A NOVEL CROSS-SECTIONAL ANALYSIS OF INSULIN SENSITIVITY AMONG ADOLESCENTS AND YOUNG ADULTS WITH TYPE 1 DIABETES, MODY2, AND NORMAL CONTROLS: THE CONTRIBUTION OF HYPERINSULINEMIA VS. HYPERGLYCEMIA TO INSULIN RESISTANCE

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A NOVEL CROSS-SECTIONAL ANALYSIS OF INSULIN SENSITIVITY AMONG ADOLESCENTS AND YOUNG ADULTS WITH TYPE 1 DIABETES, MODY2, AND NORMAL CONTROLS: THE CONTRIBUTION OF HYPERINSULINEMIA VS. HYPERGLYCEMIA TO INSULIN RESISTANCE

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1.0 Background

Type 1 diabetes mellitus (T1DM) is a chronic condition in which autoimmune pancreatic islet destruction leads to deficient insulin secretion resulting in hyperglycemia. Current therapy largely focuses on using intensive insulin regimens to reduce hyperglycemia to prevent micro- and macrovascular disease while minimizing hypoglycemia. Although these comorbidities are well-recognized,(1-7) insulin resistance (IR) is a consistent(8-12) and under-appreciated finding in T1DM, even among patients who lack traditional IR risk factors. In studies utilizing the insulin clamp to assess IR, insulin sensitivity was consistently lower in T1DM patients compared to their age, gender, and BMI matched controls without diabetes by 35-57%.(8-13) Because IR has been strongly and independently correlated with macrovascular disease in T1DM,(14-16) an increased understanding of its root cause is needed. While earlier investigations attributed T1DM IR to hyperglycemia, (17-22) more recent studies have shown little to no correlation between hyperglycemia and insulin sensitivity.(10, 11, 13, 23) Thus, the degree to which hyperglycemia contributes to IR in T1DM is unclear, and this uncertainty suggests that other factors contribute prominently. The proposed research will provide a framework for future therapeutic strategies to mitigate T1DM IR and associated macrovascular disease.

2.0 Rationale and Specific Aims

The following research plan will determine whether IR in T1DM is predominantly an effect of chronic hyperglycemia, as is commonly accepted,(17-22) or a consequence of iatrogenic hyperinsulinemia in the peripheral circulation, as I alternatively hypothesize. Despite its independent contribution to micro- and macrovascular disease,(14, 16, 24) the underlying cause of IR has not been established nor have strategies to mitigate it been developed.

Insulin therapy in T1DM attempts to achieve euglycemia but does so in an "unphysiologic" way, by delivering insulin into the subcutaneous tissue as compared to physiologic delivery directly into the hepatic portal circulation. Thus, peripheral insulin delivery must balance competing factors: 1) a sufficient amount of insulin must reach the liver to prevent unrestrained hepatic glucose production and resultant hyperglycemia and 2) simultaneously, an excess of insulin in the peripheral circulation must be avoided to prevent hypoglycemia, as I recently reported.(25) Although life-saving, peripheral insulin delivery in T1DM results in a loss of the normal insulin distribution; the physiologic state maintains insulin at 3-fold higher concentrations in the portal circulation compared with the peripheral circulation.(26-29) IR in T1DM could therefore occur in response to peripheral hyperinsulinemia, a mechanism that would protect against hypoglycemia and ensure adequate glucose delivery to the CNS.

This protocol is designed to test these hypotheses:
1) IR in T1DM is a homeostatic response to increased peripheral insulin concentrations resulting from peripheral insulin delivery and *not* significantly attributable to hyperglycemia, and
2) IR in T1DM results primarily from peripheral tissue IR (especially muscle) and *not* primarily from hepatic IR.

To test this hypothesis, I plan to utilize the hyperinsulinemic, euglycemic clamp (insulin clamp) to assess IR in a cross-sectional study of 3 groups of subjects:

1) non-diabetic control subjects,
2) patients with well controlled T1DM, and
3) patients with glucokinase (GCK) mutations causing mature-onset diabetes of the young, type 2 (MODY2), a population that has hyperglycemia without hyperinsulinemia.

As summarized in **Table 1** and discussed in the following proposal, key metabolic differences between these 3 groups will enable distinction to be made between the relative contributions of peripheral hyperinsulinemia vs. hyperglycemia to IR in T1DM.

