated with three demographic markers available for all patients, age, BMI, and sex. For each correlation graph, statistics were completed on the whole data set, females only, and males only. Females n=4, Males n=5. Graphs A,B,D,E) Significance set at P<0.05, Statistics: Two factor correlation. Best fit lines in corresponding colours created using linear regression to aid in visualization only, black lines indicate best fit of all data regardless of sex. Graphs C,F) mean ± SEM. Significance set at P<0.05, Statistics: Unpaired t-test. 2-AAA, 2-Aminoadipic Acid.

**Figure 14**

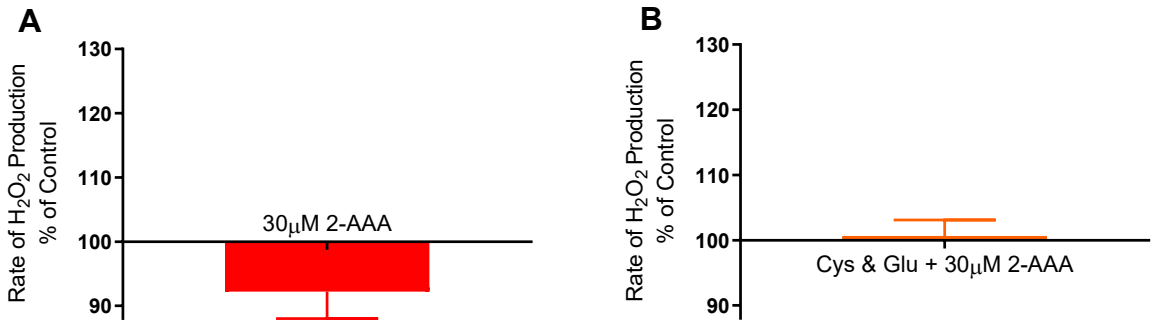


**Figure 14. Rate of $H_2O_2$ production from human islets treated with 20mM glucose in buffer.** A,B) No significant difference observed. Human islets are picked and then cultured overnight in DMEM complete media at 37°C and 5% $CO_2$. Islets are moved individually into wells containing 20mM glucose buffer. The buffer of experimental groups also contained 100μM 2-AAA. A) Buffer contained no added cystine or glutamate. B) Buffer also contained 25μM cystine and 40μM glutamate. Islets were then cultured at 37°C and 5% $CO_2$ for 8 hours. 2 hours of Amplex Red fluorescence over time was then measured individually from each islet on a plate reader. Increased fluorescence indicates increased ROS production and release from islets. Percent changes in fluorescence over time were compared to control, this indicates the rate of $H_2O_2$ production compared to control. All graphs are mean ± SEM. n=4. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate.

**Figure 15**

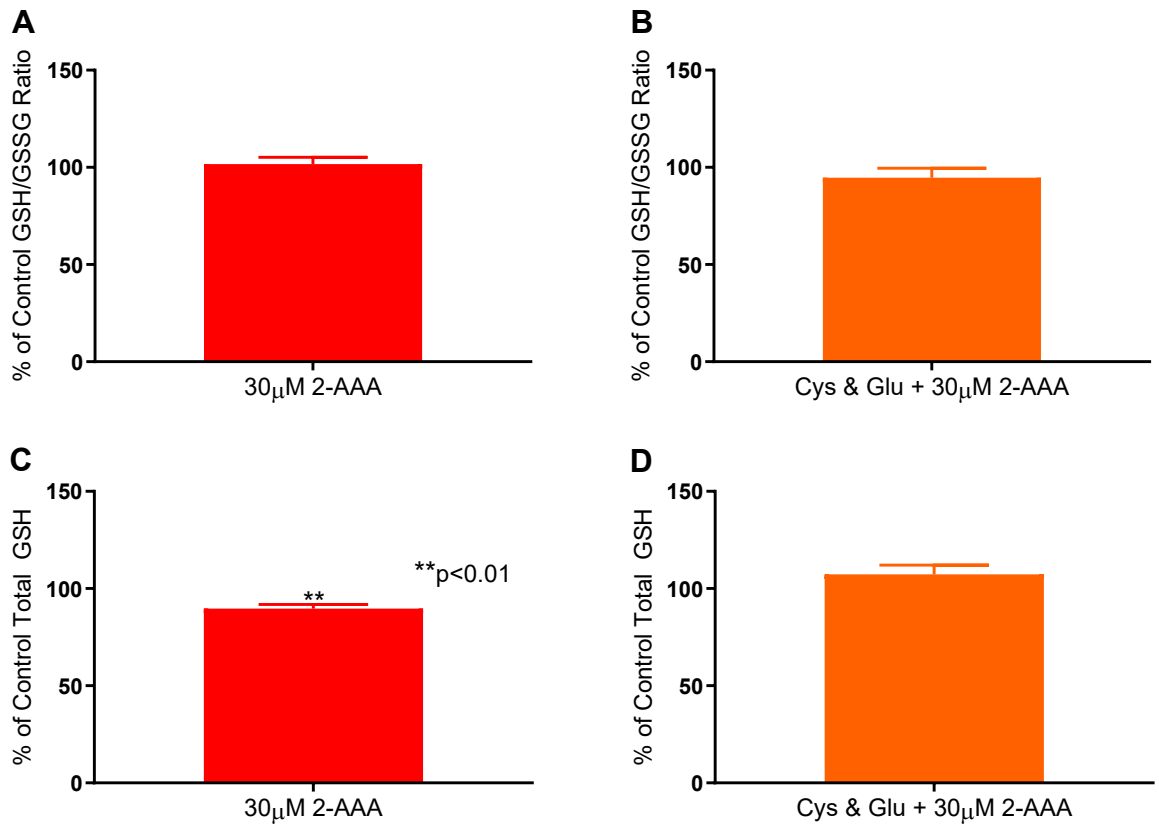


**Figure 15. Glutathione/GSSG and total glutathione levels of human islets treated with high glucose.** A,B) No significant difference observed. C) Islets in buffer not containing cystine and glutamate showed significantly decreased total glutathione levels with 30μM 2-AAA treatment compared to INS-1 cells treated only with 20mM glucose. D) No significant difference observed. Human islets are picked and then cultured overnight in DMEM complete media at 37°C and 5% CO2. Islets are moved into dishes containing 20mM glucose buffer. The buffer of the experimental group also contained 30μM 2-AAA. A,C) Buffer contained no added cystine or glutamate. B,D) Buffer also contained 25μM cystine and 40μM glutamate. These islets were cultured at 37°C and 5% CO2 for 8 hours. After all treatments islets were washed with 4x ice cold PBS and frozen with liquid nitrogen. Islets were sonicated and then glutathione and GSSG levels were measured using a GSH/GSSG Glo assay. All graphs are mean ± SEM. n=3. Significance set at $P<0.05$, Unpaired t-test. 2-AAA, 2-Aminoadipic Acid; $H_2O_2$, Hydrogen Peroxide; Cys & Glu, Cystine and Glutamate; GSH, Glutathione; GSSG, Glutathione Disulphide.

**Figure 16**

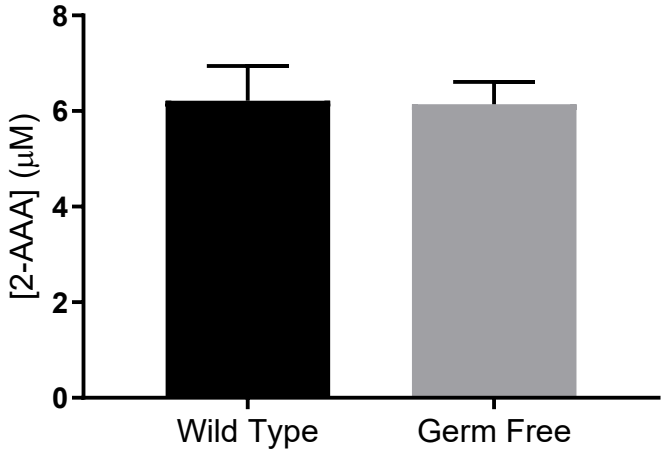


**Figure 16. 2-AAA serum levels of wild-type and germ-free mice.** No significant difference in 2-AAA levels observed. Serum samples retrieved from 5-week-old old mice. Mice were either wild-type or germ-free. Serum 2-AAA levels were determined by The Metabolomics Innovation Centre in Edmonton, Alberta. Mean ± SEM. n=5 for both groups. Significance set at $P<0.05$, Unpaired t-test.

**Figure 17**

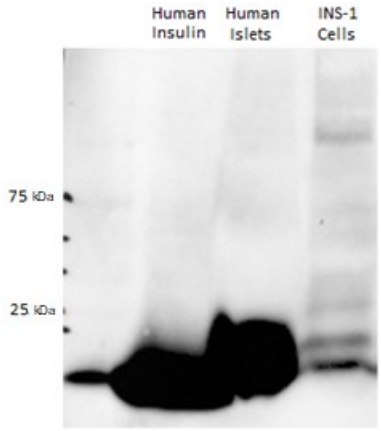


**Figure 17. Characterization INS-1 cell line insulin content by western blot.** INS-1 cells showed detectable insulin production compared to human islets and commercial insulin. Islets and cells were lysed in RIPA buffer. Samples were run through a electrophoresis gel and proteins were transferred to a nitrocellulose membrane. This was treated with a primary anti-insulin antibody (1:100 dilution) overnight, and then a secondary anti-mouse antibody (1:5000 dilution) for 1 hour. Membrane developed by ECL. Exposure time 63 seconds. See section *2.5* for more detail. No statistics completed, qualitative observation only. Experiment performed by Dr. Wentong Long of the Light lab, University of Alberta.

