Revised on 6/8/2016

2013-2191: METABOLIC CHARACTERISTICS OF LEAN DIABETES IN RURAL AND SEMI-URBAN INDIA AND IN THE UNITED STATES.

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METABOLIC CHARACTERISTICS OF LEAN DIABETES IN RURAL AND SEMI-URBAN INDIA AND IN THE UNITED STATES.

1 INTRODUCTION

BASIC INFORMATION:

1.1 THERAPEUTIC AREA:

India has the world's highest prevalence of diabetes, expected to rise to 80 million by 2030. This includes many patients with lean diabetes, with low body mass index (BMI) and severe complications of diabetes. Despite its prevalence in India and other countries and its dire health consequences to individuals with the disease, little is known about the aetiopathogenesis and the genotype-phenotype relationship, lean diabetes being a cluster of low birth weight individuals, MODY, lipododystrophic disease, type 1 diabetes and fibrocalcific disease. While rudimentary tests have suggested defects in both insulin secretion and action, no comprehensive metabolic studies have been performed. It is therefore unclear as to how these patients should be treated. The optimal therapeutic adjunct whether a suitable oralantidiabetic agent or insulin is not yet determined. Defining the metabolic defects of 'lean' diabetes should have tremendous therapeutic benefit for millions of patients with this elusive condition.

1.2 BACKGROUND AND SIGNIFICANCE:

1.2. Significance of Diabetes in Indian population and South Asians living in the United States:

Diabetes is rapidly becoming a global epidemic. India has the world's highest prevalence of diabetes, expected to rise to 80 million by 2030 ([1]). As a consequence of rapid Westernization, many Indians are developing type 2 diabetes (T2DM). Of note, T2DM incidence in Indians rises sharply when body mass index (BMI) rises above 22 kg/m^2 ([2]).

Additionally, the diabetes population in India and other LMICs includes many patients with low BMI and severe complications of diabetes. Various reports suggest that these patients are surprisingly insulin resistant considering their lean body habitus. Some of these individuals have Fibrocalculous Pancreatic Diabetes (FCPD), in whom pancreatic calculi and/or ductal dilatation may contribute to the etiology of their disease ([3]). Many, however, are classified as **Lean Diabetes,** identified clinically by early onset, high insulin requirements, absence of pancreatic calculi or ductal dilation, and low BMI (typically $\leq$17kg/m^2 at presentation). The majority of young adults with diabetes in India are likely to have FCPD or lean diabetes (3). Both conditions differ from type 1 diabetes by persistent C-peptide response to nutrient stimuli, and absence of ketosis and pancreatic beta cell autoimmunity markers ([4]). Lean diabetes tends to present in young adults between the ages of 20 and 40 and afflicts men and women at a 2:1 ratio. Despite its sizeable prevalence, lean diabetes remains a relatively unknown disease that stirs conflicting opinions regarding its classification, diagnostic criteria, and pathogenesis ([5]). Of note, patients with lean diabetes appear to have elevated VLDL and triglycerides as well as insulin resistance based on insulin tolerance tests ([6]).

It has increasingly become apparent that marked ethnic variability in the clinical features of diabetes exists. Variability in the prevalence and pattern of disease among ethnic groups

can be secondary to both genetic and environmental modifying factors. South Asians in the United States have a higher prevalence of diabetes than non-Hispanic whites, despite lower BMI and being younger in age (7). Studies in the United Kingdom have shown that migrant Indians have increased insulin resistance and hyperinsulinemia compared to Europeans (8). However, comprehensive metabolic studies of these individuals are lacking in the United States. *Careful metabolic 'phenotyping' and genotyping of South Asian individuals with lean diabetes would be very helpful to better understand this condition and develop appropriate therapeutic approaches.* Our study aims to define the ethnic variability in clinical and metabolic characteristics of diabetes in South Asian population living in United States.

Insulin resistance in individuals with lean diabetes: There are currently very few publications on the entity known as 'lean' or 'malnutrition' diabetes. Numerous case reports, however have described a high degree of insulin resistance in some of these individuals ([7], [8]). Nonetheless, there is a debate to whether the mechanisms are attributable to the pancreatic beta-cell, skeletal muscle, adipose tissue, or other tissues.

1.2.2 Significance of fat mass in the etiology of insulin resistance:

It is a well-recognized yet intriguing concept that individuals at both ends of the weight spectrum have abnormal fat metabolism impacting insulin sensitivity. In obesity, increased fat mass promotes fatty acid flux to nonadipose tissues ([9]). In apparent contradiction, similar metabolic features are observed in lipodystrophy, including insulin resistance, hepatic steatosis, hyperlipidemia and diabetes ([10]). In both obesity and lipodystrophy, a storage defect within adipocytes (due either to engorgement with lipid or a paucity of adipocytes) leads to excess accumulation of triglyceride in non-adipose tissues. Increased free fatty acid (FFA) availability in both conditions inhibits the effects of insulin on hepatic and peripheral glucose metabolism ([11],[12]). Indeed, systemic insulin resistance is associated with increased adipose tissue lipolysis and resistance to the anti-lipolytic effects of insulin ([13],[14]). Increased hepatic fat content is receiving particular attention as a potential causative factor of insulin resistance ([15]). In addition to the well-established associations between IHTG and hepatic insulin resistance ([16]), our preliminary data (below) demonstrate an intriguing association with peripheral insulin resistance. *Based upon the relationships described above, we hypothesize that deficient fat stores in individuals with lean diabetes may promote hepatic triglyceride storage, leading to elevated circulating fatty acids and insulin resistance.*

1.3 SIGNIFICANCE:

It is highly intriguing, in the twenty-first century, to encounter a condition as prevalent as lean diabetes yet about which so much remains to be learned. The diabetes literature is dominated by studies conducted in high income countries, where this condition is never encountered. *These would be the first truly comprehensive studies defining the body composition and metabolic features of lean diabetes*. Scientifically, this application will explore a novel frontier in the intriguing dynamic between fat metabolism and insulin sensitivity.