Specific Aim 1: Determine whether whole-body IR in T1DM is secondary to peripheral insulin delivery (as opposed to hyperglycemia). This study is designed to select T1DM subjects with matched glycemic control to that of MODY2 subjects. Importantly, because MODY2 patients retain pancreatic insulin secretion, the key factor influencing a difference in insulin sensitivity between these two groups will be that T1DM patients have iatrogenic peripheral hyperinsulinemia and portal hypoinsulinemia, while the insulin distribution remains normal in MODY2 (**Table 1**). Additionally, because there is no difference in insulin distribution between MODY2 and control subjects, the key factor influencing a difference in insulin sensitivity between these two groups is the presence of hyperglycemia in MODY2 and euglycemia in control.

Table 1: Key Between-Group Differences between control, MODY2, and T1DM subjects. Pe=peripheral, Po=portal. Shading is used to highlight similarities and differences between groups. Note: 1) MODY2 and T1DM subjects will be selected to have similarly mild hyperglycemia 2) Insulin distribution between peripheral and portal circulations will be normal in control and MODY2 subjects, but altered in T1DM.

Subject Group	Chronic Glycemia	Insulin Distribution	Risk of CAD	Muscle IR	Hepatic IR	Key Between-Group Differences Affecting IR
Control	Normal	Normal	Normal	Normal	Normal	
MODY2	↑	Normal	Normal	Unresolved	Unresolved	Mild hyperglycemia; Mild hyperglycemia AND Pe. hyperinsulinemia
T1DM	↑	Pe. hyperinsulinemia Po. hypoinsulinemia	↑↑↑	↑↑	Unresolved	Pe. hyperinsulinemia; Mild hyperglycemia AND Pe. hyperinsulinemia

Specific Aim 2: Establish the tissue-specific distribution of IR in T1DM (muscle, fat, and liver). The tissue-specific site of IR in T1DM has not been well established. By utilizing a 2-step clamp procedure, I will be able to simultaneously assess whether T1DM IR is predominantly an effect of muscle IR (as I hypothesize) or whether hepatic IR also contributes to the whole body IR seen in this condition.

A variation of the hyperinsulinemic, euglycemic clamp, the "pancreatic clamp technique" will be employed for the first time in T1DM and MODY2 subjects. The technique employs a short-term somatostatin infusion to suppress endogenous insulin and glucagon release while these hormones are simultaneously replaced at a prescribed infusion rate. Importantly, this approach ensures that insulin and glucagon, the key hormones influencing glucose metabolism, are equal between cohorts. A failure to match these hormones would confound the study results, particularly the assessment of hepatic insulin resistance. Somatostatin is a naturally occurring hormone, commercially

available, and has been used in metabolism studies in the U.S. (30-36) and Europe (37-41), but an FDA approval for clinical use in humans has never been pursued (largely because of the more favorable pharmacokinetic profile of its analog, octreotide). An Investigational New Drug (IND) application to use somatostatin in this study is presently being reviewed by the FDA (IND #[redacted]).

The proposed research will lay the groundwork for future testing of novel therapeutic strategies to restore the normal portal to peripheral insulin distribution can normalize insulin sensitivity in T1DM (e.g. hepatopreferential insulin analogs, intraperitoneal insulin delivery). Further, it will clarify the pathophysiologic processes underlying IR in T1DM at a tissue-specific level.

3.0 Animal Studies and Previous Human Studies

Many have thought that hyperglycemia *per se* was the predominant contributor to IR in T1DM. Yki-Järvinen , Koivisto, et al., showed that short bouts of hyperglycemia (24 hours) significantly reduced glucose uptake by ≈20% during clamp studies of T1DM patients compared to euglycemia of equal duration (18, 19). The same group reported glucose disposal during hyperinsulinemic, clamp studies was inversely related to HbA1c in a cross sectional study of T1DM 53 patients with disease durations ranging from 2-32 years (17). The Pittsburg Epidemiology of Diabetes Complications Study found that HbA1c was one of the best predictors of insulin resistance in T1DM (42).