**Figure 18**

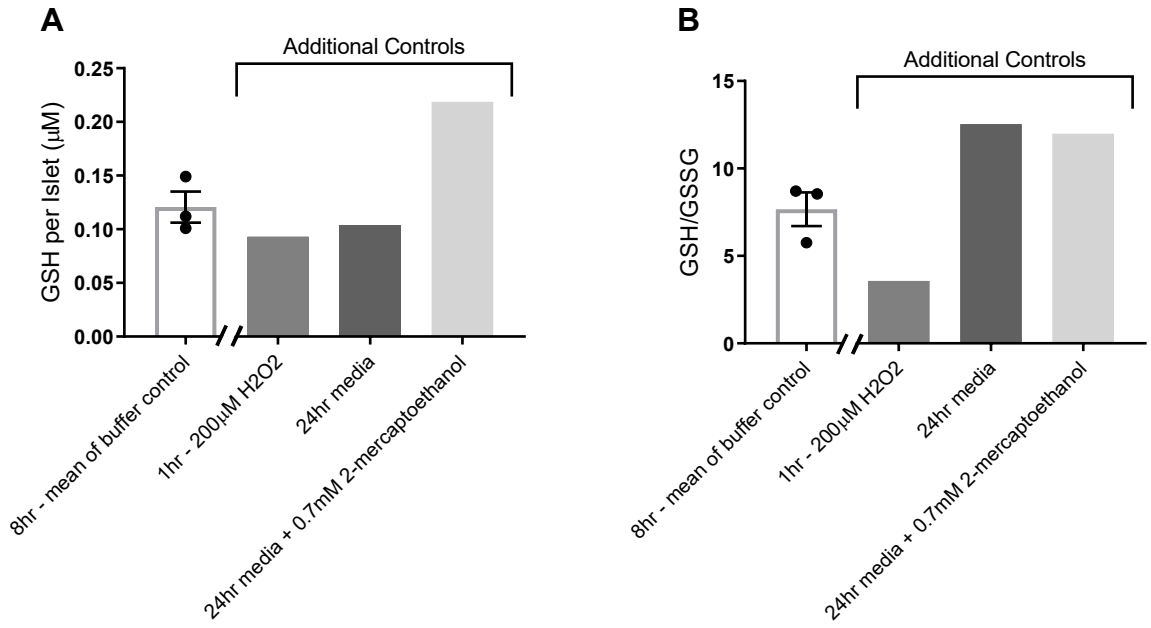


**Figure 18. Characterization of the Glutathione/GSSG Glo assay for human islet use.** A,B) Human islet total glutathione levels and glutathione/GSSG ratios under different treatments matched expected results based on previous findings (Janjic & Wollheim, 1992). Human islets are picked and then cultured for 1 hour in DMEM complete media at 37°C and 5% $CO_2$. Then islets were then given various treatments: 200µM $H_2O_2$ was added to the media for 1 hour, media treatment was continued for 24hr, media treatment was continued for 23hr with 0.7mM 2-mercaptoethanol added then islets were washed 4x with PBS wash to remove 2-mercaptoethanol then were treated with 1 additional hour of media treatment. After all treatments islets were washed with 4x ice cold PBS and frozen with liquid nitrogen. Islets were sonicated and then glutathione and GSSG levels were measured using a GSH/GSSG Glo assay. These glutathione levels and ratios were compared with the buffer control values determined for figure 13. All graphs are mean ± SEM. n=1 for characterization treatments. n=3 for buffer control data. Statistics could not be completed due to lack of n-values. GSH, glutathione; GSSG, glutathione disulphide.

## 4. Discussion

### *4.1 Comparing the detrimental effects of 2-AAA on β-cells and glial cells*

2-AAA treatment leads to significantly increased cellular necrosis of the INS-1 rat β-cell line when co-treated with $H_2O_2$ (Fig 3A). This may reflect the 2-AAA toxicity mediated by glutamate and cystine transport block found in previous studies investigating glial cells (Kato et al., 1992; Murphy, Miyamoto, Sastre, Schnaar, & Coyle, 1989). These results are also consistent with the apoptotic cell death observed when GLT-1 and Xc- transporters were pharmacologically inhibited in β-cell lines and human islets (Di Cairano et al., 2011).

2-AAA was also found to significantly increase the rate of $H_2O_2$ production from INS-1 cells co-treated with 20mM glucose to induce increased metabolic stress. This data is in line with 2-AAA's previously observed effects on increasing ROS levels and markers in glial cells (da Silva et al., 2017). It is also in line with the increasing ROS marker levels when the GLT-1 and Xc- transporters of β-cells were blocked (Di Cairano et al., 2011).

The addition of cystine and glutamate to these experiments appeared to reduce the toxic effect of 2-AAA. The increase in INS-1 cellular necrosis compared to control with 2-AAA treatment was both lower and not significant when cystine and glutamate were included in the buffer (Fig 3B). While 2-AAA treatment significantly increased the rate of $H_2O_2$ production from INS-1 cells when compared to control with added cystine and glutamate, the increased rate of production was less than that of the cystine and glutamate free buffer treatment (110% vs 127%, Fig 5A,B). Using a similar Ringer buffer to that used in this thesis, Reichelt et al., 1997 found that the addition of cystine and glutamate increased glutathione levels in glial cells. Investigating this possibility in our INS-1 β-cell model, I observed that cystine and glutamate treatment along with chronic 20mM glucose and acute $H_2O_2$ treatment lowered intracellular ROS levels compared to control (Fig 6). 2-AAA treatment along with the cystine and glutamate treatment did reverse the protective cystine and glutamate effect to some extent but did not increase the ROS levels completely back to control values (Fig 6).

It might have been expected that the presence of cystine and glutamate in the buffer, rather than lower the toxic effect of 2-AAA, would have been required to observe the toxic effect of 2-AAA. That is, if 2-AAA's main effect is mediated through inhibition of GLT-1 and Xc-, and there is little to no cystine or

glutamate in the buffer, no effect would be seen. As this is not the case, two explanations can be made to account for this. First, the inclusion of bovine serum albumin to the buffer may have added enough cystine and/or glutamate to the buffer to observe some 2-AAA inhibition of GLT-1 and Xc-. Secondly, 2-AAA's effect in β-cells may be significantly mediated through inhibition of the downstream GCL. Due to the cell's lack of glutamate and cystine supply it is possible that this enzyme's function can be more effectively inhibited by 2-AAA. These explanations are not mutually exclusive.

Finally, I observed that 2-AAA treatment significantly decreased total glutathione levels in INS-1 cells co-treated with 20mM glucose to induce increased metabolic stress (Fig 7C,D). The same treatment did not significantly reduce the glutathione/GSSG ratio of INS-1 cells (Fig 7A,B). These results were expected, as it was predicted that 2-AAA should reduce de-novo production of glutathione and therefore reduce the total amount of glutathione, while not necessarily affecting the glutathione/GSSG ratio. 2-AAA has not been confirmed to directly affect glutathione recycling. The observed reduction in total glutathione levels in INS-1 cells matches the reduction in glutathione levels with 2-AAA treatment described in the glial cell literature (da Silva et al., 2017; Kato et al., 1993; Reichelt et al., 1997).

*4.2 The effect of 2-AAA on human islets*

*4.2.1 2-AAA's effect on human islets may be mediated by β-cell level and placement.* I observed a decrease in total glutathione levels in islets co-treated with 2-AAA and 20mM glucose when compared to controls (Fig 15C). This effect was only observed when islets were treated with a buffer that does not contain cystine and glutamate. The lack of a significant decrease in total glutathione levels with 2-AAA treatment when cystine and glutamate were included in the buffer may be a part of cystine and glutamate's apparent protective effect seen in the INS-1 cell experiments (Fig 3B,5B,6,15D).

Despite observing a decrease in glutathione levels with 2-AAA treatment in human islets, a concurrent effect on increased $H_2O_2$ production was not observed (Fig 14). No significant changes in $H_2O_2$ production were observed when 2-AAA was co-treated with 20mM glucose (Fig 14). In addition, despite 2-AAA previously being reported to increase insulin secretion from mouse islets and a n=3 set of human islets, no significant increase in insulin secretion was observed in human islets (Fig 8,11) (T. J. Wang et al., 2013; Xu et al., 2018). In particular, no increase in low glucose insulin secretion was observed, both in the dynamic (Fig 8) and static (Fig 11) insulin secretion experiments. The addition of cystine and glutamate

to the buffer did not significantly modify 2-AAA's insulin secretion effect (Fig 8C-D,11C-D). Considering these islet experiments were completed using increased n-numbers, observation of insulin secretion dynamically not just statically, additional conditions, and in the more relevant model of human islets, I believe that it must be concluded that the insulin secretion effects of 2-AAA in mouse islets could not be repeated in human islets.

Why might this be the case?

As mentioned in the introduction, one of the most defined differences between mouse and human islets is the lower percentage of β-cells in human islets, ~75% and 55% respectively, and the higher percentage of α-cells, 15% and 35% respectively (Brissova et al., 2005; Cabrera et al., 2006). Also, when compared to the 55% β-cell population of human islets, the 100% β-cell population of a pure INS-1 cell population has an even more extreme β-cell percentage disparity. Additionally, α-cells have been shown to be resistant to cellular dysfunction and death induced by GLT-1 and Xc- block and therefore human islets may again be even less susceptible to 2-AAA induced cellular damage (Di Cairano et al., 2011).

Along with this, human islets are also more heterogenous, with α-cells, β-cells, and other cells being distributed throughout the islet (Brissova et al., 2005; Cabrera et al., 2006). Mouse islets instead have α-cells and other cells in a roughly 1 or 2 cell thick layer on the outside surface of the islet with the rest of the center of the islet filled almost exclusively by β-cells (Brissova et al., 2005; Cabrera et al., 2006). This means that more β-cells in mouse islets are surrounded by other dysfunctional β-cells in mouse islets. This may lead to lowered intercellular buffering of cell membrane permeable $H_2O_2$ by α-cells with unimpaired glutathione production.

