Type 1 Diabetes Diagnostic Assay

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Type 1 Diabetes Diagnostic Assay

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ABSTRACT

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Type 1 Diabetes Diagnostic Assay

Directors of Thesis: Kelly D. McCall

The objectives of this thesis research project were to (i) develop an assay sensitive enough to detect dead or dying pancreatic beta cells and to (ii) use this assay to predict which at-risk individuals will go on to develop T1DM. *In vitro* and *in vivo* approaches were utilized for its validation. This novel assay differentiated between beta cells and non-beta cells with higher analytical and diagnostic sensitivity than previous approaches. With the novel application of mathematical models the assay was also able to retrospectively predict which at-risk individuals would go on to develop T1DM with high sensitivity and specificity. This assay has the potential to be applied alone or in combination with islet antibodies to identify individuals in the very early stages of T1DM to allow for earlier intervention and perhaps halt beta cell destruction and thus prevent this disease.
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<td>Demethylation index</td>
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<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<td>CT</td>
<td>Critical threshold</td>
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<td>sqPCR</td>
<td>Semi-quantitative PCR</td>
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<td>NOD.SCID</td>
<td>Non-Obese diabetic mouse with severe combined immune deficiency</td>
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Definitions

Demethylation Index: The ratio of demethylated: methylated DNA.  
Critical Threshold: The intersection between an amplification curve and a threshold line. It is used as a relative measure of the concentration of target in the PCR reaction. A lower CT value indicates a more abundant amount of target. A higher CT value indicates a less abundant amount of target.  
NOD.WT: Non-Obese diabetic mouse that has a genetic predisposition to spontaneously develop T1DM.  
NOD.SCID: Non-Obese diabetic mouse with severe combined immune deficiency that does not develop T1DM due to an absence of T and B cells.  
DI slope: Absolute value of the change in DI value from one week to the next.  
Change in DI slope: The change in max DI slope values.  
Critical number: A critical number is the number that the DI, DI slope, or change in DI slope must be the same or greater for the assay to predict an animal that will develop T1DM.  
Analytical sensitivity: to the ability of an assay to measure on particular organism or substance, rather than others, in a sample.  
Diagnostic sensitivity: The proportion of positives correctly identified. Calculated as (true positives) / (true positives and false negatives).  
Diagnostic specificity: The proportion of negatives correctly identified. Calculated as (true negatives) / (true negatives and false positives).
CHAPTER ONE: INTRODUCTION OF TYPE 1 DIABETES

Diabetes mellitus (DM) currently affects 25.8 million people in the United States with a growing prevalence\textsuperscript{35}. Of those diagnosed with DM 1.9 million people have type I diabetes mellitus (TIDM), with a reported constant global rise in the incidence of T1DM\textsuperscript{7}. Individuals with T1DM are at risk for developing a barrage of diabetic complications in addition to a lifetime of insulin treatment regimes. Although there is some genetic linkage, most individuals with new-onset T1DM have no family history of the disease\textsuperscript{23}, and current methods for diagnosis involve measuring elevated glucose levels \textbf{after} the disease has occurred. Attempts at predicting risk for developing T1DM in family members with the disease by detecting the presence of islet autoantibodies are helpful but even with the genetic predisposition less than half of individuals with these autoantibodies develop T1DM. Approximately only 41\% of those with persistent autoantibodies actually develop T1DM\textsuperscript{41} and 20-30\% of those diagnosed with T1DM do not have autoantibodies. Due to the relatively low predictability of these islet autoantibodies, there is a significant need for more specific predictors of diabetes risk.

The successful development and implementation of the assay tested in these studies may allow for earlier detection of the pancreatic beta cell death/loss that precedes clinical onset of symptoms (i.e., hyperglycemia) as well as a better predictive test for determining which at-risk individuals will develop the disease, and thus allow earlier intervention aimed at protecting the remaining pancreatic beta cell mass from further destruction and prevent the progression to T1DM (insulin deficiency).
T1DM is characterized by the destruction of functional pancreatic beta cells that produce insulin, and is known as insulin-dependent diabetes. One-third of patients are diagnosed as children, one third as adolescences, and one third as adults. Since pancreatic beta cells are solely responsible for the production and release of insulin, their destruction renders the patient insulin deficient, and thus diabetic.  

1.1 Insulin

The insulin protein is produced as a zymogen; it is synthesized by the pancreatic beta cells as pre-proinsulin and through cleavage and post-translational modifications of disulfide bonds, the functional hormone is generated. Insulin is produced and secreted into the blood stream to be used by the body to regulate and maintain glucose homeostasis. C-peptide, which is produced as a product of the zymogen cleavage of proinsulin into the active insulin molecule and C-peptide, can also be used to evaluate the amount of residual pancreatic beta cells and their functionality.

Insulin is a hormone that allows the cells to utilize circulating glucose. Insulin signals to cells in the body and allows their uptake of glucose. Without insulin, glucose cannot be utilized properly by most of the cells of the body. This can result in chronic and dangerously high blood glucose levels (i.e., hyperglycemia). Once the disease has manifested, patients must receive exogenous insulin to normalize blood glucose levels. Chronic hyperglycemia from T1DM results in major complications including microvascular complications such as retinopathy, nephropathy, and neuropathy. It can also lead to macrovascular complications such as cardiovascular disease and peripheral
vascular disease. Individuals with T1DM also have an elevated risk of lower extremity amputation, osteoporosis, pregnancy complications, and skin and mouth infections.

1.2 Forms of T1DM

There are two major forms of T1DM: type 1A and type 1B. Type 1B is idiopathic and consists of five main factors that include abrupt onset of the disease, short duration of diabetic symptoms, prior to diagnosis ketoacidosis at diagnosis, elevated serum pancreatic enzyme levels, and negativity for pancreatic beta cell-related autoantibodies. Type 1B is thought to be primarily virus-mediated, wherein the pancreatic beta cells are rapidly destroyed through viral-mediated destruction. It has been observed that both pancreatic beta and alpha cells (islet cell types) are destroyed in subjects with type 1B, but only the beta cells are destroyed in the pancreas of subjects with type 1A. Type 1A is autoimmune-mediated and patients with this form of T1DM typically have beta cell autoantibodies. Type 1A accounts for the vast majority of T1DM cases (~85%), and viral infection can also play a role in its pathology through acceleration of the immune process via the up-regulation of pro-inflammatory cytokines and chemokines.

Toll-like receptor 3 (TLR3) has been implicated in both forms of T1DM. Viral infection and induction of pancreatic beta cell death can result in the release of beta cell-specific antigens triggering cell- and antibody-mediated autoimmune destruction of pancreatic beta cells through its activation of TLR3. TLR3 plays a role in innate immunity but when upregulated in non-immune cells, can be detrimental since it can trigger an abnormal immune response that is partially dependent on the homo-
dimerization of IRF3 (interferon regulatory factor 3) and its translocation to the nucleus which induces the transcription of pro-inflammatory cytokines and chemokines. The production of pro-inflammatory cytokines and chemokines in the inflamed islets (insulitis) trigger pancreatic beta cell apoptosis and autoimmune pancreatic beta cell destruction. The objective of this project was to design and validate an assay that can quantify the pancreatic beta cell damage/destruction very early in the disease process [during the initial inflammatory process, which is causing pancreatic beta cell loss (death)], and prior to the onset of clinically evident T1DM. Reliable detection of very early beta cell death would potentially allow earlier detection and intervention with anti-inflammatory agents (TLR3 antagonists, etc.) which inhibit the production of pro-inflammatory cytokines and chemokines that are important in the pathogenesis of T1DM. This would dampen the heightened aberrant immune response, and potentially prevent total pancreatic beta cell loss, insulin deficiency, and overt diabetes.

1.3 Insulitis

Insulitis is the lymphocytic infiltration of the islets of Langerhans that often precedes both forms of T1DM. Insulitis classically consists of a peri- or intra-islet accumulation of immune cells, mainly of the CD3^+CD8^+ lymphocyte subtype, together with CD68^+ macrophages, CD3^+CD4^+ T-helper cells and CD20^+ B-lymphocytes. Insulitis persists longer in the autoimmune-mediated type 1A form since beta cells are destroyed through a more indolent, less fulminant chronic autoimmune process (months to years), whereas in type 1B the pancreatic beta cells are destroyed very rapidly by an intense innate immune destructive process that is thought to be triggered by viral
infection\textsuperscript{12}. Although the duration of insulitis varies in these two forms of T1DM this new PCR-based diagnostic assay has the potential to detect the disease early (in the insulitis phase) in both forms. For type 1A, this assay, coupled with measuring islet autoantibodies, could be used to detect (potentially quantify) actual beta cell death and increase both the diagnostic specificity and sensitivity of detection of the disease to allow for earlier and more specific treatment.