The notion that hyperglycemia is the primary cause of IR in T1DM has been questioned in recent years, however. No correlation was found between HbA1c or continuous glucose monitoring (CGM) data and peripheral glucose uptake in hyperinsulinemic, euglycemic clamps in the Coronary Artery Calcification in Type 1 Diabetes study (13). In a later study, linear regression analysis showed that hyperglycemia accounted for only 6.3% of the decrease in glucose uptake in their T1DM subjects compared to matched controls (10). In a study of adolescents with T1DM, (11) no relationship between IR and HbA1c was found and in another (23) the correlation was weak. Thus, the degree to which hyperglycemia contributes to IR in T1DM is unclear and this uncertainty suggests that other factors likely contribute prominently.

<u>IR in T1DM: A Homeostatic Response to Peripheral Hyperinsulinemia</u>
IR in T1DM can be alternatively hypothesized to be a homeostatic response to increased insulin concentrations. Current therapy relies on subcutaneous insulin injection into the peripheral circulation to restore euglycemia in T1DM. Although life-saving, a reversal of the normal insulin distribution occurs when insulin is delivered into the peripheral circulation, rather than more physiologically into the hepatic portal circulation with higher insulin concentrations in the peripheral circulation and lower insulin levels in the hepatic portal blood (26-28). IR in T1DM could therefore be a compensatory downregulation of the glucose transport system to protect against hypoglycemia. Two key studies support this explanation for T1DM IR:

- Del Prato, DeFronzo, et al. examined the effect of sustained, physiologic euglycemic hyperinsulinemia on IR in 8 healthy subjects (43). After performing baseline hyperinsulinemic, euglycemic clamps, subjects underwent 72-96 hours of continuous IV insulin infusion at 0.25 mU/kg/min with variable glucose

infusion to maintain fasting plasma glucose at 86 ± 2 mg/dL. This led to a 3.3-fold increase in the fasting insulin concentration. After the 72-96 hour period, the hyperinsulinemic, euglycemic clamps were repeated. During the 3-steps of the clamps, insulin sensitivity decreased by 25% ($P<0.01$), 40% ($p<0.001$), and 20% ($p<0.02$) compared with the pre-treatment clamps. These studies show that a relatively short period of physiologic hyperinsulinemia with sustained euglycemia can lead to a significant increase in IR.

- Carpentier et al. studied insulin action in 16 T1DM patients who were recipients of combined kidney-pancreas transplantation with anastomosis of the pancreatic vein into either the systemic (KPT-S) or portal (KPT-P) circulation (44). All study patients had normal fasting blood glucose (BG) levels and HbA1c and were matched for age, BMI, T1DM duration, immunosuppressant doses, and GFR. Using a stepwise, hyperglycemic clamp, KPT-S subjects had a ≈40% higher total area under the insulin secretion compared to the KPT-P and non-diabetic control groups. *Notably, insulin area under the curve was practically the same for KPT-P and control*. A second group of experiments utilized a 2-step hyperinsulinemic, euglycemic clamp. Insulin sensitivity was 26% ($p=0.07$) and 32% ($p=0.008$) higher, respectively, in the KPT-P group than the KPT-S group. These data suggest that when baseline glycemic control and pulsatile insulin secretion are essentially equal (both T1DM groups had pancreas transplants), chronic insulin secretion into the portal circulation led to near-normalization of insulin sensitivity that was significantly better than chronic secretion into the peripheral circulation.

Taken together, these two studies show that 1) peripheral hyperinsulinemia within the physiologic range induces whole-body IR independent of glycemia and 2) a restoration of pancreatic insulin secretion into the portal circulation normalizes IR in T1DM.

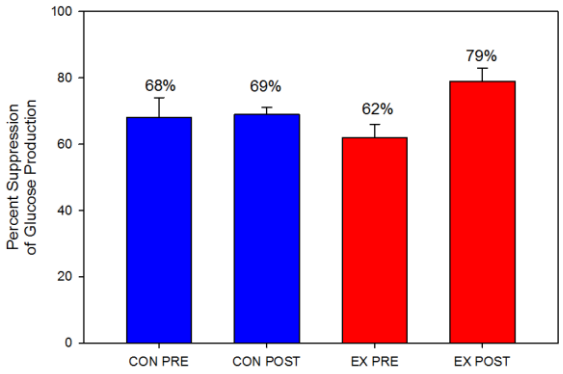

Figure 1: Hepatic insulin sensitivity before and after an exercise intervention program, quantified as the percent suppression of glucose production for a fixed insulin infusion rate. This data is intended to illustrate my ability to conduct the proposed experiments. CON=control subjects, EX=exercise subjects, PRE= pre-exercise intervention, POST= post-exercise intervention.