Another potential problem is that the center of isolated islets, particularly large islets, in culture media or buffer often has some hypoxic stress due to the lack of functional blood vessels (Komatsu et al., 2017). The center of mouse islets is entirely β-cells, whereas the center of human islets is a mixture of cells. Therefore, a higher percentage of the already higher total percentage of β-cells will be situated in the high hypoxic stress center of the mouse islet compared to a human islet.

A lower β-cell percentage, with unaffected α-cells interspersed among the β-cells, with a islet core containing a lower amount of β-cells, along with a generally higher tolerance for oxidative stress and a higher level of antioxidant enzymes, means human islets are likely to be less susceptible to oxidative

stress than mouse islets or INS-1 cells (Welsh et al., 1995). If this is the case, then the lack of an increase in $H_2O_2$ production from the human islets in this study is not surprising (Fig 14). If there is no increase in $H_2O_2$ levels in the β-cells, then we would expect no subsequent increase in $H_2O_2$ mediated insulin secretion at low or high glucose, which is what I observed in figures 8 and 11 (Fig 2).

*4.2.2 2-AAA's effect on human islets may be mediated by treatment time.* It is also possible that islets did not receive a long enough treatment time to fully show the effects of 2-AAA on human islets. This question of time could include both that human islets require more time to become fully stressed and that human islets need a longer treatment time with 2-AAA before 2-AAA's full effects can be seen. Both possibilities may link into human islet's higher tolerance to 2-AAA effects described above.

The original biomarker study does suggest that it may take years, possibly well over a decade, for 2-AAA to have a considerable effect in humans (T. J. Wang et al., 2013). Recent biomarker studies in 2019 also support this long-term effect case (Lee et al., 2019; Razquin et al., 2019). It is possible that human islets are not an ideal model for the measurement of 2-AAA's potential long-term effects in the human pancreas. General damage and deterioration over longer culture times may overshadow the toxic effects of 2-AAA, particularly when a buffer is used instead of media to prevent potential interactions with unphysiologically high glutamate or cystine levels found in media.

Observing the long-term effect of 2-AAA on islet health over a lifetime may be possible with mice. After a lifelong consistent treatment of 2-AAA mouse islets could be isolated and tested. However, treating humans with 2-AAA and waiting for organ donation to measure changes in islets is not ethical, particularly when the ramifications of 2-AAA's toxicity on glial cells and β-cells are considered.

*4.3 2-AAA should not be used as a diabetes treatment*

This ethical problem of 2-AAA treatment and toxicity in humans would likely remain a theoretical stumbling block for future investigations into the long-term effects of 2-AAA in humans, if it were not for a recent paper. This recent paper describes the potential use of 2-AAA, or therapies that will prevent 2-AAA breakdown in the body, as a cost-effective treatment during diabetes to increase insulin secretion and reduce blood glucose (Xu et al., 2019). Our results would strongly caution against use of 2-AAA as a potential treatment for diabetes. 2-AAA's effect on increasing insulin secretion from mouse islets is not reflected in our human islet insulin secretion measurements (Fig 8,11). Even if only previous mouse data

is considered, this increase in insulin secretion seems to mainly occur during low glucose (T. J. Wang et al., 2013; Xu et al., 2018). Increasing basal hyperinsulinemia with 2-AAA is not advised, as basal hyperinsulinemia may lead to further insulin insensitivity and worse diabetic disease outcomes in the long term (Dankner et al., 2012; Shanik et al., 2008).

Inducing insulin secretion without regard to glucose levels has also been found to be detrimental to β-cell health in the long term. As mentioned in the introduction, this is most often observed clinically with sulphonylurea treatment. The constant insulin secretion induced by sulphonylureas leads to short and medium term increases in insulin secretion and decreases in blood glucose, followed by a "secondary failure" due to decreases in maximum β-cell insulin secretion capacity (Matthews et al., 1998; H. Wang et al., 2017). This decrease in the maximum β-cell insulin secretion capacity is due to increased β-cell dysfunction and death, as well as increased insulin resistance due to constant basal hyperinsulinemia. This results not just in sulphonylurea therapy no longer being an effective treatment for type 2 diabetes, but the patient being worse off in the progression of the disease. The patient may be require transitioning to exogenous insulin therapy as a result of the β-cell loss.

Drugs targeting only high glucose insulin secretion or lowering glucose levels through other mechanisms have been the focus of more recent efforts of pharmacological drug development. These efforts have already been successful. Several classes of drugs which increase high glucose insulin secretion only already exist, such as GLP-1 mimetics or DPP-4 inhibitors (Pfeiffer & Klein, 2014). Other drugs such as the SGLT inhibitors or metformin lower glucose levels by allowing for urinary excretion or increasing insulin sensitivity respectively (Pfeiffer & Klein, 2014).

There is no reason to propose the use of 2-AAA as a new treatment for type 2 diabetes that, as suggested by our INS-1 cell line data, may directly damage β-cells (Fig 3A,5,6,7). Short term increases in insulin secretion at the cost of β-cell health and insulin sensitivity are detrimental to long term outcomes of diabetic patients. Several better pharmaceutical options which do not increase low glucose insulin secretion already exist. Even if these newer options are ignored, sulphonylureas may be used as an inexpensive treatment which increases insulin secretion both at high and low glucose (Pfeiffer & Klein, 2014). Therapies that increase blood 2-AAA levels should not be introduced as a new treatment for diabetes.

### *4.4 Possible additional toxic effects of 2-AAA*

Interestingly, the data presented by Xu et al., 2019 suggests that 2-AAA may show additional dangerous side effects. In their Figure 1B, the standard chow representative mouse shown treated with 2-AAA is not only much thinner than their non-treated control, but is also noticeably shorter in length than controls. This is attributed mainly to increased energy expenditure shown in their supplemental figures.

However, an increase in energy expenditure does not necessarily imply that this effect is a non-toxic one. Exposure to the toxic pesticide dichlorodiphenyltrichloroethane does reduce energy exposure, but other toxins such as lead or oil and gas related endocrine disruptors have been shown to increase energy expenditure (Balise et al., 2019; Faulk et al., 2014; La Merrill et al., 2014).

Also, this increase in energy expenditure does not account for the lowered body length of the mouse (Xu et al., 2019). This decrease in length may imply growth retardation, specifically because 2-AAA treatment began at 1 month, before the mice were fully grown. The cause of any such growth retardation is currently unknown. Considering 2-AAA's inhibitory effect on glutamate and cystine uptake into glial cells and β-cells, it is possible other cells may be affected. Any knock-on decrease in glutathione levels may lead to increased cellular death in any susceptible cell types and, if these cells were associated with growth and maturation of the animal, then a knock-on reduction in growth. Less drastically, it may simply be that during maturation certain susceptible cells require a considerable amount of glutamate or cystine uptake for protein production. Limiting this uptake may be sufficient for reduced growth and maturation of the animal.

It is possible that the mouse shown in the figure 1B is not representative and this is merely the leanest and smallest mouse that could be found to show maximal contrast (Xu et al., 2019). Body length measurements and femur length measurements would be required to definitively establish if any growth retardation may have taken place. Reductions in lean mass seen with 2-AAA treatment in standard chow fed mice do indicate that it is possible that some non-fat tissue may have suffered degeneration.

Future studies investigating 2-AAA treatment in animals should consider measurement of body and bone lengths to determine if any growth retardation may be caused by dosing animals with 2-AAA.

### *4.5 2-AAA's effects on glucose tolerance*

One additional effect of 2-AAA seen in mice is its effect on increasing glucose tolerance that was suggested by T. J. Wang et al., 2013 and then seen in Xu et al., 2018. Increased glucose tolerance is

usually the result of either increased high glucose insulin secretion or increased insulin sensitivity. 2-AAA has not been seen to increase high glucose insulin secretion in mouse islets previously and 2-AAA does not increase high glucose insulin secretion in human islets in our study (Fig 8) (T. J. Wang et al., 2013). This data suggests that if 2-AAA does have a glucose tolerance increasing effect it is likely a peripheral effect.

This is interesting as the original 2013 biomarker paper suggested that there was likely no change in insulin sensitivity (T. J. Wang et al., 2013). However, two recent papers do suggest 2-AAA has an effect on insulin sensitivity, though they disagree on the direction of 2-AAA's effect in this regard (Lee et al., 2019; Xu et al., 2019).

In the mouse study by Xu et al., 2019, the increase in apparent insulin sensitivity is linked to 2-AAA increasing energy expenditure in the fat. This may imply that the increased glucose tolerance is the result of increased energy expenditure rather than increased insulin secretion or sensitivity. As has previously been explained, it is possible that other toxic effects of 2-AAA may be influencing this result.

The recent study by Lee et al., 2019 suggests that insulin resistance is increased with higher blood 2-AAA levels in children and that initial blood 2-AAA levels were predictive of insulin resistance measured at a two-year follow-up. Interestingly, it was observed that basal insulin levels were also increased with higher blood 2-AAA levels and that 2-AAA levels were predictive of this basal insulin increase measured at a two-year follow-up. This suggests that in humans 2-AAA is associated with a decrease in insulin sensitivity and an increase in insulin secretion. This disagrees with the mouse data suggesting increased glucose tolerance with 2-AAA treatment and the data in this thesis suggesting human islets do not show increased insulin secretion with 2-AAA treatment (Fig 8,11) (Xu et al., 2019).