Clinical relevance: Given India's high prevalence of diabetes, of which a substantial yet poorly defined proportion is likely to have lean diabetes, this proposal would have considerable clinical relevance for India and many other countries. In the absence of

sufficient information about this condition and its management, many lean diabetes patients are labeled as type 1 diabetes and are prescribed insulin. Unfortunately, inconsistent food availability among poor patients results in many insulin-induced hypoglycemic deaths. A better understanding of the metabolic defects of this condition should be very helpful in pointing towards more appropriate therapeutic approaches. Specifically, being able to clearly demonstrate that these subjects are insulin resistant would favor the use of safer, cheaper oral insulin sensitizing agents. This application therefore seeks to shift current clinical practice paradigms for underweight patients with diabetes, by studying a novel hypothesis concerning the pathophysiology of lean diabetes. *Defining the metabolic defects of lean diabetes could therefore have tremendous therapeutic benefit for millions of patients with this elusive condition.*

1.4 PRELIMINARY STUDIES

Moderate elevations in FFA levels characteristic of T2DM impair glucose effectiveness: *These studies were performed at Einstein, and highlight the impact of abnormal fatty acid flux on glucose metabolism.* In previously reported studies, FFA levels were elevated in poorly controlled T2DM subjects (377±46 mM) during glucose clamp studies, relative to age- and BMI-matched nondiabetic subjects (167±12 mM)([17]). To determine the extent to which the ~2-fold elevations in FFA characteristic of poorly controlled T2DM contribute to defects in glucose metabolism, we infused nicotinic acid at 0.015 mg/kg.min to lower FFA to nondiabetic levels in n=9 poorly controlled T2DM subjects (HbA_{1C} = 10±1%, age = 49±3yr; BMI = 27±1 kg/m^2)([18]). We established steady state hormone and tracer conditions over the first four hours at normoglycemic glucose levels (90 mg/dl) and then abruptly raised glucose levels to 180 mg/dl for the final 2 hours of the studies. Normalizing FFA levels essentially restored the endogenous glucose production (EGP) response to hyperglycemia. Indeed, EGP was reduced by 41±8% with the onset of hyperglycemia when FFA were lowered with nicotinic acid ($p<0.001$), which was comparable to the response in nondiabetic individuals. *These data indicate that moderately increased FFA levels could contribute substantially to abnormal glucose metabolism in T2DM.*

Increased Intrahepatic Triglyceride (IHTG) Is Associated with Peripheral Insulin Resistance: *These studies were performed at Einstein.* A total of 56 nondiabetic subjects (49 M) ranging in age from 19 to 78 yrs underwent five hour hyperinsulinemic (40 mU/m^2.min), euglycemic (90 mg/dl) 'clamp' studies, using 3-^{3}H-glucose to quantify insulin-mediated glucose uptake. Mean glucose uptake was 10.2$\pm$0.4 mg/kg.min among normal weight subjects (BMI<25; n=15) vs. 8.0$\pm$0.8 mg/kg.min in overweight-to-obese subjects (BMI>25; n=41; ~22% reduction, $p<0.001$), with a significant, inverse correlation between BMI and glucose uptake ($r=-0.386$, $p=0.004$). *As noted above, altered fat deposition may contribute to insulin resistance in humans*. Since visceral (VF) and deep subcutaneous fat (SF) depots may contribute to IHTG via hepatic delivery of free fatty acids (FFA), we simultaneously measured intramyocellular lipid (IMCL), IHTG, VF and abdominal SF in a subgroup of n=12 nondiabetic, nonobese individuals using noninvasive ^{1}H magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI). Positive correlations were observed among IMCL, IHTG and VF. There were remarkably tight correlations between IHTG and peripheral (non-hepatic) insulin sensitivity ($r = -0.87$, p <0.001). Intriguingly, however, there was a lack of correlation of insulin-stimulated glucose uptake with IMCL or other fat depots. These observations are consistent with studies in rodents in which decreasing IHTG levels improved muscle insulin action ([19]). Although the IHTG levels in these nonobese subjects were substantially lower than levels

reported in obesity and T2DM, the inverse correlation of IHTG with insulin sensitivity was exceptionally tight. This was presumably aided by the sensitive measures generated by in vivo ^{1}H MRS and insulin 'clamps'. *This association between IHTG and insulin resistance at normal body weights demonstrates that fatty acid flux and tissue triglyceride content are important determinants of insulin sensitivity, even in the absence of obesity.*

Deleterious Metabolic Effects of Low Birth Weight: *These studies were performed at Christian Medical College India (CMC) in collaboration with University of Copenhagen.* Nutrition in rural areas of India is still less than satisfactory. Hence, pregnant women often have insufficient protein intake, and the prevalence of low birth weight in India is endemic (26% of the babies born in rural India). These babies are more prone to diabetes in adulthood. A population-based cohort of 60 low birth weight (LBW) subjects and 60 normal birth weight (NBW) subjects, aged 18 to 22 years, was recruited from a rural area near Vellore. Insulin resistance was assessed by HOMA and by the glucose infusion rate required during hyperinsulinemic euglycaemic clamp studies (M-value). Body composition was measured by DEXA, and liver triglyceride content by MRS. Among these young subjects, with comparable BMI's in both groups (~18 kg/m^2), LBW was associated with a reduction in insulin sensitivity as assessed by HOMA (LBW=1.02$\pm$0.13 vs. NBW=0.75$\pm$0.10, $p<0.05$) and lower total body fat content (LBW=7654$\pm$642 vs. 8559$\pm$542 g, $p<0.05$). However, the M-value with this clamp technique was highly variable, and did not show a difference between groups. IHTG analysis is pending. *While these studies only included nondiabetic subjects, these data suggested a relationship between LBW and insulin resistance, consistent with the increased propensity for diabetes in malnourished individuals later in life.*

1.5 Relevance to Project:

These data indicate that moderately increased FFA levels could contribute substantially to abnormal glucose metabolism in T2DM. The association between IHTG and insulin resistance at normal body weights demonstrates that fatty acid flux and tissue triglyceride content are important determinants of insulin sensitivity, even in the absence of obesity. While these studies only included nondiabetic subjects, these data suggested a relationship between LBW and insulin resistance, consistent with the increased propensity for diabetes in malnourished individuals later in life.

2.0 OBJECTIVES:

2.1 Objective 1

(i) To characterize body composition and hepatic lipid deposition in individuals with lean diabetes.

2.2 Objective 2

(ii) To characterize insulin's ability to regulate glucose fluxes and inhibit lipolysis in individuals with lean diabetes.

2.3 Objective 3

(iii) To assess the insulin secretory response to meal challenge in individuals with lean diabetes

2.4 Objective 4

(iv) To characterize and study the gene expression profile of adipose tissue and skeletal muscle in individuals with lean diabetes.

2.5 Objective 5

(v) To understand the immunologic determinants of diabetes in the lean.