\textit{1.4 Mouse Models}

The NOD.WT mouse model has a genetic predisposition to spontaneously develop T1DM and is the most commonly used model to study this disease. The timing of insulitis and diabetes development in the NOD.WT mouse is well characterized. In NOD.WT mice the pathophysiology is consistent with human T1DM in that it involves the breakdown in immune regulation, resulting in the activation and expansion of auto-reactive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, (white blood cells that are part of the adaptive immune system that infiltrate and attack the islets) and auto-reactive B lymphocytes (that produce the islet autoantibodies). The NOD.SCID is a mouse line developed on the NOD background, but is deficient in B and T cells. With the major modulators of the adaptive immune system responsible for diabetes development being absent, the NOD.SCID mice do not develop diabetes.
CHAPTER TWO: DIAGNOSTIC METHODS

There are several commonly used methods for the general diagnosis of diabetes (type 1 or 2): fasting blood glucose test, random blood glucose test, oral glucose tolerance test, and the hemoglobin A1c (HbA1C) test. The fasting blood glucose test takes a blood sample after a period of fasting for at least eight hours. Normal fasting blood glucose levels are between 70 to 110 mg/dl, and a measurement of 126 mg/dl or higher is considered diabetic \(^{34, 35}\). Each test is usually performed a second time to confirm the initial diagnosis of T1DM/T2DM. The random blood glucose test doesn’t involve a fasting period, and a blood glucose reading above 200 mg/dL is considered diabetic. This is a quicker process that allows for immediate diagnosis in cases where a patient may be going into a diabetes-induced coma \(^{34, 35}\). In the oral glucose tolerance test (OGTT) following a fasting period a baseline measurement is taken then the patient is made to consume a sugary beverage in order to observe how the pancreas manages the glucose. The beverage is consumed over twenty minutes and then glucose measurements are taken every thirty minutes for 2 hours \(^{34, 35}\). A normal OGTT result would display glucose levels rising no higher than 200 mg/dl then falling returning to 70-140 mg/dL since the body naturally produces insulin to maintain glucose homeostasis. In a patient with T1DM, a sharp rise and a sustained high level of glucose in the blood is observed because the pancreas is unable to deliver the needed insulin to lower the blood glucose level. In T1DM, the HbA1c measures the level of glycosylated hemoglobin caused by hyperglycemia, due to the lack of insulin present to facilitate its transport into the cell. In the HbA1c test, a glycation measure of 6.5 % or higher indicates diabetes \(^{34}\).
Additional diagnostic methods used to characterize the type 1A form of diabetes are the islet autoantibodies (ICA, GAD65, IAA and IA2) that are found in individuals with type 1A. The major limitations of autoantibody detection is that approximately only 41% of those with persistent autoantibodies actually develop T1DM\(^1\) and 20-30% of those diagnosed with T1DM do not have autoantibodies. The presence or absence of islet autoantibodies and the severity of insulin deficiency at diagnosis are currently used to differentiate Type 1A from Type 1B. Residual C-peptide secretion is also used as a predictor. Measurement of stimulated C-peptide levels (i.e., measure of endogenous insulin secretory capacity) following a glucose challenge or a mixed carbohydrates meal is the most sensitive and clinically validated method to evaluate residual pancreatic beta cell function\(^2\). The stimulated C-peptide will measure the pancreatic beta cell’s acute response to a glucose or meal challenge, but stimulated C-peptide levels are usually much lower when there is total destruction of pancreatic beta cells, as usually seen in pattern 1B (Figure 1). A problem with using C-peptide levels as a diagnostic method is that interpretation of the changes in C-peptide responses may be more complicated in T1DM at diagnosis since pancreatic beta cells are often “stunned” (beta cell glucose insensitivity) from the insulitis and glucotoxicity (toxic effects of hyperglycemia on beta cell function) and should be assessed after the patient is well controlled on exogenous insulin.
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Figure 1: Natural history of type 1 diabetes. The role of genetic, immunologic, and metabolic markers in defining the risk of the disease. This figure illustrates the relatively brief time period that is targeted by clinical trials. Improved predictive assays may identify individuals before significant beta cell destruction and metabolic impairment. Figure adopted from Lebatchi J., Herold K. (2012). Immunologic and Metabolic Biomarkers of B-Cell Destruction In The Diagnosis Of Type 1. Cold Spring Harbor Perspectives in Medicine. (6), a007708.

Another use of the islet autoantibody assays is to predict the development of type 1A in high-risk individuals (first degree relatives of patients with type 1A). This is of interest as there are multiple clinical research trials attempting to preserve beta cell function and prevent this form of diabetes before it occurs. The difficulty of relying on these assays is that only 41% of high risk individuals who are autoantibody positive will progress to type 1A. A majority of the patients with newly diagnosed T1DM have no family history of the disease and there is no way to determine risk or monitor for early insulitis. The symptoms of T1DM do not occur until more than 60-70% of the beta cell mass has been irreversibly destroyed so a more sensitive and specific diagnostic of actual
pancreatic beta cell loss is needed to allow for early intervention. Recently, in efforts to combat this problem, a number of studies have reported their attempts to develop new assays that utilize epigenetics (study of heritable changes in gene activity that are not caused by changes in the DNA sequence) in order to detect the onset of T1DM\textsuperscript{2, 9, 13}. The assay built upon previous\textsuperscript{1, 8, 12} studies using a PCR-based method and the demethylated CpG sites within the promoter of the INS gene, but was modified to improve the assay. The assay can detect dead or dying pancreatic beta cells that are shed by the pancreas in either form of T1DM and it may be able to do so earlier than current diagnostic methods (i.e., at the onset of insulitis). Using my assay in combination with islet autoantibodies could also increase the diagnostic specificity and sensitivity of detection of patients at risk for developing T1DM. An earlier and/or more specific diagnostic is beneficial and could allow for earlier therapeutic intervention and possibly the preservation of functional beta cell mass, thus preventing the onset of this devastating disease.
CHAPTER THREE: EPIGENETICS

Epigenetics is the study of heritable changes in gene activity that are not caused by changes in the DNA sequence. In this project, the heritable change of interest is the methylation pattern contained in the DNA. The DNA sequence of a particular subject remains consistent throughout every cell (except for occasional mutations) but in an act to conserve energy the cell only expresses the genes needed for a particular function.

3.1 Methylation

Mammalian gene promoters contain cytosine-guanosine dinucleotide (CpG) sites which play a pivotal role in the control of gene expression. The methylation of a CpG site simply means that the cytosine within the CpG site contains a methyl group. Methylation of the cytosine residues at these CpG sites regulates transcription directly by inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl CpG binding proteins and their associated repressive chromatin remodeling activities. These epigenetic alterations are responsible for modulation of developmentally-regulated and tissue-specific gene expression. Methylation of DNA sequences occurs in CpG sites to maintain a transcriptionally repressive chromatin configuration, whereas demethylation results in a transcriptionally permissive configuration.

By looking at specific methylation pattern differences in the DNA derived from various cells contained in blood samples one can differentiate their function. Since insulin production is unique to pancreatic beta cells they can be identified by a mostly demethylated INS promoter region. Since other cells do not produce insulin they will have a mostly methylated INS promoter region. Dead/dying beta cells that are being...
shed into the circulation can be identified on the basis of the demethylation of CpG sites within the INS gene promoter region.

A notable attribution to the justification behind the development of this PCR-based assay is the fact that the amino acid methionine is involved in DNA methylation and very young children who develop islet autoantibodies by two years of age have a two-fold lower amount of methionine compared with those who develop autoantibodies by eight years of age, or those that remained autoantibody negative. In addition to being involved in DNA methylation, methionine is suggested to be a potentially relevant amino acid for developing islet autoantibodies in early infancy.

Recent *in vitro* and *in vivo* studies identified differentially methylated regions of the mouse INS2 promoter. The mouse genome consists of two insulin genes, INS1 and INS2. INS1 is a functional retrotransposon that is not linked to INS2. My research utilized the INS2 gene since it has the closest structural and functional similarity to the single human insulin gene and codes for the functional insulin protein. CpG within the insulin promoter region of the INS2 gene are differentially methylated in pancreatic beta cells compared to all other non-insulin producing tissues. The CpG sites are methylated in the non-insulin producing tissues and demethylated in beta cells. My study used the -182 bp CpG site in the mouse INS2 promoter region. Previous studies have confirmed that pancreatic beta cell death produces a corresponding increase in the detection of demethylated insulin gene DNA in serum.
3.2 Deamination with Bisulfite

DNA consists of the bases: adenine, guanine, cytosine and thymine. RNA consists of bases: adenine, guanine, cytosine and uracil. The uracil base differs from the cytosine base by having a double bonded oxygen (=O) in place of an amine (NH$_2$) at the C4 position. Bisulfite treatment of the DNA deaminates the cytosines to uracils by removing the “NH$_2$” group resulting in a double-bonded oxygen in the C4 position (Figure 2). The change to uracil does not alter the sugar group allowing us to retain the advantageously stable properties of DNA while utilizing an RNA base.

![Figure 2: Cytosine Deamination. Illustration of a cytosine undergoing deamination of the base to uracil during a bisulfite treatment reaction](image)

Bisulfite treatment of a single DNA sequence can produce two different DNA products depending on the methylation status. Bisulfite treatment will consistently deaminate all cytosines not within a CpG site but if a cytosine is within a CpG site it then has the potential to be deaminated into a uracil or remain a cytosine, depending on whether the cytosine is methylated or demethylated. A methylated cytosine within a CpG site is protected from deamination upon bisulfite treatment and will remain a cytosine. A demethylated CpG site will be deaminated upon bisulfite treatment, resulting in a change from a cytosine to a uracil. The methylated cytosines within CpG sites are unchanged.
because the bulky methyl group prevents its deamination. Bisulfite treatment can cause two different changes to the sequences of DNA depending on the methylation status. The original target DNA sequence is as follows: 5’- ggTgTTgA CpG TCCAATgAg -3’. After bisulfite treatment, if the CpG site indicated in red was methylated prior to bisulfite treatment, it is protected from deamination, however all cytosines outside of the CpG site are deaminated to uracils and the resulting sequence is as follows:

5’-ggTgTTgACpGUUAATgAg – 3’.

If the CpG site indicated in red was demethylated prior to bisulfite treatment, it is not protected from deamination it is thus deaminated into uracil just as the rest of the cytosines that are not contained within a CpG site are. The resulting sequence is as follows:

5’-ggTgTTgAGpG UUAATgAg – 3’.
CHAPTER FOUR: POLYMERASE CHAIN REACTION (PCR)

PCR results in the exponential amplification of a target sequence of DNA. Semi-quantitative PCR (sqPCR) exponentially amplifies the DNA sequence but does NOT allow for absolute quantification of it. Real-time PCR (qPCR) exponentially amplifies the DNA sequence AND allows for absolute quantification of it in real time. There are 2 different main types of real-time PCR chemistries to choose from: Taqman and SYBR green. SYBR green employs a green fluorescent dye that incorporates itself into double stranded DNA. This chemistry has a higher amount of background noise than Taqman, and results in false negatives since the dye can incorporate its self into double stranded DNA that is not specific to the DNA target sequence (e.g., primer dimers, etc). Taqman consists of a forward primer, a reverse primer and probe(s). The forward primer anneals to the (+) strand and the reverse primer anneals to the (–) strand on an upstream portion of the sequence. They both replicate in the 3’-5’ direction toward the probe(s) which anneals to the middle portion of the target sequence. The probe contains a quencher attached to a reporter dye. The reporter dye is silenced as long as it is in close proximity to the quencher, as the DNA polymerase replicates in the direction of the probe(s) the reporter dye is cleaved from the probe and fluoresces since it is no longer in close proximity to the quencher. The fluorescent signal is read by the real-time PCR machine in real time and reported in the form of critical threshold values.