How might this be explained? It is also possible that given a "treatment time" of years with high blood 2-AAA levels, islet insulin secretion would be increased substantially unlike what was seen with the shorter ex-vivo treatment in this study (Fig 8,11). The reduction of glutathione that was seen in Figure 15 may lead in the long term to the increased $H_2O_2$ production that was not observed in Figure 14. This increased $H_2O_2$ could signal for the increased basal insulin levels observed by Lee et al., 2019 (Leloup et al., 2009; Pi et al., 2007). Unlike what was observed in mice, the effect of increased blood 2-AAA levels on increasing energy expenditure in the fat cells of humans does not seem to be very substantial (Lee et al.,

2019; Xu et al., 2019). Worsening of nearly all adiposity and lipid parameters were associated with higher 2-AAA levels, and the initial blood 2-AAA level predicted these parameters to be worse in a two year follow-up (Lee et al., 2019).

2-AAA treatment in mice may have a glucose tolerance increasing effect that is peripheral and likely due in part to increased energy expenditure in the fat. (Xu et al., 2019) However, in humans the current literature does not suggest any increase in energy expenditure in the adipose tissue and in fact suggests a decrease in glucose tolerance due to insulin resistance. While no increase in insulin secretion from human islets was observed in this study, it is possible that high blood levels of 2-AAA over years may lead to the increases in insulin secretion observed by Lee et al., 2019 (Fig 8,11).

*4.6 Correlation of 2-AAA with age and it's implication on mouse studies*

It is interesting that the only significant correlation in figures 12 and 13 was in figure 13A. Here we observe a negative correlation between donor age and the ratio between 2-AAA and control 3 hour low glucose insulin secretion from islets treated in buffer containing cystine and glutamate (Fig 13A). When compared to current mouse data, this finding may introduce a separate though possibly related reason for the difference in 2-AAA mediated insulin response between mice and mouse islets in previous studies and human islets in this study.

In the original biomarker paper mouse islets for insulin secretion experiments were obtained from male mice and whole mouse studies examining insulin secretion were completed on 6 week old male mice only (T. J. Wang et al., 2013). In another paper examining 2-AAA treatment, islets were obtained from 3 month old male mice (Xu et al., 2018). It is unclear from the methods of this paper exactly how old the male mice in the whole mouse studies were; however, their figure 4 implies that mice were treated with 2-AAA for 8 or 12 weeks and then had a glucose or insulin tolerance test preformed at 16 weeks of age (Xu et al., 2018). Therefore, it is likely these mice were approximately 4 or 8 weeks of age at the beginning of the study (Xu et al., 2018). It does state that mice in their supplemental figure 6 were treated with 2-AAA beginning at the age of 1 month (Xu et al., 2018). The most recent paper examining 2-AAA treatment in whole mice began treatment at the age of 1 month and all mice involved in the study were male (Xu et al., 2019).

In all these mouse studies, only young male mice were involved. Referring to the relevant reference material, 1-3 month-old mice represent approximately 20-30 year old humans (Fox et al., 2006). This means that the oldest mice beginning in these studies would represent an animal relatively younger than the youngest male donor, age 43, represented in figure 13A.

Extrapolating the correlation of figure 13A backwards to these younger ages, it is then not surprising that these young male mice showed increased low glucose insulin secretion when treated with 2-AAA. We may reasonably expect that if the correlation from figure 13A maintains at younger ages, human islets from donors aged 18-25 would respond with increased low glucose insulin secretion if treated with 2-AAA. This would be in line with the more recent biomarker study which found that in children/youth, those with high blood 2-AAA levels had increased fasting plasma insulin and that increased 2-AAA levels could predict increased future fasting plasma insulin levels (Lee et al., 2019).

Admittedly this reasoning depends on extrapolating the correlative result from human islet data in figure 13A backwards to ages not represented in my data set. However, using this same reasoning, the mouse studies on 2-AAA have implicitly speculated that their findings in young male mice would be translatable to all humans with or at risk of developing diabetes. This itself is an extrapolation, as patients at risk of type 2 diabetes tend to be middle aged or older and, while the exact sex ratio of diabetes incidence depends on the specific population in question, the sex ratio is generally no lower than 1:2 female to male and can be nearly as high as 2:1 (Gale & Gillespie, 2001; Magliano et al., 2019). Therefore, until mouse studies are repeated on mice of an older age and in females, I do not think the observations of 2-AAA's actions on insulin secretion are applicable to the relevant human patient populations.

*4.7 Source of 2-AAA*

*4.7.1 Germ-free mice have normal 2-AAA levels.* No change was observed in serum sample 2-AAA levels from germ-free mice compared to wild-type mice (Fig 16). I expected that if bacteria in the gut had lysine breakdown pathways linked to salt stress, as mentioned in the introductory chapter, the complete removal of all bacteria including these would lead to reduced 2-AAA levels overall (Neshich et al., 2013). This was not the case. Basal production of 2-AAA from the gut under a normal chow diet does not seem to significantly affect 2-AAA levels in the blood. This does not imply directly that 2-AAA is not produced from gut bacteria in substantial levels under certain extreme diets. Particularly, diets high in salt and including

lysine may still lead to a substantially increased prominence of lysine metabolizing bacteria in the gut microbiome. The impact of such a diet on blood 2-AAA levels in mice and humans is a possible next step of investigation that will be expanded upon in part *4.8.2*.

Considering that 2-AAA may not be produced in substantial amounts from the gut, what else may lead to increased production of 2-AAA in the mammalian and particularly the human body?

*4.7.2 Literature investigation into a possible inflammatory source of 2-AAA.* During inflammation macrophages and related leukocytes such as neutrophils activate, this activation results in higher release of the enzyme myeloperoxidase (MPO) (Lin et al., 2017; Rodrigues, Rodriguez, Russo, & Campa, 2002). The major function of MPO in this context is the conversion of $H_2O_2$ into hypochlorous acid (Rodrigues et al., 2002). Increased MPO levels and activity can be seen in areas of increased inflammation such as atherosclerotic lesions, where MPO activity can be colocalized with macrophage staining within the lesion itself (Daugherty, Dunn, Rateri, & Heinecke, 1994).

Fascinatingly, if MPO, $H_2O_2$, and hydrochloric acid are combined in a solution containing proteins and amino acids normally found in the body, which can be imitated in-vitro using human serum albumin, there is production of 2-AAA (Lin et al., 2017). All three components, MPO, $H_2O_2$, and hydrochloric acid, are necessary for production of 2-AAA (Lin et al., 2017). All three components are released by activated macrophages and neutrophils (Lin et al., 2017). In fact, interrupting the production or release of any of these components in activated human neutrophils almost completely prevented them from producing 2-AAA (Lin et al., 2017). In mice injected with bacterial and fungal antigens to induce a leukocyte activation response, 2-AAA levels rose significantly unless a MPO knockout mouse was used (Lin et al., 2017). Human aortic tissue was also examined and 2-AAA levels were seen to be ~5x higher in human aortic tissue with sclerotic lesions (Lin et al., 2017). This matches the MPO and macrophage staining seen in atherosclerotic lesions in previous studies (Daugherty et al., 1994). In ~60 year old male diabetics this trend has also been seen when blood levels of 2-AAA were measured, with 2-AAA being corelated with previous CVD and also being a strong predictor of atherosclerosis (Koska et al., 2018; Saremi et al., 2017).

Serum MPO levels themselves have been observed to be higher in patients with impaired fasting glucose (Agarwal et al., 2017). The MPO levels also correlated with both increased fasting plasma insulin and increased fasting plasma glucose levels (Agarwal et al., 2017). Though the n-numbers were

substantially lower in this study than in the original biomarker study, it suggests that MPO itself may be potential biomarker for diabetes (T. J. Wang et al., 2013). If MPO leads to 2-AAA production, then this would not be surprising. Additionally, it has also been observed that MPO activity is significantly higher in diabetic patients and in both non-diabetic and diabetic patients with higher BMI (Sujatha, 2015).

Interestingly, mice with granulocyte macrophage–colony stimulating factor production knocked out genetically, which leads to a drop in the population of macrophage and dendritic cells, show a dramatic reduction in 2-AAA levels (D. H. Kim et al., 2008; Plubell et al., 2018). This granulocyte macrophage–colony stimulating factor knockout has been shown to decrease dendritic cell levels at sites of inflammation, including at the site of atherosclerotic plaques, the same site mentioned previously to show upregulation in 2-AAA levels during inflammation (Lin et al., 2017; Zhu, Chen, Jongstra-Bilen, & Cybulsky, 2009).

The dramatic reduction in 2-AAA levels with the KO coincided with an upregulation of the DHTK1 gene which does regulate the breakdown of 2-AAA; however, considering the literature cited above, the knockout of these immune cells would likely also prevent MPO mediated 2-AAA production (Plubell et al., 2018). Therefore, it is possible in this case too, that immune cell activity is leading to 2-AAA production via MPO release.

*4.7.3 2-AAA breakdown vs production.* Many recent studies on 2-AAA focus on its breakdown through a DHTK1 mediated breakdown pathway, whether increasing DHTK1 levels (Plubell et al., 2018) or decreasing them (Xu et al., 2019, 2018). It is clear that in disease states such as Charcot-Marie-Tooth disease, genetic disruption of 2-AAA breakdown pathways and subsequent increases in 2-AAA levels and action can cause or worsen the disease state (Xu et al., 2018). However, the inter-mouse variability in DHTK1 activity seems to be quite low (Plubell et al., 2018). It is also clear that in the original biomarker paper, most patients had very low levels of 2-AAA, 0.5-1μM, and a subset of persons have much higher levels, 2-8μM (T. J. Wang et al., 2013).