Because of the complexity of insulin clamp studies in humans, I have learned this methodology in collaboration with Dr. Jason Winnick, a research assistant professor at Vanderbilt. I have performed nearly 50 insulin clamp studies to evaluating an exercise intervention program's effect on hepatic IR in T2DM subjects. My experience in this study (as shown in **Figure 1**) illustrates my ability to detect changes in insulin sensitivity in diabetic patients with accuracy and precision using this approach. The experience gained from performing these studies makes me confident in my ability to perform the following proposed studies, under the continued guidance of my co-mentors, Drs. Naji Abumrad and Alan Cherrington.

4.0 Inclusion/Exclusion Criteria

Inclusion and exclusion criteria for study participants are listed in **Table 2** and will be determined at the initial screening visit. Subjects will be recruited from the Vanderbilt Eskind Diabetes Clinic, a large academic center that sees 4,000 patients with T1DM annually. T1DM subjects will be recruited to match MODY2 subjects within a HbA1c ± 0.3%, age ± 5 years, and BMI ± 1.5 kg/m^2. Control subjects will match MODY2 subjects within a BMI of ± 1.5 kg/m^2 and age ± 5 years.

Table 2: Inclusion and Exclusion Criteria for Study Participants

Inclusion Criteria		Exclusion Criteria	
All subjects			
BMI	Age 13-18: 15%-85% percentile Age 18-51: 19-28 kg/m^2	Severe hypoglycemia	≥1 episode in the past 3 months or diagnosis of hypoglycemia unawareness
		Diabetes comorbidites	- ≥1 trip to emergency room for poor glucose control in the past 6 months - New York Heart Association Class II-IV cardiac functional status - SBP >140 mmHg and DBP > 100 mmHg - Fasting triglycerides > 400 mg/dL - Liver transaminases > 2 times upper limit of normal - Renal transplantation or serum creatinine > 1.5 mg/dL
		Medications	Any systemic glucocorticoid, any antipsychotic, atenolol, metoprolol, propranolol, niacin, any thiazide diuretic, any OCP with > 35 mcg ethinyl estradiol, growth hormone, any immunosuppressant, any antihypertensive, any antihyperlipidemic
		Other	Pregnancy, Tanner stage < 5, peri- and post-menopausal women
T1DM subjects			
Age	13-51 years	Medications	Any diabetes medication except insulin
T1DM duration	1-20 years	C-peptide	> 0.7 ng/mL (fasting)
HbA1c	5.9-7.5%		
MODY2 subjects			
Age	13-51 years		
HbA1c	5.9-7.5%		
Positive GCK sequencing			
Control subjects			
Age	18-51 years		
HbA1c	<5.5%		

5.0 Enrollment/Randomization

To test the aims of this proposal, the hyperinsulinemic, euglycemic clamp will be used to assess IR in three groups of subjects:

1) non-diabetic control subjects,

2) patients with well-controlled T1DM, and
3) patients with GCK mutations causing MODY2.

This is a novel paradigm using a cross-sectional study design comparing these three groups.

After initial contact with interested individuals suggests they might be candidates for enrollment in the study, they will be invited for an initial screening visit in the Vanderbilt Clinical Research Center (CRC). A history and physical exam will be performed and baseline clinical factors will be assessed. If the potential subject meets exclusion/inclusion criteria for enrollment, they will be invited to enroll in the hyperinsulinemic, euglycemic clamp study as outlined in section 6.0. The clamp study will occur within one month of the screening study. In the event a participant successfully meets exclusion/inclusion criteria for study and the clamp study is started but a technical issue precludes completion of the clamp study (e.g. unable to maintain IV access), an abbreviated screening will be pursued as outlined in section 6.2.2.5.

A detailed description of special protection for adolescents as research subjects follows in section 7.1 below.

