PROHORMONE PROCESSING IN PANCREATIC ISLET TRANSPLANTATION

by

AGNIESZKA MAGDALENA KLIMEK
B.Sc., The University of Victoria, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2010

© Agnieszka Magdalena Klimek, 2010
ABSTRACT

Islet transplantation is a promising treatment for diabetes; however, most transplant recipients exhibit progressive loss of graft function. Islet function in transplant recipients shares similarities with subjects with type 2 diabetes including impaired glucose-stimulated insulin secretion, decreased beta-cell mass associated with amyloid formation, and defective proinsulin processing resulting in disproportionate secretion of intact proinsulin and proinsulin intermediates. We hypothesized that processing of the beta-cell prohormones, proinsulin and pro-islet amyloid polypeptide (proIAPP), will be impaired in islet transplant recipients as in patients with type 2 diabetes.

Human islet transplant recipients were found to have impaired proinsulin processing manifest as elevated proinsulin/C-peptide ratios (TP/CP). The TP/CP ratio was significantly elevated in both islet allo- and auto-transplant recipients relative to controls. Furthermore, the TP/CP was greater in those recipients that received sub-optimal numbers of islets transplanted, suggesting that beta-cell dysfunction is exacerbated in the face of increased secretory demand due to insufficient islet mass. In a mouse model of islet transplantation, proinsulin processing was found to decline over time following transplantation, resulting in elevated proinsulin/insulin ratios. Amyloid deposits, a common pancreatic lesion in type 2 diabetes, were also found in human islet transplants and were associated with reduced beta-cell mass.

Since IAPP, like insulin, is also processed within the beta cell from its precursor proIAPP, and since proinsulin processing is impaired in islet transplants and type 2 diabetes, we hypothesized that proIAPP processing will also be impaired in these conditions. To quantify proIAPP levels in humans, an immunoassay was developed. Circulating proIAPP levels in normal subjects were found to be in the low picomolar range and the ratio of proIAPP/IAPP was approximately 30%. In a small cohort of type 2 diabetic subjects, the proIAPP/IAPP ratio tended to be lower.

These studies demonstrate that impaired proinsulin processing is a characteristic of transplanted islets and that the proinsulin/insulin or proinsulin/C-peptide ratios may serve as markers of graft dysfunction in islet transplantation. In addition, the proIAPP/IAPP ratio can now also be evaluated as a marker of beta-cell dysfunction in islet transplants and type 2 diabetes.
PREFACE

Ethics approval was obtained for collection and use of human samples from the UBC C&W Research Ethics Boards (Ethics Certificates # H06-03112, #H03-70453).

The study presented in Chapter 3 of this work was published online in the American Journal of Transplantation on August 20, 2009 (submitted February 27, 2009, revised April 24, 2009, accepted for publication May 18, 2009). Klimek AM designed the study, performed the research and data analysis, and wrote the manuscript.


A portion of the study presented in Chapter 4 contributed to a manuscript published the Proceedings of the National Academy of Sciences. Klimek AM monitored the transplant experiments, harvested samples and immunostained samples that contributed to a figure that is presented as Figure 18 of this manuscript in section 4.2.5.


A portion of the study presented in Chapter 4 contributed to a manuscript published in Cell Transplantation. Klimek AM provided a figure for the manuscript that is presented as Figure 21 in section 4.2.6 of this manuscript.

TABLE OF CONTENTS

ABSTRACT .............................................................................................................................. ii
PREFACE ................................................................................................................................... iii
TABLE OF CONTENTS .............................................................................................................. iv
LIST OF TABLES ........................................................................................................................ vii
LIST OF FIGURES .................................................................................................................... viii
ABBREVIATIONS ..................................................................................................................... x
ACKNOWLEDGEMENTS ........................................................................................................... xii
DEDICATION ............................................................................................................................ xiii

CHAPTER 1. INTRODUCTION ................................................................................................ 1

1.1 Diabetes mellitus .............................................................................................................. 1
  1.1.1 Background ................................................................................................................ 1
  1.1.2 Genetics of diabetes mellitus .................................................................................... 2
  1.1.3 Environmental component of diabetes mellitus ......................................................... 4
  1.1.4 Relationship between insulin sensitivity and insulin secretion ............................... 4
  1.1.5 Beta-cell mass in health and type 2 diabetes ............................................................ 6
  1.1.6 Causes of progressive beta-cell loss and dysfunction in type 2 diabetes ............... 8
  1.1.7 Islet amyloid, IAPP and type 2 diabetes ................................................................. 12
  1.1.8 Processing of proinsulin in health and type 2 diabetes ........................................... 13
  1.1.9 Normal processing of pro islet amyloid polypeptide .............................................. 15
  1.1.10 Progression of type 2 diabetes ............................................................................... 18

1.2 Pancreatic islet transplantation ....................................................................................... 18
  1.2.1 Background .............................................................................................................. 18
  1.2.2 Current status of clinical islet transplantation ....................................................... 19
  1.2.3 Factors contributing to islet graft failure ............................................................... 21

1.3 Beta-cell dysfunction in type 2 diabetes and islet transplantation ................................ 30

1.4 Thesis hypothesis and objectives .................................................................................... 31

CHAPTER 2. MATERIALS & METHODS ............................................................................ 32

2.1 Chemicals and buffers .................................................................................................... 32
2.2 Animals ............................................................................................................................ 33
2.3 Islet transplantation ......................................................................................................... 33
2.4 In-vivo immunosuppression.................................................................34
2.5 Clinical study subjects................................................................35
2.6 Assays...............................................................................................36
2.7 Immunohistochemistry and thioflavin-S staining.................................38
2.8 Antibodies.........................................................................................39
2.9 Time-resolved fluorescent proIAPP immunoassay (TR-FIA) ...............41
2.10 Meso Scale Discovery electrochemiluminescent proIAPP immunoassay (ECL).....41
2.11 Standards and samples for proIAPP immunoassay...............................42
2.12 Calculations and statistical analysis..................................................43

CHAPTER 3. IMPAIRED PROINSULIN PROCESSING IS A CHARACTERISTIC OF
TRANSPLANTED ISLETS............................................................................44
3.1 Background........................................................................................44
3.2 Results................................................................................................45
  3.2.1 Clinical characteristics of study subjects........................................45
  3.2.2 Fasting levels of insulin, C-peptide, total proinsulin, intact proinsulin and
       proinsulin fragments in non-diabetic controls and islet transplant recipients....45
  3.2.3 Proinsulin/insulin, proinsulin/C-peptide and proinsulin fragments/total
       proinsulin ratios in islet transplant recipients............................................46
  3.2.4 Fasting levels of circulating beta-cell hormones in subjects with type 2 diabetes53
  3.2.5 Correlation of total proinsulin, C-peptide and the total proinsulin/C-peptide ratio
       in recipients of islet allo- and autografts....................................................53
  3.2.6 Impact of transplanted islet mass on total proinsulin/C-peptide ratios........55
3.3 Discussion..........................................................................................56

CHAPTER 4. INSUFFICIENT TRANSPLANTED BETA-CELL MASS CONTRIBUTES
TO IMPAIRED PROINSULIN PROCESSING IN ISLET GRAFT RECIPIENTS.......61
4.1 Background........................................................................................61
4.2 Results................................................................................................61
  4.2.1 Transplanted islet mass impacts on return to normoglycemia and on the
       efficiency of proinsulin processing..........................................................61
  4.2.2 Insulin secretion and proinsulin processing are reduced in normoglycemic
       recipients of human islet transplants........................................................63
  4.2.3 Association of hyperglycemia and proinsulin processing in graft failure......69
  4.2.4 Amyloid formation is associated with human islet graft failure..............71
4.2.5 Amyloid formation is associated with a lower islet transplant mass in normoglycemic islet grafts.................................................................72
4.2.6 Immunosuppressant effects on in vivo graft survival ........................................75
4.3 Discussion...........................................................................................................76

CHAPTER 5. PRO-ISLET AMYLOID POLYPEPTIDE IMMUNOASSAY
DEVELOPMENT ...................................................................................................'80
5.1 Background.......................................................................................................80
5.2 Results.............................................................................................................80
  5.2.1 Polyclonal rat-IAPP capture/monoclonal human NH2-terminal proIAPP
detection antibody TR-FIA. ..................................................................................81
  5.2.2 Monoclonal anti-human NH2-terminal proIAPP capture/polyclonal anti-human
IAPP detection antibody TR-FIA. .........................................................................87
  5.2.3 Meso Scale Discovery® electrochemiluminescent proIAPP immunoassay.......91
  5.2.4 Limits of sensitivity of TR-FIA and ECL immunoassays.................................94
5.3 Discussion.......................................................................................................94

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS ..................................99
REFERENCES........................................................................................................101
APPENDICES .........................................................................................................115
  Appendix A: Laboratory and ethics board certificates of approval....................115
  Appendix B: ProIAPP immunoassay development calculations..........................120
  Appendix C. Publications.....................................................................................122
LIST OF TABLES

Table 1. Cross-reactivities of commercially available immunoassays for human insulin, C-peptide, total and intact proinsulin.................................................................37
Table 2. Cross-reactivities of commercially available mouse insulin and proinsulin immunoassays............................................................................................................38
Table 3. Clinical characteristics of non-diabetic controls and islet transplant recipients....45
Table 5. Detection limits of TR-FIA and ECL proIAPP immunoassays........................................94
LIST OF FIGURES

Figure 1. Proinsulin processing in healthy pancreatic beta cells.........................................................14
Figure 2. Pathway for processing of proIAPP in beta cells...............................................................17
Figure 3. Potential factors limiting pancreatic islet graft function and survival.................................23
Figure 4. Binding regions of IAPP and proIAPP antibodies used in human proIAPP time-resolved fluorescent and electrochemiluminescent immunoassays.................................................40
Figure 5. Fasting serum levels of beta-cell hormones in non-diabetic controls and islet transplant recipients ..........................................................................................................................48
Figure 6. Proinsulin/insulin and proinsulin/C-peptide ratios islet transplant recipients ....49
Figure 7. Proinsulin/C-peptide ratios in insulin-dependent and insulin-independent allotransplant recipients ..................................................................................................................50
Figure 8. Proportion of circulating proinsulin comprised of proinsulin intermediates in serum of islet transplant recipients ..........................................................................................51
Figure 9. Pre- and 1-year post-transplant fasting insulin, C-peptide, proinsulin/insulin and the proinsulin/C-peptide ratios in autologous islet transplant recipients .........................................52
Figure 10. Relationship between fasting total proinsulin, C-peptide and the proinsulin/C-peptide ratios in islet transplant recipients ..................................................................................54
Figure 11. Recipients of > 10,000 IE/kg have lower proinsulin/C-peptide ratios .........................55
Figure 12. Glycemic control in STZ-induced diabetic C57Bl/6 mice transplanted with syngeneic donor islets ..................................................................................................................62
Figure 13. Proinsulin/insulin ratio is elevated in normoglycemic C57Bl/6 mice transplanted with a lower islet mass ..................................................................................................................65
Figure 14. Glycemic control in STZ-induced diabetic NOD.scid mice transplanted with human islets ............................................................................................................................66
Figure 15. Changes in circulating levels of insulin, proinsulin and the proinsulin/insulin ratios at 2 and 6 weeks post-transplant in NOD.scid mice transplanted with human islets.67
Figure 16. Proinsulin/insulin ratios at 2 and 6 weeks post-transplant in normoglycemic mice transplanted with human islets ..................................................................................................68
Figure 17. Marginal mass islet grafts that maintain normoglycemia have a lower proinsulin/insulin ratio .....................................................................................................................................70
Figure 18. Amyloid formation is associated with failed human islet grafts .................................71
Figure 19. Decreased beta-cell area and increased amyloid area are associated with lower islet transplant mass.................................................................73
Figure 20. Proinsulin/insulin ratio in STZ-induced diabetic NOD.scid mice transplanted with islets expressing hIAPP.................................................................74
Figure 21. Immunosuppressant effects on the function of human islets transplanted into diabetic recipient mice. .........................................................................................................................75
Figure 22. Typical proIAPP TR-FIA standard curves ..................................................81
Figure 23. Effect of serum on the proIAPP TR-FIA standard curve..........................82
Figure 24. Biological forms of human proIAPP are detectable by TR-FIA...............83
Figure 25. Human proIAPP in murine models of impaired proIAPP processing ........84
Figure 26. Proposed proIAPP cleavage by DPP-IV and inhibition by the DPP-IV inhibitor, P32/98. .................................................................................................................................85
Figure 27. Inhibition of proIAPP degradation in human serum using a DPP-IV inhibitor, P32/98. .................................................................................................................................86
Figure 28. IAPP sequences are well conserved across species while the NH₂-terminal flanking peptide region of proIAPP is not.................................................................87
Figure 29. Effect of serum on proIAPP standard curves..................................................88
Figure 30. Typical standard curves for human proIAPP TR-FIA........................................89
Figure 31. Effects of antibody orientation reversal in the proIAPP TR-FIA. .......................90
Figure 32. Representative ECL proIAPP standard curve................................................91
Figure 33. Detection of proIAPP in human serum samples in subjects with type 2 diabetes and non-diabetic controls.................................................................93
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADOPT</td>
<td>A Diabetes Outcome Progression Trial</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIRglucose</td>
<td>Acute insulin response to glucose</td>
</tr>
<tr>
<td>AlloTX</td>
<td>Allotransplant</td>
</tr>
<tr>
<td>AutoTX</td>
<td>Autotransplant</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor-6</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAPN-10</td>
<td>Calpain-10</td>
</tr>
<tr>
<td>CFRI</td>
<td>Child and Family Research Institute</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CP</td>
<td>C-peptide</td>
</tr>
<tr>
<td>CPE</td>
<td>Carboxypeptidase E</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FK506</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>GAD-65</td>
<td>Glutamic acid decarboxylate-65</td>
</tr>
<tr>
<td>GK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>Glucose transporter-2</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Hemoglobin A1c</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HHEX</td>
<td>Hematopoietically expressed homeobox</td>
</tr>
<tr>
<td>hIAPP</td>
<td>Human islet amyloid polypeptide</td>
</tr>
<tr>
<td>HNF-1α</td>
<td>Hepatocyte nuclear factor-1 alpha</td>
</tr>
<tr>
<td>HNF-1β</td>
<td>Hepatocyte nuclear factor-1 beta</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostasis model assessment-insulin resistance</td>
</tr>
<tr>
<td>I</td>
<td>Insulin</td>
</tr>
<tr>
<td>IA-2</td>
<td>Insulinoma-associated protein-2</td>
</tr>
<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
</tr>
<tr>
<td>IBMIR</td>
<td>Instant blood-mediated inflammatory response</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IE/kg</td>
<td>Islet equivalents per kilogram body weight</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IRE-1α</td>
<td>Inositol-requiring endoplasmic reticulum-to-nucleus signal kinase-1α</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDLT1D</td>
<td>Long-duration type 1 diabetes</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemo-attractant protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>ND</td>
<td>Non-diabetic</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD.scid</td>
<td>Non-obese diabetic/severe combined immunodeficiency mouse</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl glycine α-amidating mono-oxygenase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>PC1/3</td>
<td>Prohormone convertase 1/3</td>
</tr>
<tr>
<td>PC2</td>
<td>Prohormone convertase 2</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic duodenal homeobox 1</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PF</td>
<td>Proinsulin fragments</td>
</tr>
<tr>
<td>PI</td>
<td>Proinsulin</td>
</tr>
<tr>
<td>PI/PI+I</td>
<td>Proinsulin/insulin ratio</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>proIAPP</td>
<td>Pro-islet amyloid polypeptide</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDB</td>
<td>Sample dilution buffer</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLC30A8</td>
<td>Gene encoding zinc transporter ZnT8</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans golgi network</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TP</td>
<td>Total proinsulin</td>
</tr>
<tr>
<td>TP/CP</td>
<td>Total proinsulin/C-peptide ratio</td>
</tr>
<tr>
<td>TP/TP+I</td>
<td>Total proinsulin/insulin ratio ratio</td>
</tr>
<tr>
<td>TR-FIA</td>
<td>Time-resolved fluorescence immunoassay</td>
</tr>
<tr>
<td>TX</td>
<td>Transplant</td>
</tr>
<tr>
<td>UCP-2</td>
<td>Uncoupling protein-2</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type mouse</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
<tr>
<td>ZNT8</td>
<td>Zinc transport family member 8</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I had never planned to do a doctorate and I find myself in disbelief that it is coming to an end. It has been a long journey full of successes and disappointments, which was both draining and exciting. Aside from the science, this process has helped me grow as an individual and shaped me into the person that I am today.

My sincerest thanks, first off to Ray Pederson, without whom I would not be where I am now, with this big piece of science in hand. I cannot thank you enough for your encouragement to reach for higher ground and your friendship throughout the years. Bruce C. Verchere, my supervisor and mentor - I am so grateful that you were able to see past the “less than stellar grades” and took the plunge in taking me on as a student. I hope I have made you proud. You have provided me with an environment that thrives on scientific freedom and independence and have shown me the true meaning of balance in life. I could not have done this without your understanding and constant support, and above all your sense of humor.

I would also like to thank members of my committee, Chris McIntosh, Geoff Hammond and Cheryl Wellington for your guidance and support. Constadina Pangiotopoulos, thank you for always pushing me and including me in your studies that will be a memorable part of my PhD.

The road of a PhD is sometimes arduous and I am indebted to members of the Verchere lab for being there whenever I needed help. In particular, Derek Dai, thank you for the countless hours of transplants – you are an “animal”! Also, Galina Soukhatcheva – you have been integral not only to my research and the countless “mouse parties”, but also in helping me maintain some semblance of sanity when I really didn’t think I had much left. Finally, I wish to acknowledge those individuals whose close friendship, love and support through the past 5 years and beyond have made this quest possible. You know who you are – Melissa Mallett, Thea Robertson, Natalie Prystajecky – you ladies have kept me sane and have reminded me that there is wine and life outside the lab. My “twin”, Meredith Hutton – you have been more than just a colleague; you’ve been a friend when I needed it the most.

To my family - Mamo, Tato and Doda - thank you so much for your enthusiasm and always believing in me. I have been so fortunate to be raised by two amazing people who are so open to new experiences and adventures, and whose value of education had pushed me to succeed.

Finally, to my husband Tim, you have been a constant source of support in life and especially during this PhD. Thank you from the bottom of my heart for your love, patience and encouragement. You have been my biggest fan and I cannot imagine what I would have done without you.
DEDICATION

To my parents, Violetta & Andrzej Klimek
To my husband, Tim Abercrombie
CHAPTER 1. INTRODUCTION

1.1 Diabetes mellitus

1.1.1 Background

Diabetes mellitus is a complex metabolic disorder with hyperglycemia as the underlying feature resulting from defective insulin secretion, action or both [1]. Two major forms of diabetes exist: type 1 and type 2. Type 1 diabetes, formerly known as insulin-dependent diabetes mellitus or “juvenile-onset” diabetes, typically presents in children under the age of 20 years. It is an autoimmune disorder that results in the destruction of insulin-producing pancreatic beta cells and its progression is marked by varying levels of insulitis and eventual diabetes primarily as a result of absolute insulin deficiency [2]. Type 2 diabetes, also know as “adult-onset” diabetes or non-insulin dependent diabetes mellitus, is a progressive disease that results in chronic hyperglycemia. Most individuals with type 2 diabetes are over the age of 30; however, more recently, there is increasing prevalence of type 2 diabetes in children and adolescents [3, 4]. Type 2 diabetes is also characterized by relative insulin deficiency and by the presence of insulin resistance in peripheral tissues resulting in defective insulin action.

Chronic hyperglycemia in both type 1 and type 2 diabetes can lead to several long-term macrovascular and microvascular complications. Diabetes has been shown to elevate risk for macrovascular complications such as coronary artery disease and stroke, and microvascular complications such as retinopathy, nephropathy and neuropathy can lead to blindness, renal failure and limb amputation [5, 6].

The incidence of diabetes is increasing worldwide. According to the World Health Organization, type 2 diabetes accounts for 90% of all cases [7]. It was estimated that 150 million people worldwide had type 2 diabetes in the year 2000 and the number of cases was predicted to reach 300 million by 2025 [4]. Approximately 2 million Canadians are afflicted with this disease [8]. As a result, diabetes has a significant economic impact on individuals, families and the health care system and by the year 2020 it has been estimated that the cost to the Canadian health care system will average $16.9 billion per year [9]. Both genetic and environmental factors in concert contribute to the etiology of type 2 diabetes; however, the specific nature of these interactions is yet to be fully elucidated.
1.1.2 Genetics of diabetes mellitus

A strong genetic component of type 2 diabetes has been found in family studies and certain ethnic groups have been shown to have a higher risk of developing disease. First-degree relatives of subjects with type 2 diabetes have up to a 3.5-fold higher risk of developing disease and monozygotic twins have close to a 100% concordance rate of type 2 diabetes development [10]. In addition, although there is significant variation among populations, certain ethnic and indigenous backgrounds also have increased susceptibility for development of type 2 diabetes. For example, individuals of African, Southeast Asian, Hispanic or Native (e.g. Pima Indians) descent in the United States have a 2-50% greater prevalence of type 2 diabetes than Caucasian populations [11-13]. In Canada, First Nations populations have a 3- to 5-fold greater risk of developing type 2 diabetes [11, 14].

In a small percentage of cases, individual genes have been directly linked to causing type 2 diabetes. Maturity-onset diabetes of the young (MODY), which accounts for 2-5% of diabetes cases [15], is an example where single gene mutations exert strong effects resulting in a type 2 diabetes-like syndrome. Most forms of MODY result from mutations of transcription factors, which are involved in insulin synthesis and secretion. Classification of MODY is dependent on mutations in genes such as glucokinase (GK; MODY2) and hepatocyte nuclear factor 1α (HNF1α; MODY3) and 1β (HNF1β; MODY5) [15]. Several mutations within critical regions of the insulin precursor, preproinsulin, have been shown to result in impaired proinsulin processing and abnormal protein folding. These mutations result in neonatal diabetes and are associated with defective insulin secretion and ER stress [16, 17]. A compound heterozygous mutation in the prohormone convertase enzyme, PC1/3, that is involved in insulin and pro-opiomelanocortin (POMC) maturation has been shown to induce obesity and abnormal glucose homeostasis in one human female proband [18]. In rodent models of type 2 diabetes, mutations in the genes encoding leptin, a hormone synthesized by white adipose tissue that is involved in the regulation of food intake and energy expenditure, or the leptin receptor have been associated with overt obesity, hyperglycemia and diabetes [19]. However, mutations in leptin or its receptor are very rare in humans and may not be associated with development of type 2 diabetes [20].

In the vast majority of cases, type 2 diabetes is a polygenic disease where several genes in concert likely play a role in disease pathogenesis. It has been difficult to identify single contributing gene polymorphisms, as there is significant variability among families and ethnic groups. However, through genome-wide association studies, several genes and genetic loci have
been identified as presenting greater risk for development of type 2 diabetes; the majority appear to affect the beta cell directly.

Human linkage studies have led to the identification of several genes associated with the most common form of type 2 diabetes and include CAPN10, PPARγ, HHEX, SLC3A08 and TCF7L2. For example, variants in the HNF1β (MODY5) gene have been associated with common type 2 diabetes in a Finnish population [12, 21], and have also been reported in UK and Danish populations [12, 21]. In a Mexican-American population, 30% of type 2 diabetes cases were linked to polymorphisms in the CAPN10 [10] which have since been confirmed in several other populations and ethnicities [12]. CAPN10 has been shown to play a role in glucose-stimulated insulin secretion, proinsulin conversion and beta-cell apoptosis [22]. Single nucleotide polymorphisms in the peroxisome proliferator-activated receptor gamma (PPARγ) gene, a nuclear receptor that regulates glucose and lipid metabolism as well as adipocyte differentiation, have been implicated in reducing insulin sensitivity. One variant in the PPARγ gene, the Pro12Ala polymorphism has been shown to confer a greater risk of type 2 diabetes in obese subjects with impaired glucose tolerance [23]. In the Oji-Cree, a First Nations population of northern Ontario, 40% of subjects with type 2 diabetes have mutations in HNF1α, the MODY3 gene [24]. The zinc transport family member 8 (ZnT8) is highly expressed in beta cells and polymorphisms in ZnT8, specifically SLC3A08, have also been associated with an increased risk of type 2 diabetes [25]. ZnT8<sup>−/−</sup> mice have normal insulin content and rates of insulin biosynthesis and secretion, however, when challenged with a high fat diet, develop glucose intolerance and diabetes; these mice also have a predominance of immature secretory granules [26]. Mice with a beta-cell specific knockout of ZnT8 have decreased levels of the proinsulin processing enzyme transcripts and atypical insulin granules as well as several abnormalities typical of type 2 diabetes including glucose intolerance, decreased first phase glucose-stimulated insulin secretion and elevated levels of proinsulin [27].

Another gene associated with type 2 diabetes and implicated in glucose sensitivity is the hematopoietically expressed homeobox (HHEX) gene, which encodes a transcription factor that is expressed during embryogenesis and regulates cell proliferation and development of the ventral pancreas and liver [25]. To date, however, the gene encoding transcription factor 7-like 2 (TCF7L2), represents the most convincing diabetes risk gene across several populations and ethnicities [12]. TCF7L2 encodes a portion of the transcription factor complex and is involved in pathways required for beta-cell proliferation and survival as well as glucose- and incretin-stimulated insulin secretion [12, 28], and polymorphisms in this gene have been associated with
changes in proinsulin conversion resulting in elevated proinsulin/insulin ratios during an oral glucose tolerance test in a Spanish population [29].

1.1.3 Environmental component of diabetes mellitus

Mutations in a number of candidate genes that may confer increased susceptibility for development of type 2 diabetes have been identified in several populations and across different ethnicities. However, despite the successes in identifying these genes, on their own each appears to have only modest effects on beta-cell function and type 2 diabetes progression. With the increasing incidence and prevalence of type 2 diabetes, it has become evident that diabetes arises from multi-gene interactions that are exacerbated in the appropriate environment.