Considering that obese, pre-diabetic, and diabetic patients tend to have high basal inflammation, it is very possible that the increased inflammation overall may lead to a subsequent increase in the 2-AAA levels overall (Esser, Legrand-Poels, Piette, Scheen, & Paquot, 2014; Lontchi-Yimagou, Sobngwi, Matsha, & Kengne, 2013). 2-AAA in this case would be a biomarker of inflammation. However, our data suggests that 2-AAA also has a detrimental effect on β-cell health (Fig 3,5-7,15). Therefore,

2-AAA may be a potential direct molecule link between general inflammation and β-cell damage in diabetes development.

There is also possibility that this inflammation and increased 2-AAA levels are more localized to the pancreas. While it is known that auto-immune activation and infiltration of the pancreas is a main cause of type 1 diabetes, several papers do indicate infiltration of macrophages in type 2 diabetes as well, though to a lesser extent. Ehses et al., 2007 shows evidence of islet-associated macrophages in the islets of both type 2 diabetic patients and high fat diet mice. Later papers repeat the human data, also showing macrophage infiltration into type 2 diabetic islets (Kamata et al., 2014; Richardson, Willcox, Bone, Foulis, & Morgan, 2009). Recent data from insulin resistant juvenile primates also shows increased macrophage infiltration into islets in a pre-diabetic model (Nicol et al., 2013). It seems that there is clearly an increase in macrophage infiltration with the onset of diabetes, and according to mouse and primate data, possibly even before the onset of diabetes. This could lead to a localized increase in 2-AAA values consistent with the high 2-AAA levels observed in the pancreas of mice and particularly high fat diet mice in the original biomarker study (T. J. Wang et al., 2013).

A strong case can be made from the literature that 2-AAA is produced during inflammation from immune cells such as macrophages. 2-AAA may be a good biomarker for diabetes in part due to its increased production during the increased inflammatory state common to many diabetes risk factors such as obesity. 2-AAA production due to inflammation before and during diabetes may be pronounced in the pancreas. 2-AAA production, particularly accumulation in the pancreas, could lead to increased β-cell ROS levels and death (Fig 3,5-7,15).

*4.7.4 2-AAA breakdown and production.* Finally, a point on the possible interplay of 2-AAA production and breakdown. DHTKD1 levels are higher in the adipose tissue of mice with reduced leukocytes numbers due to knock out of granulocyte macrophage–colony stimulating factor (Plubell et al., 2018). It is possible that the opposite, highly activated or a higher number of leukocytes such as macrophages, will lead to lower levels of DHTKD1. This would decrease 2-AAA breakdown at the same time 2-AAA production is increased by MPO release. Future studies should keep in mind that both processes may be occurring at the same time and may, in a currently unknown way, be directly or indirectly linked.

### *4.8 Future directions*

*4.8.1 Further investigations into the mechanism of 2-AAA.* As stated previously, the cultured human islet model may not be a long term enough model to measure the full effects of 2-AAA on islet and β-cell health and insulin secretion. Besides further population studies, how could we specifically examine β-cells and islets to understand 2-AAA's mechanism of action in the islet more fully?

One technique to begin to get a better idea of the long-term effect of 2-AAA treatment on islets, is to dose mice with 2-AAA over many weeks or months in a similar way to previous studies then remove and test their islets (T. J. Wang et al., 2013; Xu et al., 2019, 2018). Insulin secretion, ROS production, glutathione levels, and susceptibility of the islets to injury and apoptosis/necrosis could all be measured in these islets. Currently 2-AAA treatment in mice has only examined effects on mice physiology during treatment or has removed islets from non-2-AAA-treated mice and treated the islets with 2-AAA ex-vivo (T. J. Wang et al., 2013; Xu et al., 2019, 2018). While not examining the long-term effect on human islets, it may allow us to get an idea of how 2-AAA affects the mouse islets over a long period of time.

To examine the effects of 2-AAA on β-cells and α-cells of mouse or human islets individually, CellROX Deep Red ROS measurements of individual cells from dispersed islets could be completed. β-cells and α-cells could be identified by insulin or glucagon immunohistochemistry. These dispersed cells could be sorted into pure β-cell and α-cell populations (Russell, Noel, Files, Ingram, & Rabinovitch, 1984). β-cell and α-cells could also be dispersed and seeded in a mixed population, with their fluorescent markers being measured at the same time as CellROX Deep Red fluorescence. As long as the fluorescence profile of each component did not overlap, the cell identity and ROS measurement could be compared. CellROX Deep Red dye can function under live or fixed conditions. It would be expected that isolated β-cells would show increased ROS levels with 2-AAA treatment compared to α-cells.

Another possible way to examine the potential impact of the islet β-cell to α-cell ratio on 2-AAA susceptibility would be to detect the ratio of β-cells to α-cells on an islet by islet basis and compare this with the islet's susceptibility to 2-AAA. Amplex Red measurement of $H_2O_2$ produced from single islets could be measured. After the Amplex Red experiments on individual islets, these islets could be fixed and the β-cell to α-cell ratio could be measured by immunohistochemistry. It would be expected that human islets with a high with β-cell to α-cell ratio would produce $H_2O_2$ at a faster rate.

Another way to examine the impact of the β-cell to α-cell ratio in whole islet susceptibility to 2-AAA toxicity, may be constructing a human islet that is constituted more like a mouse islet, as to make it more susceptible to 2-AAA mediated damage like a mouse islet. Pseudo islets can be created by dissociating islets and then reassociation the islet cells back together into an islet shape (Zuellig et al., 2017). While often used to create islets of uniform size for study and possibly transplantation, another application of this technology is the formation of islets with a modified cell population or composition compared the original islet cell population by only adding back specific subsets of the dissociated cells (Liu et al., 2019; Yu et al., 2018). A pseudo human islet constructed mainly of β-cells like a mouse islet may become more susceptible to 2-AAA effects on glutathione levels and insulin secretion. As a proof of concept, a human pseudo islet could also be constructed completely of β-cells. We would expect that this all β-cell human pseudo islet would be more susceptibility to the effects of 2-AAA, as all cells would be targets for 2-AAA glutathione depletion, similar to what was seen in the pure population of INS-1 β-cells (Fig 3,5-7).

The pseudo islet technique could also be used to examine mouse islets, if human islet supply became a practical impediment to these experiments. A completely β-cell pseudo mouse islet may be slightly more susceptible to 2-AAA damage. More interestingly, it is possible that a pseudo mouse islet of β-cells and α-cells in a similar ratio and composition to that found in human islets could be constructed. This could examine if mouse islets would be less susceptible to 2-AAA's effects if their constituent cells were more like that of a human islet.

As a final test it could be possible to construct a pseudo islet of mainly α-cells using either human or mouse islet cell material. This could test if increasing the islet α-cell population leads to increased protection from 2-AAA mediated islet toxicity.

All pseudo islet tests could be compared to both control isolated islets and control pseudo islets made with the same ratio of α-cells and β-cells as the original isolated islets. This would allow us to observe the effect of pseudo islet creation on the health and insulin secretion ability of the pseudo islets.

Another important point would be the examination of other prominent cell types within the islet, such as the delta cells (δ-cells). These cells may need to be taken into consideration when producing pseudo islets. It is currently unknown if δ-cells or other cells of the islet are a targets for 2-AAA. Current

investigations into the importance and characteristics of individual cell types in both mouse and human islets may allow us to better predict their importance to 2-AAA's effect in islets (Camunas-Soler et al., 2019).

Examining the long-term effects in human islets is difficult as it would not be ethical to prospectively treat humans with 2-AAA now that I have shown its potential toxic effects on β-cells (Fig 3,5-7,15). Alternatively, mice could be treated with streptozotocin to destroy their mouse β-cells and then a xenotransplant of human islets into the mouse could be completed. Xenotransplants can be maintained for weeks, during which time the mouse could be dosed with 2-AAA as in previous studies (Pepper et al., 2017; T. J. Wang et al., 2013). Islets could then be removed and insulin secretion, ROS production, glutathione levels, and susceptibility of the islets to injury and apoptosis/necrosis could be measured. This may allow long-term measurement of the effects of prospective 2-AAA treatment on human islets in a physiological system.

It is important to note the possibility that human β-cells are simply more resilient overall than rodent β-cells (Lenzen et al., 1996; Welsh et al., 1995). If true we would not expect that the different β-cell compositions would have a considerable effect on 2-AAA islet toxicity. If this is the case, the longer term xenotransplant studies may be the only of the above proposed studies which would be sufficiently long enough to observe the effect of 2-AAA on the more resilient human islets.

*4.8.2 Further investigations into the source of 2-AAA.* Additional population-based studies, while unlikely to produce a considerable amount of additional information on 2-AAA's mechanism of action, may be very useful in determining the source of increased 2-AAA levels in humans.

As suggested previously, links between salt stress survival pathways and lysine metabolism pathways in bacteria may lead to increased survival of bacteria containing these linked pathways in the gut of a subject with a high salt diet (Neshich et al., 2013). Long term mouse studies with varying diets, particularly those with a high salt and/or lysine diet, could be completed to investigate the resulting effect on blood 2-AAA levels. Pancreas and other organ samples could also be taken to compare relative organ 2-AAA levels in a similar manner to the original biomarker study (T. J. Wang et al., 2013). Fecal samples would be an ideal secondary measurement to examine potential modification of bacterial populations to a more salt stress resistant population. As a control to ensure bacterial production of 2-AAA, germ-free mice could be treated with the same diets.

If a link between salt stress resistant bacteria and 2-AAA is established in the mouse studies, human subjects could be examined. A prospective study with a low salt and/or lysine diet intervention could be completed to examine the effect on 2-AAA blood levels. Due to the danger of high salt intake and the potential danger of high 2-AAA production it would be unethical to prospectively increase patient salt intake to measure long term increases in 2-AAA. However, we may be able to observe patient salt intake in a population study and correlate this with blood 2-AAA levels. Salt intake from diet could be examined with diet history and blood sodium levels could be measured alongside 2-AAA levels. As in the mouse studies, fecal samples would be an ideal secondary measurement to examine bacterial populations.