2.6 Objective 6

(vi) To sequence the genes involved in monogenic forms of diabetes to identify the genetic variants and their association with lean diabetes

3.0 RATIONALE

3.1 RATIONALE FOR THE TRIAL FOR OBJECTIVE 1:

Of note, these studies aim to carefully characterize a condition about which little is known. Since there are no rigorous definitions differentiating lean diabetes from T2DM, we will classify the DM subject groups based upon their BMI ranges. To further increase the likelihood of correctly identifying lean diabetes, we will elicit a history of low birth weight and documented malnutrition in the lean diabetes subjects. We will also carefully exclude subjects with features of type 1 diabetes, excluding individuals who test positive for anti-CAD, insulin and/or IC2 antibodies.

The lack of weight matching between the two DM groups is an inevitable feature of the different pathophysiology of these conditions. To minimize the impact of a sizeable difference in BMI, we will select a T2DM group at the lower BMI range for this condition. As noted above, risk of T2DM among ethnic Indians begins to increase sharply above a BMI of 22 kg/m^2.

To increase the likelihood that the “nondiabetic comparators” will have a normal metabolic profile, we will select subjects with normal birth weight and no documented history of malnutrition, despite having a BMI in the target range. Of note, it is possible that these individuals will also demonstrate some metabolic consequences of decreased fat mass. If we document insulin resistance in the underweight nondiabetic group, we will consider adding a fourth group of normal weight, nondiabetic subjects that would be BMI-matched to the T2DM group (BMI 22-25 kg/m^2).

DEXA scanning will quantify truncal fat and total body fat. If significant relationships are discovered between truncal fat content and metabolic endpoints, future studies will include a more comprehensive analysis of visceral vs. subcutaneous abdominal fat using CT scanning.

3.2 RATIONALE FOR THE TRIAL FOR OBJECTIVE 2:

Dr. Hawkins and her research group have now performed more than eight hundred clamp studies in human subjects, and thus have considerable experience both in the execution of the studies and in data analysis. Indeed, the human liver is known to be exquisitely

sensitive to small changes in plasma insulin levels, such that small increases in insulin rapidly decrease glucose production yet have little effect on peripheral glucose uptake. Particular benefits of the proposed study design include the proposed *stepped insulin clamp studies* for measuring both hepatic and peripheral insulin action, and the pancreatic clamp study design to maintain stable hormone levels. The latter is particularly relevant to the low insulin phase of the study in which hepatic insulin action is determined.

Insulin will be infused overnight prior to the studies in both DM groups, to correct the confounding effects of glucose-induced insulin resistance. A variable low-dose infusion of insulin will be initiated at 10 pm, and will be adjusted overnight based upon hourly glucose measurements to attain plasma glucose concentrations of 100 - 120 mg/dl prior to the start of the clamp study. This will ensure that all subjects will be studied under normoglycemic conditions. *This gradual lowering of plasma glucose is intended to avoid activation of counter-regulatory responses*. Indeed, the phenomenon of 'glucose toxicity' is recognized to cause reversible resistance to insulin at the level of the liver, muscle and adipocyte ([20]).

To what extent do increased FFA contribute to insulin resistance in the DM groups? As described above, there is substantial evidence that increased FFA levels in T2DM and obesity contribute to insulin resistance. Response to insulin's anti-lipolytic effects will be explored in this proposal by following FFA levels during the stepped insulin clamp studies, with the hypothesis that defective fat storage in lean diabetes will increase FFA levels and contribute to insulin resistance.

It could be argued that the proposed measures of body composition and insulin sensitivity, while highly sensitive and 'state-of-the-art', will be observational and will not prove causality. However, given the tremendous need for more knowledge about lean diabetes, it is essential to first do a thorough 'metabolic phenotyping' of individuals with lean diabetes.

3.3 RATIONALE FOR THE TRIAL FOR OBJECTIVE 3:

While rudimentary tests in individuals with lean diabetes have suggested defects in both insulin secretion and action, no comprehensive metabolic studies have been performed. It is therefore unclear as to how these patients should be treated.

3.4 RATIONALE FOR THE TRIAL FOR OBJECTIVE 4:

It is a well-recognized yet intriguing concept that individuals at both ends of the weight spectrum have abnormal fat metabolism impacting insulin sensitivity. In obesity, increased fat mass promotes fatty acid flux to nonadipose tissues ([21]). In apparent contradiction, similar metabolic features are observed in lipodystrophy, including insulin resistance, hepatic steatosis, hyperlipidemia and diabetes ([22]). In both obesity and lipodystrophy, a storage defect within adipocytes (due either to engorgement with lipid or a paucity of adipocytes) leads to excess accumulation of triglyceride in non-adipose tissues. Increased free fatty acid (FFA) availability in both conditions inhibits the effects of insulin on hepatic and peripheral glucose metabolism ([23],[24]). Indeed, systemic insulin resistance is associated with increased adipose tissue lipolysis and resistance to the anti-lipolytic effects of insulin ([25],[26]). Increased hepatic fat content is receiving particular attention as a potential causative factor of insulin resistance ([27]). In addition to the well-established associations between IHTG and hepatic insulin resistance ([28]), our preliminary data (below) demonstrate an intriguing association with peripheral insulin resistance. *Based upon the relationships described above, we hypothesize that deficient fat stores in individuals with lean diabetes may promote hepatic triglyceride storage, leading to elevated circulating fatty acids and insulin resistance.*

4.0 OUTCOME

4.1 OUTCOME OF OBJECTIVE 1:

To define body composition (DEXA) and liver triglyceride content (MRI) as well as determine the presence of fibrocalcific pancreatic disease, in young adults who have lean diabetes, based upon a history of established diabetes and malnutrition, without features of type 1 diabetes.

4.2 OUTCOME OF OBJECTIVE 2:

Hepatic insulin action: this will be determined as the ability of low infusion rates of insulin (*20 mU/m*^{2}.min above basal requirements) to suppress glucose production during the "low insulin phase" of the clamp studies.

Peripheral insulin action: this will be determined as the ability of high infusion rates of insulin to stimulate glucose uptake during the "high insulin phase" of the clamp studies. For the final two hours of the study, insulin infusion rates will be raised by a larger increment *(80 mU/m^2.min)* above basal insulin requirements, to determine peripheral insulin sensitivity under maximally stimulated conditions.

Glucose kinetics: Rates of glucose appearance (Ra) and disappearance (Rd) and other indices of glucose turnover will be estimated using Steele equations ([29]). Endogenous glucose production will be determined by subtracting the rates of glucose infusion from the tracer-derived Ra.

Plasma hormone and substrate determinations: plasma insulin, free-insulin, C-peptide, and glucagon concentrations will be measured by Core Analytical Lab at Albert Einstein College of Medicine ([30]). Plasma lactate, FFA and glycerol concentrations will be measured using spectrophotometric or colorimetric techniques ([31],[32],[33]).