4.1 Critical Threshold

The critical threshold (CT) is the intersection between an amplification curve and a threshold line. It is used as a relative measure of the concentration of target in the PCR
reaction. A lower CT value indicates a more abundant amount of target in the sample. A higher CT value indicates a less abundant amount of target in the sample. A CT ≥ 40 is accepted as minimal to no target detection.

4.2 Taqman Primers and Probes

Taqman single nucleotide polymorphism (SNP) probes have a high degree of specificity, allowing for the detection of a single nucleotide difference. Taqman SNP primer/probe set had 1 primer set that consisted of a forward and a reverse primer that annealed to the flanking regions of the target DNA sequence. In our case the target DNA sequence was the -182 bp CpG site. It also had 2 probes, each with a different nucleotide in one specific position (at the -182 bp CpG site), a U if the DNA was demethylated and a C if it was methylated. Taqman consisted of 2 probes, one to detect the demethylated sequence and one to detect the methylated sequence. A VIC or FAM labeled reporter dye was on the end of each probe. A VIC label is on the probe that detected the demethylated DNA and a FAM label was on the probe that detects the methylated DNA. As the reporter dye was cleaved a fluorescent signal specific to the VIC or FAM label was recorded by the qPCR machine as a CT values specific to that probe. If the DNA sequence of interest differed in as little as one nucleotide from the sequence specific to the VIC or FAM probe then the reporter dye remained quenched and neither a signal nor a CT value was recorded. This allowed for the quantification of the two SNP differences.
4.3 PCR Application after Bisulfite Treatment

The bisulfite reaction produces different DNA sequences from one original DNA sequence depending on the methylation status of each sequence. This property allowed for probes to be designed that detect SNP differences in one location as a result of bisulfite treatment. As previously mentioned, bisulfite treatment will consistently deaminate all cytosines not within a CpG site but if a cytosine is within a CpG site it then has the potential to be deaminated into a uracil or remain a cytosine depending on whether the cytosine is methylated or demethylated. A methylated cytosine within a CpG site is protected from deamination and will remain a cytosine. A demethylated CpG site will be deaminated, resulting in a change from a cytosine to a uracil. After bisulfite treatment, the CpG site of interest for this project consists of a one SNP difference. It will have a uracil in place of the cytosine if it was demethylated at the CpG site. This allowed for the design of probes specific to this SNP difference. The FAM probe detected the SNP difference representative of methylated DNA and the VIC probe detected the SNP difference representative of demethylated DNA. The CpG site of interest for this study is in the INS2 promoter region and methylation of the cytosine in this site is characteristic of non-beta cell DNA, while a demethylated cytosine at this site is characteristic of and specific to beta cell DNA. The CT values recorded by the qPCR machine for the FAM and VIC probes will individually indicate the abundance of methylated (FAM probe) and demethylated (VIC probe) INS2 DNA (Figure 3).

Both pancreatic beta cell-derived DNA and non-beta cell-derived DNA will contain mixtures of methylated and demethylated forms of the target INS2 -182 bp CpG
site of interest. However, there should be a higher amount of the demethylated INS2 -182 bp CpG site of interest in blood samples containing more pancreatic beta cell-derived DNA, and a higher presence of the methylated INS2 -182 bp CpG site of interest in blood samples containing more non-beta cell-derived DNA. Since every sample, no matter the origin, will have a mixture of methylated and demethylated forms of the INS2 DNA, the previously described demethylation index (DI; $2^{\text{demethylated CT-methylated CT}}$)\textsuperscript{1, 8, 12} was utilized to compare the amount of the demethylated in comparison to the methylated targeted DNA sequence in each sample. The increased presence of the demethylated DNA results in a lower CT value from the VIC probe and is consistent with the increased presence of beta cell DNA and results in a higher demethylation index.

4.4 PCR Primer and Probe Design

Our targeted CpG site was -182 bp upstream from the transcription start site within the promoter region of the INS2 gene. A sqPCR primer pair that was complimentary to sequences flanking the -182 bp CpG site (site of interest) was used to amplify the target sequence in samples prior to real-time PCR. The DNA sequences specific to the sqPCR primers did not contain any CpG sites. The sqPCR was used as a “pre-amplification” step to increase the amount of target DNA template for later amplification and differentiation by qPCR. The qPCR primer pair annealed to regions flanking the -182 bp CpG site that also did not contain any CpG sites. The qPCR probe was specific to the -182 bp CpG site which was the only CpG site contained within the targeted sequence. The qPCR probes quantified the presence of methylated (FAM-labeled probe) and demethylated (VIC-labeled probe) DNA by utilizing the SNP
difference induced by bisulfite treatment. Bisulfite treatment, sqPCR and qPCR were performed for each sample.

Figure 3: Taqman qPCR Primers and Probes.
The sqPCR primer set annealed to the outer flanking region of the -182 bp CpG site and amplified the target region. The qPCR primer set annealed to the inner flanking region of the -182 bp CpG site, and the probes annealed to the target -182 bp CpG site. The VIC or FAM probe annealed to methylated or demethylated DNA (allele 1 or 2) differentiating the SNP difference.
CHAPTER FIVE: MATERIALS

5.1 Cell Culture

The βTC6 and 3T3L1 cell lines were purchased from the American Type Culture Collection (ATCC). Both cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose from Thermo Fisher Scientific. 3T3L1 cells were cultured in 10% calf bovine serum purchased from Thermo Fisher Scientific. βTC6 cells were cultured in 15% heat inactivated fetal bovine serum (FBS) purchased from Lonza. 1% penicillin/streptomycin was used to supplement both medias and was purchased from Lonza.

5.2 Mice

Thirty eight (3 week old) female NOD.WT (001976) and 10 NOD.SCID (001303) mice were obtained from the Jackson Laboratory and maintained in sterile environments. They were screened for hyperglycemia every week from 4 to 22 weeks of age. Mice with two consecutive blood glucose measurements above 200 mg/dL were considered diabetic. This work was conducted in accordance with approval from the Ohio University Institutional Animal Care and Use Committee.

5.3 Blood Collection, DNA Extraction/Purification and Bisulfite Treatment

Blood was collected from the tail vein of mice weekly from 4 weeks of age until 21 weeks of age in EDTA capillary tubes. DNA from whole blood was extracted using a sodium hydroxide and tris-HCL solution. The DNA was purified using chloroform. The DNA was deaminated with bisulfite treatments from the Zymo EZ DNA methylation kit.
5.4 Semi-Quantitative PCR and Purification

The semi quantitative PCR reaction was methylation-independent and it increased
the amount of the target DNA in the samples by amplifying the flanking regions of the
target DNA. The bisulfite-treated samples were amplified using Extaq chemistry,
confirmed by gel electrophoresis, and then purified using the Qiagen PCR purification
kit, in preparation for real-time PCR.

5.5 Real-Time PCR

PCR-purified samples from the semi-quantitative PCR reaction were used as
templates for real-time PCR. Custom Taqman chemistry single nucleotide polymorphism
(SNP) assays, specific to the target DNA, were designed and used with the Taqman
Genotype master mix.
CHAPTER SIX: METHODS

The overall process of the assay consisted of genomic DNA first being extracted from a blood or a cell culture sample. The DNA was then purified using chloroform extractions and then exposed to bisulfite treatment. This produced two different forms of the targeted DNA sequence containing the -182 bp CpG site, depending on the original methylation status. After the bisulfite treatment, the DNA was subjected to a sqPCR step where the target DNA was amplified by primers specific to the outer flanking region of the sequence. This was to amplify the signal of interest. The product was then subjected to gel electrophoresis to confirm the presence of the amplicon, and then the PCR product was cleaned up with a PCR clean-up kit. After the DNA was cleaned up, qPCR was performed in order to quantify and compare the amount of each form of targeted DNA (Figure 4). The demethylation index was then calculated from the qPCR CT values and analyzed to compare the relative abundance of the two forms of the DNA sequences present in the samples. Predicative parameters were then retrospectively applied to the demethylation index (DI) in order to predict the development of T1DM.

The sqPCR is an added step to amplify the signal since relative to the total genomic DNA, the DNA sequence of interest is likely present in very small quantities. This step was needed since the blood samples will contain more than just the targeted DNA region. The target DNA will be in the blood at a very low concentration in relation to the total DNA population. This step allowed for the amplification of the very small target signal, thus improving the analytical sensitivity of detection of the target sequence upon qPCR.
Cell culture (In vitro)

OR  \(\rightarrow\) gDNA extraction \(\rightarrow\) Bisulfite treatment \(\rightarrow\) sqPCR \(\rightarrow\) qPCR \(\rightarrow\) CT values \(\rightarrow\) DI \(\rightarrow\) Predictive parameters

Whole blood (In vivo)

*Figure 4: Overview of Methods.*

A diagram of the order in which each of the methods were performed. DNA was extracted and purified followed by bisulfite treatment, sqPCR then qPCR. DI values were calculated from the qPCR CT values and predictive parameters were retrospectively applied to the data.