The Vanderbilt CRC is located [redacted] Nashville, TN [redacted]

6.0 Study Procedures

6.1 Recruitment

Multiple tools available to VUMC investigators will be utilized to identify prospective study participants. These include Research match, Subject locator, social media platforms, and My Research at Vanderbilt. Flyers will be posted and an institution-wide mass emailing will be used. Intramural and extramural endocrinology colleagues at Vanderbilt and in the region will be informed of this study and asked to refer potential subjects. The Vanderbilt Eskind Diabetes Clinic is a large academic center that sees approximately 4,000 patients with T1DM annually. Also, we will post our research flyer on social media platforms (e.g. Facebook and Twitter) to target further potential T1DM participants. For example, the JDRF Facebook pages have a large number of followers across the state of Tennessee who might be interested in participating in our research.

If unable to recruit an adequate number of MODY2 subjects from Vanderbilt and other endocrinology clinics in Tennessee with which we have relationships established, a collaborative relationship will be pursued with colleagues at the University of Chicago, the leading institution in the study of monogenic diabetes in the U.S. Additionally, we will post our research flyer on social media platforms (e.g. Facebook and Twitter) to target potential MODY2 participants. For example, the Monogenic

Diabetes Awareness Facebook page is followed by over 1,000 individuals who might be interested in participating in our research.

Once the research team becomes aware of a potentially interested subject, a phone call will be made to make an initial assessment of whether the subject might be eligible to participate in the study. Study personnel will ask the potential subject to share his or her:

- diagnosis of T1DM, MODY2, or that the individual is interested in being a control subject
- age
- weight
- height
- most recent HbA1c (if applicable)
- diabetes duration (if applicable),
- medications
- whether the potential subject has had severe hypoglycemia in the past 3 months
- whether the potential subject has had any trips to the emergency department for poor glucose control in the past 6 months
- if the patient has had GCK genetic sequencing confirming a diagnosis of MODY2 (if applicable)

If this conversation reveals no reasons the potential subject should be excluded based on the criteria in table 2, he or she will be invited to participate in an initial screening visit.

6.2 Study visits: Each subject will participate in two study visits.

6.2.1 Study visit 1: Initial screening

All subjects will be asked to arrive at the CRC at 8 AM on the morning of study visit 1. Participants will be asked to begin fasting except for water 8 hours before the visit. T1DM subjects will be instructed beforehand that if they have hypoglycemia during the overnight fast to use the commonly known "Rule of 15" with glucose tablets (http://www.joslin.org/info/how_to_treat_a_low_blood_glucose.html) to correct their hypoglycemia.

Upon arrival, the following will take place:

- **Consent/Assent**: The PI or designated Key Study Personnel will obtain consent/assent from all participants. Consent/assent will be obtained in a private room in the CRC prior to beginning study procedures. The consent and assent form will be provided to the subject and family (if applicable) for review prior to the visit. The PI or designated Key Study Personnel will review the consent/assent forms with the family

in detail and provide time for questions to be answered. A copy of the consent/assent form will be provided.

- **History and Physical Exam:** Each subject's clinical history will be reviewed by the PI (or a designated KSP who is an M.D., Nurse Practitioner, or Physician's Assistant) and a physical exam will be performed. Anthropometric measurements will be taken with the assistance of CRC staff or KSP. If at this point the subject continues to meet inclusion and criteria (table 2), he or she will proceed with the remainder of visit 1.

- **Resting Metabolic Rate (RMR):** Resting metabolic rate (RMR) will be measured using indirect calorimetry (45). Subjects will recline in a hospital bed during this test. When the rate of oxygen consumption changes by less than 2% over a five minute period, it will indicate that the RMR has been achieved.

- **Blood and urine tests**: each subject will have labs drawn following their 8-hour overnight fast. These include a complete metabolic panel (screening for hepatorenal disease), hemoglobin (screening for anemia), c-peptide, insulin, HbA1c, lipid panel, and urine pregnancy test (for females).

- **DEXA:** Lean body and fat mass will be measured using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy, enCore software version 10.5, GE Medical Systems).