6-6 D-glucose determinations: Initially, it is likely that these determinations will be performed at Einstein. Plasma samples for Gas Chromatography-Mass Spectroscopy (GC-MS) will be derivatized after protein precipitation to the aldonitrile pentacetate with hydroxylamine hydrochloride-acetic anhydride. GC/electron impact-mass spectrometry analysis will be performed on an Agilent model 6890/5973 with a 7673 Agilent autosampler ([34]).

4.3 OUTCOME OF OBJECTIVE 3:

Mixed meal tests have been proposed to have similar sensitivity and reproducibility to oral glucose tolerance testing (OGTT) for the detection of hyperglycemia and diagnosis of glucose intolerance ([35],[36]) and we have substantial experience in performing these tests in older subjects. Indeed, a standard mixed meal challenge, rather than an oral glucose challenge, represents a more physiologically relevant stimulus in that it may more closely mimic the metabolic changes experienced by individuals in daily life, and is better tolerated than an OGTT. It also represents a more integrated measure of nutrient metabolism than intravenous challenge tests, since it includes the contribution of the incretin hormones.

We will study subjects following an overnight fast and after a test meal of 100 g CHO, 20 g protein, and 20 g fat. Blood sampling will be performed through an indwelling intravenous catheter, fasting and 15, 30, 45, 60, 90, 120 and 180 minutes following the meal, for measurement of glucose, insulin and C-peptide levels. Plasma triglycerides and FFA levels will be measured fasting and at 60, 120 and 180 minutes. Subjects will be provided with a standard meal and snack to consume at home the night prior to the SMT and will

fast after 10 pm, in order to minimize metabolic variability between tests. Glucose and insulin levels will be used to calculate the area under the curve (AUC) using the trapezoidal method.

Glucose-stimulated insulin secretion can be assessed using a variety of techniques, including hyperglycemic "clamp" studies and the frequently sampled intravenous glucose tolerance test. However, these procedures could be considered to be less physiologic, as they do not capture the contribution of incretin hormones, and may be more burdensome for older subjects. Standard mixed-meal tests with frequent blood sampling and application of mathematical modeling have been used to evaluate insulin secretion, and have been reported to produce results comparable to more invasive and less physiologic tests ([37],[38]).

In collaboration with our colleague, Dr. Daniel Stein, indices of β-cell function will be estimated from plasma glucose and C-peptide concentrations using the *oral minimal model of C-peptide secretion*, with kinetics calculated from deconvolution of plasma C-peptide concentrations measured during the standard mixed meal ([39]) and using parameters for C-peptide kinetics and volume of distribution as measured by Van Cauter et al ([40]). Insulin secretory dynamics can be broken down into dynamic and static components of β-cell responsivity to glucose. The dynamic responsivity index, termed $\Psi_{dynamic}$, measures the β-cell secretory response to the rate of change of glucose concentration. This measure approximates the first phase of insulin release and defines the response to a given increment in glucose. Conversely, the static responsivity index (Ψ_{static}) measures the beta cell secretory response to a given glucose concentration. This static measure is believed to represent the provision of new insulin into the secretory pool. The two Phi factors can be combined into a Ψ_{total}. The appropriateness of the insulin secretion for the prevailing degree of insulin resistance will be determined by the disposition index (DI) calculated as the $\Psi_{dynamic}$, Ψ_{static} and Ψ_{total} times the insulin sensitivity ([41]), which will be determined by the method of Matsuda**Error! Bookmark not defined.**, allowing calculation of a DI for subjects who do not undergo the euglycemic clamp.

4.4 OUTCOME OF OBJECTIVE 4:

Biopsies: Participants will have fat biopsies performed in the periumbilical region *approximately one month following the clamp study.* A small ~1-2 cm cutaneous incision will be performed under local anaesthesia (Lidocaine 1%) and 1-2 g of adipose tissue will be obtained *by open biopsy technique. A skeletal muscle biopsy of ~50-100 g will be obtained with a spring-loaded biopsy needle (Bard Instruments) in the mid-thigh region following local anesthesia extending into the muscle area.*

Adipose tissue specimens- Whole adipose tissue for analysis by rt-PCR will be immediately homogenized in Trizol (Life Technologies, Bethesda, MD) with a Tissumizer homogenizer. Remaining fresh adipose biopsy specimens will be immediately digested with collagenase type 1. Adipocytes will be subsequently separated from stromal cells by centrifugation and filtration. Macrophages will be separated from stromal cells by CD14+ specific antibody coated beads. Subsequent mRNA extraction and analysis of gene expression by real-time QPCR of each adipose tissue fraction will be performed..

Gene expression profile of following array of genes would be studied in adipose tissue and muscle.

1. Carbohydrate Metabolism
2. Lipid Metabolism
3. Insulin signaling pathway
4. Adipogenesis
5. Energy metabolism
6. Inflammation

We will quantify adipose tissue macrophages with **immunohistochemistry**. To measure macrophage content in adipose tissue by immunohistochemistry, samples will be fixed and embedded in paraffin ([42]). For **immunofluorescence**, tissue sections will be stained with primary antibodies to human CD68 and PAI-1 followed by fluorescent secondary antibodies, and images quantified.

FACS analysis: After digestion with collagenase, the pellet of stromal cells will be treated with red blood cell lysing buffer followed by incubation with saturating amounts of FITC-labeled human CD14+ or CD163+ antibody, then washed and analyzed immediately by using a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA). **Intracellular cytokine staining** will involve a modification of basic flow cytometry that will be used for the simultaneous analysis of surface molecules and intracellular antigens at the single-cell level. Cells will be stained for surface antigens, then fixed to stabilize the cell membrane, and then permeabilized with the detergent saponin to allow anti-cytokine antibodies to stain intracellularly.

Muscle tissue specimens: The muscle biopsy specimens will be obtained as described above, immediately homogenized in Trizol at the bedside, to inhibit any RNAase activity, and will subsequently be stored at –80 ˚ C. They will be analyzed for the following:

- gene expression of GLUT-4
- gene expression of PPAR-y

This will be done in the endocrine molecular laboratory.

Epigenomics: The field of epigenomics can be defined as the study of heritable changes in gene expression that occur without a change in DNA sequence, principally through DNA methylation, histone post-translational modifications and chromatin organization. These processes provide an important additional layer of transcriptional control that regulates how genes are expressed under various physiological and environmental conditions. We anticipate that variable nutritional intake during development will induce specific metabolic changes in adipose tissue not only resulting from changes in substrate flux and availability but also due to changes in protein expression regulating metabolism.