A diabetes-risk genotype only predisposes subjects to the development of type 2 diabetes; environmental factors also contribute to disease progression. A sedentary lifestyle resulting in poor physical fitness is an associated risk factor for type 2 diabetes, as is obesity and cigarette smoking [30]. Consumption of a high fat diet is another risk factor for type 2 diabetes in humans [31] and is mirrored in several animal models of type 2 diabetes [1, 32-34]. For example, the gerbil Psammomys obesus in its native environment lives on a low calorie diet of sagebrush and when fed a high-calorie diet rapidly develops insulin resistance, hyperglycemia, beta-cell dysfunction and diabetes as a result of the inability of its beta-cells to produce sufficient amounts of insulin [19, 28].

1.1.4 Relationship between insulin sensitivity and insulin secretion

The interplay between genetic predisposition and environmental factors is complicated. However, it is well accepted that type 2 diabetes is a progressive disease presenting as chronic hyperglycemia that results from both insulin resistance and beta-cell dysfunction [35]. Insulin resistance in type 2 diabetes is observed in peripheral tissues such as liver, skeletal muscle and adipose and can be seen early during progression of disease and before overt hyperglycemia becomes evident [28]. Insulin resistance is also seen during puberty, the late stages of pregnancy as well as during ageing and obesity [30]; however, hyperglycemia is not always a characteristic of these states. Not all obese and insulin resistant individuals progress to type 2 diabetes due to the ability of their beta cells to compensate for changes in insulin sensitivity, indicating other factors must be present for progression of this disease. In fact, although excess weight represents a major risk factor for development of type 2 diabetes, some patients (10%) with type 2 diabetes display normal weight and are not insulin resistant [36].
Beta-cell function varies depending on the degree of insulin sensitivity, and for any change in sensitivity there must be a compensatory change in insulin secretion such that normoglycemia is maintained. In healthy individuals, a hyperbolic relationship exists between insulin sensitivity and insulin secretion defined as the ‘disposition index’ [10, 30, 37]. The disposition index is an indicator of an individual’s metabolic status and is a product of insulin sensitivity and the acute insulin response to glucose (S_I x AIRglucose) [38]. A model established by Weir et al. summarizes the progression of beta-cell compensation to decompensation and loss of glycemic control in the pathogenesis of type 2 diabetes [39].

Hyperglycemia, characteristic of type 2 diabetes, arises when the compensatory mechanism for increasing insulin resistance fails. Several groups at high risk of developing type 2 diabetes, such as obese subjects with impaired glucose tolerance, first-degree relatives of subjects with type 2 diabetes, and women with gestational diabetes or polycystic ovary syndrome, all have disposition indices that fall outside the normal range despite maintenance of normoglycemia [37, 38, 40], indicating that baseline beta-cell function is already impaired. This is supported by a landmark study of Pima Indians where progressors were shown to have a decline in their disposition index that was linked to decreases in insulin sensitivity and secretion as they progressed from normal glucose tolerance to impaired glucose tolerance and type 2 diabetes [36]. Unlike the progressive nature of beta-cell dysfunction in type 2 diabetes, the degree of insulin sensitivity does not change significantly [35, 36, 38].

According to the United Kingdom Prospective Diabetes Study (UKPDS), 50% of beta-cell function is lost at the time of diagnosis for type 2 diabetes [41]. Despite the use of various therapies for treatment of type 2 diabetes such as sulfonylureas or insulin, progression to beta-cell failure and hyperglycemia are almost inevitable [38, 42]. Disease progression is marked by several abnormalities in pancreatic beta-cell function that can already be observed in the early stages leading to disease. Beta-cell dysfunction can manifest as reduced glucose and non-glucose stimulated insulin secretion, impaired pulsatile and oscillatory patterns of insulin secretion as well as impairments in proinsulin processing.

Insulin secretion occurs in a biphasic manner with an acute first-phase occurring within 10 minutes of stimulation; this is followed by a later, second-phase secretion. Diminished first-phase response to glucose is already observed in individuals with impaired fasting glucose, and this declines further with increases in glycemia [43]. Subjects with impaired glucose tolerance and type 2 diabetes have a greatly attenuated acute insulin response and a reduced second-phase response as indicated by both oral and intravenous glucose tolerance tests [30, 44]. Additionally,
these subjects exhibit impairments in insulin responses to non-glucose secretagogues such as glucagon-like-peptide-1 (GLP-1) [45]. This latter observation is also secretagogues that are evident in vitro, where islets from patients with type 2 diabetes exhibit a complete loss of response to glucose that is associated with reduced responses to non-glucose stimuli such as arginine and GLP-1 [46].

Insulin is secreted from the beta cell in both a pulsatile and oscillatory pattern [38] that is characterized by rapid oscillations superimposed on ultradian (slower) oscillations, which are tightly coupled to plasma glucose oscillations [47]. The pulsatile release of insulin is related to intra-islet Ca$^{2+}$ concentrations that regulate insulin granule exocytosis [48]. Defects in pulsatile insulin secretion are seen early on during impaired glucose tolerance, as well as in type 2 diabetes, and are characterized by a reduction or absence of the regular rapid insulin secretory peaks and ultradian oscillations [47, 49, 50]. Pulsatility of insulin delivery has been shown to be important for maintaining normal insulin sensitivity and continuous delivery of insulin has been associated with insulin resistance [38].

Type 2 diabetes is characterized by both absolute and relative insulin deficiency, in part as a result of impaired proinsulin processing (Section 1.1.8). The efficiency of proinsulin processing is reduced prior to disease onset and worsens with time resulting in the hypersecretion of partially processed proinsulin intermediates, predominantly des-31,32-proinsulin [51]. Elevated proinsulin levels have been shown to be an independent predictor of type 2 diabetes development in several longitudinal and prospective studies in various populations [52-55]. As a result, the proinsulin/insulin ratio, a marker of beta-cell processing and secretory capacity, is elevated 2- to 3-fold in type 2 diabetes, indicative of impaired proinsulin processing which correlates with hyperglycemia and decreased beta-cell function [56, 57]. Alterations in the efficiency of proinsulin conversion likely occur well before clinical diagnosis of type 2 diabetes.

1.1.5 Beta-cell mass in health and type 2 diabetes

Beta-cell mass is a dynamic process that is regulated by several mechanisms including beta-cell neogenesis from precursor, replication of existing beta-cells, changes in beta-cell size and beta-cell apoptosis. At any given moment, beta-cell mass is represented by the sum of neogenesis, replication and size minus the rate of apoptosis. The contribution of each process is variable, may be species specific, related to different stages of life and dependent on metabolic demand [58]. Factors such as glucose, FFAs, incretins and islet innervation can all mediate changes in beta-cell mass [59, 60]. Glucose has been shown to contribute to beta-cell mass
expansion via the insulin receptor substrate-2, and incretins, such as GLP-1, have also been shown to promote expansion and reduce apoptosis [60].

In the healthy state, changes in insulin sensitivity induce changes in insulin secretion by adaptation of beta-cell mass [61]. Obese subjects who are insulin resistant but do not progress to type 2 diabetes are able to compensate for the metabolic load by increasing beta-cell proliferation and expansion of beta-cell mass by up to 50% [62]. During pregnancy, for example, a doubling of the maternal beta-cell population can occur that is driven by both prolactin and placental lactogen [63-65]. This increase in overall beta-cell mass is thought to occur via enhanced replication, neogenesis and increased cell size [58, 64] and may be accompanied by small increases in apoptosis [64]. In type 2 diabetes, the decline in insulin secretory function is paralleled by reductions in beta-cell mass.

Landmark studies by Opie and others [66-68] have indicated that beta-cell volume was significantly reduced in type 2 diabetes as a result of failed adaptation to an increased metabolic load [64] with a marked reduction in beta-cell mass as a result of increased apoptosis [48, 62]. Although much supporting evidence has been shown for this phenomenon, there is still some controversy in the field regarding whether decreases in beta-cell mass contribute to the progression of type 2 diabetes [61]. As it is not yet possible to measure human pancreatic beta-cell mass in vivo, most of the findings to date have been inferred from rodent models of type 2 diabetes or human pancreatic autopsy samples. One rodent model of type 2 diabetes is the Zucker Diabetic Fatty (ZDF) rat [69]. These animals have a mutation in the leptin receptor rendering them obese and resulting in a type 2 diabetes-like syndrome that includes hyperglycemia. In the ZDF rat, beta-cell mass expansion due to insulin resistance is inadequate, and is associated with increased beta-cell apoptosis [69] as well as the activation of pro-apoptotic markers, caspase-3 and caspase-8 [61].

Decreases in beta-cell mass can already be seen prior to development of type 2 diabetes, suggesting that the deficit in beta-cell mass in at-risk subjects occurs well before disease onset. Obese humans with impaired fasting glucose already have a 50% reduction in beta-cell mass [62]. Subjects with type 2 diabetes, irrespective of obesity, have a 41-63% reduction in beta cell mass [62] with a frequency of beta-cell apoptosis 3-10 fold higher than in respective control groups [48]. The rate of apoptosis is also increased in isolated islets exposed to high glucose levels [48], suggesting that hyperglycemia per se may induce beta cell death.

Consequences of reduced beta-cell mass have been shown to mimic some metabolic abnormalities present in type 2 diabetes, although the impact of reduced beta-cell mass on beta-
cell function is still questionable. For example, a 50% hemi-pancreatectomy in humans causes impaired glucose tolerance and insulin secretion suggesting that ~50% of beta-cell mass is insufficient for maintenance of normoglycemia [60, 70]. Studies have shown that pharmacological or surgical reduction of beta-cell mass in several animal models result in reductions in both basal and stimulated insulin secretion. In healthy rats with a 60% pancreatectomy, however, hyperglycemia is only evident only upon addition of sucrose to the drinking water suggesting that there is a minimal threshold beta-cell mass required for maintenance of normoglycemia [60]. Reduction of beta-cell mass in Göttingen mini-pigs treated with streptozotocin (STZ) and nicotinamide [71], dogs with a 50% surgical reduction [72] and primates treated with STZ [46] present with reduced insulin secretion that is associated with a decrease in insulin pulse mass [73] as well as impairments in glucose tolerance and diabetes following pancreatectomy [59, 72].

Based on the above observations, addition of an external stimulus or removal of close to 90% of pancreas from healthy animals is necessary for hyperglycemia to ensue [46, 74]. A simple reduction in beta-cell mass is insufficient to bring on hyperglycemia typical of type 2 diabetes, and hence, an underlying beta-cell defect must also be present, which is exacerbated by diminished beta-cell mass as well as other external factors such as insulin resistance.

1.1.6 Causes of progressive beta-cell loss and dysfunction in type 2 diabetes

Loss of beta-cell mass and diminished islet function has been attributed to several factors including glucotoxicity, lipotoxicity, inflammation, ER stress and amyloid.

**Glucotoxicity.** Progressive increases in glucose are characteristic of type 2 diabetes pathogenesis. Chronic elevation of glucose has been shown to have an impact on beta-cell mass and function in several in vivo and in vitro models [75]. Glucose-induced beta-cell apoptosis has been shown in diabetic Psammommys obesus (*P. obesus*) [76] and the ZDF rat [69], two animal models of type 2 diabetes. *P. obesus* are susceptible to concentration-dependent DNA fragmentation by glucose [77]. The ZDF rat is initially able to adapt to increased obesity and insulin resistance; however, once adaptation fails hyperglycemia ensues resulting in increased beta-cell apoptosis [78]. Apoptosis as a result of upregulated pro-apoptotic genes, Bid and Bad, and down-regulated pro-survival gene Bcl-X<sub>L</sub> has been shown in human islets cultured in the presence of high glucose [75, 79], which can be reversed with administration of GLP-1 [61].

During prolonged high glucose culture rat islets and beta-cell lines show an initial decrease in insulin gene expression, insulin content and glucose-stimulated insulin secretion as
well as decline in promoter activity and pancreatic and duodenal homeobox 1 (PDX-1) and MafA binding [75], key transcription factors in beta-cell development and function. The effects could be reversed by lowering blood glucose, however, reversal was time-dependent [75]. Chronically elevated glucose levels have been shown to increase glucose metabolism by oxidative phosphorylation [79], resulting in mitochondrial dysfunction and production of reactive oxygen species (ROS). Beta-cells by nature have very low levels of antioxidants and are therefore more susceptible to ROS damage [79]. In vivo, ZDF rats have elevated markers of oxidative stress and treatment with anti-oxidants such as N-acetylcysteine or aminoguanidine prevent elevation in ROS and reduce hyperglycemia [75]. Islets from antioxidant-treated ZDF rats also had increased PDX-1 and MafA binding activities with increases in insulin mRNA and insulin content [80]. Markers of oxidative stress are also significantly higher in islets from type 2 diabetic subjects and correlate with the degree of hyperglycemia [78]. ROS have also been implicated in insulin resistance [75], and hence may be an early contributor to beta-cell dysfunction and death.

Two pro-apoptotic mechanisms have been associated with hyperglycemia-induced pathogenesis of type 2 diabetes: glucose-induced synthesis of the pro-inflammatory cytokine IL-1β and endoplasmic reticulum (ER) stress [75]. Exposure of human islets to elevated glucose concentrations induces IL-1β production by islets and macrophages as well as NF-kB activation, Fas upregulation and beta-cell apoptosis by way of FasL binding on neighboring cells [81, 82]. Increased IL-1β has also been associated with decreased proinsulin conversion [83], an effect enhanced by the presence of other cytokines including TNF-α[84].

Overstimulation of the beta-cell secretory machinery may also contribute to beta-cell dysfunction by activating the ER stress pathway. Evidence of ER stress in type 2 diabetes has been shown in isolated islets from type 2 diabetic pancreata with increased immunostaining for the pro-apoptotic ER stress marker C/EBP homologous protein (CHOP) as well as increased ER size [78]. ER stress is thought to result from failure of the unfolded protein response (UPR) which functions to alleviate ER stress under conditions of increased protein synthesis and maintain cellular homeostasis by decreasing translation. When the UPR fails an apoptotic cascade is triggered [78] and up-regulation of several pro-apoptotic modulators occurs: inositol-requiring endoplasmic reticulum–to–nucleus signal kinase-1α (IRE-1α), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase (PERK) [78]. In the Akita mouse model, a folding mutation in the proinsulin gene (C96Y) activates the ER stress response.
resulting in diabetes and decreased beta-cell mass [28]. In humans, this condition results in the development of permanent neonatal diabetes.

**Lipotoxicity.** Obesity is part of the metabolic syndrome and is the main risk factor for development of type 2 diabetes. It is characterized by dyslipidemia and elevated levels of inflammatory adipocytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1RA) and leptin, factors that may impact both insulin sensitivity and beta-cell survival and function. Leptin has been shown to stimulate beta-cell proliferation in pancreatic beta-cell lines [85]; however, human pancreatic islets exposed to leptin are associated with apoptosis mediated by IL-1β, and reduced glucose-stimulated insulin secretion [82, 86].

In health, circulating FFAs play an important role in maintaining normal basal and glucose-stimulated insulin secretion rates. The dyslipidemia in obese subjects with type 2 diabetes is characterized by elevated levels of free fatty acids (FFAs) and triglycerides (TG) that may contribute to the overall beta-cell dysfunction and death.

Functionally, prolonged (>48hrs) in vitro exposure of beta cells to elevated FFAs results in increased basal insulin release as well as decreased stimulated secretion of insulin in vivo in rats and humans [75]; in mouse studies the effect is primarily seen during the late phases of the insulin secretory pathway where granule exocytosis at the fusion pore is affected [87]. A decrease in insulin secretion may also be mediated by uncoupling protein-2 (UCP-2), a ubiquitously expressed mitochondrial carrier involved in ATP synthesis. FFAs have been shown to increase expression of UCP-2 in animals fed a high fat diet or islets exposed to high levels of FFAs. Beta-cell overexpression of UCP-2 has been shown to impair insulin secretion while in a UCP-2 knockout mouse model, more circulating insulin is present and mice are protected from both genetic and nutritionally induced diabetes [75].

The roles of FFAs on insulin gene expression have also been studied and have been associated with decreases in insulin gene expression, but only in the presence of high glucose. Elevated levels of FFAs and lipoproteins also affect insulin gene expression. FFAs have been shown to decrease both proinsulin processing and prohormone convertase processing [88]. More recently, palmitate has been implicated in reducing levels of carboxypeptidase E (CPE), an enzyme essential for the proper proinsulin processing and degradation of CPE has been shown to contribute to beta-cell ER stress and apoptosis [89]. Culture of islets in the presence of palmitate is associated with increased ceramide production that results in direct inhibition of glucose induced insulin promoter activity by decreased binding of both PDX-1 and MafA [75]. By
inducing production of ceramide as well as ROS, palmitate was shown to decrease cell proliferation and increase beta-cell death, and was associated with caspase pathway activation and reduction of the anti-apoptotic Bcl-2 protein [61]. Based on some studies, only in combination with hyperglycemia was palmitate able to impact on beta cell function [90]. Thus the results of some studies suggest that lipotoxicity alone does not adversely affect beta-cell function [75], although others show lipotoxicity in the absence of hyperglycemia [91].

**Cholesterol.** According to the 2008 Canadian Diabetes Association Clinical Practice Guidelines subjects with type 2 diabetes display a dyslipidemic pattern that includes increased TG, abnormally low plasma high-density lipoprotein (HDL) particles and an abundance of small, dense low-density lipoprotein (LDL) particles [92]. More recently, changes in HDL and LDL have been implicated in beta-cell mass and function, suggesting a potential role for lipoprotein particles in type 2 diabetes pathogenesis [93]. HDL and LDL have been shown to have an impact on beta-cell function and survival *in vitro* and *in vivo*. Human islets exposed to low- (LDL) and very low-density lipoproteins (VLDL) have reduced insulin mRNA levels and decreased beta-cell proliferation and apoptosis [94] as well as inhibition of insulin secretion [95]. Conversely, HDL increases acute insulin secretion and protects against the effects of oxidized LDL, which causes decreased insulin secretion in cultured beta cells [96]. HDL has also been shown to decrease beta-cell apoptosis induced by LDL and cytokines such as IL-1β [97]. In a recent human study of subjects with type 2 diabetes, infusion of reconstituted HDL resulted in reductions of plasma glucose levels, increased plasma insulin levels and improvements in beta-cell function [93, 98].

**Islet Amyloid.** The islet in type 2 diabetes is characterized by what were initially described as hyaline deposits [66], but were later referred to as islet amyloid [99]. Islet amyloid is a characteristic pathology of type 2 diabetes and has been found in > 90% of subjects with the disease [100, 101]. The presence of islet amyloid in type 2 diabetes is associated with hyperglycemia [102], a progressive loss of insulin secretion and decline in beta-cell mass in human and non-human primates [103-106]. Amyloid deposits have also been associated with the severity of disease in humans [104, 107] and are thought to form in pre-diabetic stages of the disease [108]. In mice transgenic for human islet amyloid polypeptide, formation of amyloid deposits is associated with the development of hyperglycemia [102] suggesting that islet amyloid may play a role in promoting early beta-cell dysfunction. However, it remains debatable whether amyloid is a cause or consequence of beta-cell loss and disease progression.
1.1.7 Islet amyloid, IAPP and type 2 diabetes

Islet amyloid is composed primarily of a 37 amino acid peptide called islet amyloid polypeptide (IAPP or amylin). Islet amyloid formation occurs by aggregation of IAPP; however, not all species develop amyloid due to the differential amyloidogenicity of species-specific IAPP isoforms. The ability of IAPP to aggregate depends on its primary sequence (Figure 28). A beta-sheet region in the mid-portion of the IAPP molecule (amino acids 20-29) has been suggested to confer its amyloidogenicity and cytotoxicity [109, 110]. This amyloidogenic region is conserved between species that develop amyloid. Spontaneous diabetes seen in cats and monkeys which is characterized by older age of onset, obesity, impaired glucose tolerance that progresses to hyperglycemia and insulin dependence, is associated with progressive increases in amyloidosis [111]. Unlike humans, non-human primates and cats, rodent forms of IAPP do not form amyloid. This is due to the presence of three proline substitutions within the amyloidogenic IAPP\textsubscript{20-29} region, which act as beta-sheet breakers [109, 110, 112, 113].

IAPP is localized in the beta-cell granules and is co-secreted with insulin in a molar ratio of \( \sim 1:100 \) of IAPP:insulin in response to glucose and non-glucose secretagogues. Proposed biological roles for IAPP include effects on insulin secretion and action, food intake and gastric emptying [114, 115]. Under normal conditions, IAPP circulates in soluble form at concentrations in the low picomolar range [114]; however, in states of increased insulin requirement IAPP levels, like those of insulin are elevated. Increased secretion of IAPP is likely to be insufficient on its own for amyloid formation suggesting that other factors may be involved. Chronic hyperglycemia and hyperlipidemia associated with type 2 diabetes may enhance amyloid formation. Expression of human IAPP (hIAPP) in mouse models alone does not induce amyloid formation. For example, in hIAPP mice fed a high fat diet [33] or crossed onto an obese and hyperlipidemic background [116, 117] amyloid deposition is accelerated. In humans, a missense mutation in the IAPP gene (S20G; glycine substitution for serine at amino acid 20), which has been shown to exacerbate amyloidogenicity and cytotoxicity [118] results in impaired insulin secretion and a severe, early onset form of type 2 diabetes [119].

Human islets [120] and islets from mice with beta-cell overexpression of hIAPP [102, 121], develop amyloid in a glucose-dependent manner \textit{in vitro}, which correlates with beta-cell apoptosis and an increased ratio of alpha cells to beta cells within the islet, that can be reduced by IAPP siRNA [120] and peptide inhibitors of IAPP aggregates [122]. Advanced glycosylation end-product-modification of IAPP increases its ability to form amyloid suggesting that hyperglycemia can potentiate the effects of IAPP on beta-cell function and mass [123, 124].
Prefibrillar forms of IAPP are more toxic to beta cells, and may be the primary toxic form, while mature fibrils are more inert and less likely to reduce viability [125]. Unlike human IAPP, soluble rodent IAPP is not toxic to beta cells in vitro because it does not form fibrils or prefibrillar aggregates. Recently, other regions of human IAPP, specifically 8-20 and 30-37 have been suggested to contribute to amyloid formation [109, 126, 127].

1.1.8 Processing of proinsulin in health and type 2 diabetes

Many neuroendocrine hormones are synthesized as inactive precursors that undergo post-translational processing by proteolytic enzymes to biologically active forms. Similarly, proinsulin must be processed prior to its release into the circulation for full biological activity. In a healthy beta cell, proinsulin transcription and translation are regulated by glucose and cyclic AMP. Insulin synthesis begins with the transcription of the insulin gene followed by translation of proinsulin mRNA on the beta-cell rough endoplasmic reticulum (RER). Proinsulin is then transported from the RER to the cis- and trans-Golgi network (TGN) in small vesicles prior to release into the secretory granules. Ninety percent of proinsulin is processed in the secretory granules and in healthy subjects, proinsulin accounts for only 3-5% of total insulin immunoreactivity [128].

In the pancreatic beta cell, proinsulin processing occurs primarily in clathrin-coated early (immature) secretory granules derived from the TGN. The conversion of proinsulin to insulin involves the action of two prohormone convertases, PC1/3 and PC2. Prohormone convertases (PCs) are proteolytic enzymes that activate precursor proteins into their active forms by limited endoproteolysis at pairs of basic amino acids [129, 130]. Like proinsulin, PC1/3 and PC2 are synthesized in the ER as proproteins and undergo proteolysis and membrane association while transiting through the regulated secretory pathway in maturing dense core granules. Initial cleavage of the PCs occurs early in the ER (PC1/3) when proinsulin mRNA is translated in response to hyperglycemia [131] or later in the maturing granule (PC2) [132]. In the beta-cell, both PC1/3 and PC2 are present in secretory granules, specifically localized to membrane-lipid rafts [133, 134], and their function is dependent on intraorganellar calcium concentrations and an acidic milieu [135]. Additionally, the activity of PC2 requires the presence of a chaperone, 7B2, a neuroendocrine protein, also present in the secretory granule.

Processing of proinsulin is initiated in the immature secretory granule by PC1/3 that preferentially cleaves intact proinsulin at the B-chain/C-peptide junction between amino acid residues 32 and 33 (Arg-Glu) (Figure 1) resulting in split 32,33 proinsulin. Removal of the
Figure 1. Proinsulin processing in healthy pancreatic beta cells.
dibasic amino acids by carboxypeptidase E (CPE) results in des 31,32 proinsulin, the major conversion intermediate detectable in circulation along with proinsulin. Des 31,32 proinsulin is further cleaved by PC2 between amino acids 65 and 66 at the C-peptide/A-junction resulting in mature insulin and C-peptide [136, 137]. Following each cleavage by PC1/3 and PC2, the dibasic amino acid pairs are efficiently removed by the action of CPE [138]. In the absence of PC1/3, proinsulin is partially converted to insulin, albeit at a slower rate, and results in the accumulation of greater amounts of split 65,66 proinsulin and des 64,65 proinsulin [137]. Unlike des 31,32 proinsulin, the des 64,65 proinsulin intermediate does not account for a significant proportion of total proinsulin in circulation of humans.