The possible inflammatory pathway of 2-AAA production could also be examined. Starting with mice, long term studies could be completed on mice induced or genetically pre-disposed to have considerably high basal inflammation, particularly models such as cecal ligation and puncture, which could induce long term activation of macrophages and/or other leukocytes (Seemann, Zohles, & Lupp, 2017). These models would then allow us, after taking blood and organ samples, to determine any possible increase in 2-AAA production with an increased inflammatory state. Models that target leukocytes to the pancreas including both acute and chronic pancreatitis models using duct ligation would be particularly useful, to observe any increases in 2-AAA levels in the pancreas with increasing inflammation (Aghdassi et al., 2011; Su, Cuthbertson, & Christophi, 2006).

If these models are established and an increase in 2-AAA production was observed, interventions to prevent the 2-AAA production could be employed. Antioxidant or anti-inflammatory treatment would be first candidates for reducing 2-AAA production via macrophages or neutrophils. If a proof on concept is required, treatment with anti-rejection medication such as alemtuzumab to significantly reduce lymphocyte mediated leukocyte activation may be employed.

In humans it is unlikely that prospective studies inducing inflammatory states could be completed. Therefore prospectively, only antioxidant or anti-inflammatory treatments would likely be approved. Prospective or retrospective observational population studies examining the extremes of inflammation may be a useful source of data for this investigation. Blood samples from patients with extreme inflammatory conditions such as multiple sclerosis or patients with extreme reductions in any inflammation such as those receiving alemtuzumab anti-rejection therapy for an auto-immune condition could be

examined. Patients with constantly activated leukocytes would be expected to have much higher 2-AAA production and patients lacking most of their leukocyte population would be expected to have much lower 2-AAA production. If clear differences in 2-AAA levels are observed in these most extreme states of inflammation, then less extreme but more common inflammatory conditions such as obesity could be examined for changes in 2-AAA production. If changes in 2-AAA levels are found, then prospective studies examining potential effects of antioxidant or anti-inflammatory treatments that were promising in mouse studies can be tested in obese or pre-diabetic patients to see if they reduce 2-AAA levels.

Many of these studies have the potential benefit of being completable alongside other investigations. They may even be investigated retroactivity if a study has an appropriate population and blood samples already collected (T. J. Wang et al., 2013) or if the study had an appropriate intervention. (Razquin et al., 2019).

## Conclusion

2-AAA is a long-term biomarker for the development of diabetes. This work has investigated why that is the case. As would be suggested by 2-AAA's effects in glial cells of the nervous system, 2-AAA treatment reduces cultured β-cell health and this effect is likely due to a reduction in total glutathione levels. Contrary to previous results in mice, no change in insulin secretion was seen with 2-AAA treatment in human islets, though it is possible that this result is partially dependent on the donor ages of the islets used in this study. Reductions in human islet total glutathione levels with 2-AAA treatment was observed, but no concurrent increase in $H_2O_2$ production was observed. The cellular composition and oxidative resilience of human islets compared with mouse islets may account for the lack of increase in $H_2O_2$ production. This lack of increased $H_2O_2$ production may account for the lack of an insulin secretion effect.

It is possible that cultured human islets may not be an ideal model for studying the effect of 2-AAA in humans, as the time till the effect could be seen may be longer than the viability time of most cultured human islets. Future studies could examine 2-AAA treatment of xenotransplanted human islets to examine a longer treatment time or 2-AAA treatment of high β-cell content human pseudo islets to examine a model of potentially higher ROS susceptibility. Due to the negative correlation found between age and 2-AAA induced low glucose human islet insulin secretion from male donors, future studies on 2-AAA in mice should consider using older mice of both sexes. To examine the source of 2-AAA production, future studies may consider modifying salt and lysine levels in the diet or modifying the inflammatory state, particularly leukocyte activation, of the model in question.

This project is one step towards a fuller understanding of 2-AAA's effects on the development of diabetes, all to work toward a point where it is possible to prevent the subset of non-diabetic patients with high 2-AAA levels from developing diabetes in the future.

**References**


Agarwal, A., Hegde, A., Yadav, C., Ahmad, A., Manjrekar, P. A., & Srikantiah, R. M. (2017). Association of Interleukin-6 and Myeloperoxidase with Insulin Resistance in Impaired Fasting Glucose Subjects. *Indian Journal of Clinical Biochemistry*. https://doi.org/10.1007/s12291-016-0567-8

Aghdassi, A. A., Mayerle, J., Christochowitz, S., Weiss, F. U., Sendler, M., & Lerch, M. M. (2011, December 1). Animal models for investigating chronic pancreatitis. *Fibrogenesis and Tissue Repair*, Vol. 4, p. 26. https://doi.org/10.1186/1755-1536-4-26

Atkinson, M. A., Eisenbarth, G. S., & Michels, A. W. (2014). Type 1 diabetes. *The Lancet*, Vol. 383, pp. 69–82. https://doi.org/10.1016/S0140-6736(13)60591-7

Balise, V. D., Cornelius-Green, J. N., Parmenter, B., Baxter, S., Kassotis, C. D., Rector, R. S., … Nagel, S. C. (2019). Developmental Exposure to a Mixture of Unconventional Oil and Gas Chemicals Increased Risk-Taking Behavior, Activity and Energy Expenditure in Aged Female Mice After a Metabolic Challenge. *Frontiers in Endocrinology*, *10*, 460. https://doi.org/10.3389/fendo.2019.00460

Bourne, R. R. A., Stevens, G. A., White, R. A., Smith, J. L., Flaxman, S. R., Price, H., … Taylor, H. R. (2013). Causes of vision loss worldwide, 1990-2010: A systematic analysis. *The Lancet Global Health*, *1*(6). https://doi.org/10.1016/S2214-109X(13)70113-X

Brissova, M., Fowler, M. J., Nicholson, W. E., Chu, A., Hirshberg, B., Harlan, D. M., & Powers, A. C. (2005). Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *Journal of Histochemistry and Cytochemistry*, *53*(9), 1087–1097. https://doi.org/10.1369/jhc.5C6684.2005

Cabrera, O., Berman, D. M., Kenyon, N. S., Ricordi, C., Berggren, P. O., & Caicedo, A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(7), 2334–2339. https://doi.org/10.1073/pnas.0510790103

Camunas-Soler, J., Dai, X., Hang, Y., Bautista, A., Lyon, J., Suzuki, K., … MacDonald, P. E. (2019). Pancreas patch-seq links physiologic dysfunction in diabetes to single-cell transcriptomic phenotypes. *BioRxiv*, 555110. https://doi.org/10.1101/555110

Cannon, A., Handelsman, Y., Heile, M., & Shannon, M. (2018). Burden of Illness in Type 2 diabetes mellitus. *Journal of Managed Care and Specialty Pharmacy*, *24*(9), S5–S13. https://doi.org/10.18553/jmcp.2018.24.9-a.s5

Cox, A. R., Lam, C. J., Rankin, M. M., King, K. A., Chen, P., Martinez, R., … Kushner, J. A. (2016). Extreme obesity induces massive beta cell expansion in mice through self-renewal and does not alter the beta cell lineage. *Diabetologia*, *59*(6), 1231–1241. https://doi.org/10.1007/s00125-016-3922-7

da Silva, J. C., Amaral, A. U., Cecatto, C., Wajner, A., Dos Santos Godoy, K., Ribeiro, R. T., … Wajner, M. (2017). α-Ketoadipic Acid and α-Aminoadipic Acid Cause Disturbance of Glutamatergic Neurotransmission and Induction of Oxidative Stress In Vitro in Brain of Adolescent Rats. *Neurotoxicity Research*, *32*(2), 276–290. https://doi.org/10.1007/s12640-017-9735-8

Dankner, R., Chetrit, A., Shanik, M. H., Raz, I., & Roth, J. (2012). Basal state hyperinsulinemia in healthy normoglycemic adults heralds dysglycemia after more than two decades of follow up. *Diabetes/Metabolism Research and Reviews*, *28*(7), 618–624. https://doi.org/10.1002/dmrr.2322

Daugherty, A., Dunn, J. L., Rateri, D. L., & Heinecke, J. W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *Journal of Clinical Investigation*, *94*(1), 437–444. https://doi.org/10.1172/JCI117342

de Ritter, R., de Jong, M., Vos, R. C., van der Kallen, C. J. H., Sep, S. J. S., Woodward, M., … Peters, S. A. E. (2020, January 3). Sex differences in the risk of vascular disease associated with diabetes. *Biology of Sex Differences*, Vol. 11, p. 1. https://doi.org/10.1186/s13293-019-0277-z

DeFronzo, R. A. (2004, July). Pathogenesis of type 2 diabetes mellitus. *Medical Clinics of North America*, Vol. 88, pp. 787–835. https://doi.org/10.1016/j.mcna.2004.04.013

Del Prato, S. (1992). [Hyperinsulinism. Causes and mechanisms]. *Presse Medicale (Paris, France : 1983)*, *21*(28), 1312–1317. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1332021

Di Cairano, E. S., Davalli, A. M., Perego, L., Sala, S., Sacchi, V. F., La Rosa, S., … Perego, C. (2011). The glial glutamate transporter 1 (GLT1) is expressed by pancreatic β-cells and prevents glutamate-induced β-cell death. *Journal of Biological Chemistry*, *286*(16), 14007–14018. https://doi.org/10.1074/jbc.M110.183517

Ehses, J. A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J. A., Maor-Cahn, R., … Donath, M. Y. (2007). Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes*, *56*(9), 2356–2370. https://doi.org/10.2337/db06-1650