This study differed from previous studies conducted in four major ways; (i) the Taqman chemistry used, (ii) the CT values obtained, (iii) the portion of the blood sample used, and lastly (iv) the use of predictive parameters. Two of the previous studies utilized SYBR green PCR chemistry\(^1,12\), and one used Taqman chemistry\(^9\), which minimizes background “noise” and decreases the potential for false negatives. The study that utilized Taqman chemistry\(^9\) reported results with CT values >40 for their *in vivo* studies, which are values that are too high to be considered reliable. Giddings, et al., reported CT values ranging from 35-40 even for their artificial cell culture system\(^9\) while the assay described herein had CT values of 12-13 with as little as 0.001ng of template DNA. The study by Giddings, et. al., did not use sqPCR to amplify their target sequence prior to qPCR. The CT values obtained with this assay were far superior to those reported in the literature\(^1,8,12\). All prior studies also used serum or plasma samples in efforts to detect the pancreatic beta cell loss. This study used whole blood in order to minimize the loss of possible beta cell DNA, and thus increase the analytical sensitivity of the
assay. Moreover, past studies have used streptozotocin (STZ)\textsuperscript{9} in order to induce an acute pancreatic insult\textsuperscript{9}. STZ use results in beta cell death. The previously described studies\textsuperscript{1,8,12} reported that they were able to detect a higher demethylation index after the STZ insult, supporting the concept that the demethylation index should rise as pancreatic beta cells are destroyed and subsequently released into the blood circulation\textsuperscript{1,8,12}. However, this type of insult is not indicative of the insults that occur in most cases of T1DM and is not likely to be a useful predictive model. For this study NOD.WT and NOD.SCID mice were used and serial blood samples from the mice were collected to use as samples for the assay validation and to characterize the changes in the demethylation index over time in order to correlate these demethylation indices with the timing of onset of diabetes. The NOD mouse model was used since this model most closely mimics the insults that lead to T1DM in humans. Predictive parameters were then applied retrospectively to the data to predict which individuals would develop T1DM.

6.1 Statistical Analyses

Each sample was processed in duplicate for real-time PCR. Data sets are represented as mean +/- the standard error of the mean (SEM) for each sample. Boxplots were generated using the standard inter-quartile range (IQR) parameters. The outliers are indicated by asterisks and are measurements > 1.5 times the IQR.

6.2 Power Analysis

The number of mice needed for this study was determined in consultation with the Ohio University Heritage College of Osteopathic Medicine biostatistician, Dr. Masato
Nakazawa. Dr. Nakazawa used previous data from NOD mouse studies conducted in the labs of Drs. McCall and Schwartz to calculate the number of mice needed for this study, taking into account the approximate number of mice that become diabetic during this experimental time frame to obtain a power of 0.8.

6.3 Mathematical Models

The relative abundance of demethylated DNA was expressed using the demethylation index (DI; $2^{methylated\ CT}$-demethylated CT). The DI slope was determined by the absolute value $D_{ly} - D_{lx}$ that occurred each week. The change in DI slope was determined by $\max_1 (D_{ly} - D_{lx}) - \max_2 (D_{ly} - D_{lx})$ and $\max_2 (D_{ly} - D_{lx}) - \max_3 (D_{ly} - D_{lx})$. A critical number is the number that the demethylation index, weekly slope, or change in weekly slope must be the same or greater for the assay to predict an animal that will develop T1DM. The predictive model incorporated a critical value of 2 for the demethylation index to predict the development of diabetes, indicating a 2:1 ratio of beta cell to non-beta cell DNA. The crucial value for the DI slope was also 2, indicating a doubling of the demethylation index from the previous week’s measurements. The critical value for the change in slope was 0.5, indicating a half magnitude decrease in the subsequent maximum slope.
CHAPTER SEVEN: SPECIFIC AIMS

The aim was to develop and validate an improved Polymerase Chain Reaction (PCR)-based assay to measure demethylated fragments of the insulin gene (INS) which was sensitive enough to detect dead beta cells being shed from pancreatic islets into the blood earlier than previously described PCR-based assay methods and to evaluate if this assay might be more useful to predict the very early onset of T1DM in the non-obese diabetic wild-type (NOD.WT) mouse model of T1DM. Insulitis (i.e., inflammation of pancreatic islets) precedes T1DM in both humans and the NOD.WT mouse. The NOD.WT mouse has a genetic predisposition to spontaneously develop T1DM and is the most commonly used model to study T1DM. The timing of insulitis and subsequent diabetes in the NOD.WT mouse is well characterized. In this research thesis we detected the presence of dead or dying pancreatic beta cells that are shed into the circulation prior to and during the active insulitis phase in these mice using an improved PCR-based assay that is theoretically capable of identifying their presence in blood samples at very early stages of insulitis that precede the onset of T1DM (earlier than the best current assays).

Impact statement: This assay could allow the detection of early pancreatic beta cell death in the initial phases of the insulitis that precedes both forms of T1DM. Coupled with islet autoantibody testing it could add specificity for prediction of actual development of T1DM-1A and enable earlier intervention with disease modifying treatments to prevent total pancreatic beta cell loss and development of T1DM. For pattern T1DM-1B, this assay, coupled with acute phase viral diagnostic testing during enteroviral infections, could potentially determine patients at risk for developing diabetes,
which would also allow intervention with an antiviral or anti-inflammatory medication and possibly prevent acute beta cell destruction and the onset of T1DM-1B.

Objective: The objective of this research was to develop a Polymerase Chain Reaction (PCR)-based assay that is more sensitive than current assays at detecting circulating, shed pancreatic beta cells in the blood and to validate if this assay can be used to predict the very early onset of T1DM in NOD.WT mice. Thus, this work addressed the following 3 main aims:

1. **Establish a PCR-based assay that differentiates between pancreatic beta cell DNA and non-pancreatic beta cell DNA**

2. **Detect the pancreatic beta cell DNA (shed beta cells) in the whole blood during the course of the natural history of insulitis and T1DM onset in the NOD.WT mouse model (21 week experiment).**

3. **Use mathematical modeling to validate the PCR-based assay as a diagnostic assay to predict which individuals with a genetic risk will go on to develop T1DM.**
CHAPTER EIGHT: RESULTS

8.1 In vitro

The 3T3L1 cell line is a murine fibroblast cell line that served as the methylated DNA control (negative control) since they do not express insulin. As such, the -182 CpG site within the INS2 promoter region should be mostly methylated. The βTC6 cell line is a murine pancreatic beta cell line that served as the demethylated DNA control (positive control) since they actively express insulin. As such, the -182 CpG site within the INS2 promoter region should be mostly demethylated. Positive and negative controls are necessary since all DNA, no matter the source, will contain a mixture of both methylated and demethylated forms of the target sequence. The abundance of demethylated DNA was quantified using the DI. An increase in the amount of demethylated DNA coincides with an increased DI \( \frac{2^{\text{methylated CT}} - \text{demethylated CT}}{\text{methylated CT}} \) and is indicative of an increased amount of pancreatic beta cells in the sample. Utilizing these cell lines, I established that (i) the target sequences were detected (Figure 5), (ii) it is necessary to include an initial sqPCR step in the assay (Figure 6A), (iii) the lower limit of sensitivity was 1 ng (1 pg) (i.e., it can detect as little as 1 pg of target DNA within a sample) (Figure 6A), and (iv) the qPCR probes differentiated between βTC6 and 3T3L1 DNA, indicating that they differentiated between demethylated and methylated DNA and thus allowed for relative quantification of beta cell DNA (Figure 6B). Each concept is expanded on below.

The data confirmed that the targeted sequences of DNA (Figure 5). DNA was extracted from βTC6 cells, subjected to bisulfite treatment, sqPCR, then qPCR. The CT values from the qPCR were in the 5-13 range for demethylated and methylated DNA with
starting template amounts ranging from 1 to 100 ng of DNA. CT values below 40 are considered reliable. The fact that the CT values ranged from 5-13 indicate that the assay detected both methylated and demethylated DNA within the sample with high sensitivity. The CT values also decreased as the amount of starting template DNA increased, indicting that changes in the amount of template in the samples were detected.

Figure 5: qPCR Results of βTC6 Cells. DNA was extracted from βTC6 cells, purified, then subjected to bisulfite treatment. After the bisulfite treatment, sqPCR was performed to amplify the amount of target DNA in the sample. qPCR was then performed on 1 ng, 5 ng, 10 ng, 15 ng, 20 ng and 100 ng of the sqPCR product. CT values in the 5-13 range indicate that the assay can detect both target forms of the sequence with high sensitivity.

Next, I questioned whether the sqPCR step was necessary for detection. DNA was extracted from each cell type and subjected to bisulfite treatment. One sample of each bisulfite treated βTC6 and 3T3L1 DNA sample was then subjected to sqPCR followed by
qPCR. As can be seen in Figure 6A indicates that sqPCR prior to qPCR is a necessary step. The target INS2 -182 bp CpG site was amplified by sqPCR primers designed to amplify this region of interest. When 100 ng of DNA that had not been previously subjected to sqPCR was used for qPCR, the results resembled that of the negative control [i.e., have similar and very high (~30) CT values, indicative of very little target DNA being present in the sample] (Figure 6A). The 1 sample that was subjected to sqPCR prior to qPCR resulted in CT values below 15 for 0.001-1 ng of starting sqPCR-treated template (Figure 6A). Samples of 100 ng of sqPCR-treated template resulted in CT values less than 6 (Figure 5). Taken collectively, this validated the need for the sqPCR step prior to qPCR.

After establishing the assay methodology, all subsequent samples were subjected to sqPCR treatment prior to qPCR.