In type 2 diabetes, proinsulin processing is inefficient and results in the disproportionate secretion of proinsulin and des 31,32 proinsulin relative to insulin [57]. The ratio of proinsulin to insulin (PI/PI+I) provides a measure of the efficiency of proinsulin conversion and has been used as a marker of beta-cell dysfunction. In healthy subjects this ratio is 3-fold lower than in patients with type 2 diabetes, where it can range between 20-50% [57]. Several mechanisms may explain the increased proinsulin/insulin ratio observed. Beta cells may be under increased demand for insulin resulting in a shorter residence time of proinsulin in the granules [139]. Defects in PC1/3 and PC2 synthesis, processing and/or activity may also be contributing factors. Lack of CPE, which is controls in part the ability of PC1/3 and PC3 to cleave their substrates, may also impact processing. Missorting of proinsulin into the constitutive secretory pathway may also result in hyperproinsulinemia since the constitutive pathway does not contain PC1/3 or PC2 [140]. Islet cell tumors (insulinomas) secrete newly synthesized proinsulin via a constitutive mechanism and are characterized by hyperproinsulinemia [108]. Although the constitutive secretory pathway contains an endopeptidase, furin, it is unable to fully process proinsulin as seen in vitro [141]. Prolonged exposure of islets and beta-cell lines to environmental triggers, such as elevated levels of glucose [139], FFAs [88] or cytokines [83, 84] have all been implicated in inducing impaired proinsulin processing.

1.1.9 Normal processing of pro islet amyloid polypeptide

Like insulin, IAPP is derived from a larger precursor peptide, proIAPP which is ~ 8kDa in size and is processed at two sets of dibasic residues (Lys-Arg; K-R) found at both the amino- and carboxy-termini of proIAPP [142]. Early in vitro studies indicated that both PC1/3 and PC2 also play a role in cleavage of proIAPP [143]. Several mouse models have enabled better understanding of the proIAPP processing pathway. ProIAPP processing is blocked completely at
the NH₂-terminus in mice lacking PC2 (PC2\(^{-/-}\)). PC2\(^{-/-}\) mice had no detectable levels of mature IAPP with elevated levels of the 8kDa unprocessed proIAPP and the 6kDa NH₂-terminally extended proIAPP intermediate [144]. When PC2\(^{-/-}\) mice were crossed with hIAPP transgenic mice, the lack of PC2 led to amyloid formation and beta cell death [145]. Islets from mice lacking PC1/3 (PC1/3\(^{-/-}\)) have elevated levels of the COOH-terminally unprocessed form of proIAPP, but normal levels of mature IAPP [146] suggesting that in the absence of PC1/3, PC2 can cleave proIAPP at both the NH₂ and COOH-termini. As in proinsulin processing, CPE is also required for the removal of dibasic residues [147]. Based on in vitro and in vivo studies on proIAPP, a model has been proposed for proIAPP processing in normal beta cells (Figure 2). In the immature secretory granule, proIAPP processing is initiated by PC1/3 at a pair of well-conserved dibasic residues within the COOH-terminal region of proIAPP (Lys\(^{50}\)-Arg\(^{51}\); KR) resulting in the NH₂-terminally extended proIAPP intermediate. Completion of proIAPP processing occurs when PC2 cleaves at a second set of dibasic residues (Lys\(^{10}\)-Arg\(^{11}\); KR) within the NH₂-terminal region. Mature IAPP formation is completed by amidation of IAPP by peptidyl glycine α-amidating mono-oxygenase complex (PAM).

There is some evidence that amyloid deposits contain proIAPP and that defective processing of proIAPP may play a role in the pathogenesis of 2 diabetes [148, 149]. As described previously, hyperglycemia and hyperlipidemia are characteristic of the type 2 diabetic milieu. Exposure of islets to high glucose levels has been shown to result in increase relative proportions of proIAPP or the partially processed NH₂-terminal proIAPP [150]. Prolonged exposure of islets to high levels of FFAs has been shown to impact on proinsulin processing by decreasing PC1/3 and PC2 activation [88], and since both proinsulin and proIAPP are processed in a similar fashion, it is possible that proIAPP and its processed forms may be increased. Another plausible mechanism for defective beta-cell function resulting in increases in proIAPP and its intermediates may be due to altered sorting of proteins into the constitutive or constitutive-like pathway resulting in a greater proportion of unprocessed proIAPP being secreted.
Figure 2. Pathway for processing of proIAPP in beta cells. Pro-IAPP processing is initiated by cleavage at its COOH terminus preferentially by PC1/3 followed by cleavage of the NH$_2$-terminally unprocessed proIAPP intermediate by PC2. After cleavage by PC1/3, the COOH-terminal dibasic residues (KR) are removed by the action of CPE. This step is essential for removal of Gly$^{49}$ and amidation of IAPP at the COOH terminus by the PAM complex. 

G, Gly; K, Lys; R, Arg.
1.1.10 Progression of type 2 diabetes

Several longitudinal studies have examined the progression of type 2 diabetes in human subjects and the associated loss of glycemic control. The progressive nature of type 2 diabetes was most strikingly depicted by the landmark UKPDS study [6], and was supported by others such as the A Diabetes Outcome Progression Trial (ADOPT) [151] and Belfast diet intervention studies [152]. All suggested that although initial treatments arms may have had some therapeutical success, the progression of the disease resulted in a deterioration of beta-cell function that could not be ameliorated with either dietary or lifestyle interventions, or treatments with medications. The UKPDS study demonstrated in a group of individuals with recently diagnosed type 2 diabetes, subjects on monotherapy with metformin, sulfonylureas or insulin had a 13%, 24% and 42% success rate at 9 year follow-up, respectively, of maintaining optimal glucose control [153]. With dietary intervention, only 8% of subjects were successfully managed [153]. This observation was recapitulated in the Belfast diet intervention study [152]. Recently diagnosed individuals with type 2 diabetes in the ADOPT study treated with rosiglitazone, metformin and glyburide also had similar results with respect to glycemic maintenance [151]. Results from these studies underlie the importance of managing progression of disease at a much earlier stage, prior to diagnosis. In the Diabetes Prevention Program, it was shown that individuals with pre-diabetes were able to delay the progression to full-blown type 2 diabetes with lifestyle modification (diet and exercise) or with therapy to enhance insulin action with a concomitant decrease in blood glucose levels.

1.2 Pancreatic islet transplantation

1.2.1 Background

Type 1 diabetes is a chronic and progressive autoimmune disease characterized by reduced beta-cell mass as a result of immune-mediated destruction. Insulin therapy is the primary modality for treatment of type 1 diabetes. However, despite its ability to improve glycemic control in patients, exogenous insulin administration is unable to prevent the long-term macro- and microvascular complications. Currently, the only therapy available for re-establishing endogenous insulin production is through the replacement of beta cells by transplantation of whole pancreas or pancreatic islet transplantation.

Pancreas transplantation reestablished long-term normoglycemia and insulin independence in more than 80% of patients at 1 year follow-up [154], and is especially
successful in patients receiving simultaneous pancreas and kidney transplants. Although the success of this procedure warrants its use for therapy, risks associated with the invasive surgery and life-long immunosuppression prevent it from being used for the majority of patients, especially young patients who have not yet developed complications [155]. A promising alternative is the transplantation of isolated islets as it is a less invasive procedure. Islets for transplantation may be obtained from the recipient's own pancreas (autotransplantation) or from pancreata from cadaveric multiorgan donors (allotransplantation) that are digested with collagenase to release the islets. Once the islets are isolated, they are purified and cultured prior to transplantation into the recipient by injecting the islet preparations via the hepatic portal vein and hence depositing the islets within the liver.

1.2.2 Current status of clinical islet transplantation

The first pre-clinical evidence that islet transplantation could provide a “cure” for type 1 diabetes was performed in a rodent model with induced diabetes that could be reversed with transplanted islets [156, 157]. Since then much effort has been made into the optimization of the procedure for use as treatment for type 1 diabetes in humans. Initial efforts indicated a 66% success rate of insulin independence in allograft recipients which progressively declined to only 2% at 5 years post-transplant although graft survival rates, indicated as baseline C-peptide levels greater than 0.5 ng/ml (>166.5 pM), remained at 41% [156]. Improved transplant outcomes in patients occurred between 1974 and 1999, where from over 450 islet allotransplant procedures as reported by the Islet Transplant Registry, even though 28% of patients had sustained C-peptide secretion, only 10% remained insulin independent at 1 year post-transplant [158]. Several factors including beta cell or islet mass transplanted and the immunosuppressive regimen contributed to the poor outcomes seen in these patient groups.

Not until the year 2000 and the advent of the “Edmonton Protocol” did clinical islet transplantation change radically following results from a study describing seven poorly controlled and severely hypoglycemic type 1 diabetic patients, who underwent islet allotransplantation [159]. The Edmonton protocol changes included: transplantation of a greater number of freshly prepared and high-grade islets from an average of two donors (>10,000 IE/kg), steroid-free immunosuppression therapy using a combination of sirolimus and low-dose tacrolimus as well as induction with anti-IL-2 receptor antibodies for prevention of acute rejection in the immediate post-transplant phase [159]. Insulin independence was gained in 82% of patients at 1 year post-transplant, however, the proportion of patients able to maintain insulin
independence in the long-term declined to less than 10% at 5 years post-transplant [160]; however, graft survival was maintained in the majority (80%) of patients resulting in decreased insulin requirements, less frequent hypoglycemia and improved glucose control.

Following the Edmonton results in 2000, another trial initiated at nine international centers, found that 44% of subjects gained insulin independence at 1 year post-transplant and only 14% maintained insulin independence after 2 years [161]. Although much work needs to be done to increase the insulin-independence rate in transplant recipients, restoring islet function has been shown to protect against long-term diabetic complications associated with type 1 diabetes [162] and evidence suggests that islet transplantation can slow the progression of these complications beyond that of best medical therapy [163].

Whole or segmental pancreas resection and islet autotransplantation is performed in subjects with chronic pancreatitis, which is a progressive inflammatory disease causing irreversible damage to the pancreatic (exocrine) parenchyma and has been implicated in decreased endocrine function leading to diabetes mellitus [164]. To date, more than 300 islet autotransplants have been performed at various centers worldwide including the University of Cincinnati and the University of Minnesota. Approximately 50% of patients achieve insulin independence with one case report of an insulin-free graft survival for 13 years [165]. Islet autotransplantation in combination with pancreatectomy can be performed in both adults as well as young patients since there is no need for immunosuppression [164]. In a pediatric population at 1 year post-transplant, 56% showed insulin independence and 22% had partial graft function [166]. In adults, results indicate that long-term insulin independence is possible with islet autotransplantation where 46% of subjects remaining insulin independent at 5 years and 28% at 10 years post-transplant [167]. Outcomes of insulin independence and graft function were far superior for islet autograft recipients than they were for recipients of islet allografts. For example, of subjects who had functional grafts immediately following transplantation, only 66% of allograft recipients maintained graft function compared to 85% of autograft recipients. A similar trend was seen with respect to achievement and maintenance of insulin independence, such that at 2 years post transplant only 46% of allograft recipients maintained insulin-independence compared to 76% of autograft recipients [167].

Despite the greater success of autotransplants in maintenance of insulin independence, progressive loss of graft function is evident in both allo- and autotransplantation and improvements must be made prior to broadening the availability of islet transplantation as therapy.
1.2.3 Factors contributing to islet graft failure

Graft dysfunction in islet transplantation has been associated with a loss of functional beta-cell mass [168]. Although loss of beta-cell mass is primarily mediated by both allo- and autoimmune responses, non-immune mechanisms and inflammation also may contribute to early and long-term graft failure (Figure 3). Early graft failure comprises both primary islet dysfunction as well as the rapid loss of graft function and survival in the early post-transplant period. Long-term graft failure occurs when a once functional graft loses function over a period of years. Factors specific to both the donor and the recipient, procedures of islet isolation, engraftment and revascularization, immunosuppressive drugs and the loss of beta-cell mass may exacerbate beta-cell dysfunction and apoptosis in the transplant milieu.

**Donor tissue.** Several factors related to the donor can impact islet viability prior to transplantation including donor age, health status and the cold ischemic time of the pancreas. Organs allocated for whole pancreas transplants typically come from donors who are younger and have a low BMI. These account for about 10% of pancreata isolated from cadaveric organ donors that are eligible for pancreas transplantation. Islets isolated for the purpose of islet transplantation, therefore, tend to come from a greater source of donors who are generally older than 50 years of age and many of whom are obese.

Donor age is thought to directly correlate with islet function in whole pancreas transplantation or isolated islet transplantation. Once the donor age exceeds 45 years, there is a greater risk of poor glycemic control and early graft dysfunction in whole pancreas recipients. Mice transplanted with islets from older donors matched for glycemia had a lower diabetes reversal rate, decreased glucose stimulated insulin release and islet ATP content regardless of the mass of islets transplanted [169].

Islets from obese donors are less difficult to isolate and therefore provide a higher yield for islet transplantation. Obesity plays a role in beta-cell mass proliferation, hypertrophy and beta-cell density within the islet [62] and although initially islets from obese donors may perform better in the transplanted graft, it is possible that increased demand within the transplant environment may trigger metabolic exhaustion and long-term dysfunction. Shorter graft survival has been reported in association with high donor BMI in islet transplant recipients. Islet size has been suggested to be important in transplantation outcomes. *In vitro* functional assays and *in vivo* islet transplants in rats with a marginal islet volume indicate that smaller islets (<125μm) have better function and are able to restore normoglycemia in 80% of cases, while larger ones
(>150µm) fail to do so [164]. In human islet transplants, small islets were also found to be more functional in terms of in vitro insulin secretion and showed a higher survival rate [170].

The glycemic state of the donors is also an important factor for graft function. Hyperglycemia has been associated with poor outcomes in recipients of pancreas transplants [158] and transplantation of islets from type 2 diabetic subjects show impairments in glucose stimulated insulin secretion and failure to reverse hyperglycemia in transplanted mice [168].

Cause of death is also pertinent for future graft function. For islet allotransplanation, islets are procured from brain-dead patients. Brain death is associated with the activation of pro-inflammatory cytokines within the pancreas such as TNF-α, IL-1β and IL-6, that are known to impact on islet function and survival, and can therefore induce cell death and graft dysfunction post-transplant [156, 171]. Post-isolation culture and incubation with anti-inflammatory agents including the IL-1 receptor antagonist, Anakinra [172], may be effective at reducing levels of these and other cytokines prior to transplantation.
Figure 3. Potential factors limiting pancreatic islet graft function and survival. Adapted from Harlan et al. Diabetes 58, 2009.
**Islet viability following isolation and culture.** Following pancreas retrieval, the organ is stored in conditions designed to mimic the physiological environment prior to the islet isolation procedure. During islet isolation, islets are damaged as a result of osmotic, chemical, ischemic and enzymatic processes [174, 175]. Following organ isolation, the pancreas is perfused, cut into sections and transferred to a 37°C Ricordi chamber containing collagenase which allows islets to be separated from exocrine tissue by disruption of the islet-to-exocrine tissue adhesive contacts. Islet function highly depends on the integrity of the islets as well as the interaction with non-beta cells within the islet. Enzymatic digestion leads to reduced islet viability and subsequent apoptosis immediately following isolation [156]. Post-isolation culture can improve islet function by increasing the ATP/ADP ratio and reducing pro-apoptotic signaling in islets [176, 177], improving islet viability and morphology, although, at the expense of losing islet mass [178]. Post-isolation culture has also been shown to decrease expression of tissue factor which is a glycoprotein that initiates the extrinsic coagulation system and triggers clotting, and can activate intracellular signals resulting in inflammation [173]. Administration of anti-tissue factor antibodies to non-primate recipients of human allografts with a marginal mass of islets transplanted, improved islet engraftment and function significantly [173].

Cold ischemic time has been associated with loss of islet mass [178] and islets are highly susceptible to hypoxia since they have very low levels of anti-oxidants [79]. Islet viability is also severely affected by hypoxia to the cells in the inner islet core [155], which may result in necrosis and therefore decreased insulin secretion.

Purity of islets is also a factor that impacts graft function. Post-isolation culture with slightly impure islet preps that partially consist of extracellular matrix components or co-culture with ductal epithelial cells, enhances islet viability and function [155]. In humans for example, islets from subjects with chronic pancreatitis that were subsequently used for autotransplantation are fibrous and the digestion process of the pancreas is often incomplete and results in less than 40% of total islets isolated per pancreas [164]. Of 12 islet autotransplant recipients receiving these islet preparations, 8 achieved insulin independence [164].

**Site of implantation.** The site of implantation is also an important determinant for the survival of the islet graft. In humans, islets are predominantly transplanted in the liver via the portal vein, while in rodent models of transplantation, the subcapsular renal space is primarily used. Transplantation into the liver is a relatively non-invasive procedure that allows unrestricted access to oxygenated blood, although at lower arterial pressure, and intrahepatic islets avoid the systemic hyperinsulinemia seen in some pancreas allograft recipients [173]. Although
complications from portal infusions are rare there are reports of bleeding, portal venous thrombosis and portal hypertension [155], as well as hepatic steatosis [156]. Liver necrosis around the islet area can be seen as early as 1 day post-transplant, but resolve within the following week [179]. Despite the potential complications, the liver has served as a reasonable transplant site for islets. Importantly, upon engraftment islets are able to achieve pulsatile insulin secretion in response to hyperglycemia [180] and maintain glucose transporter 2 (GLUT-2) expression. However, when compared to whole pancreas recipients insulin content, glucose-stimulated insulin secretion, insulin biosynthesis, glucose oxidation rate and glucagon responsiveness to hypoglycemia are all markedly decreased [181].

Islet damage can occur due to non-specific activation and dysfunction of hepatic sinusoidal cells as well as intrahepatic host endothelial cells. Kupffer cells and host endothelial cells have been implicated as the primary mediators of inflammation-mediated loss of islets when transplanted into the liver [155]. Upon implantation of the islets in the hepatic sinusoids, both activated Kupffer cells and host endothelial cells release inflammatory cytokines in the vicinity of the transplanted islets [182]. Release of inflammatory cytokines such as IL-1β, TNF-α or IFN-γ, from the transplanted islets or from neighboring cells contributes to early beta-cell loss. Blockade of all three cytokines (IL-1β, TNF-α ad IFN-γ) with neutralizing antibodies can reduce the number of islets required for achievement of normoglycemia in mice [183]. In addition, endothelial cells up-regulate intracellular adhesion molecule (ICAM-1) and P-selectin and produce nitric oxide (NO).

The liver is a heterologous transplant site that does not mimic the native islet milieu with respect to metabolite concentrations or growth factors. Insulin is known to be lipogenic, and hence, islets transplanted into the liver may be exposed to elevated lipids. Insulin released locally from intrahepatic islets has been shown to stimulate triglyceride synthesis and accumulation of lipogenic enzymes in adjacent hepatocytes [184]. Elevated serum triglycerides have been associated with decline in graft function [185] and chronic FFA stimulation leads to beta-cell dysfunction and toxicity [78, 186, 187]. Lipids, in conjunction with elevated levels of glucose (glucolipotoxicity) act synergistically in promoting beta-cell dysfunction and death [75]. Recently, lipotoxicity was associated with loss of beta-cell mass following islet transplantation that could be reversed by lipid-depletion therapy with diet or by leptin overexpression [184]. In addition, because of the intestinal absorption of orally administered drugs, the portal vein has elevated concentrations of not only glucose and its metabolites, but also immunosuppressive
drugs which can be 2-3 fold higher than in the periphery and impact islet function (discussed below).

**Islet revascularization.** Islets in the pancreas are highly vascularized for optimal delivery of nutrients and oxygen supply to the inner islet core. Conversely, transplanted islets generally have poor vascular supply and density. Although islets only constitute 1% of the pancreas weight, they receive 5-15% of the total blood flow [155]. Loss of viable islets during the immediate post-transplant phase is of great importance, as only about 25-30% of the infused islet mass actually engrafts in the liver of the recipient [158]. Rapid revascularization of the islets within the liver is crucial to their functionality in the early post-transplant phase and several factors may contribute to non-engraftment such as local inflammation, blood-clotting resulting in islet apoptosis, and hypoxia.

Islet grafts are able to regenerate their microvasculature within a period between 10 to 14 days following islet infusion [155]. During this period, islets are exposed to a hypoxic environment, which may induce islet apoptosis. Following culture, endothelial cells are lost making islet endothelial expansion essential for revascularization. Islets secrete vascular endothelial growth factor (VEGF), an angiogenic factor secreted from islets in response to hypoxia which promotes revascularization with the surrounding host-tissue, creating a chimera of donor and recipient endothelium, and in effect has been shown to improve graft function [179]. The proportion of islets that restore their original endothelium, however, vary and may be limited. Final vascular graft density in transplanted islets is likely only half of native islets and the capillaries that do form may not always penetrate into the core of the islets, which predominantly consist of beta cells, and therefore result in insufficient oxygen and nutrient delivery [188]. Additionally, the liver has very low oxygen tension which may be inadequate to support the high metabolic demands imposed on the transplanted pancreatic islets [189]. Transplant recipients have persistent hyperglycemia and elevated portal levels of glucose that can lead to further decreases in oxygen tension [189]. With the limited amount of oxygen present in the post-transplant period, hyperglycemic overstimulation of beta cells can result in greater oxygen consumption and promotion of apoptosis [190].

The instant blood-mediated inflammatory reaction (IBMIR) is characterized by the activation of coagulation and complement systems, islet infiltration of recipient leukocytes and the binding of host platelets to islets [155]. Once islets come in contact with ABO-compatible blood in the portal vein, IBMIR occurs contributing to the early loss of islets [179]. The IBMIR is triggered by expression and secretion of tissue factor from the transplanted islets that in turn
generates thrombin leading to fibrin encapsulation of the islets [179]. Islets also express macrophage chemo-attractant protein-1 (MCP-1) that plays a role in leukocyte infiltration. In vitro inhibition of neutrophil elastase and thrombin activity by treatment with the protease inhibitor, α1-antitrypsin, has been effective by protecting mouse islets from deleterious effects of IL-1β and IFN-γ by increasing viability and reducing nitric oxide production [179]. Moreover, in vivo administration of α1-antitrypsin to mice significantly improves islet survival [179].

**Alloimmunity, recurrent autoimmunity and graft rejection.** Diabetes is characterized by the presence of beta-cell reactive autoantibodies and T cells in patients. Although recent studies suggest that cellular autoimmunity determines the outcome of clinical islet transplantation, the ability to predict graft outcomes is debatable. Increasing levels of islet autoantigens, glutamic acid decarboxylase 65 (GAD65) and insulinoma-associated protein (IA-2), in whole pancreas transplant have been reported to correlate with graft loss [173]. Insulin independence in islet allograft recipients was correlated with pre-transplantation levels of both GAD65 and IA-2 [191]. Subjects positive for the autoantibodies exhibited delayed insulin independence at one-year post transplant and had lower levels of circulating C-peptide [191].

**Recipient immunosuppression.** The goal of administering immunosuppressive drugs is to induce graft tolerance without promoting damage to the transplant. Like solid organ transplants, islet allotransplants trigger immune-mediated graft rejection which is controlled by immunosuppressive drugs. Unlike islet allograft recipients, autograft recipients are free from immunosuppression. Introduction of the Edmonton protocol significantly improved the success of islet transplantation outcomes with increasing insulin independence rates in recipients. The Edmonton immunosuppressive regimen employed an induction and maintenance phase of immunosuppression. Anti-IL-2 receptor antibody (daclizumab) was administered immediately following transplantation and was later discontinued while maintenance therapy continued with the calcineurin inhibitor, tacrolimus, and mTOR inhibitor, sirolimus [159]. Induction with daclizumab has not been shown to be deleterious to beta cells and does not impact either insulin synthesis or secretion, and hence is amenable to use in the induction phase of the immunosuppressive protocol [192]. Immunosuppressive agents in use currently include tacrolimus, sirolimus and mycophenolate mofetil (MMF) and all have been shown to impact on glycemic control, beta-cell function and apoptosis.

Sirolimus is a macrolide that inhibits T-cell activation by forming a complex with FKBP-12 cytosolic protein inhibiting mTOR [193] and blocks IL-2 mediated T-cell proliferation [192]. mTOR inhibition results in the blockade of protein translation which is critical in increasing cell
cycle progression and proliferation [193]. Results of studies on the effects of sirolimus on beta-cell function and survival have been equivocal. In a study of autologously transplanted dogs, no impairment in islet function was observed and fasting plasma insulin levels were elevated [194]. In a more recent study with minipigs and isolated human islets, increased basal and stimulated insulin secretion was observed in vivo; sirolimus had no demonstrable effects on islet function in vitro at concentrations targeted for immunosuppression in humans and was associated with increase in insulin content and cellular ATP [193]. In contrast, in a syngeneic mouse transplant model, sirolimus was found to impair blood glucose profiles and reduce glucose-stimulated insulin secretion. These correlated with reduced intragraft insulin content and decreased vascular density suggesting that sirolimus plays a role in the early post-transplant stage by impairing islet revascularization and engraftment as well as beta-cell function. Furthermore, sirolimus was also associated with dyslipidemia [195]. In the same study, culture of murine islets in the presence of sirolimus showed diminished glucose-stimulated insulin secretion coinciding with reduction in PDX-1 and GLUT-2 expression [195]. These observations were replicated in studies with HIT-T15 cells [192] and were also seen in human islets cultured in the presence of supraphysiological concentrations of immunosuppressive drugs which may be more representative of the portal milieu. In marginal transplanted islet grafts, sirolimus has been shown to impair proliferation and increase apoptosis [196]. It has also been implicated in inducing insulin resistance [173].

Tacrolimus is a calcineurin inhibitor capable of blocking IL-2 gene transcription [192] and has been implicated with the development of post-transplant diabetes mellitus [197, 198]. Along with sirolimus it has been implicated in inducing nephrotoxic effects resulting in the development of hyperlipidemia and hypertension [199]. In a group of pancreas transplant recipients, treatment with tacrolimus in some cases was associated with hyperglycemia, and morphological changes that included cytoplasmic swelling and vacuolization with a marked decrease or absence of dense-core secretory granules [200]. Tacrolimus impairs insulin secretion in vivo [197] and decreases insulin gene transcription and translation [173]. In an early study of immunosuppressive drugs in pancreas transplant recipients, fasting glucose homeostasis was obtained at the expense of impaired insulin secretion with a greater proportion of proinsulin intermediates being secreted [201].