- **Peripheral artery tonometry**: Reactive hyperemia-periopheral artery tonometry (RH-PAT) endothelial function testing will be used (Endo-PAT, Itamar Medical Ltd.) to assess risk of endothelial dysfunction and future premature cardiovascular disease. The subject will sit in a reclining chair with the hands at heart level and propped in a comfortable position such that the fingers are hanging freely. Fingertip probes are placed on both index fingers and pulse wave amplitudes are recorded for the duration of the study. After 5 min of baseline measurement, arterial flow to the non-dominant arm is occluded for 5 min using a BP cuff inflated to 40 mmHg above systolic pressure. After the 5-min occlusion, the cuff is rapidly deflated to allow for reactive or flow-mediated hyperemia. Pulse wave amplitudes are recorded for at least 5 min after the cuff is deflated. An integrated software program compares the ratio of arterial pressure in the two fingers before and after the occlusion to calculate the RH-PAT score.(46)

- **VO_2 max Testing:** Exercise testing will be performed to assess VO_2 max.The Bruce protocol will be used, as described elsewhere (47). Participants will be asked to perform a progressive treadmill test for about 10 minutes (or until fatigued). Attainment of a valid measure for VO_2 max will be if two of the following three criteria are met: 1) a respiratory exchange ratio of 1.1 or greater, 2) attainment of a maximal measured heart rate within 10 beats/ min of the age predicted maximum (220-age), or 3) no change in oxygen consumption despite an increase in work load. T1DM participants will check their blood glucose prior to exercising. If glucose is < 120 mg/dL, a 15-30 gm carbohydrate snack will be provided.

Following VO_2 max testing, the subject will be provided with a meal from the CRC kitchen. T1DM subjects will be instructed to resume their routine insulin regimen. Patients will then be discharged home. Once the data is collected from the screening visit and inclusion/exclusion criteria are confirmed as being met, the subject will be contacted. Study visit 2 will be scheduled within one month of study visit 1.

Premenopausal females will be scheduled for their study during the follicular phase of their menstrual cycle (day 2-10) to reduce a potential confounder of insulin sensitivity.(48)

T1DM and MODY2 subjects will be asked to perform an 8-point self-monitored BG profile (before meals, 2 h. post-prandial, bedtime, and 2 AM) for three days leading up to the clamp study. These participants will record this information on a glucose log designed for the study (see appendix). All participants will refrain from vigorous exercise and consume an isocaloric diet. MODY2 subjects from out of the region (if needed) may be required to consume the standard diet for less than 3 days to accommodate travel needs.

6.2.2 Study visit 2: Hyperinsulinemic, euglycemic clamp

Study subjects will undergo a two-step clamp as depicted in **figure 2**.