Next, the lower limit of sensitivity was established for the assay (Figure 6A). To accomplish this, qPCR was performed on 0.001 ng, 0.01 ng, 0.1 ng, 1 ng and 5 ng of TC6 and 3T3L1 DNA and the resulting CT values were re-analyzed. For 0.001 ng of DNA the CT values of both the demethylated and methylated form of the target DNA sequence from TC6 and 3T3L1 cells were below 15. This indicated that the assay was able to detect the target DNA sequences in both cell lines with high sensitivity (1 pg of DNA) (Figure 6A). This is significant because it demonstrated that the assay could detect very small quantities of target reliably. It was critical to establish the lower limit of detection since the target sequence in the blood samples may be minute in the very early stages of T1DM (i.e., the early insulitis phase).
After determining the lower limit of sensitivity the DI or DI (2\textsuperscript{methylated CT-demethylated CT}) was employed to compute the relative abundance of the demethylated DNA in the samples. A DI higher than 1 indicated the presence of more demethylated than methylated DNA in a sample. Since βTC6 cells are pancreatic beta cells and 3T3L1 cells are fibroblasts it was expected that the βTC6 cells would have a higher DI than the 3T3L1 cells if the assay and DI were able to relatively quantify the amount of demethylated target DNA and correctly correlate that to the amount of beta cell DNA in the sample. The DI was greater than 1 for all of the βTC6 cell samples and lower than 1 for all of the 3T3L1 cell samples (0.001 ng to 5 ng) (Figure 6B). This confirms that the assay could detect DNA that is demethylated or methylated at the -182 bp CpG site of the INS2 gene (indicated by the CT values) and allow for relative quantification of beta cell DNA.
DNA was extracted from the cells, purified and then subjected to bisulfite treatment. After the bisulfite treatment sqPCR was performed to amplify the amount of target DNA in the sample to allow for detection. The 100 ng sample did not receive sqPCR amplification prior to qPCR. Figure 6A illustrates the CT values obtained. Samples 0.001ng-5ng were first subjected to sqPCR then to qPCR. The CT values indicate that the assay is able to detect the target sequence in the DNA. The CT values also indicated a concentration-dependent result for both the negative and positive control, i.e., the CT values decreased as the amount of DNA placed in the qPCR system increased. The CT values of the 100 ng DNA sample that was not subjected to sqPCR prior to qPCR resembled that of the control. This indicated that the assay required the sqPCR amplification step prior to qPCR quantification in order to detect the low levels or target DNA in the samples. Figure 6B represents the DI $2^{(methylated\ CT-demethylated\ CT)}$ calculated from the experiment. The DI is an indicator of the relative amount of beta cell (demethylated) DNA in the sample compared to non-beta cell DNA (methylated DNA). The graph illustrates a DI greater than 1 for all of the sqPCR-treated βTC6 cell samples (0.001ng-5 ng) and less than one for all of the sqPCR-treated 3T3L1 cells (0.001 ng- 5 ng). This indicates that the
assay was able to effectively differentiate between the two forms of DNA present (methylated and demethylated) when the target regions are first amplified using sqPCR. Moreover, these results confirm that the assay requires the sqPCR amplification step prior to qPCR quantification in order to effectively differentiate between the two forms of differentially methylated DNA present in a sample. This is evidenced by the 100 ng unamplified sample displaying similar results to that of the negative control.

8.2 Preliminary In Vivo

Before moving into the NOD mouse model, additional in vitro experiments were conducted to determine whether the assay retained the same or similar ability to amplify the INS2 -182 bp demethylated and methylated INS2 CpG site and to allow for relative quantification of beta cell DNA when isolated from whole blood. To accomplish this 3 week old NOD mouse blood was spiked with βTC6 cells (0, 100, and 1000 cells) and then subjected to bisulfite treatment, sqPCR and qPCR. The assay was able to detect the demethylated and methylated -182 INS2 CpG site in all of the blood samples, indicated by the CT values below 16 (Figure 7A). Whole blood spiked with 100 βTC6 cells had a significantly higher DI than the blood not spiked with cells (P <0.01). In addition, the DI increased from 0.5 to 1 when the blood was spiked with 100 BTC6 cells. The whole blood spiked with 1000 cells had a significantly higher DI than samples spiked with 100 cells (P<0.05) and resulted in a DI that increased from 1 to 2. This demonstrated that the DI of blood samples spiked with βTC6 cells increased as the amount of cells increased. As the amount of beta cells (beta cell DNA) in the sample increased, the DI increased, allowing for relative quantification of beta cell DNA. As the quantity of beta cell DNA increased the DI approached that of the βTC6 cell culture control samples (Figure7B). It should also be noted that the DI of 3 week old NOD mouse blood that was
not spiked with cells was already higher than that of the 3T3L1 cells, indicating that even at 3 weeks some degree of beta cell shedding occurred in the NOD mouse and was detectable. Taken all together this indicated that the assay could detect the target demethylated and methylated DNA sequence when the DNA was extracted from whole blood. By applying the DI to the CT values I was again able to show that the assay allowed for the relative quantification of beta cell DNA (Figure 7B).
Figure 7A and B: qPCR Results of NOD Mice Blood Samples Spiked with βTC6 Cells. ‘0’, ‘100’ and ‘1000’ labels correspond to blood samples from NOD mice spiked with βTC6 cells. O was used as the no spike control. 100 was spiked with 100 βTC6 cells and 1000 was spiked with 1000 βTC6 cells.

Figure 7A illustrates the CT values of the blood samples. The CT values indicate that the assay is able to detect the methylated and demethylated target sequence in the DNA extracted from blood samples.

Figure 7B represents the DI calculated from the experiment. The graph illustrates a concentration-dependent increase in the DI after the blood was spiked with βTC6 cells at a significance level of 0.01 and 0.05 (one-way anova). This indicates that the assay can quantify the amount of beta cells in blood using the demethylation index.
To evaluate the use of whole blood in preference to serum whole blood was separated into two portions; serum and cell pack portions. The whole blood was centrifuged and the serum was collected. The remainder of the sample is referred to here as the “cell pack”. Both portions were then separately subjected to bisulfite treatment, sqPCR and qPCR and the demethylation indices were calculated from the resultant CT values. Figure 8 represents the demethylation indices of the serum and cell pack portions of the whole blood samples. The majority of the beta cell DNA in the samples was detected in the cell pack portion indicating that the use of serum only does not allow the complete capture of beta cell destruction, greatly decreasing the analytical sensitivity of the assay.

![Relative Presence of Beta Cell DNA (demethylated CpG site) in Serum and Cell Pack Portions of Whole Blood](image)

**Figure 8**: Demethylation Index of Serum and Cell Pack Portions Of Whole Blood. *Figure 8* represents the DI calculated from the CT values (15-20) for the serum and cell pack portion of whole blood samples. Samples of whole blood were centrifuged and the serum was separated from the remaining sample, which is referred to here as the “cell pack”. This indicates that the majority of the detectable beta cell DNA is within the cell pack portion of blood.
8.3 In Vivo

Next, the natural history of beta cell destruction was investigated in NOD mice using the validated assay. A percentage of the NOD.WT mice spontaneously develop T1DM throughout their lifetime, while NOD.SCID mice do not develop T1DM due to their lack of T and B cells. As described above, my previous studies established that the assay was able to detect the presence of the demethylated and methylated -182 bp INS2 CpG site allowing for the relative quantification of the amount of beta cell DNA in whole blood (Figure 7B). This enabled an analysis of beta cell destruction in whole blood in NOD.WT and NOD.SCID mice from 4- to 21 weeks of age, something which has not yet been done. To this end, the assay described herein was used to examine beta cell destruction in these mice from 4 to 21 weeks of age. The results of this analysis indicated that: (i) NOD.WT beta cell shedding peaked at 8 weeks (Figure 9A), (ii) all NOD.WT mice experienced beta cell shedding throughout their lifespan (Figure 9B), (iii) NOD.SCID mice experienced beta cell shedding throughout their lifespan (Figure 10), (iv) the NOD.SCID mice experienced a minor 8-week peak in the DI, but it was significantly lower in magnitude than the NOD.WT mice (P<0.05) (Figure 10, 11A and Table 1) and the NOD.SCID mice also experienced significantly less cumulative demethylation (beta cell shedding) than the NOD.WT mice (P<0.05) (Figure 11B), (v) the change in beta cell shedding patterns is higher in NOD.WT pre-diabetic animals than in non-diabetic NOD.WT and NOD.SCID mice (Figures 12, 13,14). These findings are expanded upon below.
The DI was calculated from the CT values of whole blood that was subjected to bisulfite treatment, sqPCR then qPCR. A boxplot representation of the even-week DI results are illustrated below for all NOD.WT mice (Figure 9A). Fifteen of the NOD.WT mice became diabetic (hyperglycemic) by the end of the study and are referred to as “pre-diabetics” and 23 did not and are referred to as “non-diabetics”. The data indicated that the NOD.WT mice constantly shed beta cells throughout their lifespan with a peak in shedding of beta cells at 8 weeks (P<0.01) (Figure 9A), The 8 week spike was detected up to 6 weeks earlier than previously reported\(^1\). This indicated that my assay had higher analytical sensitivity than previously described assays.

By breaking the data into categories by diabetic status it was found that all NOD.WT mice experienced beta cell shedding throughout their lifespan regardless of whether they were pre-diabetic or non-diabetic (Figure 9B). This suggests that NOD mice may have a predisposition to shed an increased amount of beta cells (increased beta cell turnover) regardless of whether or not they develop diabetes.
Figure 9: The Natural History of NOD.WT Beta Cell Shedding. DNA was extracted from whole blood, purified, and then subjected to bisulfite treatment, sqPCR and then qPCR. The graph illustrates the DI that was calculated from the qPCR CT values.

**Figure 9A** illustrates the even-week DI of NOD.WT mice (4 weeks to 20 weeks of age). The trend of DI is indicative of the trend of beta cell shedding. This trend indicates the natural history of beta cell shedding. The DI indicates that the beta cell shedding peaks at 8 weeks of age with a significance level of P < 0.01 (one-way ANOVA).

**Figure 9B** illustrates the even week DI of NOD.WT mice (age 4 weeks to 20 weeks). The green represents the NOD.WT non-diabetics N= 23. The blue represents the NOD.WT pre-diabetics, N = 15. This indicates that the beta cell shedding peaks at 8 weeks of age. After 6 weeks of age, the NOD.WT shedding of the pre-diabetics was higher on average than the non-diabetics.
It was also shown that all NOD.SCID mice also experienced beta cell shedding throughout their lifespan (Figure 10). This suggested that mice of the NOD background, and not just the NOD.WT mice, may have a genetic predisposition to shed beta cells regardless of whether or not they develop diabetes or have T or B cells present. Even though the NOD.SCID mice also experienced beta cell shedding they experienced a significantly (P<0.05) smaller peak at 8 weeks than the NOD.WT mice (Figure 10 and Table 1).

Figure 10: Natural History of NOD.SCID Beta Cell Shedding.
Figure 10 illustrates the DI of NOD.SCID mice from age 4 weeks to 20 weeks, N=10. The trend of DI is indicative of the trend of beta cell shedding. This trend indicates the natural history of beta cell shedding in the NOD.SCID mouse model. The DI indicates that the beta cell shedding peaked at 8 and 10 weeks of age, indicating that an elevated basal amount of beta cell shedding occurs without involvement of the adaptive immune system.