Mycofenolate mofetil (MMF) prevents the proliferation and function of T and B-lymphocytes by inhibiting the enzyme inosine monophosphate dehydrogenase upon which lymphocytes are dependent on purine production. Insulin secretion from cultured human islets
was not affected by the presence of MMF, whereas, exposure of HIT-T15 cells or Wistar rat islets to the compound resulted in decreased insulin secretion [192]. In another study, MMF was found to decrease islet cell viability by downregulating anti-apoptotic factors in cultures of freshly isolated human islets [202].

Proliferation of pancreatic beta cells and maintenance of graft mass are critical for graft survival. incretin signaling has been shown to be an important mediator in promoting beta-cell viability and secretory function and there is evidence to support a positive role of incretin therapy in islet transplantation. *In vitro* culture of MIN-6 cells in the presence of sirolimus, tacrolimus and MMF resulted in a reduction of insulin secretion with a greater proportion of apoptotic and necrotic cells [203] which could be ameliorated by transfection of GLP-1 that was shown to have significant anti-apoptotic properties, that included upregulation of pro-survival genes such as Bcl-2 [204]. Similar to the effects observed with GLP-1, transduction of islets with the anti-apoptotic X-linked inhibitor of apoptosis (XIAP) ameliorated the negative effects of decreased insulin secretion as well as reduced insulin gene transcription in the presence of the immunosuppression cocktail, by enhancing islet viability and beta-cell function[202]. Although immunosuppression is mandatory for prevention of graft rejection, further development of novel immunosuppressive agents is necessary to overcome their toxic effects on beta cells.

**Transplanted islet mass.** A typical pancreas has approximately 1 million islets, however only about 40% of these can be successfully isolated and therefore more than one donor pancreas is necessary for achievement of normoglycemia and insulin independence in islet allotransplant recipients. In human allotransplant recipients, >10,000 Islet Equivalents (IE)/kg body weight isolated from two or more donor pancreata are required for achievement of normoglycemia and long-term graft survival and function [205]. In contrast, islet autotransplant recipients receive on average between 2000-7000 IE/kg and are able to achieve normoglycemia and prolonged insulin independence [206]. In both situations, fewer islets are less likely to achieve recipient normoglycemia and insulin independence, especially since most of the transplanted mass is lost in the early post-transplant period. While most groups see correlations between insulin free status and beta-cell mass there was one report of an autotransplant patient receiving only 954 IE/kg and maintaining insulin independence for 4 years following transplantation [164].

Since only about 30% of transplanted islets engraft [158], there may be increased metabolic demand on the residual islets impacting survival and function of the graft. Factors such as the insulin resistant state of the recipient, local levels of triglycerides and glucose can all contribute to a decrease in graft mass and function, especially on a marginal mass graft. Earlier
islet graft decline was associated with higher baseline levels of triglycerides in allotransplant recipients [185]. In a syngeneic rodent model of transplantation, islet mass correlated inversely with the time for glycemic normalization to occur [207] as well as short- and long-term survival, indicating that glucose levels are key determinants of graft survival, especially in marginal mass transplants. Marginal mass islet recipients have fasting hyperglycemia and hypoinsulinemia when fed a high sucrose diet for several weeks [208]. This is also supported by the finding that 50% pancreatectomy in humans leads to increased fasting glucose and decreased insulin and C-peptide levels following a stimulus and is associated with the severity related to the surgical procedure and the portion of the pancreas excised [209].

The above studies suggest that regardless of the mass transplanted, progression to graft failure occurs in the majority of patients, despite residual C-peptide secretion, and may be the result of several factors including glucolipotoxicity, immunosuppressive drug toxicity or the continual assaults by autoimmunity and allorejection.

1.3 Beta-cell dysfunction in type 2 diabetes and islet transplantation

Recipients of pancreatic islet grafts and subjects with type 2 diabetes exhibit multiple similarities with regards to beta-cell function and metabolic stability. Insulin resistance has been demonstrated in subjects with type 1 diabetes [162] which may increase the metabolic load on the transplanted islets and tacrolimus has been shown to induce insulin resistance post-transplant. Although not all insulin resistant subjects progress to diabetes, insulin resistance is a characteristic in many individuals who go on to develop type 2 diabetes. In islet transplant recipients, the beta cell must function appropriately to maintain normoglycemia and depends greatly on the peripheral insulin sensitivity.

In the case of an insufficient mass, the demand placed on beta cells may be too much such that dysfunction ensues. Impaired glucose-stimulated insulin secretion is also present in islet transplant recipients and manifests as defective first-phase insulin secretion [210]. The endoplasmic reticulum plays a crucial role in insulin biosynthesis and prolonged ER stress can result in cellular dysfunction and decreased survival. Transplanted islets are not only exposed to increased metabolic demand, but also to elevated levels of cytokines, NO and ROS, as well as immunosuppressant drugs such as MMF, sirolimus and tacrolimus, all of which may promote ER stress responses. Like type 2 diabetic islets, transplanted islets have elevated levels of pro-apoptotic markers that are associated with beta-cell death.
Reduced islet mass seen in type 2 diabetes is associated with amyloid deposits and has recently been demonstrated in human recipients of transplanted islets. Westermark et al. were the first to report on the presence of amyloid in human islets transplanted into the subcapsular renal space of diabetic nude mice which was evident already at 2 weeks post-transplantation and was localized to beta cells [211]. This study described a rapid process unlike that seen in islets from type 2 diabetic subjects. To verify that it was not an artifact of the transplantation site, human islets were transplanted into the liver and amyloid deposits were also observed [212]. More importantly, amyloid deposits were found in islets transplanted into a type 1 diabetic patient only 5 years post-transplant, implicating amyloid as a potential cause of islet graft dysfunction and beta-cell loss [213].

1.4 Thesis hypothesis and objectives

In light of the metabolic and functional similarities observed in recipients of pancreatic islet transplants and subjects with type 2 diabetes, we hypothesized that as in type 2 diabetes, processing of prohormones, proinsulin and possibly proIAPP in islet grafts, are likely also impaired. To address this hypothesis, the following objectives were pursued.

1.4.1 Objective 1: To determine whether prohormone processing is impaired in islet transplantation

1.4.1.1 Aim 1: To determine whether human islet transplant recipients have impaired prohormone processing

1.4.1.2 Aim 2: To determine whether transplanted islet graft mass impacts prohormone processing

1.4.2 Objective 2: To develop a sensitive and specific two-antibody sandwich immunoassay to enable measurement of proIAPP in human serum from islet transplant recipients and subjects with type 2 diabetes
CHAPTER 2. MATERIALS & METHODS

2.1 Chemicals and buffers

Reagents used in experiments were of analytical grade and suppliers are noted for each. All solutions were brought to room temperature before use.

The following buffers were used for the time-resolved fluorescent immunoassay (TR-FIA). Antibody coating buffer contained 0.5M (42 g/L) NaHCO₃ (Sigma-Aldrich, St. Louis, MA). Blocking buffer contained 20 mM (2.4 g/L) Tris-HCl (Promega, Madison, MI) and 150 mM (8.8 g/L) NaCl (Fisher), pH 8. Before use, 2% bovine serum albumin (BSA; Sigma-Aldrich) was added and the solution filtered with a 0.45 µm syringe-top filter (Acrodisc Cat# PN4614; Pall Corporation MI). Sample dilution buffer (SDB) contained 50 mM (6.1 g/L) Tris-HCl and 150 mM (8.8 g/L) NaCl, pH 7.5. Prior to use, 1% BSA was added and the solution filtered. SDB was used as the zero standard for proIAPP standard curves as well as diluent for proIAPP standards, human and mouse islet lysates and human serum samples to be assayed. For assessment of proIAPP in serum samples, serum from long-duration type 1 diabetic patients (LDT1D) diluted 1:5 in SDB was used for preparation of proIAPP standards. LDT1D was obtained from subjects at BC Children’s Hospital (BCCH) who had type 1 diabetes for a period of 10 years or more. Subjects with LDT1D are thought to have little residual beta-cell function and therefore, no proIAPP or minimally detectable amounts. Wash buffer contained 50 mM (6.1 g/L) Tris-HCl, 150 mM (8.8 g/L) NaCl, pH 7.5 and 0.01% Tween 20 (Sigma-Aldrich). DELFIA® enhancement solution for signal generation was purchased from Perkin-Elmer (Cat # 1244-105).

The following buffers were used in the electrochemiluminescent assay (ECL). Phosphate buffered saline pH 7.4 (PBS) was used as diluent for coating the plate with capture antibody. MSD® 5X Blocker A (Cat # R93BA-1) diluted in MSD® Phosphate Buffer (Cat # R93SA-1) was used to block plates (Meso Scale Discovery, Inc., Gaithersburg, Maryland). The blocking buffer is a proprietary cocktail of proteins in a PBS-based buffer optimized by the manufacturer. Assay diluent used for preparation of proIAPP standards or serum samples consisted of 5 ml fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, ON), 4.9 ml of 5X MSD® Blocker A diluted to 1X in PBS-T, 0.1 ml of 10% Triton X-100 (Sigma-Aldrich) and up to 1000 KI U/ml aprotinin (Sigma-Aldrich). For dilution of primary and secondary antibodies, 1% MSD® Blocker
A in PBS-T was used. Wash buffer was comprised of 0.05% Tween-20 in 1X PBS. MSD® 4X Read Buffer T, a Tris-based buffer containing tripropylamine (TPA) as a co-reactant for light generation, was diluted 4-fold with ddH₂O and used for generation of signal.

2.2 Animals

All mouse and human islet transplant experiments were approved by the UBC Animal Care Committee and were performed at the University of British Columbia in compliance with institutional guidelines and guidelines of the Canadian Council on Animal Care. All surgeries were performed under isofluorane anesthesia except during procedures performed at the termination of the experiments during which time, Avertin (0.02 ml/g body weight, i.p.) was used.

All donor and recipient animals used for experiments were 8-10 week old male mice. Unless otherwise noted, all mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice homozygous for the severe combined immune deficiency spontaneous mutation Prkdc<sup>scid</sup> (NOD.scid; NOD.CB17-Prkdcscid/J) were further bred in-house at the Child and Family Research Institute Animal Care Facility, according to institutional guidelines. NOD.scid mice were used as recipients of human islet allografts and donor islets from transgenic mice expressing human IAPP (hIAPP; FVB/N-Tg(Ins2-IAPP)RHFSoel/J). C57BL/6J mice were purchased and used directly from the supplier for syngeneic islet transplants. hIAPP mice were purchased as a heterozygous breeding pair, bred in-house and genotyped for the presence of hIAPP according to protocols provided by the Jackson Laboratory. Wild-type littermates were used as controls in all experiments.

2.3 Islet transplantation

NOD.scid mice receiving allogeneic human islet transplants or transplants of hIAPP islets were rendered diabetic by a single intraperitoneal (i.p.) injection of 200 mg/kg streptozotocin (STZ) (Sigma-Aldrich) in citrate buffer. C57Bl/6J mice receiving syngeneic islet grafts were rendered diabetic with a single dose of 180 mg/kg STZ. Islet transplant recipients were transplanted 3 days after STZ injection, once blood glucose levels were above 20 mM.

Human islets transplanted into diabetic NOD.scid mice were isolated from 7 cadaveric donors as previously described [214]. To allow for recovery, islets were incubated overnight in CMRL (5.5 mM glucose) (Mediatech, Manassas, VA) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 50 µg/ml gentamycin. The following day, human islets were washed
and hand picked into groups of 200, 300 or 500 islets of similar size and transplanted under the kidney capsule of the diabetic and hyperglycemic NOD.scid recipient mice. The kidney is one of the most vascular organs within the body and therefore contributes to more efficient tissue engraftment and viability following transplantation [215]. This transplant site is also relatively easy to access such that the experimenter is able to not only implant the islets but also retrieve the graft for histological analysis.

Donor mouse islets were isolated from C57Bl/6J or hIAPP transgenic mice using retrograde injection of the common pancreatic bile duct with collagenase Type XI (1000 units/mL in HBSS; Sigma Aldrich, St Louis, MO), followed by incubation at 37°C to allow for digestion and purification on a dextran gradient [216]. After gradient centrifugation all islets were pooled, washed 3 times, hand-picked and cultured overnight in Ham’s F-10 media (Gibco, Invitrogen Canada, Burlington, ON) supplemented with 5% BSA. The next day, islets were split into groups of 200 and 300 for C57Bl/6J donors and 250 for hIAPP<sup>tg/wt</sup> and hIAPP<sup>wt/wt</sup> littermate control donors and transplanted under the kidney capsule of diabetic C57Bl/6J or NOD.scid recipient mice, respectively.

Random blood glucose measurements of transplanted mice were taken between 0900 and 1200h via the tail vein using a OneTouch Ultra Glucometer (Life Scan) daily for the first week and weekly for the remaining 5 weeks until sacrifice at 6 weeks post-transplant. Mice were qualified as having a failed or non-functional graft when glycemia was above 15 mM at 6 weeks post-transplant and on one or more occasions over the duration of the experiment.

Plasma from transplant recipients fed ad libitum was collected at 2 and/or 6 weeks post-transplant. Blood was withdrawn via the saphenous vein without anesthesia (2 weeks) or with anesthesia and heart puncture (6 weeks). Blood was collected into plastic tubes containing Na<sub>2</sub>EDTA (Microvette® CB300, Sarstedt) and kept on ice prior to centrifugation (5000 rpm, 6 min, 4°C). After separation, plasma was immediately frozen and stored at -80°C until assay. Samples were assayed for human insulin and proinsulin (human islet recipients) and mouse insulin and proinsulin (C57Bl/6J and hIAPP islet recipients) using commercially available immunoassays described in Section 2.5.

### 2.4 In-vivo immunosuppression

Five hundred hand picked human islets were transplanted under the kidney capsule of diabetic recipient NOD.scid mice as described in Section 2.3 and blood glucose was monitored regularly for 3-4 weeks. Post-transplant, mice were injected i.p. daily with the following
immunosuppressive medications: sirolimus (50 mg/g body weight; Wyeth, Montreal, QC), FK506 (30 mg/g body weight; also known as tacrolimus; Astellas, Markham, ON), mycophenolate mofetil (1.5 mg/g body weight; Hoffmann-La Roche, Mississauga, ON) or saline.

2.5 Clinical study subjects

A cross-sectional study of human islet transplant recipients was approved by the Clinical Research Ethics Board at the University of British Columbia, the Research Review Committees at BC Children’s Hospital and Vancouver General Hospital, and the University of Cincinnati Institutional Review Board. Written informed consent was obtained from each subject prior to participation.

Thirteen islet allotransplant recipients were recruited from the Vancouver Islet Transplant Program at Vancouver General Hospital. Subjects received a total of 25 islet infusions (6 subjects received one infusion, 3 subjects received two infusions, 3 subjects received three infusions and 1 subject received four infusions) for an average of 13,235±1,632 (range 6,837–24,973) IE/kg. On average, subjects were 398±130 days post transplantation at the time of study (range: 5-1224 days). Twelve subjects were receiving treatment with tacrolimus (Fujisawa Healthcare Ins., Deerfield, IL) and mycophenolate mofetil (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and one subject was receiving treatment with both tacrolimus and sirolimus (Wyeth, Madison, NJ). Subjects were receiving the following therapeutics: pioglitazone and exenatide (7), pioglitazone (3), exenatide (2); one subject was not taking either medication. Of those same subjects, 8 were on insulin therapy and 5 were insulin independent. Creatinine (111±10 µmol/L; range: 67–193 µmol/L) and blood urea nitrogen (BUN; 9±1 mg/dL; range 4.7-13.7 mg/dL) measurements were retrospectively obtained from clinical charts for the islet transplant recipients, and indicated that some of these subjects had mildly impaired renal function.

Twelve subjects who had received islet autotransplants following total pancreatectomy as treatment for chronic pancreatitis were recruited from the University of Cincinnati. No information regarding the origin of pancreatitis or the presence/absence or duration of diabetes were available at time of study. These subjects had blood sampled prior to surgery and islet autotransplantation, and one year following islet transplantation. These subjects received 5,642±1,324 IE/kg (range 138-19,590).
We also studied a representative population of subjects with type 2 diabetes (n=14), in order to validate the immunoassays used throughout the study. These subjects tended to be older and had a higher body mass index (BMI) than the non-diabetic controls or islet transplant recipients. The mean hemoglobin A1c (HbA1c) levels were 6.5±0.2% with a fasting blood glucose level of 7.0±0.3 mM. Some subjects were taking oral hypoglycemic medications: metformin (8), glyburide (5), gliclazide (2); 5 subjects took no medication). None of the subjects with type 2 diabetes were being treated with insulin. Seventeen healthy non-diabetic subjects were also recruited to participate as controls.

All anthropometric measurements were taken in the morning after an overnight fast. Body weight was measured to the nearest 0.1 kg and height was measured to the nearest 0.1 cm. Body mass index (BMI) was calculated as the weight (kg) divided by height (m) squared.

Venous blood samples for analysis of glucose, insulin (I), C-peptide (CP), total (TP) and intact proinsulin (IPI) were collected between 0700 and 0900 h after an overnight fast and centrifuged (3700rpm) at 4°C for 20 min. After addition of 2µM DPP-IV inhibitor (P32/98 (Isoleucine thiazolidide) Probiodrug, Halle Germany; gift from Dr. C. McIntosh, UBC), serum was frozen at -80°C until further analysis. HbA1c was measured using a point-of-care testing glycohemoglobin analyzer (Bayer DCA®2000; Bayer, Canada). Islet transplant patients treated with insulin had their last injection at least 12h prior to blood sampling.

In addition, we also analyzed levels of islet amyloid polypeptide (IAPP) and pro-islet amyloid polypeptide (proIAPP) in several non-diabetic control samples as well as samples from subjects with type 2 diabetes.

2.6 Assays

Serum from human islet transplant recipients was assayed using commercially available enzyme linked immunoassays (ELISA) to determine levels of insulin (Mercodia, Uppsala, Sweden), C-peptide (Mercodia), total proinsulin (Linco Research, St.Charles, MO), and intact proinsulin (Linco). The cross-reactivities of these assays with other proinsulin-derived molecules are provided by the supplier and are listed in Table 1. A schematic representation of intact proinsulin and proinsulin intermediates, as well as mature insulin and C-peptide can be found in Figure 1. To validate our proinsulin values and proinsulin/insulin and proinsulin/C-peptide ratios, total proinsulin levels were re-measured using an ELISA from Mercodia for which cross-reactivities are listed in Table 1. Proinsulin fragments were calculated as total proinsulin minus intact proinsulin using assays obtained from Linco.
Plasma from mice transplanted with human donor islets was assayed for insulin (Cat # EZHI-14K, Linco Research, St.Charles, MO) and total proinsulin (Linco) and cross-reactivities of each are presented in Table 1.

Table 1. Cross-reactivities of commercially available immunoassays for human insulin, C-peptide, total and intact proinsulin.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Intact Proinsulin (Linco)</th>
<th>Total Proinsulin (Linco)</th>
<th>Total Proinsulin (Mercodia)</th>
<th>Insulin (Mercodia)</th>
<th>Insulin (Linco)</th>
<th>C-peptide (Mercodia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>n/a</td>
<td>n/a</td>
<td>&lt; 0.03 %</td>
<td>100%</td>
<td>100 %</td>
<td>&lt; 0.0006 %</td>
</tr>
<tr>
<td>C-peptide</td>
<td>n/a</td>
<td>n/a</td>
<td>&lt; 0.0006 %</td>
<td>&lt; 0.01 %</td>
<td>0 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Intact Proinsulin</td>
<td>100 %</td>
<td>100 %</td>
<td>100%</td>
<td>&lt; 0.01 %</td>
<td>0 %</td>
<td>&lt; 1.8 %</td>
</tr>
<tr>
<td>Des 31,32 Proinsulin</td>
<td>n/a</td>
<td>100 %</td>
<td>95 %</td>
<td>&lt; 0.05 %</td>
<td>0.3 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Split 32,33 Proinsulin</td>
<td>n/a</td>
<td>n/a</td>
<td>95 %</td>
<td>&lt; 0.05 %</td>
<td>n/a</td>
<td>2 %</td>
</tr>
<tr>
<td>Des 64,65 Proinsulin</td>
<td>36 %</td>
<td>81 %</td>
<td>84 %</td>
<td>98 %</td>
<td>117 %</td>
<td>74 %</td>
</tr>
<tr>
<td>Split 65,66 Proinsulin</td>
<td>n/a</td>
<td>n/a</td>
<td>90 %</td>
<td>n/a</td>
<td>n/a</td>
<td>10 %</td>
</tr>
</tbody>
</table>

For the determination of plasma levels of insulin and proinsulin in mice transplanted with C57Bl/6 islets or transgenic hIAPP islets, commercially available assays from Alpco Diagnostics Inc. (Salem, NH) were used. The cross-reactivities for the ultrasensitive mouse insulin ELISA (Cat # 80-INSMSU-E01) and mouse proinsulin ELISA (Cat # 80-PINMS-E01) are listed in Table 2. Although not noted on the manufacturer’s insert, the mouse insulin ELISA is believed to cross-react 30-40% with mouse proinsulin (personal communication from Collin Shaw and Craig LaMarca, Alpco Diagnostics) (Table 2).
Table 2. Cross-reactivities of commercially available mouse insulin and proinsulin immunoassays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ultrasensitive Mouse Insulin</th>
<th>Mouse Proinsulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>100 %</td>
<td>n/a</td>
</tr>
<tr>
<td>C-peptide I</td>
<td>&lt; 0.01 %</td>
<td>&lt; 0.05 %</td>
</tr>
<tr>
<td>C-peptide II</td>
<td>&lt; 0.01 %</td>
<td>&lt; 0.05 %</td>
</tr>
<tr>
<td>Proinsulin I</td>
<td>n/a</td>
<td>100 %</td>
</tr>
<tr>
<td>Proinsulin II</td>
<td>n/a</td>
<td>65.6 %</td>
</tr>
</tbody>
</table>

For the determination of IAPP levels in human serum, a commercially available assay from Linco was used. Human IAPP was measured using the Total Amylin ELISA kit (Cat # EZHAT-51K). According to the manufacturer, the cross-reactivity of this assay with other human peptides is as follows: < 1% glucagon, < 1% GLP-1, < 1% insulin, < 1% pancreatic polypeptide, 1% adrenomedullin, < 1% calcitonin, < 1% calcitonin gene-related peptide.

All fasting human serum samples were assayed in duplicate and high- and low-level controls were run with each assay. The intra- and inter-assay CVs for all assays were < 10%. Glucose was measured using GLU micro-slides with the VITROS 950 Chemistry System (Ortho-Clinical Diagnostics). Plasma samples obtained from mice transplanted with human islets were assayed as single wells due to the limited availability of samples. Plasma samples from mice transplanted with mouse islets were assayed in duplicate.

### 2.7 Immunohistochemistry and thioflavin-S staining

At 6 weeks post-transplant all human and mouse islet grafts were excised, fixed in Z-Fix (Anatech Ltd., Battle Creek, MI), and processed for histology. Paraaffin-embedded graft sections (5 μm) were deparaffinized in xylene, hydrated though 95% and 70% ethanol and rinsed with distilled water. All antibody incubations were performed at room temperature.

Human islet grafts were blocked in PBS containing 2% normal goat serum (Vector Laboratories). Slides were then incubated with guinea pig anti-insulin antibody (Dako) at 1:100 dilution in PBS containing 1% BSA for 1h, followed by incubation with Alexa Fluor 594 goat anti-guinea pig secondary antibody (Invitrogen; Cat # A-11076) for 1h. Slides were then immersed in 0.5% thioflavin S solution for 2 minutes, rinsed with 70% ethanol and wet-mounted with Vectashield mounting medium (Vector Laboratories; Cat # H-1000).
Mouse islet grafts isolated from C57Bl/6J mice underwent heat-induced epitope retrieval performed in antigen retrieval buffer (Dako Target Retrieval Solution, Cat # S1699) for 45 min using a steamer. Slides were then blocked in PBS containing 10% normal goat serum (Vector Laboratories) for 30 min followed by incubation with mouse anti-insulin (Cat# E54071M, Meridian Life Science Inc, Saco ME) and rabbit anti-glucagon (Cat#A0565, DAKO) antibodies at 1:100 and 1:75 dilution, respectively, in PBS containing 1% BSA for 1h. Secondary antibody incubations were performed for 1h with Alexa Fluor 594 goat anti-mouse (Invitrogen; Cat # A-11076) and Alexa Fluor 488 goat anti-rabbit both at 1:100 dilution (Invitrogen; A-11034). Slides were viewed and final images captured using an Olympus Bx61 fluorescent microscope and analyzed with ImagePro 6.2 software (Media Cybernetics, Inc.) for determination of amyloid positive area.