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J., & Paquot, N. (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Research and Clinical Practice*, Vol. 105, pp. 141–150. https://doi.org/10.1016/j.diabres.2014.04.006

Faulk, C., Barks, A., Sánchez, B. N., Zhang, Z., Anderson, O. S., Peterson, K. E., & Dolinoy, D. C. (2014). Perinatal lead (Pb) exposure results in sex-specific effects on food intake, fat, weight, and insulin response across the murine life-course. *PLoS ONE*, *9*(8), e104273. https://doi.org/10.1371/journal.pone.0104273

Fox, J., Barthold, S., Davisson, M., Newcomer, C., Quimby, F., & Smith, A. (Eds.). (2006). *The Mouse in Biomedical Research* (2nd ed.). Retrieved from https://www.elsevier.com/books/the-mouse-in-biomedical-research/fox/978-0-12-369454-6

Gale, E. A. M., & Gillespie, K. M. (2001). Diabetes and gender. *Diabetologia*, Vol. 44, pp. 3–15. https://doi.org/10.1007/s001250051573

Gnatiuc, L., Herrington, W. G., Halsey, J., Tuomilehto, J., Fang, X., Kim, H. C., … Zhang, X. H. (2018). Sex-specific relevance of diabetes to occlusive vascular and other mortality: a collaborative meta-analysis of individual data from 980 793 adults from 68 prospective studies. *The Lancet Diabetes and Endocrinology*, *6*(7), 538–546. https://doi.org/10.1016/S2213-8587(18)30079-2

Gruessner, A., & Sutherland, D. E. (1996). Pancreas transplantation in the United States (US) and non-US as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR). *Clinical Transplants*, 47–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9286558

Guthrie, R. A., & Guthrie, D. W. (2004). Pathophysiology of Diabetes Mellitus. *Critical Care Nursing Quarterly*, Vol. 27, pp. 113–125. https://doi.org/10.1097/00002727-200404000-00003

Higashino, K., Fujioka, M., & Yamamura, Y. (1971). The conversion of L-lysine to saccharopine and alpha-

aminoadipate in mouse. *Archives of Biochemistry and Biophysics*, *142*(2), 606–614. https://doi.org/10.1016/0003-9861(71)90525-x

Janjic, D., & Wollheim, C. B. (1992). Effect of 2-mercaptoethanol on glutathione levels, cystine uptake and insulin secretion in insulin-secreting cells. *European Journal of Biochemistry*, *210*(1), 297–304. https://doi.org/10.1111/j.1432-1033.1992.tb17421.x

Jing, X., Chen, J., Dong, Y., Han, D., Zhao, H., Wang, X., … Ma, J. (2018). Related factors of quality of life of type 2 diabetes patients: A systematic review and meta-analysis 11 Medical and Health Sciences 1117 Public Health and Health Services 11 Medical and Health Sciences 1103 Clinical Sciences. *Health and Quality of Life Outcomes*, *16*(1), 189. https://doi.org/10.1186/s12955-018-1021-9

Kamata, K., Mizukami, H., Inaba, W., Tsuboi, K., Tateishi, Y., Yoshida, T., & Yagihashi, S. (2014). Islet amyloid with macrophage migration correlates with augmented β-cell deficits in type 2 diabetic patients. *Amyloid : The International Journal of Experimental and Clinical Investigation : The Official Journal of the International Society of Amyloidosis*, *21*(3), 191–201. https://doi.org/10.3109/13506129.2014.937857

Kato, S., Ishita, S., Sugawara, K., & Mawatari, K. (1993). Cystine/glutamate antiporter expression in retinal Müller glial cells: implications for DL-alpha-aminoadipate toxicity. *Neuroscience*, *57*(2), 473–482. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7906874

Kato, S., Negishi, K., Mawatari, K., & Kuo, C. H. (1992). A mechanism for glutamate toxicity in the C6 glioma cells involving inhibition of cystine uptake leading to glutathione depletion. *Neuroscience*, *48*(4), 903–914. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1352866

Kim, D. H., Sandoval, D., Reed, J. A., Matter, E. K., Tolod, E. G., Woods, S. C., & Seeley, R. J. (2008). The role of GM-CSF in adipose tissue inflammation. *American Journal of Physiology - Endocrinology and Metabolism*, *295*(5), E1038-46. https://doi.org/10.1152/ajpendo.00061.2008

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., … Bolton, E. E. (2019). PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research*, *47*(D1), D1102–D1109. https://doi.org/10.1093/nar/gky1033

Komatsu, H., Cook, C., Wang, C. H., Medrano, L., Lin, H., Kandeel, F., … Mullen, Y. (2017). Oxygen environment and islet size are the primary limiting factors of isolated pancreatic islet survival. *PLoS ONE*, *12*(8), e0183780. https://doi.org/10.1371/journal.pone.0183780

Koska, J., Saremi, A., Howell, S., Bahn, G., De Courten, B., Ginsberg, H., … Reaven, P. D. (2018). Advanced glycation end products, oxidation products, and incident cardiovascular events in patients with type 2 diabetes. *Diabetes Care*. https://doi.org/10.2337/dc17-1740

La Merrill, M., Karey, E., Moshier, E., Lindtner, C., La Frano, M. R., Newman, J. W., & Buettner, C. (2014). Perinatal exposure of mice to the pesticide DDT impairs energy expenditure and metabolism in adult female offspring. *PLoS ONE*, *9*(7), e103337. https://doi.org/10.1371/journal.pone.0103337

Lee, H. J., Jang, H. B., Kim, W. H., Park, K. J., Kim, K. Y., Park, S. I., & Lee, H. J. (2019). 2-Aminoadipic acid (2-AAA) as a potential biomarker for insulin resistance in childhood obesity. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-49578-z

Leloup, C., Tourrel-Cuzin, C., Magnan, C., Karaca, M., Castel, J., Carneiro, L., … Pénicaud, L. (2009). Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. *Diabetes*. https://doi.org/10.2337/db07-1056

Lenzen, S., Drinkgern, J., & Tiedge, M. (1996). Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radical Biology and Medicine*, *20*(3), 463–466. https://doi.org/10.1016/0891-5849(96)02051-5

Libert, D. M., Nowacki, A. S., & Natowicz, M. R. (2018). Metabolomic analysis of obesity, metabolic syndrome, and type 2 diabetes: amino acid and acylcarnitine levels change along a spectrum of metabolic wellness. *PeerJ*, *6*, e5410. https://doi.org/10.7717/peerj.5410

Lin, H., Levison, B. S., Buffa, J. A., Huang, Y., Fu, X., Wang, Z., … Hazen, S. L. (2017). Myeloperoxidase-mediated protein lysine oxidation generates 2-aminoadipic acid and lysine nitrile in vivo. *Free Radical Biology and Medicine*. https://doi.org/10.1016/j.freeradbiomed.2017.01.006

Linnemann, A. K., Baan, M., & Davis, D. B. (2014). Pancreatic β-Cell Proliferation in Obesity1,2. *Advances in Nutrition*, *5*(3), 278–288. https://doi.org/10.3945/an.113.005488

Liu, W., Kin, T., Ho, S., Dorrell, C., Campbell, S. R., Luo, P., & Chen, X. (2019). Abnormal regulation of glucagon secretion by human islet alpha cells in the absence of beta cells. *EBioMedicine*, *50*, 306–316. https://doi.org/10.1016/j.ebiom.2019.11.018

Lontchi-Yimagou, E., Sobngwi, E., Matsha, T. E., & Kengne, A. P. (2013). Diabetes mellitus and inflammation. *Current Diabetes Reports*, *13*(3), 435–444. https://doi.org/10.1007/s11892-013-0375-y

Magliano, D. J., Islam, R. M., Barr, E. L. M., Gregg, E. W., Pavkov, M. E., Harding, J. L., … Shaw, J. E. (2019). Trends in incidence of total or type 2 diabetes: Systematic review. *The BMJ*, *366*, l5003. https://doi.org/10.1136/bmj.l5003

Malone, J. I., & Hansen, B. C. (2019, February 1). Does obesity cause type 2 diabetes mellitus (T2DM)? Or is it the opposite? *Pediatric Diabetes*, Vol. 20, pp. 5–9. https://doi.org/10.1111/pedi.12787

Matthews, D. R., Cull, C. A., Stratton, I. M., Holman, R. R., & Turner, R. C. (1998). UKPDS 26: Sulphonylurea failure in non-insulin-dependent diabetic patients over six years. *Diabetic Medicine*, *15*(4), 297–303. https://doi.org/10.1002/(SICI)1096-9136(199804)15:4<297::AID-DIA572>3.0.CO;2-W

Melo, E. P., Lopes, C., Gollwitzer, P., Lortz, S., Lenzen, S., Mehmeti, I., … Avezov, E. (2017). TriPer, an optical probe tuned to the endoplasmic reticulum tracks changes in luminal H2O2. *BMC Biology*, *15*(1), 24. https://doi.org/10.1186/s12915-017-0367-5

Murphy, T. H., Miyamoto, M., Sastre, A., Schnaar, R. L., & Coyle, J. T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron*, *2*(6), 1547–1558. https://doi.org/10.1016/0896-6273(89)90043-3

Nathan, D., Genuth, S., Lachin, J., Cleary, P., Crofford, O., Davis, M., … Siebert, C. (1993). The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus. *New England Journal of Medicine*, *329*(14), 977–986. https://doi.org/10.1056/NEJM199309303291401

Neshich, I. A. P., Kiyota, E., & Arruda, P. (2013). Genome-wide analysis of lysine catabolism in bacteria reveals new connections with osmotic stress resistance. *The ISME Journal*, *7*(12), 2400–2410. https://doi.org/10.1038/ismej.2013.123

Nicol, L. E., Grant, W. F., Comstock, S. M., Nguyen, M. L., Smith, M. S., Grove, K. L., & Marks, D. L. (2013).