Figure 11A and Table 1 depict a side-by-side comparison of beta cell shedding in NOD.SCID, pre-diabetic NOD.WT and non-diabetic NOD.WT mice. This illustrates that the peak at 8 weeks that is largest in the NOD.WT pre-diabetic mice. After 8 weeks, the NOD.SCID beta cell shedding was comparable to that of the NOD.WT beta cell.
shedding. Next, the cumulative shedding over the lifespan of each NOD.SCID and NOD.WT mouse was evaluated. Overall, the cumulative amount of shedding was significantly greater in NOD.WT pre-diabetic mice than it was in NOD.SCID mice (Figure 10B) (P < 0.05) and in NOD.WT non-diabetics. This illustrated the increased beta cell death in the NOD.WT pre-diabetics that accompanied their development of T1DM.
**Figure 11:** Natural History of NOD. SCID, Non-Diabetic NOD.WT and Pre-Diabetic Beta Cell Shedding.

*Figure 11A* illustrates the DI \(^{(\text{methylated CT-demethylated CT})}\) of NOD.SCID and NOD.WT mice from age 4 weeks to 20 weeks. The DI is indicative of beta cell shedding. The trend indicates the natural history of beta cell shedding. The red represents NOD.SCID mice, N= 10. The green represents NOD.WT mice that did not develop diabetes by 22 weeks of age, N= 23. The blue represents the NOD. WT mice that developed diabetes ‘pre-diabetic’ N = 15. At 8 weeks of age, the degree of beta cell shedding in NOD.WT mice was significantly greater than the NOD.SCID mice with a significance level of P < 0.05 (one-way ANOVA).

*Figure 11B* illustrates the average cumulative DI of mice through 21 weeks of age. This tells us that on average a pre-diabetic NOD.WT mouse shed more beta cells than a non-diabetic NOD.WT and NOD.SCID mouse. Overall, the cumulative amount of shedding was
significantly greater in NOD.WT pre-diabetic mice than it was in NOD.SCID mice with a significance level of P < 0.05 (one-way ANOVA)

Table 1

Average DI at 6, 8, 10 and 12 Weeks of Age

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Average NOD.SCID</th>
<th>Average non-diabetic NOD.WT</th>
<th>Average pre-diabetic NOD.WT</th>
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<tr>
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<td>0.32</td>
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In an effort to analyze the changes within the magnitude of beta cell destruction that occurred for each mouse throughout their lifespan the variation, slope and change in slope was retrospectively analyzed. The variation in the beta cell shedding was variation from week-to-week (i.e., week 4 and week 5). The variation was higher in NOD.WT pre-diabetic animals than in non-diabetic NOD.WT and NOD.SCID mice (Figure 12). The DI slope was the absolute change in the DI from week-to-week. There was a larger number of NOD.WT pre-diabetic mice that had high outlier DI slopes than the non-diabetics and the NOD.SCID mice (Figure 13). The change in DI slope was the difference between maximum slopes evaluated. The DI slope was also higher in NOD.WT pre-diabetic animals than non-diabetic NOD.WT and NOD.SCID mice (Figure 14). Parameters to evaluate change depicted larger magnitudes of difference between that of the NOD.WT pre-diabetic and non-diabetics than DI alone. This illustrated how parameters to evaluate change in the DI could be applied to the DI to increase the sensitivity and specificity of detection so that this method could be used to predict which animals would develop T1DM.
**Figure 12**: DI Variation

Figure 12 illustrates the week-to-week variation of demethylation indices for NOD.SCID and NOD.WT mice from 4-20 weeks. The variation is highest in the NOD.WT pre-diabetics.

**Figure 13**: DI Slope.

Figure 13 illustrates the week-to-week DI slope of weekly demethylation indices for NOD.SCID and NOD.WT mice from 4-20 weeks. The DI slope is the change in week to week amounts of beta cell shedding and is highest in NOD.WT pre-diabetics.

**Figure 14**: Change in DI Slope.

Figure 14 illustrates the week-to-week change in DI slope of weekly demethylation indices for NOD.SCID and NOD.WT mice from 4-20 weeks. The change in DI slope is the max difference in DI slopes and is highest in the NOD.WT pre-diabetics.
CHAPTER NINE: MATHEMATICAL MODELS

The DI was calculated from the CT values using $2^{\text{methylated CT-demethylated CT}}$. The DI along with additional parameters were retrospectively applied to the data in efforts to formulate predictors that could identify those that would develop T1DM. The DI was also the basis of all other mathematical models applied to the data. The DI slope is the absolute change from one week to the next (i.e., week 4 to week 5) in the calculated DI values. It was determined by the absolute value DI$_{5}$ - DI$_{4}$ from consecutive weeks (DI of week 4 – DI of week 5). The change in DI slope was determined by looking at the maximum differences among DI slopes, so subtracting one maximum slope from another. It was measured as max$_{1}$ (DI slope) – max$_{2}$ (DI slope and max$_{2}$ (DI slope) – max$_{3}$ (Slope).

In order to use DI, DI slope and/ or change in DI slope as a predictive model, it required critical numbers to be met by the data in order for a parameter to predict T1DM development. A critical number is the number that the DI, DI slope, or change in DI slope must be the same or greater than for the assay to predict an animal to be at risk for developing T1DM during his lifetime. This predictive model required a critical number of 2 for the DI, signifying a 2:1 ratio of beta cell to non-beta cell DNA. If the DI was 2 or mo, then the beta cell DNA detected was 2 times the amount of non-beta cell DNA detected and the animal was predicted to develop T1DM at some point in their lifetime. The critical number for the DI slope was also 2, signifying a doubling of the DI value. If the DI slope was 2 or more, then the DI either experienced a 2-fold increase or decrease and the animal was predicted to develop T1DM at some point in their lifetime. The critical number for the change in slope was 0.5, signifying a half magnitude decrease
between maximum slope values. If the change in slope was 0.5 or more then the animal was predicted to develop T1DM at some point in their lifetime.

The DI, DI slope and change in slope represent one predictive model. In the predictive model at least one of the parameters have to have a critical number met in order to predict that an animal would develop T1DM. Each predictive parameter (DI, DI slope and change in DI slope) has a specific sensitivity and specificity. The more parameters that reach the critical number the higher the sensitivity and specificity. The DI critical number alone can predict with 53% sensitivity, 94% specificity and an average of 8 weeks and 4 days prior to the onset of hyperglycemia (Table 2). The DI and DI slope critical numbers together have a sensitivity that remains at 53% but the specificity is increased to 97%. Using these 2 parameters T1DM is predicted an average of 7 weeks and 5 days prior to hyperglycemia (table 2). The DI, DI slope and change in DI slope critical numbers collectively have a sensitivity of 47% but the specificity increases to 100%. Using all 3 parameters T1DM is predicted an average of 7 weeks and 4 days prior to the onset of hyperglycemia (table 2). If the change in DI slope critical number is the only parameter met it has a 60% sensitivity, 91% specificity, and the ability to predict T1DM 7 weeks and 6 days prior to the onset of hyperglycemia.

The use of a predictive model that incorporates one or more parameters demonstrates the versatility that can be modulated on an individual basis, without running additional tests on a patient, yet still allowing for personalized approached. Personalized approaches can adjust the sensitivity and specificity of an assay depending on the patient’s genetic profile, family history and autoantibody assays. The use of different
parameters could also be included or excluded based on autoantibody positivity, HLA 3/4 presence and family history

Table 2

*Prediction Capacity*

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<th>Weeks prior to hyperglycemia (DI)</th>
<th>Weeks prior to hyperglycemia (slope)</th>
<th>Weeks prior to hyperglycemia (change in slope)</th>
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Presented below are 10 case studies of NOD.WT mice that became diabetic. The graphs track the DI of each mouse until the time that they became diabetic (hyperglycemic). When a measure for the DI, DI slope or change in DI slope was at or above the critical number the assay predicted that the animal should have developed diabetes. The graphs that follow (figure 15 A-J) are in order of when the mouse developed T1DM and illustrate (with colored arrows) when critical numbers were reached for the DI, DI slope and/or the change in DI slope, thus allowing the predictive model to predict diabetes with respect to an individual mouse with certain levels of sensitivity and specificity. Each predictive parameter (DI, DI slope and change in DI slope) has a specific diagnostic sensitivity and specificity, the more parameters that reach the critical number the higher the specificity becomes. The specificity will be further discussed.

The blue arrows on the graphs indicate the time at which the animal became diabetic, the red arrows indicate the time at which the DI critical number predicted the animal to develop T1DM, the black arrows indicate the time at which the DI slope critical number predicted the animal to develop T1DM and the purple arrows indicated the time at which the change in DI slope critical number predicted the animal to develop T1DM.

NOD.WT #14 became diabetic at age 12 weeks. The blood glucose levels ranged from 81-94 mg/dL from 4 weeks of age to 11 weeks of age and spiked to 292 mg/dL on week 12. The DI began at 0.3 at 4 weeks of age, spiked to 4.3 at 5 weeks of age, decreased to 0.6 and 0.1 in the following weeks, and then spiked to 1.6 at 8 weeks of age
The DI decreased to 0.4 by 10 weeks of age, increased to 0.7 at 11 weeks and decreased back to 0.4 at 12 weeks of age as the animal became hyperglycemic. NOD #14 had a major spike in the DI at 5 weeks of age followed by 2 minor increases in DI, one being an increase at 8 weeks. The DI critical number (red arrow) and DI slope critical number (black arrow) predicted NOD.WT#14 to be at risk for developing T1DM during its lifetime at the 5 week spike, with 97% specificity and 89% precision. By 9 weeks of age the change in DI slope critical number (purple slope) predicted NOD#14 to be at risk for developing T1DM during its lifetime and increased the specificity to 100%.

**Figure 15: Diabetes Detection in NOD.WT Mouse #14**

NOD.WT mouse number 14 developed T1DM at 12 weeks of age. By age 5 weeks the assay predicted the development of T1DM with 97% specificity. By 9 weeks of age the specificity increased to 100%.