2.8 Antibodies

A schematic of the binding sites of proIAPP and IAPP antibodies used in TR-FIA and ECL immunoassays are shown in Figure 4. The monoclonal antibody directed against the N-terminal flanking peptide of proIAPP (F064) was a generous gift of Amylin Pharmaceuticals (San Diego, CA). Polyclonal anti-roden IAPP antibody (7323) was obtained from Peninsula Laboratories (Belmont, CA). Polyclonal anti-human IAPP antibody (PF07B2) was a generous gift from Dr. Paul Fraser (Toronto, ON). DELFIA® Eu³⁺- labeled anti-mouse antibody (Eu-N1; Cat # AD0124; Perkin-Elmer) is a purified polyclonal rabbit antibody that reacts with all mouse IgG subclasses. Eu-N1 was used as the secondary antibody for signal detection. When the monoclonal antibody was used as capture and the polyclonal antibody as primary, a DELFIA® Eu³⁺ - labeled goat anti-rabbit antibody was used as the secondary antibody (Cat # AD 0106, Perkin-Elmer). For the ECL assay, a MSD SULFO-TAG® labeled goat anti-rabbit was used as detection antibody (Cat # R32AB-5, Meso Scale Discovery, Gaithersburg, Maryland).
Figure 4. Binding regions of IAPP and proIAPP antibodies used in human proIAPP time-resolved fluorescent and electrochemiluminescent immunoassays.
2.9 Time-resolved fluorescent proIAPP immunoassay (TR-FIA)

DELFIA® 96-well yellow plates (Perkin Elmer; Cat # AAAND-0001) were pre-coated for 1h at room temperature then overnight at 4°C with 1:500 diluted 7323 or PF07B2 capture antibodies in 300 μL/well antibody coating buffer. The next day, plates were brought to room temperature and blocked with 300 μL/well blocking buffer for 1h. Pre-diluted samples or standards were pipetted in duplicate (200 μL/well), covered and incubated for 2h with continuous horizontal shaking at 450 rpm on an Eppendorf Theromomixer-R plate shaker. After 3 washes of 300 μL/well of wash buffer, 200 μL/well of primary monoclonal F064 antibody diluted 1:2000 in SDB were added and the plates incubated for 1h with shaking. After another 3 washes, 200 μL/well of 1:500 diluted Eu-N1 rabbit anti-mouse antibody was added and incubated with shaking for the following hour. After 6 washes, 300 μL/well of enhancement solution was added, the plate was shaken for 10 min, and the Eu³⁺ time-resolved fluorescence was measured with the VICTOR³V® 1420 Multilabel counter capable of excitation and emission wavelengths of 340nm and 613nm, respectively. All incubation steps were done at room temperature and all washes were performed with a multichannel pipette and tips pre-coated with Sigmacote® (Sigma-Aldrich, St. Louis, MA).

2.10 Meso Scale Discovery electrochemiluminescent proIAPP immunoassay (ECL)

MULTI-ARRAY® 96-well standard-bind plates (Cat # L15XA, Meso Scale Discovery, Gaithersburg, Maryland) were pre-coated with 1:2000 diluted monoclonal F064 capture antibody and incubated at 4°C overnight. The next day, wells were blocked with 150 μL/well blocking buffer with shaking for 1h at room temperature and washed 3 times with 350 μL/well of wash buffer. For incubation of samples and standards, 20 μL assay diluent and 40 μL sample or standards were added to wells and incubated for 1h with shaking. Plates were again washed 3 times and 25 μL/well of 1:500 diluted polyclonal PF07B2 was added, sealed and incubated with shaking for the next hour. Plates were again washed 3 times and MSD SULFO-TAG® labeled goat anti-rabbit secondary antibody diluted 1:500 was added and incubated with shaking for 1h. After the last 3 washes, MSD® 4X Read Buffer T® was diluted to 1X, 150 μL/well diluted Read Buffer was added and the signal was read immediately in the SECTOR® Imager 2400. All incubations were performed at room temperature unless otherwise noted. All washes were performed with a multi-channel pipette.
2.11 Standards and samples for proIAPP immunoassay

Synthetic truncated proIAPP 1-30 peptide was synthesized and 95% HPLC purified at the Peptide Array Facility/Kinexus Bioinformatics Corporation at the University of British Columbia (Vancouver, BC). Stock proIAPP was in sample dilution buffer (SDB) and stored at -20°C until use. Standards for the TR-FIA were prepared in 2% BSA-coated 1.5 ml Eppendorf tubes and tips coated with Sigmacote. Pipette tips were changed with every dilution step and all dilutions were completed on ice.

ProIAPP standards were prepared in various concentrations ranging from 0 – 180 pM in SDB, 20% LDT1D, 20% charcoal extracted human serum (CEHS; Bioreclamation, Inc., East Meadow, NY), 20% fetal bovine serum (FBS) or horse serum (Sigma-Aldrich, St. Louis, MA) as indicated in Chapter 5. All standards were assayed in duplicate and the standard curves were generated using a 4-parameter logistic curve-fit program (GraphPad Prism version 4; GraphPad Software, Inc., San Diego, CA, 2003) from which, unknown samples were quantified.

The following samples were used to assess whether the TR-FIA or ECL were capable of detecting biosynthetic proIAPP. Serum samples collected from adult subjects with normal glucose tolerance or type 2 diabetes described in Section 2.5 were diluted 5-fold unless otherwise noted and values determined from a standard curve with 20% LDT1D serum for the TRF-IA and assay diluent for the ECL assay as described in Section 2.1. These samples were also assayed for insulin, total proinsulin and IAPP using commercially available immunoassays. All samples were measured in duplicate wells in each assay.

In order to study proteolytic degradation of proIAPP in serum, synthetic proIAPP 1-30 peptide, with or without 2 µM DPP-IV inhibitor (P32/98), was incubated in buffer containing 40% normal human serum isolated from one individual with normal glucose tolerance. Samples of the peptide were removed from serum at 0, 1, 2, 3, 4, and 24h after incubation at 37°C with moderate shaking and stored at -80°C until assay. ProIAPP standards were reconstituted in buffer containing 40% CEHS and unknown samples were determined for residual proIAPP using graphing software.

Isolated human and transgenic mouse islets were also used to determine whether the TR-FIA is capable of detecting proIAPP in cell lysates. Islets were incubated in 1 M acetic acid lysis buffer containing 1% BSA for 10 min at 100°C. Lysates were spun down for 10 min at 4°C and 12,000 rpm, and supernatants collected and stored at -80°C until use. Prior to analysis of samples, standards were diluted in SDB.
2.12 Calculations and statistical analysis

Data from the clinical islet transplant study are represented as medians and ranges unless otherwise noted. Data obtained in the *in vivo* animal models of islet transplantation are presented as mean ± SEM (standard error of the mean).

Normality tests were performed on all groups to determine whether they followed a Gaussian (normal) distribution. Where data were normally distributed, Student’s t-test was used to compare between two groups or a one-way ANOVA to compare more than two groups, followed by Tukey’s post-test to adjust for multiple comparisons. For non-Gaussian distributed groups, the Kruskall-Wallis test was used to compare more than two groups, followed by Dunn’s post-test and the Mann-Whitney test was used for analysis between two groups. *P*<0.05 was considered significant.

To describe proinsulin as a proportion of total insulin immunoreactivity, the proinsulin/insulin ratio was calculated as \( \frac{\text{total proinsulin}}{\text{total proinsulin} + \text{insulin}} \) (TP/TP+I) for clinical human islet transplants and \( \frac{\text{proinsulin}}{\text{proinsulin} + \text{insulin}} \) (PI/PI+I) for mouse models of islet transplantation. In original literature, the proinsulin/insulin ratios were expressed as proinsulin/total immunoreactive insulin (PI/IRI) and we chose to follow a similar calculation by taking into account all the insulin immunoreactive species that would be present in circulation. The proinsulin/insulin and proinsulin/C-peptide ratios are expressed as a percentage (%).

Proinsulin intermediates, or fragments (PF), were calculated as total proinsulin-intact proinsulin (TP-IPI). To assess the relationships between measured (insulin, proinsulin) or calculated (proinsulin/insulin ratio) parameters and islet equivalents per kilogram body weight (IE/kg), either Pearson or Spearman correlation were used. TR-FIA and ECL assay sensitivity was assessed by the following equation: blank plus two standard deviations above the mean blank (Blank + 2 SD).

To assess the presence of insulin resistance, the homeostasis model assessment-insulin resistance (HOMA-IR) was calculated using the following formula: \( \frac{\text{non-fasting blood glucose (mM)} \times \text{non-fasting insulin (mU/L)}}{22.5} \).

All statistical analyses were performed using GraphPad Prism (version 4; GraphPad Software, Inc., San Diego, CA, 2003).
CHAPTER 3. IMPAIRED PROINSULIN PROCESSING IS A CHARACTERISTIC OF TRANSPLANTED ISLETS

3.1 Background

Islet transplantation can improve glycemic control for individuals with type 1 diabetes mellitus. Despite the initial achievement of insulin independence with near normalization of HbA1c in most islet transplant recipients, almost all experience a gradual decline in graft function as early as two years post-transplant [160, 161]. Loss of graft function may be mediated in part by both allo- and autoimmune mechanisms [217]. However, non-immune mechanisms of beta-cell loss are also probably involved [218, 219]. In addition, it is likely that an insufficient beta-cell mass is initially transplanted [160, 220, 221] and many transplanted islets fail to engraft [222]. There is also evidence that immunosuppressive drugs administered to patients to prevent rejection of grafts have detrimental effects on beta cells [223-225]. Finally, transplanted beta-cells exhibit a functional defect [205] manifest as impaired insulin secretion during a hyperglycemic clamp when compared to healthy controls or subjects with whole pancreas transplants [226, 227].

The defect observed in islet transplant recipients may thus share some commonality with the defect seen in individuals with type 2 diabetes, a disease similarly characterized by impaired insulin secretion and decreased beta-cell mass. Additionally, in type 2 diabetes conversion of the insulin precursor, proinsulin, is altered. The conversion process in normal beta-cells is very efficient; however, in type 2 diabetes, processing is impaired, resulting in increased secretion of proinsulin relative to the mature hormone, insulin [57]. Absolute or relative hyperproinsulinemia occurs in type 2 diabetes and in impaired glucose tolerance [55, 228], and is thought to be a sign of defective proinsulin conversion associated with beta-cell dysfunction. Elevated levels of proinsulin have been used as markers for beta-cell dysfunction [229] and are associated with deterioration of glucose tolerance; an increased ratio of proinsulin/insulin has been strongly correlated with impaired first-phase insulin secretion [55].

To determine whether the beta-cell defect seen in islet transplant recipients also manifests as impaired proinsulin conversion, we compared intact and total proinsulin levels among islet transplant recipients, subjects with type 2 diabetes and healthy non-diabetic controls, and determined whether the proinsulin/insulin and proinsulin/C-peptide ratios are elevated in islet transplant recipients.
3.2 Results

3.2.1 Clinical characteristics of study subjects

Characteristics of the study subjects are shown in Table 3. Allotransplant recipients and non-diabetic controls were matched for sex and BMI. Although autotransplant recipients were matched for BMI, they were younger than the non-diabetic controls and allotransplant recipients. Fasting blood glucose levels were elevated in allotransplant recipients relative to non-diabetic controls and autotransplant recipients. HbA1c levels were significantly higher in both allo- and autotransplant recipients compared to non-diabetic controls suggesting the presence of abnormal glucose control even in autotransplant subjects with seemingly normal fasting blood glucose levels.

Table 3. Clinical characteristics of non-diabetic controls and islet transplant recipients.

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>Non-Diabetic Controls</th>
<th>Allotransplants</th>
<th>Autotransplants</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>(8/9)</td>
<td>(7/6)</td>
<td>(3/9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.7 ± 1.6</td>
<td>46.8 ± 1.9</td>
<td>38.4 ± 2.7 a,b</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>25.6 ± 1.1</td>
<td>23.7 ± 0.9</td>
<td>26.4 ± 1.0</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mM)</td>
<td>5.1 ± 0.1</td>
<td>7.7 ± 0.5 c</td>
<td>5.5 ± 0.4 d</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 ± 0.1</td>
<td>7.1 ± 0.2 c</td>
<td>7.1 ± 0.3 c</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. One-way ANOVA was used to analyze differences between groups. *P*<0.05 was considered significant. a *P*<0.01 vs. non-diabetic controls, b *P*<0.05 vs. allotransplants, c *P*<0.001 vs. non-diabetic controls, d *P*<0.01 vs. allotransplants.

3.2.2 Fasting levels of insulin, C-peptide, total proinsulin, intact proinsulin and proinsulin fragments in non-diabetic controls and islet transplant recipients

Fasting levels of insulin (I) were similar between allotransplant recipients and non-diabetic controls but higher in both groups relative to autotransplant recipients (*P*<0.05 vs. allotransplant; *P*<0.01 vs. non-diabetic controls; Figure 5). However, since the majority of allotransplant recipients were on insulin therapy, the observed fasting insulin levels may be falsely elevated. To this end, we determined fasting levels of C-peptide as a surrogate measure of insulin secretion. Fasting levels of C-peptide (CP) were similar between allotransplant recipients
and non-diabetic controls but higher in both groups relative to the autotransplant recipients ($P<0.001$; Figure 5). Since some allotransplant recipients were taking pioglitazone and/or exenatide as concurrent therapy, it is possible that the finding of similar C-peptide levels may be attributed to these drugs as they have been shown to enhance insulin secretion as well as insulin sensitivity, \textit{in vitro} and \textit{in vivo}; however, although this may indeed be the case in other situations, we have no direct indication that these drugs are in fact beneficial to our cohort of allotransplant subjects.

Total proinsulin (TP) was markedly elevated in allotransplant recipients relative to non-diabetic controls [16.8 (5.5–28.8) vs. 8.4 (4.0–21.8) pM; $P<0.05$; Figure 5] and autologous transplant recipients [7.3 (0.3–82.3) pM; $P<0.05$; Figure 5]. Interestingly, although insulin levels were much lower in the autotransplant recipients compared to non-diabetic controls [22.5 (1.6–134) vs. 58.5 (24.1–124.2); $P<0.01$; Figure 5], total proinsulin levels were similar in these two groups [7.3 (0.3–82.3) vs. 8.4 (4.0–21.8); $P=\text{NS}$; Figure 5], resulting in elevated proinsulin/insulin (TP/TP+I) and proinsulin/C-peptide (TP/CP) ratios. No statistical differences were seen in the levels of intact proinsulin or proinsulin fragments (PF) among groups, although proinsulin fragments tended to be higher in the allotransplant subjects.

\subsection{3.2.3 Proinsulin/insulin, proinsulin/C-peptide and proinsulin fragments/total proinsulin ratios in islet transplant recipients}

To address whether impairment in proinsulin processing was a characteristic of our transplant population, we determined the proinsulin/insulin and proinsulin/C-peptide ratios. The fasting TP/TP+I ratio was significantly higher in autotransplant recipients compared to non-diabetic controls (35.9 ± 6.4 vs. 13.9 ± 1.4%; $P<0.001$; Figure 6A) and was similar to that observed in allotransplant recipients (24.1 ± 2.4%; $P=\text{NS}$; Figure 6A). Because a number of allo- and autotransplant recipients were receiving exogenous insulin therapy where insulin could contribute to a falsely low ratio, we also determined the TP/CP ratio in all groups. TP/CP was similarly elevated in islet autotransplant subjects compared to non-diabetic controls [8.9 (0.6–105.2) vs. 1.4 (0.5–2.6)%; $P<0.05$; Figure 6B], and was also higher in subjects with allotransplants [2.4 (0.8–8.8); $P<0.001$; Figure 6B]. Although the TP/CP ratio tended to be higher in the insulin dependent allotransplant group (3.6 ± 0.9 vs. 2.3 ± 0.4 %), this difference was not statistically significant (Figure 7). Insulin-dependent (n=8) and independent allotransplants (n=5) had similar HbA$_1c$ and fasting blood glucose levels.
The des-31, 32 proinsulin conversion intermediate is known to be the primary form of circulating proinsulin, comprising ~ 70% of total proinsulin immunoreactivity in both non-diabetic and type 2 diabetic subjects [57]. We found that the proportion of total proinsulin comprised of proinsulin fragments (PF/TP ratio) did not differ significantly between the non-diabetic controls and either of the transplant groups, although there was a tendency for a higher ratio in the islet transplant recipients (Figure 8).

Since immunosuppressive medications administered to islet allograft recipients are known to impact beta-cell function and could therefore directly influence the observed proinsulin/insulin and proinsulin/C-peptide ratios, we further examined our cohort of patients who had received islet autografts following pancreatectomy (Figure 9). We assessed serum insulin, C-peptide and the proinsulin/insulin and proinsulin/C-peptide ratios in these patients prior to total pancreatectomy and transplantation, and one year following surgery. These subjects, because they received autologous islet transplants, received no immunosuppressive treatment. At one year post-transplantation, circulating levels of both insulin (Figure 9A; \(P<0.05\)) and C-peptide (Figure 9B; \(P<0.05\)) were lower in these patients compared to the serum levels in samples taken prior to pancreatectomy and islet transplantation. In addition, both the TP/TP+I (Figure 9C) and TP/CP (Figure 9D) ratios rose markedly in these patients compared to levels prior to pancreatectomy and islet transplantation (17.0 ± 3.9 vs. 35.9 ± 6.4%; \(P<0.05\) and 3.6 ± 0.7 vs. 21.9 ± 9.2%; \(P<0.05\), respectively).
Figure 5. Fasting serum levels of beta-cell hormones in non-diabetic controls and islet transplant recipients. Beta-cell hormone levels were determined in non-diabetic controls (ND; n=17), allotransplant recipients (AlloTx; n=13) and autotransplant recipients (AutoTx; n=12). Data are represented as medians and ranges. One-way ANOVA or the Kruksall-Wallis test was used to analyze differences between groups. $p<0.05$ was considered significant.
Proinsulin/insulin (TP/TP+I) (A) and proinsulin/C-peptide (TP/CP) (B) were determined in islet allotransplant (AlloTx; n=13) and autotransplant (AutoTx; n=12) recipients and compared to non-diabetic controls (ND; n=17). Data are expressed as medians and ranges and were analyzed by Kruskal-Wallis test for significance. Total proinsulin (TP), Insulin (I), C-peptide (CP). * \( p<0.05 \) vs. ND; ** \( p<0.001 \) vs. ND.
Figure 7. Proinsulin/C-peptide ratios in insulin-dependent and insulin-independent allotransplant recipients. Subjects were separated into insulin-dependent (n=5) and insulin independent (n=8) groups based on their insulin requirement at the time of sample collection. The ratio was calculated as total proinsulin/C-peptide (TP/CP) and expressed as a percentage. Data are represented as mean ± SEM and a Student’s t-test was used to assess inter-group differences. *p*<0.05 was considered significant.
Figure 8. Proportion of circulating proinsulin comprised of proinsulin intermediates in serum of islet transplant recipients. Proportion of intermediates is calculated as proinsulin fragments/total proinsulin (PF/TP) and expressed as a percentage (%). Islet allo- (AlloTx; n=13) and autotransplant (AutoTx; n=12) recipients are compared to non-diabetic controls (ND; n=17) and data are expressed as medians and ranges. One-way ANOVA was used to determine differences between groups and \( p<0.05 \) was considered significant.
Figure 9. Pre- and 1-year post-transplant fasting insulin, C-peptide, proinsulin/insulin and the proinsulin/C-peptide ratios in autologous islet transplant recipients. Data are represented as paired observations and statistical analysis was performed using paired Student’s t-test. Insulin (I), C-peptide (CP), proinsulin/insulin (TP/TP+I), proinsulin/C-peptide (TP/CP). \( p<0.05 \) was considered significant.
3.2.4 Fasting levels of circulating beta-cell hormones in subjects with type 2 diabetes

We also measured the proinsulin/insulin and proinsulin/C-peptide ratios in a group of subjects with type 2 diabetes. Fasting levels of insulin, C-peptide, total proinsulin, intact proinsulin and proinsulin fragments in type 2 diabetic subjects are shown in Table 4. The mean TP/TP+I ratio in our type 2 diabetic subjects was 26.2±2.7%, consistent with previously reported values in subjects with type 2 diabetes [57, 230, 231]. Total proinsulin levels were also comparable to those reported. Together our data indicate that our population is representative of type 2 diabetes and indicative of an underlying defect in the beta cell.

Table 4. Fasting levels of beta-cell hormones and proinsulin/insulin and proinsulin/C-peptide ratios in subjects with type 2 diabetes.

<table>
<thead>
<tr>
<th>Levels of Beta-Cell Hormones in Type 2 Diabetes</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pM)</td>
<td>58.4 (26.5 - 224.2)</td>
</tr>
<tr>
<td>C-peptide (pM)</td>
<td>1118 (368 - 2686)</td>
</tr>
<tr>
<td>Total Proinsulin (pM)</td>
<td>25.1 (6.4 – 133.6)</td>
</tr>
<tr>
<td>Intact Proinsulin (pM)</td>
<td>7.3 (2.8 – 45.5)</td>
</tr>
<tr>
<td>Proinsulin fragments (pM)</td>
<td>14.8 (3.6 – 88.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratios in Type 2 Diabetes</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP/TP+I (%)</td>
<td>26.2 ± 2.7</td>
</tr>
<tr>
<td>TP/I (%)</td>
<td>37.5 ± 4.9</td>
</tr>
<tr>
<td>TP/CP (%)</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

TP/TP+I; TP/I = proinsulin/insulin, TP/CP = proinsulin/C-peptide.

3.2.5 Correlation of total proinsulin, C-peptide and the total proinsulin/C-peptide ratio in recipients of islet allo- and autografts

To determine whether transplanted mass (IE/kg), impacted either fasting levels of total proinsulin or C-peptide, or the proinsulin/C-peptide ratios in our transplant population we assessed the relationship by Spearman correlation. Total proinsulin levels in transplant recipients did not correlate significantly with IE/kg transplanted (Figure 10A); however, fasting levels of C-peptide showed a strong positive correlation (Figure 10B). Fasting C-peptide levels were used as a surrogate measure of insulin since some recipients were in insulin therapy. Interestingly, we also found a negative correlation between the TP/CP ratio and IE/kg transplanted (Figure 10C).
Figure 10. Relationship between fasting total proinsulin, C-peptide and the proinsulin/C-peptide ratios in islet transplant recipients. Proinsulin (A), Insulin (B) and TP/CP (C) are presented as individual data points for allotransplant (black triangles) and autotransplant recipients (pink squares). TP/CP = total proinsulin/C-peptide. Statistical analysis was performed using Spearman correlation. \( p<0.05 \) was considered significant.
3.2.6 Impact of transplanted islet mass on total proinsulin/C-peptide ratios

Most allotransplant recipients require islets from more than one donor pancreas to achieve optimal glucose control, since islets from a single transplant are usually insufficient to normalize blood glucose. A transplanted mass of 10,000 IE/kg has been reported as the minimum for achieving insulin independence [159]. To determine whether insufficient transplanted mass is associated with impaired proinsulin processing, we stratified our allotransplant cohort into two groups: those who had received an optimal number of islets (>10,000 IE/kg) and those who had received less (<10,000 IE/kg). Individuals who received transplants of less than 10,000 IE/kg had circulating TP/CP ratios approximately two-fold higher than subjects who received more than 10,000 IE/kg (4.6 ± 1.3 vs. 2.2 ± 0.2%; P<0.05; Figure 11).

![Figure 11](image_url)

**Figure 11. Recipients of > 10,000 IE/kg have lower proinsulin/C-peptide ratios.** Allotransplant recipients were stratified into those who received islet grafts of >10,000 (n=5) or <10,000 IE/kg (n=8). Data are represented as mean ± SEM and Student’s t-test was used to assess differences. P<0.05 was considered significant. TP/CP = total proinsulin/C-peptide.
3.3 Discussion

To determine whether transplanted islets have impaired proinsulin processing, we measured fasting levels of intact proinsulin, proinsulin intermediates, insulin and C-peptide in type 1 diabetic recipients of human islets. The absolute levels of total proinsulin and the proinsulin/C-peptide ratio were markedly elevated in allotransplant recipients relative to non-diabetic controls, and were close to values observed in subjects with type 2 diabetes. Interestingly, we found that islet autotransplant recipients have elevated proinsulin/insulin and proinsulin/C-peptide ratios suggesting that the increased proinsulin/C-peptide ratio observed in islet allograft recipients was unlikely to be related to beta-cell dysfunction induced by immunosuppressive drugs. These data suggest that transplanted islets have a defect in beta-cell function similar to that seen in type 2 diabetes.

Two mechanisms have been proposed to explain the disproportionate secretion of proinsulin in individuals with type 2 diabetes. First, it is possible that there is an intrinsic defect in the processing of insulin from its precursor proinsulin [229]. A second theory proposes that an increase in the demand for insulin, associated with some combination of insulin resistance, hyperglycemia and decreased beta-cell mass, results in insufficient time for complete proinsulin processing to occur before granule secretion [232].

The mechanism underlying the elevated TP/CP ratio in allo- and autotransplants may be unique to each. Autotransplant recipients had lower absolute insulin levels due to a lower islet mass transplanted, since these subjects receive islets from a single donor. Because of the lower islet mass transplanted, islets in autotransplant recipients are more likely subject to greater secretory demand. Absolute total proinsulin levels were higher in the allotransplants relative to the auto-transplants, and C-peptide levels were similar to normal subjects. In our transplant populations, beta-cell mass transplanted correlated positively with C-peptide levels and correlated negatively with the TP/CP ratio. This suggests that an optimal transplant mass is necessary for normal processing to occur. Since some patients were on immunosuppressive drugs, it is possible that, in addition to the islet defect seen in transplanted islets and the relatively insufficient islet mass transplanted, these drugs may contribute to the increased ratios. Since immunosuppressive drugs can cause both insulin resistance [233] and beta cell dysfunction [223], it is possible that they may contribute to the impaired proinsulin processing seen in our allotransplant population.
Our allotransplant recipients were on a variety of medications that may have a direct or indirect impact on proinsulin processing. Administration of insulin to islet transplant recipients may reduce the metabolic demand imposed on the transplanted beta cells to secrete insulin and potentially improve the TP/CP ratio by promoting beta-cell rest. In a study where beta-cell secretion was suppressed by somatostatin administration to subjects with type 2 diabetes, although it reduced the proinsulin/insulin ratio, it failed to normalize this measure suggesting that there is an intrinsic defect in beta-cell function in subjects with type 2 diabetes that may be aggravated by metabolic demand [234]. We found that in our allotransplant group, there was no difference in the TP/CP ratios whether subjects were on insulin therapy or not; however, there was a tendency for a higher ratio in those that were on insulin. We propose that the TP/CP ratio will be significantly higher in the insulin-dependent subjects and may be blunted by the reduced demand placed on the beta cell in the presence of insulin. Both exenatide and thiazolidinediones, such as pioglitazone and rosiglitazone, have been shown to alter (pro)insulin processing resulting in lower absolute proinsulin levels as well as a decrease in the proinsulin/insulin ratio [231, 235, 236]. Our data indicate that despite the presence of these medications, the proinsulin/insulin and proinsulin/C-peptide ratios remained elevated in the allotransplant population.