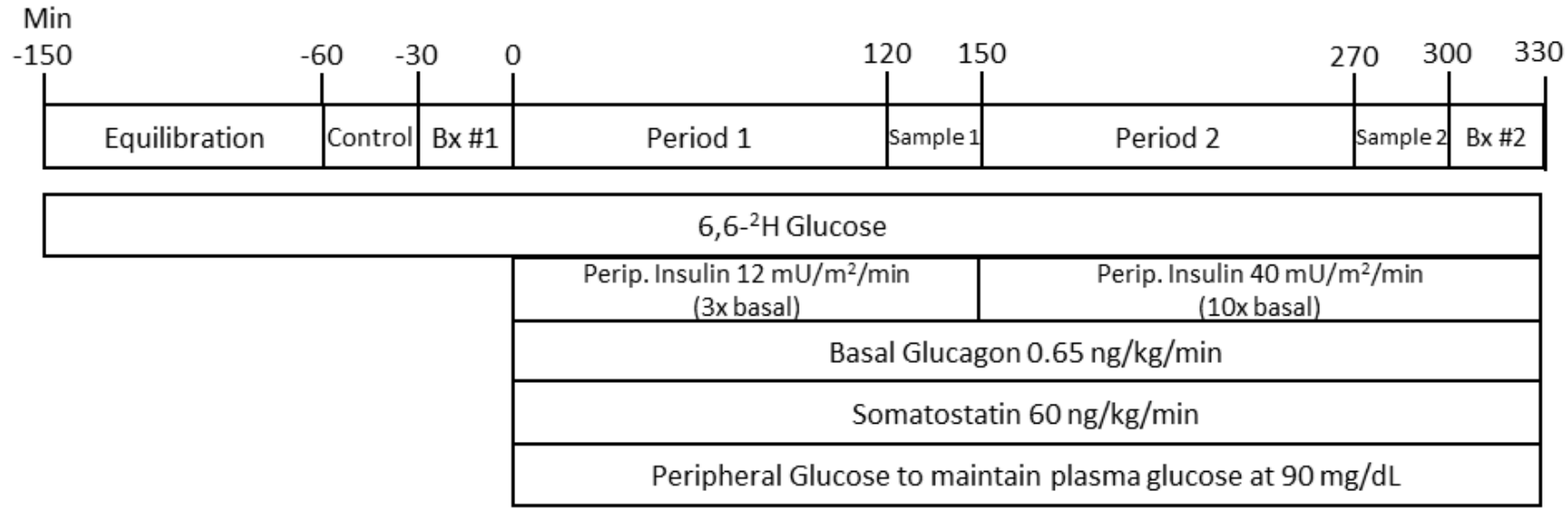

Figure 2: Hyperinsulinemic, euglycemic clamp protocol. Insulin infusion rates are predicted to cause a 3 and 10-fold rise in plasma insulin levels in control subjects. In a 70 kg, 1.73 m^2 individual, 12 $mU/m^2/min$ would approximately equal 0.3 mU/kg/min and 40 $mU/m^2/min$ would approximately equal 1.0 mU/kg/min.

6.2.2.1 Overnight CRC stay

Subjects will be admitted to the Vanderbilt CRC on the night before the study and begin fasting except for water at 10 PM. All female subjects will have a second hCG measured to confirm the absence of pregnancy upon arrival to the CRC.

T1DM and MODY2 subjects will then be given a continuous IV infusion of regular human insulin (recombinant DNA origin), titrated by the protocol of White et al. (49) as shown in **Table 3**.

Table 3: Algorithm for Maintenance of Overnight Euglycemia

Glucose reading (mg/dL)	Insulin Infusion rate (U/h)
above 250	5
201-250	4
171-200	3
141-170	2
121-140	1.5
101-120	1
81-100	0.8
61-80	0.5
under 60	0

Glucose will be checked on an hourly basis using a calibrated, hospital bedside glucose monitor. Testing this protocol in 25 subjects with T1DM, White et al. reached and maintained near-euglycemia (101 ± 2 mg/dL) in the fasted state. Glucose fell to below 60 mg/dL on six occasions and to below 50 mg/dL on only one occasion. T1DM and MODY2 study patients should be at euglycemia when the clamp study begins (matching them with non-diabetic controls) and the overnight-titrated fasting insulin infusion rate should approximate the peripheral insulin infusion rate needed for euglycemia in each patient. Should glucose fall to < 75 mg/dL the PI may elect to infuse a small dose of 20% dextrose to prevent hypoglycemia. Should glucose fall to < 55 mg/dL and the patient experience symptoms of hypoglycemia, 20% dextrose will be at bedside to treat the hypoglycemia (e.g. 75 mL of 20% dextrose delivers 15 gm of glucose). If glucose were to fall to < 55 mg/dL, the following morning's clamp study would need to be postponed to a later date

because of the confounding influence of the counter-regulatory response to hypoglycemia.

The PI will be physically present in the CRC for at least the first 3 overnight insulin infusions (T1DM or MODY2 subjects) to monitor and adjust the insulin infusion from the protocol if needed. Thereafter, the PI and CRC nurse manager will confer on the need for the PI (or a qualified M.D., N.P., or P.A.) to be physically present for the overnight insulin infusions based on the safety profile of the preceding IV insulin infusions. If the PI is not physically present, he will be readily available via telephone pager and will be within 20 minutes of the CRC.

Control subjects will be admitted on the night prior to the study, begin fasting at 10 PM, but no insulin will be infused until the start of period 1.

6.2.2.2 Hyperinsulinemic, euglycemic clamp

As depicted in figure 2, each experiment will consist of:

1. a 90 min equilibration period for [6,6-^{2}H]-glucose,
2. a 30 min basal sampling period (control),
3. a 30 min period to obtain muscle biopsy (Bx) #1; then
4. 2 consecutive, 150 min experimental periods where increasing IV infusions of regular human insulin are given, and
5. a 30 min period for Bx #2.