To determine changes in DNA methylation patterns in fat biopsies from the human subjects, we will use a modify and updated protocol of previously established HELP assay system namely HELP-tagging assay, which involved the sequencing of the short tag adjacent to the digestion site, fundamentally a similar approach to the recently-described MSCC assay. HELP-tagging is a more quantitative assay than those previously available and is more sensitive to hypomethylated loci. In addition, the HELP-tag assay has

demonstrated that polymorphic HpaII sites occur at higher frequencies in the human genome than was previously thought based upon less sensitive DNA methylation assays.

Briefly, DNA is digested with HpaII and ligated to a customised Illumina adapter with a complementary cohesive end. The adapter also contains an EcoP15I site that cuts into the adjacent sequence 27 bp away, allowing us to polish that end and ligate the other Illumina adapter for library generation by PCR. The presence of the CCGG and EcoP15I sequences at the end of the reads obtained is a useful quality metric and allows us to remove spurious sequences. Prior to sequencing, we perform quantitative real time PCR with primers that measure the proportion of adapter dimer complexes in the library, usually a very small proportion (<5%) of the total library. Following sequencing, we remove low quality or unmapped reads, pile up reads on each locus and create an output for each locus in terms of read frequency. We find some heterogeneity in the MspI representation, requiring that, as for microarray-based HELP, we normalize the HpaII signal with that of the deep sequenced MspI profiles we have generated to date. Co-investigator Dr. John Greally at Einstein will lead the continued development of the analytical pipelines supporting the use of HELP-tagging studies.

OUTCOME OF OBJECTIVE 5:

To screen for type 1 diabetes using auto-antibody testing. We will perform specific immunologic (radiobinding) assays to detect islet autoantibodies including: Islet Antigen-2 autoantibodies (IA-2A), Glutamic acid decarboxylase autoantibodies (GAD65A), and Insulin autoantibodies (IAA). In addition, auto-antibodies to Zink transporter 8 (ZnT8A) were recently identified as novel markers for type 1 diabetes diagnosis and prediction ([43], [44]) . Therefore, using dried blood spot samples (see attached protocol), we will evaluate for Zinc Transporter 8 (ZnT8) autoantibody variants: Arginine (ZnT8-RA), Tryptophan (ZnT8-WA) and Glutamine (ZnT8-QA) ([45], [46]). Importantly, measuring ZnT8A, GADA, IA-2A, and IAA has been shown to raise autoimmunity detection rates to 98% at disease onset (43). Considering that measurements of these novel auto-antibodies are currently not available in India, these determinations will initially be done at Lund University in Sweden.

Moreover, to better characterize these lean diabetic individuals we plan to examine maturity-onset diabetes of the young (MODY) genes 1-6 and the neonatal diabetes genes ABCC-8 and KCNJ-2.

5.0 STUDY POPULATION AND RECRUITMENT:

Diabetes Mellitus (DM) Subjects: The subjects with diabetes will be recruited from the community as well as from the outpatient diabetes practice at CMC India, Jacobi Medical Center and North Central Bronx hospital. Subjects with diabetes mellitus will meet the following criteria: ages 19-45 years, diabetes duration at least one year, negative GAD antibodies, present (>1.0 pmol/l) C-peptide response to Sustacal challenge, stable and moderate-to-poor glycemic control (HbA1c between 8 and 11%), with no pancreatic calcification on imaging, and not suffering from significant complications. Diabetic subjects will be otherwise in good health.

- **Lean Diabetes Subjects:** N=20 subjects with BMI 16-22.5 kg/m^2, with a history of low birth weight and malnutrition documented on at least one occasion.
- ***T2DM Subjects:*** *N=20 subjects with BMI 22.5-27 kg/m^2*
- ***T1DM Subjects:*** *N=20 subjects with BMI 16-22.5 kg/m^2*

- **Nondiabetic Subjects:** N=20 nondiabetic *with BMI 16-22.5 kg/m2* subjects *19-45 years* of age and in general good health, taking no medications, with normal glucose tolerance and no family history of diabetes. These subjects will be similar in age, ethnicity and BMI with the lean DM group, and will be recruited through various means of community outreach, including notices in shops and newspapers.

6.0 Subject Eligibility Criteria

6.1 Inclusion Criteria:

1. Age 19-45 yr
2. Diabetes duration at least one year (for the diabetes groups)
3. BMI range: 16-22.5 kg/m2 (individuals with lean and type 1 diabetes and non-diabetic controls); 22.5-27 kg/m2 in individuals with type 2 diabetes
4. Negative GAD antibodies
5. Present (>1.0pmol/l) C-peptide response to Sustacal challenge
6. Stable and moderate-to-poor glycemic control (HbA1c greater than 8%)
7. Able and willing to provide informed consent.

6.2 Exclusion Criteria:

1. Mentally disabled persons
2. Major psychiatric disorder on medication (excluding successfully treated depression)
3. HIV/AIDS
4. History of any cancer
5. Alcohol or substance abuse or toxin exposure which could be associated with neuropathy
6. Cushing's syndrome
7. Pregnancy or breast-feeding
8. Untreated or uncontrolled hypertension
9. Any Chronic illness requiring medication
10. History of bleeding disorder or with a prolonged PT or PTT
11. Renal disease
12. Liver impairment
13. Prisoners

Prior to their enrollment in the study the purpose, nature, risks and benefits of the study will be explained to all subjects and their voluntary, informed, written consent will be obtained. All subjects will have a screening visit to allow for clinical evaluation, including history, physical examination and consent procedures.

7.0 TREATMENT OF SUBJECTS:

7.1 VISIT PROCEDURE:

SCREENING VISIT (Visit 1). All subjects recruited in the United States will present to the Department of Endocrinology fasting; consequently, visits will be scheduled in the morning. Before screening takes place subject will be provided with written information about the trial and the procedures involved in accordance with local requirements. Subjects will be fully informed, orally and in writing, of their responsibilities and rights while participating in the trial, as well as of possible advantages/disadvantages when being treated with the trial medication. Subjects will have the opportunity to ask questions and have ample time to consider participation.

The informed consent process will take place at the screening visit (visit 1).
Subjects who wish to participate in the trial will sign and date the informed consent form for the trial before any trial related procedure.
All subjects will be provided with a copy of their own signed and dated informed consent form.

If the investigator is not the primary physician, the investigator will preferably notify the primary physician about the subjects trial participation. If required permission should be given by the subject.