Our finding that recipients of an optimal islet mass (>10,000 IE/kg), as recommended by the Edmonton Protocol, have lower proinsulin/C-peptide ratios is consistent with the idea that increased secretory pressure on transplanted islets contributes to the impairment in proinsulin processing. Transplanted islets have also been shown to exhibit defects in first-phase glucose-stimulated insulin secretion and a blunted response to arginine [205, 226], similar to that observed in subjects with type 2 diabetes and consistent with beta-cell dysfunction. While it remains to be determined what mechanism underlies impaired proinsulin processing in transplanted islets, there are clearly similarities to type 2 diabetes that are consistent with both increased secretory demand and beta-cell dysfunction in transplant recipients [210].

The observation that islet allo- and autotransplant recipients have similar defects in proinsulin processing suggests that islet isolation and transplantation per se cause beta-cell dysfunction. Following transplantation islet are exposed to various factors including cytokines, that are released from neighboring epithelial cells or hepatocyte Kupffer cells as a result of injury to the host cells where islets engraft. Islet isolation per se may cause cytokine production by islets. Cytokines have been implicated in impaired proinsulin processing [83, 84] and may therefore contribute to the elevated ratios observed, at least in the early post-transplant period. Another well-documented factor that contributes to impaired beta-cell function is the presence of
immunosuppressive drugs some of which are nephrotoxic [199]. The autotransplant subjects were not taking immunosuppressive medications that are administered to allotransplant recipients and might have influenced circulating proinsulin/insulin or proinsulin/C-peptide ratios, either by directly impairing beta-cell function or by impacting on renal or hepatic hormone clearance. We surmise that these two islet transplant groups may share similar defects in islet function, despite differences in the source of the islets and the treatment regimens.

These findings are in contrast with a previous report that the proinsulin/C-peptide ratio is lower in islet transplant recipients than in normal controls [237]. While the circulating insulin and C-peptide levels measured in the present study were similar to those reported by McDonald et al. [237], the proinsulin levels reported in both their transplant and control cohorts were several-fold lower than we observed. Therefore, to verify our findings, we re-measured proinsulin levels in our samples using an ELISA from Alpco Diagnostics (Salem, NH) and obtained similar results to those with the Linco assay, confirming that at least in our transplant patient cohort, proinsulin/C-peptide levels were indeed elevated similarly to type 2 diabetic patients. This elevated proinsulin/C-peptide ratio was also shown in a study by Secchi et al., where islet transplant subjects with long term graft function tended to have lower ratios than those who had early graft failure [238]. One possible explanation for the disparity between our study and that of McDonald et al. is the use of different proinsulin assays. The DAKO (Denmark) proinsulin assay was used in the previous report, however, it is no longer commercially available [237]. The DAKO total proinsulin assay was reported to cross-react only 65% with des-31,32 proinsulin, the predominant proinsulin intermediate, whereas both the Linco and Alpco assays are reported to cross-react 100% with this intermediate form.

In comparison to our study population, observations from other groups have focused on recipients with optimal glucose control, including HbA1c <6.1% and fasting blood glucose levels in the normoglycemic range. In contrast, our subjects tended to have elevated HbA1c that is an indicator of poor glycemic control. We cannot rule out that hyperglycemia contributed to the elevated TP/CP. In a study by Rickels et al., subjects under hyperglycemic clamp conditions were found to have proinsulin levels similar to normal controls [239]. We found a positive relationship between levels of total proinsulin and glycemia ($p=0.05$ and $r=0.42$) in our transplant recipients supporting the idea that hyperglycemia per se may contribute to the elevated TP/CP ratios that we observed.

Elevated glucose levels may contribute to the increased proinsulin/C-peptide ratios in several ways. Increased demand on transplanted beta cells in the milieu of chronic
hyperglycemia may result in increased proinsulin secretion that is not related to defects in proinsulin processing, but rather due to an insufficient time for processing to occur in vivo [139]. In fact, prolonged glucose exposure has been shown to increase secretion of newly formed proinsulin peptides by 7-fold [240], which could contribute to the increased ratios. Other studies have also suggested that beta-cell exhaustion is the primary cause of altered proinsulin secretion in humans and that granule maturity is key to normal processing of proinsulin, and particularly the des 31,32 proinsulin intermediate [241].

Processing of hormone precursors occurs primarily in the secretory granules of the regulated secretory pathway [132] and the enzymes responsible for maturation of prohormones are also delivered to the granules coordinately. Glucose has been shown to stimulate synthesis of PC1/3 early on within the secretory pathway, and PC2 maturation, which is also stimulated by glucose, is highly dependent on an acidic granule milieu for activation [132, 242, 243]. Changes in maturation, primarily of PC2, would result in a greater proportion of the des 31,32 proinsulin in the circulation, especially in states of elevated demand where maturation of granules may be inhibited. Cytokines such as IL-1β in combination with TNFα and IFNγ, for example, have also been shown to reduce PC1/3 and PC2 expression by 40-45% [84], which may contribute to increased proinsulin/C-peptide ratios. Therefore, regulation of prohormone processing is dependent on the expression of these endopeptidases, as well as the granular milieu within the regulated secretory pathway that may be important in the transplant setting.

The proinsulin/insulin ratio is conventionally used to assess the efficiency of proinsulin processing, and is done so as a way to normalize for the rate of insulin secretion among different metabolic states such as impaired glucose tolerance or type 2 diabetes. Since, C-peptide and insulin are co-secreted in equimolar amounts from beta cells [244], the proinsulin/C-peptide ratio may be used as a surrogate measure to determine processing efficiency. Although insulin and C-peptide are secreted together, once in the systemic circulation, their clearance rates diverge such that the majority of insulin is extracted by the liver, whereas C-peptide, which is primarily cleared by the kidney, remains in the circulation for a prolonged period of time. Like C-peptide, proinsulin is also cleared by the kidney, however, its clearance rate is 3-fold slower than that of insulin [245].

The clinical data in this study suggest that some of the islet transplant recipients have mildly impaired renal function as assessed by serum creatinine levels. Based on these results, we cannot rule out the effects of renal clearance on the proinsulin/C-peptide ratio given that the kidney is a major site of proinsulin (and C-peptide) clearance [246]. Regression analysis was
performed and indicated that neither proinsulin, C-peptide nor the proinsulin/C-peptide ratio were associated with serum creatinine levels as a measure of renal dysfunction (data not shown), suggesting that the mild impairment of renal function observed in some of our subjects was unlikely to be a cause of the elevated proinsulin/C-peptide ratios. Also, since the same organ clears both proinsulin and C-peptide, it seems likely that both hormone clearance rates would be affected to a similar degree, and as such the proinsulin/C-peptide ratio would not change significantly.

In summary, islet transplant recipients have increased proinsulin/insulin and proinsulin/C-peptide ratios that are associated with a low beta-cell mass. These findings support the hypothesis that as in type 2 diabetes, islet transplants exhibit hyperproinsulinemia, however, whether this is due to increased secretory demand or an intrinsic defect in proinsulin processing has yet to be elucidated.
CHAPTER 4. INSUFFICIENT TRANSPLANTED BETA-CELL MASS CONTRIBUTES TO IMPAIRED PROINSULIN PROCESSING IN ISLET GRAFT RECIPIENTS

4.1 Background

Transplantation of a sufficient beta-cell mass is essential for an optimal outcome of pancreatic islet transplantation and graft dysfunction has been associated with a progressive loss of beta-cell mass in the early post-transplant period. Studies in autotransplants suggest that immunological factors cannot alone explain the poor graft outcomes. Other contributing factors include lack of islet engraftment, hypoxia, apoptosis and amyloid deposition resulting in a decrease in beta-cell mass [158, 190, 211, 212]. Toxicity of immunosuppressant drugs has also been implicated in the loss of beta-cell function and apoptosis [173, 197, 203, 204]. Metabolic demand imposed on a marginal mass transplant may impact beta-cell function. In our human islet transplant study, we found that subjects receiving a suboptimal islet mass, as defined by the Edmonton Protocol to be 10,000 IE/kg, had increased TP/CP ratios supporting our hypothesis that insufficient beta-cell mass contributes to defective proinsulin processing [247]. To exclude the effects of factors such as hyperglycemia or immunosuppressive drugs, which may contribute to the observation of elevated ratios, we used a syngeneic transplant model and a model of human islet transplantation to assess whether reduced beta-cell mass results in impaired proinsulin processing, and if so whether proinsulin processing declines following pancreatic islet transplantation.

4.2 Results

4.2.1 Transplanted islet mass impacts on return to normoglycemia and on the efficiency of proinsulin processing

To determine the role of beta-cell mass in proinsulin processing, STZ-induced diabetic C57Bl/6 recipient mice were transplanted with either 200 (n=4) or 300 (n=9) C57Bl/6 donor islets under the kidney capsule. Blood glucose levels were similar in both groups over the duration of the experiment (Figure 12A). In the immediate post-transplant period, mice transplanted with 200 islets required more time to return to pre-transplant normoglycemia than 300 islet recipients (18 vs. 2 days, p<0.05; Figure 12B).
Figure 12. Glycemic control in STZ-induced diabetic C57Bl/6 mice transplanted with syngeneic donor islets. (A) Non-fasted blood glucose was measured daily for the first week and weekly for the remaining 6-week post-transplant period in recipients of 200 (n=4) or 300 (n=9) pooled syngeneic donor islets. Data are expressed as mean±SEM. (B) Survival analysis for the time required for transplanted mice to achieve post-transplant normoglycemia. $p<0.05$ was considered significant. STZ = streptozotocin, TX = transplantation.
To extend our findings from human islet transplant recipients that the total proinsulin/C-peptide ratios were higher in graft recipients who receive a lower number of IE/kg, plasma was collected from syngeneic transplant recipients at 6 weeks post-transplant and analyzed for levels of mouse insulin and proinsulin, and the proinsulin/insulin ratio was calculated. Mice transplanted with 200 islets tended to have lower insulin levels (107.7 ± 29.8 vs. 213.9 ± 61.7 pM; p=ns; Figure 13B) but similar levels of total proinsulin (9.2 ± 0.8 vs. 7.9 ± 1.3 pM; p=ns; Figure 13C) compared to mice transplanted with 300 islets. Interestingly, the proinsulin/insulin ratio was elevated in mice transplanted with a lower islet mass of 200 islets (9.1 ± 0.9 vs. 5.0 ± 0.7 %; p<0.05; Figure 13D). Insulin and glucagon immunostaining of isolated islet graft sections revealed normal islet morphology with numerous insulin-immunopositive and glucagon-immunopositive cells in both 200 and 300 islet grafts (representative image; Figure 13E).

4.2.2 Insulin secretion and proinsulin processing are reduced in normoglycemic recipients of human islet transplants

Given that impaired proinsulin processing is characteristic of islet transplants both in human subjects and in a normoglycemic mouse model of transplantation, we sought to confirm our findings in a mouse model transplanted with human islets. STZ-induced diabetic NOD.scid mice were transplanted with 200 (n=9), 300 (n=14) or 500 (n=16) hand-picked human islets from 7 donors under the kidney capsule. To exclude the potential impact of hyperglycemia on beta-cell function, only normoglycemic mice (blood glucose <15mM) were assessed. Blood glucose levels were not different among transplant groups. All mice achieved normoglycemia by 3 days post transplant and maintained normal blood glucose levels for the duration of the experiment (Figure 14).

Blood glucose levels were monitored in recipient mice for 6 weeks post-transplantation as described in Chapter 2. Plasma was collected at 2 and 6 weeks post-transplant to assess graft function by determining the levels of human insulin and total proinsulin in the circulation of recipient mice. Interestingly insulin levels declined significantly regardless of transplanted mass between 2 and 6 weeks post-transplant (200 islets: 211.1±40 vs. 75.3±16.1 pM; 300 islets: 203.8±22.8 vs. 68.3±14.7 pM; 500 islets: 261.7±27.3 vs. 93.4±17.4 pM; p<0.001 for all groups; Figure 15A). Unlike the decline in insulin, no significant changes were seen in proinsulin levels between 2 and 6 weeks post-transplant in any of the groups, although recipients of 200 islets
already tended to have more total proinsulin than either the 300 or 500 islet recipients at 2 weeks (Figure 15B).

To address whether proinsulin processing worsens over time in islet graft recipients, the proinsulin/insulin ratio was assessed at 2 and 6 weeks post-transplant. No significant differences in TP/TP+I were observed in recipients of 200 islets although there was a tendency for an increased ratio over time (34.8±5.3 vs. 47.7±6.1 %, p=ns; Figure 15C). In contrast, islet graft recipients who received either 300 or 500 islets had significant increases in TP/TP+I during the 2 to 6 week post-transplant period (300 islets: 24.5±3.9 vs. 39.7±4.9, p<0.05; 500 islets: 16.5±2.8 vs. 27.5±4.2, p=0.05; Figure 15C). Importantly, mice transplanted with 200 islets had higher TP/TP+I relative to 500 islet recipients at both 2 and 6 weeks post-transplant (34.8±5.3 vs. 16.5±2.8 %, Figure 16A and 47.7±6.1 vs. 27.5±4.2 %, Figure 16B; p<0.05 at both time points). Together, these data suggest that proinsulin processing deteriorates in the post-transplant period and that a lower islet transplant mass may contribute to the disproportionate hyperproinsulinemia observed.
Figure 13. Proinsulin/insulin ratio is elevated in normoglycemic C57Bl/6 mice transplanted with a lower islet mass. (A) STZ-diabetic mice were transplanted with 200 (n=4) or 300 (n=9) pooled syngeneic islets and plasma was collected from normoglycemic mice (A) at 2 and 6 weeks post-transplant. (B) Proinsulin and (C) insulin were analyzed by ELISA and (D) proinsulin/insulin ratio was calculated. (E) A representative islet graft section co-stained with insulin (red) and glucagon (green). Data are expressed as mean ± SEM and analyzed by Student’s t-test. p<0.05 was considered significant.
Figure 14. Glycemic control in STZ-induced diabetic NOD.scid mice transplanted with human islets. Non-fasted blood glucose was measured daily for the first week and weekly for the remaining 5 weeks post-transplant in mice transplanted with 200 (n=9), 300 (n=14) or 500 (n=16) human islets isolated from 7 human donors. Data are expressed as mean ± SEM from seven human islet donors. Normoglycemia was qualified as <15mM. STZ = streptozotocin, TX = transplantation.
Figure 15. Changes in circulating levels of insulin, proinsulin and the proinsulin/insulin ratio at 2 and 6 weeks post-transplant in NOD.scid mice transplanted with human islets. Mice were made diabetic by a single injection of streptozotocin (200mg/kg) and transplanted with 200 (n=9), 300 (n=14) or 500 (n=16) human islets under the kidney capsule. Plasma was collected at 2 and 6 weeks post-transplant and analyzed for insulin (A) and proinsulin (B) by immunoassay, and the TP/TP+I was calculated (C). Data within each transplant group (200, 300 or 500) were analyzed using Student’s t-test or Mann-Whitney test to detect differences between 2 and 6-week measures. Data are expressed as mean ± SEM and $p<0.05$ was considered significant; *$p<0.05$, **$p<0.001$. 
Figure 16. Proinsulin/insulin ratios at 2 and 6 weeks post-transplant in normoglycemic mice transplanted with human islets. NOD.scid mice transplanted with a lower islet mass tended to have a higher ratio. The proinsulin/insulin ratio in mice transplanted with 200 (n=9), 300 (n=14) or 500 (n=16) human donor islets was calculated as PI/PI+I and expressed as a percentage (%). Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. $p<0.05$ was considered significant.
4.2.3 Association of hyperglycemia and proinsulin processing in graft failure

We determined whether glucose plays a role in proinsulin processing in mice transplanted with a marginal mass of 200 human islets, in which the graft had either failed or maintained normoglycemia. Mice were categorized as having a failed or non-functional graft when glycemia was above 15mM at 6 weeks post-transplant and on two or more occasions over the duration of the experiment (failed; n=4). Mice categorized into the “functional” graft group were able to achieve normoglycemia (n=9) post-transplant and maintain it over the duration of the experiment.

The TP/TP+I was significantly higher in recipients with non-functional grafts (70.5 ± 5.4 vs. 47.7 ± 6.1 %, p<0.05; Figure 17D). Recipients with failed islet grafts were hyperglycemic (18.5 ± 1.3 vs. 5.4 ± 0.4 mM, p<0.001; Figure 17A) tended to have lower plasma insulin levels (45.3± 9.5 vs. 75.3 ± 16.1 pM, p=ns; Figure 17B) but higher plasma proinsulin levels (105.5 ± 14.8 vs. 70.1 ± 13.9 pM, p=ns; Figure 17C) although the differences were not statistically significant.

To assess the ability of the transplanted islets to compensate for increased glucose levels and therefore insulin demand, we determined the insulin/glucose ratio in failed and functional graft recipient groups. Not surprisingly, the insulin/glucose ratio was higher in mice that had functional grafts when compared with mice that had failed grafts (14.1 ± 2.4 vs. 2.5 ± 0.6 arbitrary units, p<0.05; Figure 17E) suggesting that insulin levels were inappropriate for the degree of glycemia in failed grafts.

Insulin resistance is known to be associated with increased secretory demand and within the transplant setting may be associated with defective proinsulin processing. We calculated the homeostasis model assessment–insulin resistance (HOMA-IR) and found that mice with failed 200 islet grafts were more insulin resistant than mice with sustained graft function (5.3±1.1 vs. 2.0±0.4 HOMA-IR units, p<0.05; Figure 17F) that was likely secondary to the hyperglycemia. It is possible that this extra demand placed on an already marginal mass may have in part contributed to the observed elevated ratios.
Figure 17. Marginal mass islet grafts that maintain normoglycemia have a lower proinsulin/insulin ratio. Mice were qualified as having a failed or non-functional graft when glycemia was above 15mM at 6 weeks post-transplant and on two or more occasions over the duration of the experiment. Comparisons of blood glucose (A), insulin (B), proinsulin (C), proinsulin/insulin ratio (D), insulin/glucose ratio (E) and HOMA-IR (F) between failed (n=4) and functional (n=9) groups at 6 weeks post-transplant were assessed by Student’s t-test. $p<0.05$ was considered significant. Data are presented as mean ± SEM.
4.2.4 Amyloid formation is associated with human islet graft failure

Islet amyloid forms rapidly in human islets transplanted into diabetic, immune-deficient recipients [248]. To determine whether amyloid correlates with graft dysfunction, NOD.scid mice transplanted with human islets were assessed for the presence of amyloid by thioflavin S staining in islet grafts. Prior to transplantation, no amyloid was detectable in grafts. As previously described, mice that maintained normoglycemia tended to have more functional grafts than mice that became hyperglycemic. Upon graft harvest, mice whose grafts had maintained normoglycemia throughout the duration of the experiment, tended to have a greater proportion of the graft area occupied by beta cells than those mice that failed to maintain normoglycemia (24.8 ± 3.8 vs. 16.5 ±4.3 %, p=NS; Figure 18A). The proportion of graft area occupied by amyloid was 0.7 ± 0.3 % in normoglycemic mice and 6.1 ± 3.5 % in hyperglycemic recipients (p<0.05; Figure 18B). These results demonstrate that amyloid deposition is greater in failed islet grafts in this mouse model of human islet transplantation and is associated with a loss of beta-cell mass.

Figure 18. Amyloid formation is associated with failed human islet grafts. At 6 weeks post-transplant, beta-cell area tended to be reduced (A) while amyloid area was increased (B) in recipients whose glycemia was >15mM (n=12) at time of graft harvest relative to normoglycemic recipient mice (n=31). Data are represented as mean ± SEM and Student’s t-test was used to analyze differences between groups. p<0.05 was considered significant.
4.2.5 Amyloid formation is associated with a lower islet transplant mass in normoglycemic islet grafts

To determine whether increased amyloid formation is associated with reduced transplanted islet mass, we compared normoglycemic recipients of 300 or 500 islets with respect to their graft insulin-positive beta-cell area and thioflavin-S-positive amyloid area. Recipients of 300 islet grafts had a significantly lower proportion of total graft area comprised of beta cells (12.9 ± 2.5 vs. 22.9 ± 1.9 %, \( p < 0.05 \); Figure 19A & 19C) and a greater proportion of the graft comprised of amyloid (0.5 ± 0.2 vs. 0.1 ± 0.04 %, \( p < 0.05 \); Figure 19B & 19D) relative to recipients of 500 islets. The finding suggests that islet amyloid is associated with a decreased beta-cell mass transplanted.

To further support the possibility that amyloid deposition may contribute to the decline in beta-cell function manifest as increased proinsulin/insulin ratios, we studied transplantation of human IAPP expressing mouse islets into NOD.scid mice with STZ-induced diabetes. Recipients were transplanted with 250 mouse islets expressing human IAPP (hIAPP) and plasma was collected at 6 weeks post-transplant. Both hIAPP\(^{+/wt}\) (n=9) and wild-type (hIAPP\(^{wt/wt}\)) (n=7) transplant groups maintained normoglycemia over the course of the experiment (Figure 20A). Recipients of hIAPP\(^{+/wt}\) islets tended to have less insulin (91.8 ± 7.9 vs. 141.1 ± 26.3 pM, \( p = 0.07 \); Figure 20B) and similar amounts of proinsulin (4.0 ± 0.3 vs. 4.2 ± 0.8 pM, \( p = \text{ns} \); Figure 20C) as wild-type islet recipients, although they were not significantly different. Interestingly, the PI/PI+I was higher in the hIAPP\(^{+/wt}\) islet recipients (4.3 ± 0.4 vs. 2.8 ± 0.3 %, \( p < 0.05 \); Figure 20D) relative to wild-type controls.
Figure 19. Decreased beta-cell area and increased amyloid area are associated with lower islet transplant mass. Islet grafts from mice transplanted with 300 or 500 islets were stained for insulin (A) and amyloid (B) and analyzed for positivity as a percentage of total graft area. Transplants of 300 islets (n=7; C) tended to have more amyloid than grafts with 500 islets (n=15; D) (representative images). Arrows indicate amyloid as assessed by Thioflavin S staining. Data are expressed as mean ± SEM and the Mann-Whitney test was used to assess differences between groups. p<0.05 was considered statistically significant.
Figure 20. Proinsulin/insulin ratio in STZ-induced diabetic NOD.scid mice transplanted with islets expressing hIAPP. Male diabetic NOD.scid mice were transplanted with 250 hIAPP-expressing mouse islets (hIAPP+/wt; n=9) or littermate-control islets (hIAPPwt/wt; n=7) under the kidney capsule of the recipient mice. Plasma was collected from normoglycemic mice (A) at 6 weeks post-transplant for analysis of insulin (B) and proinsulin (C), and the proinsulin/insulin ratio was calculated (D). Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test. p<0.05 was considered significant.
4.2.6 Immunosuppressant effects on *in vivo* graft survival

To determine whether immunosuppressive drugs impact on graft survival and function, STZ-induced diabetic NOD.scid mice transplanted with 500 human islet grafts were monitored by measurement of blood glucose levels over a 3-4 week period. Recipients attained rapid post-transplant normoglycemia (<5 days). Over a period of 30 days post-transplant, mice were administered with daily injections of FK506, sirolimus or MMF. FK506 resulted in graft failure and hyperglycemia at 20 days post-transplant when mice became ill and experienced rapid weight loss. No differences were seen with MMF treatment relative to controls (Figure 21). Like MMF, no difference was seen in glycemia in recipients treated with sirolimus (data not shown). Together these results suggest that FK506 induced impairments in beta-cell function *in vivo* and/or induced beta-cell apoptosis, resulting in hyperglycemia [203].

![Graph showing blood glucose levels post-transplant](image_url)

**Figure 21.** Immunosuppressant effects on the function of human islets transplanted into diabetic recipient mice. NOD.scid mice were made diabetic with streptozotocin (arrow-STZ), and then transplanted (arrow-TX) with 500 human islets (two separate donors are represented in the data). Mice were subsequently injected intra-peritoneally with saline (n=2), 30 mg/g FK506 (n=4), or 1.5 mg/g MMF (n=3) each day. Data are represented as mean ± SEM except for saline controls which are individual mice transplanted on two different days.
4.3 Discussion

To determine whether transplanted mass contributes to defective proinsulin processing, we measured non-fasting levels of insulin and proinsulin in a syngeneic islet transplant model and a model of human islet transplantation in the context of normoglycemia. Additionally, we determined whether amyloid formation in human islets transplanted into immune-deficient mice, was associated with graft failure and reductions in beta-cell mass.

In the syngeneic mouse model, mice transplanted with a lower islet mass required more time to return to normoglycemia, had similar proinsulin levels but elevated proinsulin/insulin ratios compared to mice transplanted with an optimal islet mass. Immune-deficient mice transplanted with human islets exhibited no differences in return to normoglycemia and showed a progressive reduction in insulin levels irrespective of beta-cell mass transplanted. Proinsulin/insulin ratios were greater with a lower islet mass transplanted and they were exacerbated by the presence of hyperglycemia. Finally we demonstrated that graft failure was associated with amyloid deposition in the contexts of hyperglycemia or reduced beta-cell mass. Together these data suggest that transplanted islets have defects in proinsulin processing similar to those observed in our human islet transplant subjects, which are unrelated to the presence of immunosuppressive drugs or hyperglycemia, and that amyloid deposits may play a significant role in reducing transplanted human islet viability.