Figures 15-25 illustrate the DI of individual animals throughout their lifespan. The onset of diabetes (hyperglycemia) is indicated by a blue arrow. Mathematical models were retrospectively applied to the data. There are 3 mathematical parameters in this model; DI, DI slope and change in slope. Each parameter has a critical number that must be met or exceeded for the parameter to predict the animal to develop T1DM. The red arrow represents when the DI reached the critical number and was at or above 2, the black arrow represents when the DI slope reached the critical number and was at or above 2. The purple arrow indicates when the change in DI slope reached the critical number and was at or above 0.5.

NOD.WT #25 became diabetic at age 14 weeks. The blood glucose levels ranged from 78-155 mg/dL from 4 weeks of age to 13 and spiked to 252 mg/dL at 14 weeks of
age. The DI began at 2 at 4 weeks of age and dropped to 0.6 at 5 weeks (Figure 15B). The DI then increased to 1 at week 6 and decreased again to 0.4 at week 7. At 8 weeks the DI spiked to 2.8 and then decreased to 0.2 at 9 weeks. At 13 weeks the DI was at 0.5 and spiked to 3.9 at the 14 weeks time point, which coincided with the onset of hyperglycemia. NOD #25 produced 3 spikes in DI, at 4, 8 and 14 weeks and was one of only two animals to have a high DI at onset of hyperglycemia, and was also only 1 of 2 to have a DI spike at 4 weeks. The DI critical number (red arrow) predicted at 4 weeks of age with 94% specificity that NOD.WT #25 was at risk for developing diabetes during its lifetime. The major 8-week spike in the DI slope critical number (black arrow) predicted with 97% specificity that the mouse was at risk for developing T1DM during its lifetime. The change in slope DI did not exceed 0.5 so the critical number for this parameter was not met. Since it was not met this parameter did not predict the animal to develop T1DM, even though the other 2 parameters (DI and DI slope) did.

Figure 16: Diabetes Detection in NOD.WT Mouse #25
NOD.WT mouse number 25 developed T1DM at 14 weeks of age. By age 4 weeks the assay predicted the development of T1DM with 94% specificity. By 8 weeks of age the specificity increased to 97%.

NOD.WT #10 became diabetic at age 14 weeks of age. The blood glucose levels ranged from 70-112 mg/dL from 4 weeks of age to 13 and spiked to 356 mg/dL at 14
weeks. At 4 weeks the DI began at 0.6 then dropped to 0.2 at 6 weeks of age, declined to 0.1 at 7 weeks of age, then increased to 1.4 at 8 week old, followed by a drop again to 0.2 at 9 weeks old (Figure 15C). The DI increased to 1 by 11 weeks of age then back to 0.5 when the animal was 12 weeks, with a measure of 0.7 at the onset of hyperglycemia at 14 weeks of age. NOD.WT #10 produced 2 small increases in DI at 8 and 11 weeks of age. At 9 weeks of age, the change in DI slope critical number (purple arrow) predicted with 91% specificity that NOD.WT #10 was at risk for developing T1DM during his lifetime. The DI nor the DI slope exceeded 2 so the critical number for those parameter were not met. Since they were not met those parameter did not predict the animal to develop T1DM, even though the other 1 parameters (change in DI slope) did.

Figure 17: Diabetes Detection in NOD.WT Mouse #10
NOD.WT mouse number 10 developed T1DM at 14 weeks of age. By age 9 weeks the assay predicted the development of T1DM with 91% specificity.

NOD.WT #19 became diabetic at age 19 weeks of age. The blood glucose levels ranged from 66-137 mg/dL from 4 weeks of age to 18 and spiked to 299 mg/dL at 14 weeks of age. The DI began at 0.7 and decreased to 0.2 by 6 weeks of age. The DI increased to 1.6 at 8 weeks of age then decreased to 0.5 (Figure 15D). The DI remained in the 0.2-0.5 range until 18 weeks of age when it dropped to 0.08 as the onset of
hyperglycemia occurred. NOD.WT #19 produced 1 increase in DI, at 8 weeks of age. The change in DI slope critical number (purple arrow) predicted NOD.WT #19 to be at risk for developing T1DM during its lifetime at 7 weeks, with 91% specificity. The DI nor the DI slope exceeded 2 so the critical number for those parameter were not met. Since they were not met those parameter did not predict the animal to develop T1DM, even though the other 1 parameters (change in DI slope) did.

Figure 18: Diabetes Detection in NOD.WT Mouse #19
NOD.WT mouse number 19 developed T1DM at 19 weeks of age. By age 5 weeks the assay predicted the development of T1DM with 91% specificity and 77% precision.

NOD.WT #32 became diabetic at age 18 weeks of age. Its blood glucose levels ranged from 69-158 mg/dL from 4 weeks of age to 17 and spiked to 237 mg/dL at 18 weeks of age. The DI began at 2.5 at week 4 and remained high at 1.8 and 2.3 for weeks 5 and 6 (Figure 15E). The DI dropped to 0.1 at 7 weeks, then spiked to 4.1 at week 8 and remained high at 3.5. From week 11 to week 15 the DI ranged from 0.2 to 0.3, decreased to 0.06 at 16 weeks, increased to 0.15 at 17 weeks, and decreased again to 0.15 as the onset of hyperglycemia occurred at 18 weeks. NOD.WT#32 produced 4 spikes in DI at weeks 4, 5, 6, 8, 9. The DI critical number (red arrow) predicted NOD.WT #32 to be at risk for developing T1DM during its lifetime at the initial 4 weeks with 94%
specificity. The DI slope critical number (black arrow) predicted NOD.WT #32 to be at risk for developing T1DM during its lifetime at 7 weeks, with 97% specificity. The change in DI slope critical number (purple arrow) predicted the animal to be at risk for developing T1DM during its lifetime at 9 weeks and increased the specificity to 100%.

Figure 19: Diabetes Detection in NOD.WT Mouse #32
NOD.WT mouse number 32 developed T1DM at 18 weeks of age. By age 4 weeks the assay predicted the development of T1DM with 94% specificity. By 7 weeks of age the specificity increased to 97%. By week 9 the specificity increased to 100%.

NOD.WT #21 became diabetic at age 19 weeks of age. The blood glucose levels ranged from 65-137 from 4 weeks to 18 and spiked to 299 at 19 weeks. The DI began at 0.7 at 4 weeks of age then increased to 1.5 at week 5 and spiked to 2.3 at week 6. It then decreased to 0.3 at week 7 and increased to 1.3 at week 8. The DI then decreased to 0.2 at week 9 and stayed in the 0.6-1.3 range until 13 weeks when it dropped to 0.4 and then lower to 0.2 at week 14 and 0.1 at 15 weeks. The DI increased to 0.7 then dropped to 0.3 and 0.1 at weeks 17 and 18 and further down to 0.09 as the onset of hyperglycemia occurred at 18 weeks. NOD.WT#21 produced 1 spike at week 6 and a minor increase at week 8. The DI critical number (red arrow) predicted NOD#21 to be at risk for developing T1DM during its lifetime at 6 weeks with 94% specificity. The DI slope (black arrow) and change in DI slope (purple arrow) critical numbers predicted the
animal to be at risk for developing T1DM during its lifetime at 7 weeks, with 100% specificity.

Figure 20: Diabetes Detection in NOD.WT Mouse #21
NOD mouse number 21 developed T1DM at 19 weeks of age. By age 6 weeks the assay predicted the development of T1DM with 94% specificity. By 7 weeks of age the specificity increased to 100%.

NOD.WT#9 became diabetic at 21 weeks of age. The blood glucose levels ranged from 76-153 from 4 weeks of age to 20 and spiked to 363 at 21 weeks of age. The DI began at 0.7 at 4 weeks of age then increased to 0.05 and stayed in the 0.1-0.5 range until 13 weeks. The DI was 0.2 at week 14 and increased to 1 at 15 weeks then back down to 0.2. It then spiked 3.2 at week 17 then back down to 0.1. The DI decreased to 0.05 at 18 weeks then decreased further to 0.006 at week 20 and then increased to 0.19 at 21 weeks as the onset of hyperglycemia occurred. NOD.WT#9 produced 1 minor increase at week 15 and a major spike at week 17. The DI critical number (red arrow), DI slope critical number (black arrow) and change in DI slope critical number (purple arrow) all predicted the animal to be at risk for developing T1DM during its lifetime at the major 17 week spike, with 100% specificity.
Figure 21: Diabetes Detection in NOD.WT Mouse #9
NOD mouse number 9 developed T1DM at 21 weeks of age. By age 17 weeks the assay predicted the development of T1DM with 100% specificity.

NOD#22 became diabetic at 21 weeks of age. The blood glucose levels ranged from 79-219 from 4 weeks to 19 and spiked to 253 at 20 weeks, but was below 200 for the second check to confirm after 24 hours. The blood glucose then spiked again to 363 on week 21. The DI ranged from 0.5-0.9 for 4-6 weeks of age. The DI spiked to 3.7 at 7 weeks of age, then decreasing to 0.6 8 weeks of age and further to 0.08 at 9 weeks. The DI stayed in the 0.4-0.08 range until 13 weeks, then dropped to 0.2 and raised to 1.4. The DI then stayed in the 0.1-0.2 range 16-18 weeks and then remained in the 0.05-0.06 range until week 21 as the onset of hyperglycemia occurred at 21 weeks. The animal experienced 1 spike at 7 weeks. The DI critical number (red arrow) and DI slope critical number (black arrow) predicted the animal to be at risk for developing T1DM during its lifetime with 97% specificity at week 7. The change in DI slope (purple arrow) predicted NOD#22 to be at risk for developing T1DM during its lifetime at 8 weeks with 100% specificity.
Figure 22: Diabetes Detection in NOD.WT Mouse #22
NOD mouse number 22 developed T1DM at 21 weeks of age. By age 7 weeks the assay predicted the development of T1DM with 97% specificity. By 8 weeks of age the assay predicted the development of T1DM with 100% specificity.