The following will be performed and recorded during the screening visit

An initial screening visit will include a full history and physical examination, an oral glucose tolerance test and additional laboratory tests to determine eligibility, including testing for autoantibodies to Islet Antigen-2 autoantibodies (IA-2A), Glutamic acid decarboxylase autoantibodies (GAD65A), Zinc Transporter 8 (ZnT8) autoantibody variants: Arginine (ZnT8-RA), Tryptophan (ZnT8-WA) and Glutamine (ZnT8-QA), c-peptide, HbA1c, serum electrolytes, BUN and creatinine, PT/PTT, liver function tests, lipid profile, and urinalysis.

a) Signed informed consents, date and time

b) Demography ●Name,
- ● Date of Birth
- ● Sex
- ● Medical History.
- ● Concomitant Medications.

c) full history and physical examination,

d) laboratory tests to determine eligibility,

i) serum electrolytes,

ii) serum BUN and creatinine

iii) PT/PTT,

iv) liver function tests,

v) lipid profile,

vi) HbA1c,

vii) screening urinalysis

The patients will be evaluated for fibrocalcific pancreatic disease by abdominal imaging such as abdominal X-ray, ultrasound or MRI scan.

This is an observational study and hence there will be no randomization.

Schedule of proposed clinical studies:

TIMELINE:

	Year 1	Year 2	Year 3
Recruitment efforts and screening visits	50	30	20
Body composition analysis	10	25	25
Clamp studies	10	25	25
Data analysis	X	XX	XX
Manuscript preparation & presentations		X	XX

8.0 ASSESSMENTS

8.1 Physical Assessment:

Physical examination will include a careful assessment of upper extremity veins, musculo-skeletal exam as well as measurement of height, weight, waist circumference and percent body fat (by bio-impedance absorptiometry).

8.2 Laboratory assessment:

Laboratory assessment will tests to determine eligibility, including serum electrolytes, serum BUN and creatinine, PT/PTT, liver function tests, lipid profile, HbA1c, screening urinalysis, and blood pressure measurements.

8.3 In vivo clamp studies – General Conditions (Figure 6)

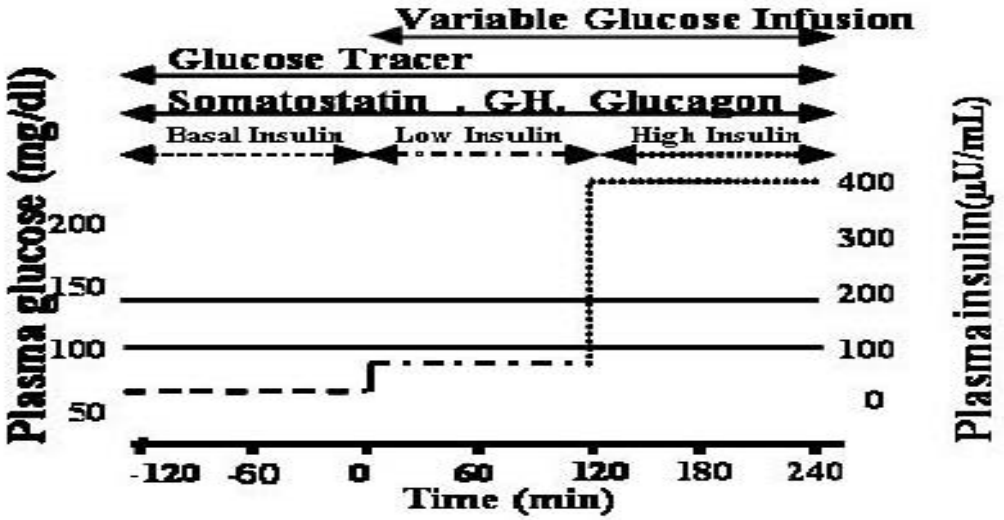
All subjects will be admitted on the day of the study to the Endocrinology Department study room after an overnight fast. At 8:00 am 2 intravenous cannulae will be established, one for infusions and a second one to be inserted in a retrograde fashion in a dorsal vein of the opposite arm for blood sampling. To obtain arterialized venous blood samples, this hand will be maintained at 65°C in a heated blanket.

A primed continuous infusion of D2G- Glucose will be initiated at t= 0 min (2 hours prior to the onset of the 'clamp' study). A bolus of D2G 200 mg/m^2 over 3 min will be followed by continuous infusion of 2 mg/min/m^2 for the entire study in all subjects (for total duration of 6 hours) to quantify glucose turnover, specifically rates of peripheral glucose disposal and hepatic glucose production.

These 'pancreatic clamp' experiments will consist of 360 min insulin/somatostatin (250 µg/hr) infusions with replacement of glucoregulatory hormones (glucagon 1 ng/kgmin; growth hormone 3 ng/kgmin). Throughout the entire 360 min the plasma glucose concentration will be maintained at basal levels (~90 mg/dl).

Basal phase: From t=0 min to t=120, optimal insulin infusion rates will be selected in each individual by making frequent (~ every 20-25 mins) adjustments to the insulin infusion rates in order to establish insulin infusion rates required to maintain euglycemia (90 mg/dl) without the requirement for exogenous glucose infusion. In our experience, this approach has resulted in our ability to detect small changes in insulin sensitivity (86)

Low phase: Following establishment of basal insulin requirements during the basal phase, at T=0 the insulin infusion rate will be increased by ~20 mU/m^2/min, and it will be maintained at this rate for 2 hours (T=120-240 min). These rates are designed to optimally assess hepatic insulin sensitivity. Plasma glucose will be maintained at euglycemic concentrations (~90mg/dl) by a variable infusion of 20% dextrose for the entire study (96).



High phase: At T=240 min, insulin infusion rate will be increased to 80 mU/m^2/min, and will be maintained at that rate for the final 2 hours of the study (T=240-360). These rates are designed to assess whole body insulin sensitivity.

All infusions will be stopped at t=360 min. The subject will be given a standard meal and plasma glucose levels will be monitored at 15-30 min intervals for the next one hour. Dextrose infusion will be continued for approximately 45 minutes after the study in order to avoid hypoglycemia. The subject will be discharged provided that his or her condition remains stable.

Measurements of plasma insulin, C-peptide, glucagon, and growth hormone are undertaken throughout the studies in order to evaluate the adequacy of the "pancreatic clamp" technique, i.e. the inhibitory effects of somatostatin on hormone secretion, and uniformity of hormone replacement. All samples will be kept on ice until centrifuged (within 30 min). Plasma samples will then be sorted and handled by the Special Assay Core, and will be stored frozen at -20°C for later assays.