Pancreatic inflammation and increased islet macrophages in insulin-resistant juvenile primates. *Journal of Endocrinology*, *217*(2), 207–213. https://doi.org/10.1530/JOE-12-0424

Pepper, A. R., Bruni, A., Pawlick, R., Wink, J., Rafiei, Y., Gala-Lopez, B., … Shapiro, A. M. J. (2017). Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation*, *101*(10), 2321–2329. https://doi.org/10.1097/TP.0000000000001638

Pfeiffer, A. F. H., & Klein, H. H. (2014, January 31). Therapie des diabetes mellitus typ 2. *Deutsches Arzteblatt International*, Vol. 111, pp. 69–82. https://doi.org/10.3238/arztebl.2014.0069

Pi, J., Bai, Y., Zhang, Q., Wong, V., Floering, L. M., Daniel, K., … Collins, S. (2007). Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes*, *56*(7), 1783–1791. https://doi.org/10.2337/db06-1601

Pines, G., Danbolt, N. C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., … Kanner, B. I. (1992). Cloning and expression of a rat brain L-glutamate transporter. *Nature*, *360*(6403), 464–467. https://doi.org/10.1038/360464a0

Plecitá-Hlavatá, L., Jabůrek, M., Holendová, B., Tauber, J., Pavluch, V., Berková, Z., … Ježek, P. (2020). Glucose-Stimulated Insulin Secretion Fundamentally Requires H 2 O 2 Signaling by NADPH Oxidase 4 . *Diabetes*, db191130. https://doi.org/10.2337/db19-1130

Plubell, D. L., Fenton, A. M., Wilmarth, P. A., Bergstrom, P., Zhao, Y., Minnier, J., … Pamir, N. (2018). GM-CSF driven myeloid cells in adipose tissue link weight gain and insulin resistance via formation of 2-aminoadipate. *Scientific Reports*. https://doi.org/10.1038/s41598-018-29250-8

Razquin, C., Ruiz-Canela, M., Clish, C. B., Li, J., Toledo, E., Dennis, C., … Martínez-González, M. A. (2019). Lysine pathway metabolites and the risk of type 2 diabetes and cardiovascular disease in the PREDIMED study: Results from two case-cohort studies. *Cardiovascular Diabetology*, *18*(1). https://doi.org/10.1186/s12933-019-0958-2

Reichelt, W., Stabel-Burow, J., Pannicke, T., Weichert, H., & Heinemann, U. (1997). The glutathione level of retinal Müller glial cells is dependent on the high-affinity sodium-dependent uptake of glutamate. *Neuroscience*, *77*(4), 1213–1224. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9130799

Richardson, S. J., Willcox, A., Bone, A. J., Foulis, A. K., & Morgan, N. G. (2009). Islet-associated macrophages in type 2 diabetes. *Diabetologia*, *52*(8), 1686–1688. https://doi.org/10.1007/s00125-009-1410-z

Rodrigues, M. R., Rodriguez, D., Russo, M., & Campa, A. (2002). Macrophage activation includes high intracellular myeloperoxidase activity. *Biochemical and Biophysical Research Communications*, *292*(4), 869–873. https://doi.org/10.1006/bbrc.2002.6724

Russell, T. R., Noel, J., Files, N., Ingram, M., & Rabinovitch, A. (1984). Purification of beta cells from rat islets by monoclonal antibody-fluorescence flow cytometry. *Cytometry*, *5*(5), 539–542. https://doi.org/10.1002/cyto.990050517

Saremi, A., Howell, S., Schwenke, D. C., Bahn, G., Beisswenger, P. J., Reaven, P. D., & VADT Investigators. (2017). Advanced Glycation End Products, Oxidation Products, and the Extent of Atherosclerosis During the VA Diabetes Trial and Follow-up Study. *Diabetes Care*, *40*(4), 591–598. https://doi.org/10.2337/dc16-1875

Seemann, S., Zohles, F., & Lupp, A. (2017). Comprehensive comparison of three different animal models for systemic inflammation. *Journal of Biomedical Science*, *24*(1). https://doi.org/10.1186/s12929-017-0370-8

Shanik, M. H., Xu, Y., Skrha, J., Dankner, R., Zick, Y., & Roth, J. (2008). Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? *Diabetes Care*. https://doi.org/10.2337/dc08-s264

Shapiro, A. M. J., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., … Lakey, J. R. T. (2006). International trial of the Edmonton protocol for islet transplantation. *New England Journal of Medicine*, *355*(13), 1318–1330. https://doi.org/10.1056/NEJMoa061267

Stevens, J. W., Khunti, K., Harvey, R., Johnson, M., Preston, L., Woods, H. B., … Goyder, E. (2015, March 1). Preventing the progression to Type 2 diabetes mellitus in adults at high risk: A systematic review and network meta-analysis of lifestyle, pharmacological and surgical interventions. *Diabetes Research and Clinical Practice*, Vol. 107, pp. 320–331. https://doi.org/10.1016/j.diabres.2015.01.027

Su, K. H., Cuthbertson, C., & Christophi, C. (2006, August). Review of experimental animal models of acute pancreatitis. *HPB*, Vol. 8, pp. 264–286. https://doi.org/10.1080/13651820500467358

Sujatha, R. (2015). *Plasma Myeloperoxidase levels in Type-2 Diabetes Mellitus.* (PSG Institute of Medical sciences and Research, Coimbatore). Retrieved from http://repository-tnmgrmu.ac.in/7560/

Sun, T., & Han, X. (2019, December 9). Death versus dedifferentiation: The molecular bases of beta cell mass reduction in type 2 diabetes. *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2019.12.002

Taddeo, E. P., Alsabeeh, N., Baghdasarian, S., Wikstrom, J. D., Ritou, E., Sereda, S., … Shirihai, O. S. (2020). Mitochondrial proton leak regulated by Cyclophilin D elevates insulin secretion in islets at nonstimulatory glucose levels. *Diabetes*, *69*(2), 131–145. https://doi.org/10.2337/db19-0379

Thomas, M. C., Cooper, M. E., & Zimmet, P. (2016, February 1). Changing epidemiology of type 2 diabetes mellitus and associated chronic kidney disease. *Nature Reviews Nephrology*, Vol. 12, pp. 73–81. https://doi.org/10.1038/nrneph.2015.173

Tsai, M. J., Chang, Y. F., Schwarcz, R., & Brookes, N. (1996). Characterization of L-alpha-aminoadipic acid transport in cultured rat astrocytes. *Brain Research*, *741*(1–2), 166–173. https://doi.org/10.1016/s0006-8993(96)00910-9

Turner, R. (1998). Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*, *352*(9131), 854–865. https://doi.org/10.1016/S0140-6736(98)07037-8

Wang, H., Ren, Q., Han, X., Chen, J., Zhou, L., Chen, Y., & Ji, L. (2017). Factors of primary and secondary sulfonylurea failure in type 2 diabetic subjects. *Journal of Diabetes*, *9*(12), 1091–1099. https://doi.org/10.1111/1753-0407.12542

Wang, T. J., Larson, M. G., Vasan, R. S., Cheng, S., Rhee, E. P., McCabe, E., … Gerszten, R. E. (2011). Metabolite profiles and the risk of developing diabetes. *Nature Medicine*, *17*(4), 448–453. https://doi.org/10.1038/nm.2307

Wang, T. J., Ngo, D., Psychogios, N., Dejam, A., Larson, M. G., Vasan, R. S., … Gerszten, R. E. (2013). 2-

Aminoadipic acid is a biomarker for diabetes risk. *Journal of Clinical Investigation*, *123*(10), 4309–4317. https://doi.org/10.1172/JCI64801

Welsh, N., Margulis, B., Borg, L. A., Wiklund, H. J., Saldeen, J., Flodström, M., … Hellerström, C. (1995). Differences in the expression of heat-shock proteins and antioxidant enzymes between human and rodent pancreatic islets: implications for the pathogenesis of insulin-dependent diabetes mellitus. *Molecular Medicine (Cambridge, Mass.)*, *1*(7), 806–820. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8612203

Xu, W.-Y., Shen, Y., Zhu, H., Gao, J., Zhang, C., Tang, L., … Wang, Z.-G. (2019). 2-Aminoadipic acid protects against obesity and diabetes. *The Journal of Endocrinology*. https://doi.org/10.1530/JOE-19-0157

Xu, W.-Y., Zhu, H., Shen, Y., Wan, Y.-H., Tu, X.-D., Wu, W.-T., … Wang, Z.-G. (2018). DHTKD1 deficiency causes Charcot-Marie-Tooth disease in mice. *Molecular and Cellular Biology*, *38*(13), MCB.00085-18. https://doi.org/10.1128/MCB.00085-18

Yu, Y., Gamble, A., Pawlick, R., Pepper, A. R., Salama, B., Toms, D., … Ungrin, M. (2018). Bioengineered human pseudoislets form efficiently from donated tissue, compare favourably with native islets in vitro and restore normoglycaemia in mice. *Diabetologia*, *61*(9), 2016–2029. https://doi.org/10.1007/s00125-018-4672-5

Zhu, S. N., Chen, M., Jongstra-Bilen, J., & Cybulsky, M. I. (2009). GM-CSF regulates intimal cell proliferation in nascent atherosclerotic lesions. *Journal of Experimental Medicine*, *206*(10), 2141–2149. https://doi.org/10.1084/jem.20090866

Zuellig, R. A., Cavallari, G., Gerber, P., Tschopp, O., Spinas, G. A., Moritz, W., & Lehmann, R. (2017). Improved physiological properties of gravity-enforced reassembled rat and human pancreatic pseudo-islets. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(1), 109–120. https://doi.org/10.1002/term.1891