NOD #36 became diabetic at 21 weeks of age. The blood glucose levels ranged from 69-163 from 4 weeks to 19 and spiked to 237 at 21 weeks. The DI began at 1.2 at age week 4 and 1.5 at week 5, it decreased to 0.8 at week 6 and then 0.2 at week 7. It increased to 1.6 at week 8. The DI then ranged from 0.3 to 0.7 until week 15 when it increased to 1.4, decreased to 0.09 and then increased again to 1.3 at week 17. The DI then decreased to 0.2 and increased to 2.1 at week 19 and remained in the 0.2 range as the onset of hyperglycemia occurred at 21 weeks. The animal produced minor increases at 4, 5, 15, 17, 19 but only 1 was a spike above 2, week 19. The DI critical number (red arrow) predicted NOD #36 to be at risk for developing T1DM during its lifetime at the week 17 spike with 94% specificity. The DI slope critical number (black arrow) predicted NOD #36 at 18 weeks, with 97% specificity. The change in DI slope predicted the animal to be at risk for developing T1DM during its lifetime at 19 weeks and increased the specificity to 100%.
Figure 23: Diabetes Detection in NOD.WT Mouse #36
NOD mouse number 36 developed T1DM at 21 weeks of age. By age 17 weeks the assay predicted the development of T1DM with 91% specificity. By 18 weeks of age the specificity increased to 97%. By week 19 the specificity increased to 100%.

NOD#2 became diabetic at 21 weeks of age. The blood glucose levels ranged from 77-188 from 4 weeks to 21 weeks and spiked to 324 at 22 weeks of age. The DI began at 0.5 at age 4 weeks, 1.1 at 5 weeks and 0.7 at 6 weeks. The DI lowered to 0.1 at week 7 and then increased to 0.4 at week 8. The DI then decreased to 0.2 at week 9 and increased to 0.6 at week 10. The DI remained in the 0.1-0.4 range until it spiked to 5.4 at week 18 and then lowered to 0.04 at week 19 and remained the 0.2-0.3 range until as the onset of hyperglycemia occurred at 22 weeks. The animal produced a major spike at week 17. The DI critical number (black arrow), DI slope critical number (black arrow) and change in DI slope critical number (purple arrow) predicted NOD#2 to be at risk for developing T1DM during its lifetime at 18 weeks, with 100% specificity.
Figure 24: Diabetes Detection in NOD.WT Mouse #2
NOD mouse number 2 developed T1DM at 21 weeks of age. By age 18 weeks the assay predicted the development of T1DM with 100% specificity.
CHAPTER ELEVEN: DISCUSSION

The preliminary *in vitro* studies indicated that the diagnostic assay could accurately and with high sensitivity detect the demethylated and methylated forms of the -182 bp CpG site within the INS2 promoter region of interest (Figure 5). The lower limit of sensitivity was 1 pg of DNA. A single cell contains 3.96 pg of DNA, which correlates to being able to detect as low as one/ fourth of the DNA content from a single beta cell shed into the bloodstream (Figure 6A). By applying the DI to the CT values from qPCR the assay was able to compute the relative abundance of insulin (beta cell) DNA in a sample which was demonstrated by the differences in demethylation indices for βTC6 (above 1) and 3T3L1 cells (below 1) (Figure 6B).

The preliminary *in vitro* studies using blood samples spiked with βTC6 cells indicated that the assay could identify beta cell-derived DNA (demethylated DNA) in whole blood by demonstrating the significant (P<0.01 and P<0.05) doubling of the DI as 100 and 1000 βTC6 cells were added to blood samples (Figure 7B). It should also be noted that even at 3 weeks of age, the NOD DI was higher than that of the 3T3L1 samples, suggesting that increased beta cell turnover may begin earlier than 4 weeks in NOD mice. This study proved that as beta cells are shed into the blood circulation *in-vivo* the demethylation index accurately measures that increase, allowing for the relative quantification of beta cells.

The importance of using whole blood in the *in-vivo* assay was verified by separating whole blood into serum and cell pack portions and evaluating the beta cell DNA content. The cell pack portion contained more beta cell DNA than the serum
portion (Figure 8), indicating that previous studies\textsuperscript{1,9,12} were unable to capture a large majority of the beta cell shedding that occurred. The use of whole blood over serum resulted in higher analytical sensitivity of this assay in comparison to previous ones. This translated to an earlier and more sensitive prediction of T1DM.

The \textit{in vivo} studies characterized the natural history of beta cell shedding in NOD.WT pre-diabetic, NOD.WT non-diabetic and NOD.SCID mice (Figures 9 and 10). All NOD.WT mice experienced elevated levels beta cell shedding throughout their lifetime (Figure 9B). The study observed a broad range of demethylation indices of magnitudes that were supported by previous reports\textsuperscript{1}. The pre-diabetic and non-diabetic mice experience constant beta cell shedding (Figure 9A and B) greater than magnitudes observe for BALBc mice\textsuperscript{1} (do not develop diabetes). This suggests that mice of the NOD.WT background have a higher basal beta cell shedding, either due to increased beta cell turnover and/or immune destruction. This is the first study to characterize the beta cell shedding patterns of NOD mice from 4 weeks of age to 21 weeks of age.

This was the assay to use whole blood, which resulted in an increased analytical sensitivity. In both the pre-diabetic and non-diabetic NOD.WT mice the DI peaked at 8 weeks of age, Akirav \textit{et al.}, reported a spike in demethylation indices of diabetic mice at 14 weeks, prior to the onset of diabetes\textsuperscript{1}. This assay detected a similar magnitude of spike 6 weeks earlier (Figures 8A and 11). The 6 weeks earlier detection is at least partly due to the use of whole blood for DNA extraction from samples, allowing our assay to capture more beta cell DNA.
A novel discovery was that the NOD.SCID mice also experienced beta cell shedding through their lifetime (Figure 10) independent of adaptive immune modulation. The demethylation indices were similar to that reported for NOD.SCID mice pre-STZ treatment\(^{12}\). The total cumulative shedding was lower than the NOD.WT mice but still far higher than the BALB/c mice. The NOD.SCID mice experienced lower shedding than NOD.WT mice until after the major 8 week peak. After the DI peak at 8 weeks of age the NOD.SCID mice average weekly DI were slightly higher than or equal to NOD.WT mice for weeks 10, 12, 14, 16 and 20 (Figure 11A). Even though NOD.SCID mice are immune deficient because they lack T or B cells they still had higher basal beta cell turnover than non-NOD models. These findings suggest that mice of the NOD background may experience higher basal beta cell turnover and/or beta cell destruction due to toll-like receptor signaling\(^{26}\) or other innate immune system modulators since beta cell shedding still occurs in the SCID model. Thus, an increased basal beta cell shedding seems to be present due to undefined mechanisms that may be important in the pathogenesis of T1DM.

The fluctuations between weekly demethylation indices led to the investigation and additional conclusion that the change in beta cell shedding patterns is higher in NOD.WT pre-diabetic animals than in non-diabetic NOD.WT and NOD.SCID mice (Figures 13, 14 and 15). The variation (Figure 13), DI slope (Figure 14), and the change in DI slope (Figure 14) illustrated larger magnitudes of difference between the pre-diabetic NOD.WT, non-diabetic NOD.WT and NOD.SCID mice when compared to the
cumulative shedding alone (Figure 11B) Thus, the DI, DI slope and the change in DI slope were all used as predictive parameters in the mathematical model.

Fifteen of the 38 mice became diabetic in our study and 10 were able to be predicted by this assay using the mathematical model with 68% sensitivity (Figure 15A-J). Figure 15A-J illustrates that this assay/approach is able to predict the very early onset of T1DM using multiple predictive parameters and critical numbers that resulted in a useful mathematical model (see mathematical models and case studies sections) to predict the development of type 1 diabetes. The parameters used were DI, DI slope (the individual week to week change in DI) and change in DI slope. A critical number is the number that the DI, DI slope, or change in DI slope must be the same or greater than for the assay to predict an animal to be at risk for developing T1DM during their lifetime.

With the use of the DI, DI slopes and change in DI critical numbers slope 10 out of 15 diabetic mice were identified to be at risk and 5 out of 10 of the mice were predicted prior to the 8 week peak in beta cell shedding, each with a high degree of diagnostic sensitivity and specificity. The incorporation of multiple parameters and critical numbers increased the diagnostic sensitivity and specificity. This demonstrates the future ability to incorporate multiple parameters and critical numbers from the assay depending on whether a patient has autoimmune antibodies present, HLA3 or 4, and/or a family history of the disease, allowing the personalization of medicine for prevention/intervention of T1DM.

Since NOD.WT mice develop diabetes from 12 weeks – 30 weeks, the fact that the study was ended before 30 weeks may account for some of the decreased specificity,
where some of the false positives may be due to early prediction and the inability to confirm the development of diabetes due to a premature completion time of the study. Thus, future studies will follow all mice until diabetes develops or death.

In conclusion, the natural history of beta cell shedding in NOD.WT and NOD.SCID mice was characterized from 4 to 21 weeks of age. This project demonstrated heterogeneity and wide variability of the beta cell shedding during the study however a consistent peak was observed in the DI weeks prior to the onset of diabetes. That peak was observed up to 6 weeks earlier than other assays\textsuperscript{1,9,12} indicating this assay had superior analytical sensitivity, which is partly due to the use of whole blood allowing the capture of more beta cell DNA versus serum. It was also a novel discovery that even in the absence of T and B cells NOD.SCID mice also demonstrated significant basal beta cell shedding indicating higher basal beta cell turnover and/or beta cell destruction due to toll-like receptor signaling or other innate immune system modulators. Overall, this assay non-invasively identified (and quantified) beta cell death as it occurred in mice and with the use of the mathematical model (DI, DI slope and change in DI slope) and critical numbers that the assay can be used as a diagnostic tool in the future to earlier identify those at risk for developing T1DM and allow for prevention/intervention of the disease process.
CHAPTER TWELVE: SIGNIFICANCE

The data generated in this study could have significant impact on the medical community. This diagnostic method could provide an improved efficient and accurate method for detecting the early onset and/or susceptibility to the onset of type 1 diabetes.
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