8.4 Plasma measurements:

From t=0 to t= 360 min., blood samples will be obtained at hourly intervals for

determination of plasma glucose, insulin, glucagon, C-peptide, and Free fatty acids (FFA). Additional samples for D2G glucose determinations will also be obtained every 15 minutes.

Plasma glucose will be measured every 5-10 min to adjust the glucose infusion rate. Plasma lipids (LDL, HDL, VLDL, total Cholesterol and Triglycerides) will be measured at t=0).

8.5 Analytical procedures:

Plasma glucose will be measured with a Beckman glucose analyzer (Fullerton, CA) by use of the glucose oxidase method (87). Plasma hormone determinations will be performed by radioimmunoassay as previously reported (98), and these determinations will be provided by the Radioimmunoassay Core of the Diabetes Research and Training Center (DRTC).

8.6 Substrate determination:

Plasma FFA levels will be determined by using an acyl-CoA oxidase based colorimetric kit (Wako, Osaka, Japan) (99, 100). Plasma lactate will be measured by fluorometric enzyme techniques (101).

Plasma for labeled glucose will be treated as previously described (102).Rates of glucose appearance (Ra) and disappearance (Rd) and other indices of glucose turnover will be estimated by using the steady-state equation of Steele (103). Endogenous glucose production (EGP) will be determined by subtracting the rates of glucose infusion from the tracer-derived rate of appearance of glucose. Data for glucose turnover represent the mean values during the final 60 min of the low insulin period (t=60-120 min), and during the final 60 min of the high insulin period (t=180-240 min).

8.7 Measurement of insulin secretory response to meal challenge:

Mixed meal tests have been proposed to have similar sensitivity and reproducibility to oral glucose tolerance testing (OGTT) for the detection of hyperglycemia and diagnosis of glucose intolerance ([47],[48]) and we have substantial experience in performing these tests in older subjects. Indeed, a standard mixed meal challenge, rather than an oral glucose challenge, represents a more physiologically relevant stimulus in that it may more closely mimic the metabolic changes experienced by individuals in daily life, and is better tolerated than an OGTT. It also represents a more integrated measure of nutrient metabolism than intravenous challenge tests, since it includes the contribution of the incretin hormones.

We will study subjects following an overnight fast and after a test meal of 100 g CHO, 20 g protein, and 20 g fat. Blood sampling will be performed through an indwelling intravenous catheter, fasting and 15, 30, 45, 60, 90, 120 and 180 minutes following the meal, for measurement of glucose, insulin, C-peptide and glucagon levels. Plasma triglycerides and FFA levels will be measured fasting and at 60, 120 and 180 minutes. Subjects will be provided with a standard meal and snack to consume at home the night prior to the MMT and will fast after 10 pm, in order to minimize metabolic variability between tests. Glucose and insulin levels will be used to calculate the area under the curve (AUC) using the trapezoidal method.

Glucose-stimulated insulin secretion can be assessed using a variety of techniques, including hyperglycemic “clamp” studies and the frequently sampled intravenous glucose

tolerance test. However, these procedures could be considered to be less physiologic, as they do not capture the contribution of incretin hormones, and may be more burdensome for older subjects. Standard mixed-meal tests with frequent blood sampling and application of mathematical modeling have been used to evaluate insulin secretion, and have been reported to produce results comparable to more invasive and less physiologic tests ([49],[50]).

In collaboration with our colleague, Dr. Daniel Stein, indices of β-cell function will be estimated from plasma glucose and C-peptide concentrations using the *oral minimal model of C-peptide secretion*, with kinetics calculated from deconvolution of plasma C-peptide concentrations measured during the standard mixed meal ([51]) and using parameters for C-peptide kinetics and volume of distribution as measured by Van Cauter et al ([52]). Insulin secretory dynamics can be broken down into dynamic and static components of β-cell responsivity to glucose. The dynamic responsivity index, termed $\Psi_{dynamic}$, measures the β-cell secretory response to the rate of change of glucose concentration. This measure approximates the first phase of insulin release and defines the response to a given increment in glucose. Conversely, the static responsivity index (Ψ_{static}) measures the beta cell secretory response to a given glucose concentration. This static measure is believed to represent the provision of new insulin into the secretory pool. The two Phi factors can be combined into a Ψ_{total}. The appropriateness of the insulin secretion for the prevailing degree of insulin resistance will be determined by the disposition index (DI) calculated as the $\Psi_{dynamic}$, Ψ_{static} and Ψ_{total} times the insulin sensitivity ([53]), which will be determined by the method of Matsuda**Error! Bookmark not defined.**, allowing calculation of a DI for subjects who do not undergo the euglycemic clamp.

8.8 Fat Biopsies and Analysis:

Biopsies: Participants will have fat biopsies performed in the periumbilical region approximately one month following the clamp study. A small ~1-2 cm cutaneous incision will be performed under local anaesthesia (Lidocaine 1%) and 1-2 g of adipose tissue will be obtained by open biopsy technique.

Initially, the fat biopsies were planned to be obtained by aspiration technique. However, given the low BMI of the lean diabetic subjects, an aspiration biopsy is likely to be very difficult. We would therefore like to perform an open subcutaneous abdominal fat biopsy in each study participant. Moreover, given the complex nature of the clamp study we propose to perform the fat biopsy on a separate date approximately one month following the clamp study.

Adipose tissue specimens- Whole adipose tissue for analysis by rt-PCR will be immediately homogenized in Trizol (Life Technologies, Bethesda, MD) with a Tissumizer homogenizer. Remaining fresh adipose biopsy specimens will be immediately digested with collagenase type 1. Adipocytes will be subsequently separated from stromal cells by centrifugation and filtration. Macrophages will be separated from stromal cells by CD14+ specific antibody coated beads. Subsequent mRNA extraction and analysis of gene expression by real-time QPCR of each adipose tissue fraction will be performed .

We will quantify adipose tissue macrophages with **immunohistochemistry**. To measure macrophage content in adipose tissue by immunohistochemistry, samples will be fixed and embedded in paraffin ([54]). For **immunofluorescence**, tissue sections will be stained with primary antibodies to human CD68 and PAI-1 followed by fluorescent secondary antibodies, and images quantified.

FACS analysis: After digestion with collagenase, the pellet of stromal cells will be treated with red blood cell lysing buffer followed by incubation with saturating amounts of FITC-labeled human CD14+ or CD163+ antibody, then washed and analyzed immediately by using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). **Intracellular cytokine staining** will involve a modification of basic flow cytometry that will be used for the simultaneous analysis of surface molecules and intracellular antigens at the single-cell level. Cells will be stained for surface antigens, then fixed to stabilize the cell membrane, and then permeabilized with the detergent saponin to allow anti-cytokine antibodies to stain intracellularly.