Islet transplantation has been shown to result in insulin independence in type 1 diabetic recipients, however despite initial graft function, progressive graft failure is characteristic in the majority of cases [161]. Since only a small portion of infused islets engraft, we and others have proposed that functional exhaustion of the residual beta-cell mass may lead to functional beta-cell defects. Marginal mass transplants, as defined by their inability to achieve or maintain normoglycemia in islet recipients, have been shown to exhibit worse function in humans. Although disproportionate hyperproinsulinemia is a characteristic of our marginal mass transplants, the observation of normal plasma proinsulin levels may be due to the lack of hyperglycemia that is known to stimulate proinsulin biosynthesis [249] and potentially elevate plasma proinsulin levels. Our observations are in agreement with Davalli et al., who similarly showed no differences in the levels of proinsulin between normoglycemic recipients of 1000 and 2000 islets, however, their observed proinsulin/insulin ratios were 3-fold greater in recipients transplanted with the lower islet mass [250]. Our results that marginal mass transplants of 200 human islets had elevated proinsulin/insulin ratios compared to mice transplanted with 500 islets
support the “demand hypothesis” which suggests that the abnormality most linked to beta-cell overstimulation is disproportionate hyperproinsulinemia. Additionally, we showed that the proinsulin/insulin ratio tended to increase with time following transplantation, suggesting that it may also be a potential marker of graft dysfunction, at least in the normoglycemic setting.

In type 2 diabetes, the proinsulin/insulin ratios can range between 20-50% and have been shown to be 3-fold greater than in healthy subjects [57], indicating inefficiency of proinsulin conversion to insulin. The ratios seen in our transplant recipients fall within the range of the ratios seen in type 2 diabetes, suggesting that the defect observed in islet transplant recipients recapitulates the defect present in type 2 diabetes. In our transplant population, the presence of an elevated proinsulin/insulin ratio suggests that there may be defects within the transplanted beta cells exacerbated by a marginal transplant mass that ultimately lead to graft dysfunction.

Donor and recipient characteristics have been shown to impact on beta-cell function and factors that may influence islet quality including donor glycemic status, BMI and donor age, have all been shown to correlate with progressive graft dysfunction. Without knowing the proinsulin/insulin ratios prior to islet isolation, it is impossible to ascertain whether the defect is related to the donor and their metabolic status, the isolation procedure, and/or the metabolic demand imposed on the islets following transplantation. It is interesting to note that in our study, a transplant of 200 human islets into immune-deficient diabetic mice was considered marginal, whereas Davalli et al. [250] found a transplant mass of 1000 islets to be marginal. Several reasons may account for this difference. It is possible that the quality of our isolated islet preparations were better. It is conceivable that islet donors in our study were younger, normoglycemic or lean, and therefore had better quality islets. Another possibility may be that the majority of islets transplanted were non-functional or degranulated, such that transplantation of a greater mass would not necessarily imply a greater functional mass. Additionally, the severity of pre-transplant hyperglycemia may also impact graft outcome. Our islet transplant recipients had a lower pre-transplant glycemia (24 mM) as compared to the recipients in the Davalli et al. study (29 mM) [250]. Therefore, it is possible that in combination with poor islet quality and higher pre-transplant glycemia there in a need for a greater islet mass to achieve similar results. Alternatively, the method of counting islets for transplantation may have been different. In our studies similar sized islets were hand picked and counted, whereas, Davalli et al. may have expressed their islet mass as islet equivalents.

Amyloid deposits are a clinical pathology of islets from subjects with type 2 diabetes and have been previously reported in human islets transplanted into immune-deficient mice [211].
Although IAPP is present in all mammalian beta cells, only a few species are capable of developing amyloid deposits due to the presence of an amyloidogenic region within the mature IAPP molecule. Our group has found that amyloid forms rapidly in cultured human islets [145] and inhibition of amyloid formation, either by suppression of IAPP by siRNA [120] or incubation of islets with short peptide inhibitors of IAPP aggregation [122] has been shown to maintain beta-cell viability with a reduction in amyloid deposition. Our finding that amyloid is associated with graft failure (defined by hyperglycemia of >15mM) underlies the importance of developing strategies to inhibit synthesis or aggregation of (pro)IAPP in transplanted human islets, and therefore improving islet viability in vivo.

Several mechanisms could explain the rapid formation of amyloid in transplanted islets. Firstly, it is conceivable that IAPP or its precursors are unable to exit the beta cell rapidly enough following stimulated secretion such that they aggregate within the pancreatic beta-cell granule. Indeed, there is evidence to suggest the presence of intracellular amyloid deposits and that pre-fibrillar IAPP oligomers which are thought to be the more toxic species, contribute to the formation of the beta-pleated amyloid deposits [115]. Impaired processing of proinsulin or the IAPP precursor, proIAPP, may be another mechanism contributing to amyloid formation. Indeed, impaired proIAPP processing has been shown to lead to amyloid formation, at least in an in vitro model [145] and proIAPP has also been shown to be a component of amyloid [149]. It is therefore possible that impaired proIAPP processing may be a trigger for initial amyloid formation. It is also conceivable that the toxic oligomers that are thought to contribute to amyloid formation, can form pores within the beta-cell granule membranes [115] resulting in Ca$^{2+}$ leakage and therefore may impact on Ca$^{2+}$ availability required for normal processing to occur.

It has also been proposed that insulin, and to a lesser degree proinsulin, is able to inhibit fibril formation [251]. Insulin was shown to progressively decline post transplantation regardless of transplanted islet mass, and therefore, it is reasonable to surmise that the lack of insulin may result in a more suitable milieu for amyloid formation to occur that would be further promoted by the demand placed on an insufficient beta-cell mass transplanted. Mice transplanted with islets expressing human IAPP had less insulin present and impaired proinsulin processing manifest as an elevated proinsulin/insulin ratio that may further support the observation that impairments in proinsulin processing, resulting in less insulin being synthesized or secreted, may be associated with the development of amyloid and beta-cell death.
Although this was not examined by the current studies it is likely that proinsulin and potentially proIAPP processing may be impacted by changes in the expression levels and activities of the prohormone convertases that are present in the immature and maturing secretory granules. PC1/3 and PC2 are synthesized coordinately with insulin in response to glucose and mature to their biologically active forms while transiting through the various compartments of the regulated secretory pathway. Incomplete maturation of the processing enzymes as a direct result of increased demand on a suboptimal beta-cell mass resulting in insufficient time for maturation to occur, may impact the processing of proinsulin and proIAPP and lead to an elevated proinsulin/insulin ratio and amyloid deposition. In support of this possible mechanism for reduced proinsulin and proIAPP processing, there is evidence from other groups of a reduction in beta-cell PC2 immunostaining in isolated human islet grafts, with a concomitant increase of PC2 presence in the α-cell [250].

In summary, recipients of islet grafts have impaired processing of proinsulin that progressively declines following transplantation regardless of the islet mass transplanted and is exacerbated by the presence of a reduced transplant mass. This defect is unrelated to the presence of immunosuppressive drugs or hyperglycemia, although the latter may exacerbate the dysfunction in beta-cells in marginal islet grafts. We also show that normoglycemic transplants that receive a lower islet mass have elevated proinsulin/insulin ratios and are associated with a greater proportion of amyloid in the grafts. These findings point to the importance of amyloid formation in transplants thereby limiting graft function and survival, and therefore strategies to prevent the deposition of amyloid may be necessary to improve transplant outcomes in the future.
CHAPTER 5. PRO-ISLET AMYLOID POLYPEPTIDE IMMUNOASSAY DEVELOPMENT

5.1 Background

Amyloid deposits are characteristic of the beta-cell pathology seen in subjects with type 2 diabetes and are associated with beta-cell loss and hyperglycemia. These deposits are primarily formed by aggregation of islet amyloid polypeptide (IAPP or amylin), but also comprise the unprocessed precursor hormone, proIAPP [149]. Recent reports suggest that amyloid deposits are also present in transplanted human islets further contributing to a decline in functional beta-cell mass [250, 252]. Since proinsulin and proIAPP are processed in parallel, and since proinsulin processing is impaired in both islet transplantation and type 2 diabetes, we predict that proIAPP will also be secreted in excess amounts relative to mature IAPP and that this will be an early event in the progression not only in type 2 diabetes but also islet transplantation. We propose that the proIAPP/IAPP ratio may have value as an early marker of beta-cell dysfunction, and that an elevated ratio may be a novel early marker of ensuing graft failure. Although sensitive assays enabling quantification of insulin, proinsulin and mature IAPP in normal and diabetic human plasma have been described, there has been no report of direct proIAPP measurement in human serum, likely due to a lack of suitable antibodies and the mid-femtomolar range of concentrations of proIAPP. To this end, we sought to develop an immunoassay capable of measuring proIAPP in human serum.

5.2 Results

The accuracy and precision of an immunoassay can be improved considerably by optimizing the use of various antibodies for antigen capture and type of antibody labeling. To develop the proIAPP immunoassay, the following parameters were evaluated: antibodies, antibody concentrations and orientations, incubation times, temperature, buffers and detection systems. Other components such as choice of blocking agents and diluents were also studied.

To determine the efficiency and reproducibility of labeling, we used the signal/background ratio calculated as the signal from each standard divided by the signal from the zero standard, as a determining factor before proceeding to further steps along the development process (Appendix B).
5.2.1 Polyclonal rat-IAPP capture/monoclonal human NH₂-terminal proIAPP detection antibody TR-FIA.

**Typical proIAPP TR-FIA standard curves.** As an initial step in the development of the TR-FIA, we determined the optimal concentration of the polyclonal anti-rat IAPP capture antibody (Peninsula 7323) together with a fixed concentration (1:2000) of the monoclonal anti-human N-terminal proIAPP antibody (F064) in a sandwich-type assay format. It was found that increasing the dilution resulted in higher background fluorescence counts and we chose to use a 1:500 dilution of 7323 for subsequent assays since we achieved the best signal/background ratio. Representative standard curves for the 7323/F064 antibody pair and TR-FIA performed with sample dilution buffer as diluent on 5 different days are shown in Figure 22. The fluorescence counts are proportional to concentrations of proIAPP standards over a large concentration range. In the range between 0–5.2 pM, the changes in signal/background ratio were not significantly different between standards making it more difficult to detect small differences in proIAPP concentrations in this range, whereas standards ranging from 5.2–167 pM tended to have a more pronounced signal/background ratios indicating greater differences between standards.

Figure 22. **Typical proIAPP TR-FIA standard curves.** Plates were coated with polyclonal anti-rat IAPP (7323) and detected with monoclonal anti-human N-terminal proIAPP (F064) antibodies. Each point on the standard curve is a mean of duplicate well measurements in each of 5 assays performed on 5 different days.
For the assessment of serum or plasma samples, which have more complex components within them, it is important to choose the appropriate diluent for the standards so as to best mimic the environment of the serum protein of interest. To this end, we used serum from patients with long-duration type 1 diabetes with little beta cell function and therefore likely to have little or no proIAPP in serum that could interfere with the assay. Comparison of typical standard curves with standards diluted in sample dilution buffer or 20% long-duration type 1 diabetic serum is shown in Figure 23. The sample dilution buffer standard curve had higher signal/background ratios for all standards compared to the 20% long-duration type 1 diabetic serum curve, which tended to fall below the sample dilution buffer curve suggesting that there is likely serum interference.

**Figure 23. Effect of serum on the proIAPP TR-FIA standard curve.** The plate was coated with polyclonal anti-rat IAPP (7323) and detected with monoclonal anti-human N-terminal proIAPP (F064) antibodies. Sample dilution buffer (SDB) or long-duration type 1 diabetic serum (LDT1D) diluted 5-fold with sample dilution buffer were used for preparation of proIAPP standards. Data are presented as means of duplicate wells.
**Biosynthesized proIAPP is detectable by TR-FIA.** To determine whether the developed TR-FIA was capable of measuring biosynthesized proIAPP, islets obtained from a cadaveric human donor and from various transgenic mice expressing human IAPP were assayed. Serially diluted human islet tended to dilute linearly (Figure 24). Human proIAPP was also detectable in islet lysates from mice expressing human IAPP and with defective proIAPP processing (Figure 25). Mice lacking the prohormone convertase enzyme 2 (PC2<sup>−/−</sup>hIAPP<sup>tg/tg</sup>) or with a mutation in the N-terminal proIAPP cleavage site (KKK<sup>+/−</sup>) had proIAPP concentrations of 3.5 pM and 0.7 pM, respectively.

**Figure 24. Biological forms of human proIAPP are detectable by TR-FIA.** Lysates of islets obtained from one cadaveric islet donor were incubated for 2h in a plate coated with polyclonal anti-rat IAPP antibody (7323) and detected with monoclonal anti-human N-terminal proIAPP (F064) antibodies. Data are presented as means of duplicate wells.
Figure 25. Human proIAPP in murine models of impaired proIAPP processing. Lysates of islets obtained from mice transgenic for proIAPP and lacking prohormone convertase 2 (PC2) (PC2^{-/-}hIAPP^{+/+}; PC2^{+/+}hIAPP^{+/+}), with a mutated proIAPP N-terminal processing site (KKK^{+/+}) or mice lacking murine IAPP(mIAPP^{-/-}) were incubated for 2h in a plate coated with polyclonal anti-rat IAPP (7323) and detected with monoclonal anti-human N-terminal proIAPP (F064) antibodies. Data are presented as means of duplicate wells from a single islet extract.
Degradation of proIAPP by dipeptidyl peptidase-IV. In the majority of samples assayed in the current TR-FIA, we were unable to detect proIAPP levels in serum. This may be due to the limited sensitivity of the assay itself or potential protein degradation by peptidases present in circulation. Dipeptidyl peptidase-IV (DPP-IV) has an important role in glucose metabolism and is responsible for the degradation of incretins such as glucose-dependent insulinotropic polypeptide (GIP) and GLP-1, neuropeptides such as neuropeptide Y (NPY) and several chemokines [253]. DPP-4 exhibits a strong preference for peptides with proline (Pro, P) or alanine (Ala, A) as the penultimate amino acid (X-Ala or X-Pro) [253]. Human proIAPP is a potentially novel substrate for DPP-IV since it has a proline residue as the desired penultimate amino acid position in its N-terminal flanking peptide (Figure 26).

![Figure 26. Proposed proIAPP cleavage by DPP-IV and inhibition by the DPP-IV inhibitor, P32/98.](image-url)
To determine whether proIAPP is degraded by DPP-IV, proIAPP peptide was incubated in human serum with or without the DPP-IV inhibitor P32/98 (Figure 27) and proIAPP was measured in samples at different time points. Over time, the proIAPP concentration in serum decreased and the addition of the DPP-IV inhibitor, P32/98 reduced peptide loss over a 24hr incubation period. In summary, the 7323/TR-FIA was able to detect human proIAPP in both islet extracts as well as human serum samples. Addition of DPP-IV inhibitor delayed proIAPP degradation.

Figure 27. Inhibition of proIAPP degradation in human serum using a DPP-IV inhibitor, P32/98. Normal human serum was spiked with synthetic human proIAPP standard and incubated at 37°C. Aliquots were frozen at 0, 1, 2, 3, 4 and 24 hrs for analysis of residual proIAPP in an assay with polyclonal rat IAPP (7323) antibody as capture and monoclonal human N-terminally specific proIAPP (F064) antibody as detection. Data are expressed as means of duplicate wells in one assay.
5.2.2 Monoclonal anti-human NH₂-terminal proIAPP capture/polyclonal anti-human IAPP detection antibody TR-FIA.

The goal of developing the proIAPP TR-FIA was to measure human forms of proIAPP in circulation. The capture IAPP antibody used in the 7323/F064 TR-FIA was rat specific and since the IAPP molecule is conserved across species (Figure 28), it is possible that some human proIAPP was captured. However, since the antibody was not raised to human proIAPP, it may have been insufficient for detection of proIAPP in our samples and may have been recognizing other antigens also. To develop a more sensitive and specific TR-FIA, we sought to assess the ability of a polyclonal anti-human IAPP antibody (PF07B2) in its ability to capture proIAPP in human serum. Prior to obtaining results on human clinical samples, capture, primary and detection antibody concentrations were optimized.

<table>
<thead>
<tr>
<th></th>
<th>N-terminal flanking peptide</th>
<th>C-terminal flanking peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>human proIAPP</td>
<td>TPIESHQVEKR</td>
<td>NAVELKREPLNYLPL</td>
</tr>
<tr>
<td>human IAPP</td>
<td>TPIESHQVEKR</td>
<td>NAVELKREPLNYLPL</td>
</tr>
<tr>
<td>rat proIAPP</td>
<td>TPVGSQPQVYDKR</td>
<td>TPIESHQVEKR</td>
</tr>
<tr>
<td>mouse IAPP</td>
<td>TPIESHQVEKR</td>
<td>TPIESHQVEKR</td>
</tr>
<tr>
<td>synthetic proIAPP standard</td>
<td>1 KNTATCATQRLANFLVHSSNNNF</td>
<td>37 KNTATCATQRLANFLVHSSNNNF</td>
</tr>
<tr>
<td></td>
<td>GAILSSTNVGSNTY</td>
<td>GAILSSTNVGSNTY</td>
</tr>
<tr>
<td></td>
<td>KCNTATCATQRLANFLVHSSNNF</td>
<td>KCNTATCATQRLANFLVHSSNNF</td>
</tr>
<tr>
<td></td>
<td>GAILSSTNVGSNTY</td>
<td>GAILSSTNVGSNTY</td>
</tr>
</tbody>
</table>

Figure 28. IAPP sequences are well conserved across species while the NH₂-terminal flanking peptide region of proIAPP is not. Adapted from Clark et al. Diabetologia 2004)
To determine the optimal assay diluent for measurement of proIAPP in serum samples, we compared various sera including serum from subjects with long-duration type 1 diabetes, charcoal extracted human serum, fetal bovine serum and horse serum. Using the signal/background ratio as an indicator of the labeling efficiency, we found long-duration type 1 diabetic serum, charcoal extracted human serum and fetal bovine serum to have comparable effects (Figure 29). As in the previous TR-FIA, the type 1 diabetic serum curve tended to fall below the sample dilution buffer curve, however, the signal/background ratios were higher (Figure 30). This assay was unable to measure proIAPP in human serum.

Figure 29. Effect of serum on proIAPP standard curve. The plate was coated with polyclonal human IAPP (PF07B2) and detected with human N-terminally specific proIAPP (F064) antibodies. Sample dilution buffer (SDB), long-duration type 1 diabetic serum (LDT1D), charcoal extracted human serum (CEHS), fetal bovine serum (FBS) or horse serum (HS), each diluted 5-fold with sample dilution buffer + 1% BSA, were used for dilution of proIAPP standards. Data are presented as means of duplicate wells.
Figure 30. Typical standard curves for human proIAPP TR-FIA. Plates were coated with polyclonal human IAPP (PF07B2) and detected with human N-terminally specific proIAPP (F064) antibodies. Each point on the standard curve is a mean of duplicate well measurements in one assay.
ELISAs are frequently based on two monoclonal antibodies. Unlike monoclonal antibodies that have specificity for a single epitope and therefore allow finer detection and measurement of small differences in the analyte of interest, polyclonal antibodies may recognize multiple epitopes and may contribute to increased background or non-specific reactions. To determine whether it was possible to improve the sensitivity of the TR-FIA, a reverse antibody orientation was examined where the monoclonal human proIAPP antibody (F064) was used as capture and the polyclonal human IAPP (PF07B2) as detection. Results from one assay are shown in Figure 31 where a titration of antibody concentrations was performed to ascertain whether a sensitive standard curve could be generated. Results show that a standard curve could be generated with this antibody orientation, however, the sensitivity of this assay (20.9 pM) was much lower that the assay where a polyclonal anti-human IAPP antibody was used for capture.

**Figure 31. Effects of antibody orientation reversal in the proIAPP TR-FIA.** Plate was coated with various dilutions of monoclonal human N-terminally specific proIAPP (F064) antibodies and detected with human IAPP antibody (PF07B2). Standards were diluted in sample dilution buffer. Data are presented as means of duplicate wells from one assay.
5.2.3 Meso Scale Discovery electrochemiluminescent proIAPP immunoassay

The Meso Scale Discovery® electrochemiluminescent (ECL) platform uses electrical stimulation for the measurement of protein levels in biological samples. Carbon electrodes are integrated into the bottom of 96-well plates onto which antibodies can be absorbed such as in traditional ELISA. Primary antibodies used for detection of the analyte of interest can be labeled with the detection molecule, ruthenium(II) tris-bipyridal (Ru(bpy)$_3^{2+}$), which upon excitation emits light at 620nm. The reaction can be enhanced with the proprietary “Read” buffer that contains tripropylamine (TPA) as a coreactant. Multiple excitation cycles are able to enhance light generation and therefore increase sensitivity, and background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light).

**Typical ECL proIAPP standard curve.** For the development of a typical ECL proIAPP calibration curve (Figure 32) the following conditions were optimized: plate type, antibody orientation and concentrations of PF07B2 and F064, blocking solutions as well as assay and antibody diluents. Using the monoclonal antibody as the capture to increase the sensitivity of the assay, there was a persistent high background as is evident on both the TR-FIA's. We found that this was primarily due to interaction between the coating monoclonal antibody (F064) and the polyclonal detection antibody (PF07B2) with some contribution to the background signal from the SULFO-TAG anti-rabbit antibody binding to F064. Little or no non-specific binding was observed for either the PF07B2 or the SULFO-TAG antibodies to the plate surface.

![Figure 32. Representative ECL proIAPP standard curve.](image)

Plate was coated with monoclonal human N-terminally specific proIAPP (F064) antibody and detected with human IAPP antibody (PF07B2) using electrochemiluminescent detection. Data are presented as means of duplicate wells from one assay.
Serum proIAPP is detectable by ECL proIAPP immunoassay. Human serum samples collected from subjects with normal glucose tolerance (ND; n=3) or type 2 diabetes (T2D; n=4) were measured in the ECL assay. In addition, we determined levels of insulin, proinsulin and IAPP were determined in samples from the same subjects, and the proinsulin/insulin and proIAPP/IAPP ratios were calculated. Insulin (Figure 33A) and IAPP (Figure 33B) both tended to be higher in subjects with type 2 diabetes (insulin: 58.7 ± 6.4 vs. 111.3 ± 41.4 pM and IAPP: 11.0 ± 1.8 vs. 13.6 ± 1.4 pM). Proinsulin also tended to be higher in subjects with type 2 diabetes (12.5 ± 1.9 vs. 45.7 ± 22.2 pM; Figure 33C) while proIAPP was lower (5.2 ± 1.3 vs. 3.7 ± 0.6 pM; Figure 33D). The proinsulin/insulin ratio (TP/TP+I) tended to be higher in subjects with type 2 diabetes (17.8 ± 3.2 vs. 31.9 ± 5.8 %; Figure 33E) while the proIAPP/IAPP ratio (proIAPP/ proIAPP+IAPP) tended to be lower (32.7 ± 9.1 vs. 23.0 ± 4.7 %; Figure 33F).
Figure 33. Detection of proIAPP in human serum samples in subjects with type 2 diabetes and non-diabetic controls. Plate was coated with monoclonal human N-terminally specific proIAPP (F064) antibody and detected with human IAPP antibody (PF07B2) using electrochemiluminescent detection. Data are represented as mean ± SEM and Student’s t-test was used to determine difference between groups. ND=non-diabetic controls (n=3); T2D=type 2 diabetes (n=4).
5.2.4 Limits of sensitivity of TR-FIA and ECL immunoassays

In assays such as those measuring hormone concentrations in serum samples, it is important to determine the limit of sensitivity so as to facilitate interpretation of results. Assay sensitivity can vary as a result of using different antibodies or detection systems. The detection limit can be defined as the smallest concentration that is able to deduce the presence of the analyte within reasonable statistical certainty [254]. We calculated the limit of detection, or sensitivity, within our assays as the mean of the measured values of the zero standard plus two times the standard deviation of the mean. Results are shown in Table 5. Of the three assays developed, the ECL was the most sensitive, followed by the TR-FIA that used the polyclonal anti-human IAPP antibody (PF07B2) as the capture antibody, the TR-FIA using the polyclonal anti-rat IAPP antibody (7323) and lastly, the TR-FIA which used the monoclonal anti-human N-terminal proIAPP antibody (F064) as capture antibody.

Table 5. Detection limits of TR-FIA and ECL proIAPP immunoassays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Capture Antibody</th>
<th>Detection Antibody</th>
<th>ProIAPP Detection Limit (pM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR-FIA</td>
<td>7323</td>
<td>F064</td>
<td>0.5 ± 1.8</td>
<td>10</td>
</tr>
<tr>
<td>TR-FIA</td>
<td>PF07B2</td>
<td>F064</td>
<td>0.4 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>TR-FIA</td>
<td>F064</td>
<td>PF07B2</td>
<td>20.9</td>
<td>1</td>
</tr>
<tr>
<td>ECL</td>
<td>PF07B2</td>
<td>F064</td>
<td>0.2 ± 0.01</td>
<td>2</td>
</tr>
</tbody>
</table>

TR-FIA = time resolved fluorescent immunoassay, ECL = electrochemiluminescent immunoassay, 7323 = polyclonal anti-rat IAPP, PF07B2 = polyclonal anti-human IAPP, F064 = monoclonal anti-human N-terminal proIAPP antibodies. N refers to the number of assays used to calculate the detection limits that are expressed as mean ± SEM.

5.3 Discussion

The aim of the study described in this section was to establish a highly sensitive and specific two-antibody immunoassay for the detection of proIAPP in human serum. Using two different immunoassay platforms we were able to detect proIAPP in human serum without extraction and in cell extracts of human islets, and islets isolated from mice transgenic for human IAPP. We also showed preliminary results suggesting that proIAPP may be degraded in serum by the enzyme dipeptidyl peptidase IV (DPP-IV).
The enzyme linked immunosorbent assay (ELISA) is the most frequently used technique for detection of circulating serum proteins that can be used as markers for disease detection. It is relatively easy to optimize analyte detection by manipulation of factors such as antibody concentrations and analyte incubation times. The ELISA is most successful for detection of more abundant proteins; however, its ability to detect proteins in the low-picomolar or mid-femtomolar concentrations may be limited. Since proIAPP is thought to circulate in the mid-femtomolar range of concentrations, we chose to use two highly sensitive detection systems that were extensions of the typical two-antibody ELISA that uses capture and detection antibodies for analyte measurement.