9. STATISTICAL ANALYSIS

Analysis of Variance (ANOVA): A one-way ANOVA will be used to compare the primary measures (eg. glucose uptake, glucose production, IHTG) among the three study groups. When the ANOVA indicates a significant difference, unpaired t-tests will be performed to distinguish which groups differ. The null hypothesis is that the primary measures will not differ between the three groups: the nondiabetic, lean diabetes and T2DM groups. These analyses will be performed in collaboration with Einstein's biostatistician .

POWER ANALYSIS

It should be emphasized that the proposed comparisons represent 'ground-breaking' studies, as such measures have not yet been performed in individuals with lean diabetes. Based on our hypotheses, we predict that both DM groups will have similarly reduced rates of insulin-stimulated glucose uptake, while glucose uptake in the lean diabetes group will differ from matched nondiabetic controls to a similar extent as the difference observed between obese and lean subjects in our preliminary data. A sample size calculation was therefore performed based on an unpaired two-tailed t-test of between-group difference. Sample size analyses were performed, using NCSS/Pass software, to calculate the minimum sample size needed to achieve at least 80% power to detect a statistically significant difference in the primary outcomes of interest. Using our preliminary data in which mean glucose uptake was 8.0 mg/kg.min with SD of 2.0 mg/kg.min in the obese group, vs. mean glucose uptake of 10.2 mg/kg.min with SD of 2.5 mg/kg.min in the lean group, this calculation yielded a sample size of 20 per group.

Potential Risks:

Potential risks to the subjects include the following:

1. Blood withdrawal: The total amount of blood sampled will not exceed 190 ml per study, less than half of that donated by a blood donor.
2. 20% dextrose: The infusion of 20% dextrose may be associated with local venous irritation. This will be minimized by use of a large-bore antecubital vein in which the glucose will be infused.
3. D2G: is a non-radioactive glucose tracer and is not associated with any known side effects at the doses used.
4. Glucagon infusion in high doses can cause gastrointestinal symptoms such as cramps and nausea, and produce hyperglycemia in persons with glucose intolerance. The doses to be employed here are intended to maintain plasma glucagon at basal levels.

5. Growth Hormone: At higher doses and for prolonged periods of exposure, growth hormone (rhGH) has either insulin-like or insulin-antagonistic effects on carbohydrate and lipid metabolism, however the proposed infusion is for physiological replacement during SRIF infusion and is unlikely to have adverse effects.
6. Hypoglycemia: is unlikely to develop in these subjects because of the desired plasma glucose targets (90 mg/dl during the normoglycemic studies), the low rates of insulin infusion, and the constant blood glucose monitoring.
7. Somatostatin: At the proposed physiological replacement doses has no known side effects.
8. Ocreotide: At the proposed physiological replacement doses has no known side effects.

Protection Against Risks:

Recruitment and Informed Consent Procedures:

Consent will be initially obtained during telephone screening performed by a research fellow or the study coordinator. Formal consent procedures which adhere to the Committee on Clinical Investigations of the Albert Einstein College of Medicine will be followed. Specifically, each subject will be verbally informed in layman's language of the purpose, benefits and possible risks of the studies. They will then read the written consent form in the presence of a member of the research team and a physician who will answer any further questions. The subject and witness shall be asked to sign the consent form which will be kept in the patient's chart. Each subject's potential participation in related experiments (for example, when each subject is asked to perform repeat studies) will be explicitly stated.

All clinical data on the subjects will be confidential. Data generated in these studies will be considered only relevant for research purposes and will not be included in any clinical databases or the patient's chart. Since the GCRC databases are coded, the study data will not be available on any clinical database system.

Procedures employed to protect subjects from undue risks include the following:

1) Blood withdrawal: Blood withdrawal during any single study will be limited to less than 190cc and subsequent studies will be separated by at least, a four week interval. The infusion and withdrawal catheters may produce infection or local hematoma, but strict aseptic technique will be observed by the experienced physician performing the procedure.
2) 20% glucose: Vein irritation with 20% glucose infusion will be prevented by use of a large-bore antecubital vein.
3) Glucagon: Symptoms of mild nausea will be treated with antiemetic medications, if needed.
4) Hypoglycemia: All of these studies will be performed by a physician, hypoglycemia will be prevented by blood glucose sampling every 5-10 minutes and if inadvertent hypoglycemia develops can be rapidly treated by glucose infusion.

Benefits

This study could be of direct benefit to you. It will help you gain a better understanding of your own metabolic processes, whole body glucose handling as well as insulin secretion in response to a standard meal.

The study can also be of benefit to others as would be very helpful to better understand this condition and develop appropriate therapeutic approaches.

Data Storage/Confidentiality

All data and records obtained in this study will be used for research purposes only. The protocol will be submitted for review and approval by the Institutional Review Board known as the Committee on Clinical Investigations of the Albert Einstein College of Medicine (CCI). The CCI is responsible to ensure human subject protections in compliance with institutional policies and federal regulations including the HIPAA privacy law.

All clinical data on the subjects will be confidential. Data generated in these studies will be considered only relevant for research purposes and will not be included in any clinical databases or the patient's chart. Since the GCRC databases are coded, the study data will not be available on any clinical database system.
The research records will be kept private, in a secure manner, and subject names will not be used in any written or verbal reports. All computer records will be password protected in the Albert Einstein College of Medicine Clinical Research Center. Only the Clinical Research Center staff, as well as the research personnel authorized by the researcher will have access to these records.

Data Safety Monitoring Board

The data safety monitoring board will include:

Dr. Jill Crandall, Professor of Clinical Medicine in the Division of Endocrinology, Director of the Diabetes Clinical Trials Unit, and co-director of the DRTC Clinical Research Facilitation Core

Dr. Nir Barzilai, director of the Institute for Aging Research at the Albert Einstein College of Medicine and the Director of the Paul F. Glenn Center for the Biology of Human Aging Research and of the National Institutes of Health's (NIH) Nathan Shock Centers of Excellence in the Basic Biology of Aging, as well as the Ingeborg and Ira Leon Rennert Chair of Aging Research, a professor in the Departments of Medicine and Genetics, and member of the Diabetes Research Center and of the Divisions of Endocrinology & Diabetes and Geriatrics.

Dr. Joel Zonszein, director of the Clinical Diabetes Center at the University Hospital of the Albert Einstein College of Medicine, a Division of Montefiore Medical Center, as well as a Professor of Clinical Medicine.

Selected Publications by Study Team:

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