The first method tested was the dissociation-enhanced lanthanide fluorescence immunoassay that uses time-resolved fluorescence (TRF) technology. TRF provides a way to distinguish between the specific signal and the non-specific background and uses probes that have longer decay times than the background signal. The technology uses lanthanide chelates such as Europium, which have much longer decay times and a larger Stokes’ shift (difference between the excitation and emission wavelengths) than traditional fluorophores, and therefore, allow for a reduction of background signal. The signal is further enhanced by dissociation of the fluorophore into a micellar structure that has even higher fluorescence.

The second technique used was the electrochemiluminescent-based immunoassay method that is also an extension of the ELISA. The ECL immunoassay uses a strong luminophore, tris(2,2'-bipyridyl)ruthenium(II) (Ru(bpy)₃²⁺) that in the presence of a co-reactant, tripropylamine, is oxidized and a signal is generated. The ECL emission intensity is therefore proportional to the amount of analyte present in the sample of interest [255]. In addition, ECL has a large dynamic range with high sensitivity, allows for better detection in complex matrices such as serum and requires a much smaller sample volume and is thought to be comparable to the sensitivity of typical radioimmunoassay.

To date, there have been no methods described for the detection of proIAPP in human serum. TR-FIA and ECL detection are widely available for various other analytes including human insulin and proinsulin [256, 257] as well as GLP-1. We were able to measure human proIAPP in serum samples using both signal detection systems, however, the ECL immunoassay was much more sensitive as determined by the background plus two standard deviations from the zero, and was able to measure proIAPP in human serum samples. Although we were able to detect circulating proIAPP in the TR-FIA, detection was inconsistent such that we were frequently unable to measure circulating proIAPP in serum samples. This may be due to the lack
of specificity of the antibodies, the sensitivity of our detection system, factors in serum that may interfere with binding of the antigen to the analyte, or degradation of proIAPP.

In the proIAPP immunoassays, a truncated synthetic proIAPP 1-30 peptide was used for the development of a proIAPP standard curve. Based on the proposed proIAPP processing pathway (Figure 2), it is reasonable to assume that this standard does precisely represent the major circulating form of proIAPP in human serum and as such an assay that detects this standard may not be able to accurately quantify human biosynthetic forms. Although we were able to measure proIAPP in human and transgenic mouse islet extracts using the TR-FIA, the proIAPP levels would have been much more concentrated than in human serum. In order to better represent the biosynthetic form of proIAPP in human serum, a full length human proIAPP or C-terminally truncated proIAPP standard could be used, however, due to the aggregation prone nature of the mature IAPP molecule this would likely prove to be more difficult in developing a robust standard curve. Other possibilities that may impede or reduce proIAPP measurement in human serum samples may be due to other protein modifications such as protein glycosylation, which can mask epitopes for antibodies. Protein glycation is known to be a feature of type 2 diabetes and advanced glycation end products are seen in proteins with low turnover [111]. Whether this is also characteristic of proIAPP has yet to be elucidated, although there is evidence for O-linked glycosylation of IAPP at positions 6 and 9 [258], which could conceivably interfere with antibody binding. Finally, another possible limitation to consistently measuring proIAPP in human serum is that it circulates in very low amounts potentially due to low secretion from the beta cell or due to rapid protein degradation.

The proIAPP processing intermediate, NH$_2$-terminally extended proIAPP (processed at the C-terminus) has been identified by immunostaining in amyloid deposits in islets from hIAPP expressing mice and in human islets transplanted under the kidney capsule into nude mice; these deposits were found intracellularly within the beta-cell granules [149]. NH$_2$-terminally proIAPP has also been shown to interact with heparan sulfate proteoglycans of the basement membrane [259, 260]. NH$_2$-terminally extended proIAPP may therefore be important in amyloid development and could occur at an earlier stage in the progression to type 2 diabetes, such as in impaired glucose tolerance. It has been proposed that proIAPP, rather than IAPP, may initiate fibril formation within the granules and serve as a nidus for further amyloid deposition, a pathology characteristic of type 2 diabetes [114, 149]. Since proinsulin and proIAPP are thought to be processed coordinately, and since elevations in proinsulin can already be seen in pre-
diabetes, it seems possible that with increased amounts of proIAPP or its NH$_2$-terminally extended intermediate, amyloid deposition may occur early in the progression to type 2 diabetes.

Increased DPP-IV activity has been reported in fasting serum of subjects with type 2 diabetes and has been associated with poor glycemic control as assessed by elevated fasting blood glucose and HbA$_1c$ levels [261]. Hyperglycemia has also been associated with increased DPP-IV activity [262] and elevated DPP-IV gene expression [261]. DPP-IV has been implicated in the degradation of the incretin hormones, GLP-1 and GIP which are important in glucose metabolism [253] and as such, N-terminally directed GLP-1 immunoassays require the inclusion of DPP-IV inhibitors for blood sample collection. DPP-IV preferentially cleaves substrates that have either a proline or alanine as the penultimate amino acid (Figure 29). ProIAPP has a penultimate proline in its N-terminal flanking peptide such that DPP-IV would be able to degrade it, resulting in a reduced antibody binding. It is therefore reasonable to surmise that our observation of lower proIAPP levels in subjects with type 2 diabetes may be a result of increased serum DPP-IV activity, and therefore addition of inhibitors, especially to samples that may have increased DPP-IV activity may improve our ability to detect levels of proIAPP with our immunoassay. We have shown a progressive degradation of proIAPP in human serum that could be alleviated with the addition of the DPP-IV inhibitor, P32/98. Based on this observation, P32/98 was added to all subsequent serum samples collected for this and other potential studies.

Another plausible explanation for our inability to consistently measure proIAPP in human serum between different assays is due to matrix interference from the serum as it contains numerous large proteins that may impact on antibody binding. In a study designed to measure serum total thyroxin, matrix interference was reduced by the streptavidin-biotin separation technique where the biotinylated capture antibody was immobilized onto the plate by interacting with pre-coated streptavidin [263]. Indirect immobilization such as this may improve an assay’s reproducibility and sensitivity. Another method to improve sensitivity was to use the N-terminal proIAPP monoclonal antibody as the capture; however, since we were very limited by its availability, another detection system was tested that exhibits higher sensitivity and reduced interference from complex matrices such as serum, and one that used much lower sample volumes than the current TR-FIA.

With the ECL immunoassay, it was possible to detect proIAPP in samples from non-diabetic control serum and serum from subjects with type 2 diabetes. We hypothesized that since proinsulin processing is impaired in type 2 diabetes, and possibly already in the early stages of disease pathogenesis, proIAPP processing may also be impaired resulting in the relative
hypersecretion of proIAPP relative to the mature hormone, IAPP. As expected, the proinsulin/insulin ratio tended to be elevated in this small sample set of subjects with type 2 diabetes. It is interesting to note that, although the number of subjects was very low, our patients with type 2 diabetes did not have higher proIAPP or proIAPP/IAPP ratios than normal controls, and if anything, the proIAPP/IAPP ratio tended to be lower. This finding raises the possibility that unlike proinsulin, proIAPP processing may not be impaired in type 2 diabetes, but we cannot rule out the possibility that it is not impaired in other metabolic states, such as impaired glucose tolerance. We must be cautious in drawing any strong conclusions with the current data since in addition to the low sample size, the assay used for measurement of human proIAPP in serum had not been optimized nor validated for parameters such as species specificity, interference, analytical recovery, dilution linearity or inter- and intra-assay variability.

In summary, we have developed immunoassays with a range of sensitivities capable of measuring proIAPP in extracts from human islets and islets from mice transgenic for human proIAPP, as well as serum proIAPP levels in non-diabetic controls and subjects with type 2 diabetes. We found that proIAPP may undergo proteolytic degradation by dipeptidyl peptidase-IV (DPP-IV) and that addition of a DPP-IV inhibitor to serum samples may be important for delaying proIAPP degradation prior to measurement by immunoassay. Interestingly, we also found that unlike the elevated levels of proinsulin and the higher proinsulin/insulin ratios observed in type 2 diabetes, proIAPP and the proIAPP/IAPP ratio were not higher in our diabetic subjects suggesting that proIAPP processing is not impaired in type 2 diabetes.
CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

Human islet transplant recipients have increased proinsulin/insulin and proinsulin/C-peptide ratios suggesting that they have impairments in proinsulin processing. From our mouse models of transplantation we conclude that the elevated proinsulin/insulin ratio is unrelated to the presence of immunosuppressive drugs or hyperglycemia, although in marginal islet grafts hyperglycemia may play a role in exacerbating the defect in beta-cell function. We also show that transplantation of a low beta-cell mass likely contributes to the progressive decline in graft function, possibly due to increased secretory demand placed on the transplanted islets. Our findings also point to the importance of amyloid formation in transplants, thereby limiting graft survival and function, and suggest that strategies to prevent the deposition of amyloid may improve islet transplant outcomes.

In the future, we propose to continue development of the proIAPP immunoassay as we believe that measuring human proIAPP in serum of islet transplant recipients or subjects with type 2 diabetes will allow us to determine the proIAPP/IAPP ratio, a measure that may serve as a marker of beta-cell dysfunction. This marker may be more pertinent to islet transplants since the pathology of amyloid formation seems to be much faster than in type 2 diabetes, where amyloid generally takes years to develop. Further development of our human proIAPP immunoassay, and in particular increased assay sensitivity, is needed before this assay can be routinely used to determine proIAPP levels in normal and diabetic human subjects. The assays still need further validation including determination of whether the antibody pair we have chosen is specific enough for the detection of human proIAPP. It seems likely that for sufficient sensitivity and specificity for the detection of serum proIAPP, other antibody pairs will need to be tested. In addition, determination of other parameters such as sample spike recovery and dilution linearity, as well as assay precision (inter- and intra-assay variability) should be assessed. For assessment of assay specificity, one should measure several closely related peptides and other islet peptides such as human IAPP, insulin, proinsulin, glucagon, pancreatic polypeptide, adrenomedulin, calcitonin and calcitonin gene related peptide.

To determine whether proIAPP levels are lower or higher in type 2 diabetes and islet transplantation, we have banked fasting serum samples from subjects with type 2 diabetes, recipients of islet transplants as well as non-diabetic controls that can be used for measurement once the assay is optimized. To exclude the contribution of clearance of IAPP or proIAPP in our subject and control groups, samples following beta-cell stimulation could be measured to
determine the magnitude of the beta-cell defect in proIAPP processing. In the future this assay could be used clinically to determine whether the proIAPP/IAPP ratios are elevated in prediabetes as is the case for proinsulin. Other experiments could examine the contribution of other factors such as obesity and insulin resistance, increased secretory demand, glucose and/or free fatty acid infusion to changes in the proIAPP/IAPP ratios.

In conclusion, the major finding of this thesis is that islet transplants, like islets in type 2 diabetes, have beta-cell dysfunction manifest as hyperproinsulinemia. Elevated proinsulin levels have been shown to be an independent risk factor in the development of type 2 diabetes and an elevated proinsulin/insulin ratio is indicative of decreased beta-cell function. These measures could be translated into the setting of islet transplantation and be used as markers of beta-cell function prior to the onset of graft failure. As such, these findings may point to new approaches for the progressive assessment of islet graft function following transplantation, and therefore allow for earlier therapeutic intervention to improve graft outcomes in vivo.
REFERENCES


[40] Larsson H, Ahren B (1996) Islet dysfunction in obese women with impaired glucose tolerance. Metabolism 45: 502-509


Brunzell JD, Robertson RP, Lerner RL, et al. (1976) Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests. J Clin Endocrinol Metab 42: 222-229


Kahn SE, Zraika S, Utschneider KM, Hull RL (2009) The beta cell lesion in type 2 diabetes: there has to be a primary functional abnormality. Diabetologia 52: 1003-1012


Kahn SE, Halban PA (1997) Release of incompletely processed proinsulin is the cause of the disproportionate proinsulinemia of NIDDM. Diabetes 46: 1725-1732


Hou X, Ling Z, Quartier E, et al. (1999) Prolonged exposure of pancreatic beta cells to raised glucose concentrations results in increased cellular content of islet amyloid polypeptide precursors. Diabetologia 42: 188-194


[228] Larsson H, Ahren B (1999) Relative hyperproinsulinemia as a sign of islet dysfunction in women with impaired glucose tolerance. J Clin Endocrinol Metab 84: 2068-2074

112
APPENDICES

Appendix A: Laboratory and ethics board certificates of approval

UBC/C&W Ethics Certificates
UBC Animal Care Certificate
UBC Biohazard Approval Certificate
**ETHICS CERTIFICATE OF MINIMAL RISK APPROVAL:**

**RENEWAL WITH AMENDMENTS TO THE STUDY**

<table>
<thead>
<tr>
<th>PRINCIPAL INVESTIGATOR:</th>
<th>DEPARTMENT:</th>
<th>UBC C&amp;W NUMBER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Bruce Verchere</td>
<td>UBC/Medicine, Faculty of Pathology &amp; Laboratory Medicine</td>
<td>H06-03112</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institution</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Vancouver Coastal Health (VCHRI/VCHA)</td>
</tr>
<tr>
<td>Children's and Women's Health Centre of BC (incl. Sunny Hill)</td>
</tr>
<tr>
<td>Other locations where the research will be conducted:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CO-INVESTIGATOR(S):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constadina Panagiotopoulos</td>
</tr>
<tr>
<td>Garth Warnock</td>
</tr>
<tr>
<td>Agnieszka Klimek</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPONSORING AGENCIES:</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Canadian Institutes of Health Research (CIHR) - &quot;Processing of ProlAPP: Role in islet amyloid formation&quot;</td>
</tr>
<tr>
<td>- Michael Smith Foundation for Health Research - &quot;Role of Amyloid in Failure of Transplanted Human Islets&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROJECT TITLE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prohormone Processing in Human Islet Transplantation</td>
</tr>
</tbody>
</table>

The current UBC Children's and Women's approval for this study expires: March 15, 2011

<table>
<thead>
<tr>
<th>AMENDMENT(S):</th>
<th>AMENDMENT APPROVAL DATE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Document Name</td>
<td>Version</td>
</tr>
<tr>
<td>Consent Forms:</td>
<td></td>
</tr>
<tr>
<td>IAPP Study Consent Form</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**CERTIFICATION:**

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Children's and Women's Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Children's and Women's Research Ethics Board.

Approved by one of:

Dr. Marc Levine, Chair
Dr. Caron Strahlendorf, Associate Chair
ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR: Garth Warnock
DEPARTMENT: UBC/Medicine, Faculty of Surgery/General Surgery
UBC CREB NUMBER: H03-70453

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

<table>
<thead>
<tr>
<th>Institution</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancouver Coastal Health (VCHRI/VCHA)</td>
<td>Vancouver General Hospital</td>
</tr>
<tr>
<td>UBC</td>
<td>Vancouver (excludes UBC Hospital)</td>
</tr>
<tr>
<td>Children's and Women's Health Centre of BC (incl. Sunny Hill)</td>
<td>Child &amp; Family Research institute</td>
</tr>
</tbody>
</table>

Other locations where the research will be conducted: N/A

CO-INVESTIGATOR(S):
- James M. Piret
- Zilang Ao
- Megan K. Levings
- Dawei Ou
- James D. Johnson
- C. Bruce Verchere
- Zehua He
- Robert Mark Meloche
- Theresa Liao
- Timothy J. Kieffer
- T. Michael Underhill

SPONSORING AGENCIES:
- Canadian Diabetes Association - "Islet Amyloid And Islet Transplant Failure"
- Juvenile Diabetes Research Foundation International - "Autocrine Survival Factors in Human Islets"
- Juvenile Diabetes Research Foundation International - "Characterization of T-cell costimulatory molecules in pancreata from IAPCD"
- Juvenile Diabetes Research Foundation International - "Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes"
- Juvenile Diabetes Research Foundation International - "Prolonging Diabetes Reversal in Islet Xenotransplantation by SHT-I4"
- Michael Smith Foundation for Health Research - "The Centre of Human Islet Transplant and Beta Cell Regeneration"
- National Centre of Excellence - "High content, high throughput screening for molecules that promote beta-cell formation"
- Roche Organ Transplant Research Foundation - "Manipulating FOXP3 to induce transplantation tolerance in humans."
- Stem Cell Network (SCN) - Networks of Centres of Excellence (NCE) - "Generating Transplantable beta-cells for Diabetes"
- VGH and UBC Hospitals Special Opportunities Research Fund - "Autocrine Survival Factors in Human Islets"
- VGH and UBC Hospitals Special Opportunities Research Fund - "Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes"

PROJECT TITLE:
Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes

To add a New title "The Centre of Human Islet Transplant and Beta Cell Regeneration"

High content, high throughput screening for molecules that promote beta-cell formation.

Manipulating FOXP3 to induce transplantation tolerance in humans.

Islet Amyloid and Islet Transplant Failure.

EXPIRY DATE OF THIS APPROVAL: May 28, 2011

APPROVAL DATE: May 28, 2010

CERTIFICATION:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by one of:

Dr. Peter Loewen, Chair
Dr. James McCormack, Associate Chair
The Animal Care Committee has examined and approved the use of animals for the

<table>
<thead>
<tr>
<th>Application Number:</th>
<th>A09-0647</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigator or Course Director:</td>
<td>C. Bruce Verchere</td>
</tr>
<tr>
<td>Department:</td>
<td>Pathology &amp; Laboratory Medicine</td>
</tr>
<tr>
<td>Animals:</td>
<td></td>
</tr>
<tr>
<td>Mice Bl/6 36</td>
<td></td>
</tr>
<tr>
<td>Mice Human proIAPP transgenic 144</td>
<td></td>
</tr>
<tr>
<td>Mice PC2 knockout 36</td>
<td></td>
</tr>
<tr>
<td>Mice Mouse proIAPP knockout 36</td>
<td></td>
</tr>
<tr>
<td>Mice ABCA1 loxp 24</td>
<td></td>
</tr>
<tr>
<td>Mice CPE knockout 24</td>
<td></td>
</tr>
<tr>
<td>Mice RIP-Cre 72</td>
<td></td>
</tr>
<tr>
<td>Mice PC2 loxp 36</td>
<td></td>
</tr>
</tbody>
</table>

| Start Date: | April 1, 2009 |
| Approval Date: | September 24, 2009 |

Funding Sources:

| Funding Agency: | Canadian Institutes of Health Research (CIHR) |
| Funding Title: | Processing of ProIAPP: Role in islet amyloid formation |

| Unfunded title: | N/A |
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: B09-0199

INVESTIGATOR OR COURSE DIRECTOR: C. Bruce Verchere

DEPARTMENT: Pathology & Laboratory Medicine

PROJECT OR COURSE TITLE: Processing of ProIAPP: Role in Islet Amyloid Formation

APPROVAL DATE: November 6, 2009 START DATE: April 1, 2009

APPROVED CONTAINMENT LEVEL: 2

FUNDING TITLE: Processing of ProIAPP: Role in islet amyloid formation
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

UNFUNDED TITLE: N/A

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093
Appendix B: ProIAPP immunoassay development calculations

Table 1. Effects of antibody concentrations on signal/background ratios in the 7323/F064 proIAPP TR-FIA.

<table>
<thead>
<tr>
<th>ProIAPP (pM)</th>
<th>1:100</th>
<th>1:250</th>
<th>1:500</th>
<th>1:1000</th>
<th>1:2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.33</td>
<td>0.97</td>
<td>0.98</td>
<td>1.07</td>
<td>1.09</td>
<td>1.02</td>
</tr>
<tr>
<td>0.65</td>
<td>0.98</td>
<td>1.03</td>
<td>1.09</td>
<td>1.07</td>
<td>1.05</td>
</tr>
<tr>
<td>1.30</td>
<td>1.00</td>
<td>1.06</td>
<td>1.16</td>
<td>1.14</td>
<td>1.05</td>
</tr>
<tr>
<td>2.61</td>
<td>2.61</td>
<td>1.05</td>
<td>1.23</td>
<td>1.3</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Table 2. Signal/background ratios in the 7323/F064 proIAPP TR-FIA standard curves on 5 different days.

<table>
<thead>
<tr>
<th>proIAPP (pM)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>0.65</td>
<td>1.24</td>
<td>1.14</td>
<td>1.09</td>
<td>1.13</td>
<td>1.05</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>1.30</td>
<td>1.50</td>
<td>1.22</td>
<td>1.11</td>
<td>1.35</td>
<td>1.17</td>
<td>1.27 ± 0.07</td>
</tr>
<tr>
<td>2.61</td>
<td>2.00</td>
<td>1.32</td>
<td>1.22</td>
<td>1.36</td>
<td>1.39</td>
<td>1.46 ± 0.14</td>
</tr>
<tr>
<td>5.22</td>
<td>2.80</td>
<td>1.72</td>
<td>1.52</td>
<td>1.63</td>
<td>1.84</td>
<td>1.9 ± 0.23</td>
</tr>
<tr>
<td>10.44</td>
<td>4.53</td>
<td>2.04</td>
<td>2.02</td>
<td>2.22</td>
<td>2.76</td>
<td>2.71 ± 0.47</td>
</tr>
<tr>
<td>20.88</td>
<td>7.49</td>
<td>3.25</td>
<td>3.06</td>
<td>3.36</td>
<td>4.44</td>
<td>4.32 ± 0.83</td>
</tr>
<tr>
<td>41.75</td>
<td>13.34</td>
<td>5.30</td>
<td>4.86</td>
<td>5.46</td>
<td>7.21</td>
<td>7.24 ± 1.58</td>
</tr>
<tr>
<td>83.50</td>
<td>18.58</td>
<td>9.56</td>
<td>7.98</td>
<td>8.79</td>
<td>11.79</td>
<td>11.34 ± 1.92</td>
</tr>
<tr>
<td>167.00</td>
<td>24.60</td>
<td>15.73</td>
<td>13.45</td>
<td>13.21</td>
<td>17.89</td>
<td>16.98 ± 2.09</td>
</tr>
</tbody>
</table>

Table 3. Effect of serum on the signal/background ratios in the 7232/F064 proIAPP TR-FIA.

<table>
<thead>
<tr>
<th>ProIAPP (pM)</th>
<th>SDB</th>
<th>20% LDT1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.17</td>
<td>1.07</td>
<td>1.11</td>
</tr>
<tr>
<td>0.84</td>
<td>1.72</td>
<td>1.54</td>
</tr>
<tr>
<td>1.67</td>
<td>2.60</td>
<td>2.38</td>
</tr>
<tr>
<td>8.35</td>
<td>10.02</td>
<td>6.77</td>
</tr>
<tr>
<td>16.70</td>
<td>17.41</td>
<td>14.33</td>
</tr>
<tr>
<td>167.00</td>
<td>93.64</td>
<td>77.76</td>
</tr>
</tbody>
</table>
Table 4. Effects of different serum matrices on the signal/background ratios in the PF07B2/F064 proIAPP TR-FIA. SDB = sample dilution buffer, LDT1D = long-duration type 1 diabetic serum, CEHS = charcoal extracted human serum, FBS = fetal bovine serum, HS = horse serum. All sera were diluted 1:5 in SDB.

<table>
<thead>
<tr>
<th>ProIAPP (pM)</th>
<th>SDB</th>
<th>LDT1D</th>
<th>CEHS</th>
<th>FBS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2.61</td>
<td>1.13</td>
<td>1.24</td>
<td>1.38</td>
<td>1.33</td>
<td>1.49</td>
</tr>
<tr>
<td>5.23</td>
<td>1.34</td>
<td>1.56</td>
<td>1.54</td>
<td>1.60</td>
<td>1.37</td>
</tr>
<tr>
<td>10.44</td>
<td>1.72</td>
<td>2.09</td>
<td>1.96</td>
<td>1.94</td>
<td>1.69</td>
</tr>
<tr>
<td>20.88</td>
<td>2.43</td>
<td>3.28</td>
<td>3.17</td>
<td>3.16</td>
<td>2.37</td>
</tr>
<tr>
<td>41.75</td>
<td>4.04</td>
<td>5.73</td>
<td>5.57</td>
<td>5.27</td>
<td>3.25</td>
</tr>
</tbody>
</table>

Table 5. Comparison of signal/background ratios in PF07B2/F064 proIAPP TR-FIA using sample dilution buffer (SDB) or long-duration type 1 diabetic serum diluted to 1:5 in SDB.

<table>
<thead>
<tr>
<th>ProIAPP (pM)</th>
<th>SDB</th>
<th>LDT1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2.61</td>
<td>1.10</td>
<td>1.22</td>
</tr>
<tr>
<td>5.23</td>
<td>1.19</td>
<td>1.17</td>
</tr>
<tr>
<td>10.44</td>
<td>1.28</td>
<td>1.49</td>
</tr>
<tr>
<td>20.88</td>
<td>1.52</td>
<td>1.94</td>
</tr>
<tr>
<td>41.75</td>
<td>2.02</td>
<td>3.02</td>
</tr>
</tbody>
</table>

Table 6. Signal/background ratios in the Meso Scale Discover electrochemiluminescent proIAPP immunoassay assay.

<table>
<thead>
<tr>
<th>ProIAPP (pM)</th>
<th>Signal/Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.19</td>
<td>1.06</td>
</tr>
<tr>
<td>0.56</td>
<td>1.09</td>
</tr>
<tr>
<td>1.67</td>
<td>1.36</td>
</tr>
<tr>
<td>5.00</td>
<td>2.13</td>
</tr>
<tr>
<td>15.00</td>
<td>5.85</td>
</tr>
</tbody>
</table>
Appendix C. Publications


Johnson, James D.1; Ao, Ziliang; Ao, Peter; Li, Hong; Dai, Long-Jun; He, Zehua; Tee, May; Potter, Kathryn J.; **Klimek, Agnieszka M.**; Meloche, R. Mark; Thompson, David M.; Verchere, C. Bruce; Warnock, Garth L. Different Effects of FK506, Rapamycin, and Mycophenolate Mofetil on Glucose-Stimulated Insulin Release and Apoptosis in Human Islets. Cell Transplantation, 2009; 18(8): 833-84.