Somatostatin will be infused into a peripheral vein to allow for a pancreatic clamp. Simultaneously glucagon will be infused into a peripheral vein at a rate chosen to approximate basal concentrations of glucagon at the liver, preventing a fall in glucagon that would otherwise occur secondary to the hyperinsulinemia and somatostatin. As noted previously, this allows for insulin and glucagon concentrations to be identical between test subjects, enabling a direct assessment of hepatic insulin sensitivity (50). In the United States, numerous groups currently use somatostatin for the purpose of "clamping" pancreatic hormone secretion (30-36) and it is used extensively in Europe for the same purpose (37-41).

Plasma glucose will be monitored every 10-30 minutes during the equilibration, control, and Bx1 periods, then every 5-10 minutes during the 2 consecutive 150 min experimental periods and Bx2. The YSI Glucose Analyzer (Yellow Springs, OH), the gold standard instrument for glucose concentration analysis, will be used during the clamp study. IV glucose will be infused to maintain plasma glucose at approximately 90-100 mg/dL throughout the study. At nine points in the study (3 times each during the control, sample 1, and sample 2 periods as in figure 2), a larger blood draw will be made from a second intravenous catheter to determine plasma concentrations of the following:

- 6,6 2H-glucose
- Insulin
- Catecholamines
- Glucagon
- Cortisol
- C-peptide
- Free Fatty Acids
- Lactate, glycerol, alanine (and possibly beta-hydroxybutyrate)
- Triglycerides

These blood draws are calculated to equal just under 160 mL in total blood volume. This includes the volume of blood that is wasted to clear the IV line with each blood draw.

6.2.2.3 Muscle biopsy

Subjects will be asked to allow the study team to perform two muscle biopsies during the study. If the subjects decline the muscle biopsy, they will still be allowed to participate in the hyperinsulinemic, euglycemic clamp.

The muscle biopsies, Bx #1 and #2, will be obtained immediately before study period 1 and immediately following study period 2, respectively (figure 2). Percutaneous biopsies will be performed on the lateral portion of the quadriceps femoris muscle (vastus lateralis) using sterile technique. A minimum distance of 2 cm proximal or distal between the two sampling sites will be used to minimize specimen artifact caused by trauma, inflammation, and/or scarring. Local anesthesia with lidocaine 0.5-1% will be applied in two steps: first within the dermis and second into the muscle fascia before the biopsy is obtained. A 4-6 mm

incision through the skin will be made with a scalpel. Then we will insert a trocar into the incision site, pushing through the muscle fascia, and advance to ensure that the cutting chamber lies fully within the muscle. We will apply suction to withdraw the muscle tissue into the cutting chamber and then we will rotate the trocar clockwise 90 degrees to maximize the amount of muscle sample obtained. We will place sterile gauze and apply pressure with a cold compress for 10 minutes to minimize bleeding, inflammation, and pain. During and after the procedure, participants will be allowed to take acetaminophen on an as needed basis to minimize pain under the supervision of the PI. Muscle samples will be immediately blotted free of visible non-muscle tissue, flash frozen in liquid nitrogen and stored at -80°C until analysis. Samples will be analyzed using immunoprecipitation/immunoblot analysis.

6.2.2.4 Completion of clamp study

Upon completion of the study, insulin will be discontinued and the study subject will be allowed to eat. Plasma glucose will be monitored every ten minutes for an additional hour and the glucose infusion rate will be steadily decreased to maintain glucose > 120 mg/dL. Patients taking insulin at home will be instructed to resume their home regimen, however they will be instructed to check glucose before dinner, bedtime, and at 2 AM following the study. These glucose readings will be recorded on a 24-hour post-study glucose log (see appendix). If glucose is < 120 at any of these checks, they will consume an additional 15-30 gm of glucose with 5-15 gm of protein to prevent hypoglycemia. As an example one Luna bar has 26 gm of carbohydrates and 9 grams of protein. By the following morning, risk of hypoglycemia as a result of participating in the clamp study will be no greater than their baseline risk.

After local anesthesia from the muscle biopsy wears off, the subject may experience soreness for less than a week; during this time the study team will recommend subjects use acetaminophen 650 mg every four hours as needed for pain or discomfort, with no more than 2,000 mg in a 24 hour period. The study team will instruct the participants and parents of adolescent participants on post-procedural wound

management. We will recommend that subjects minimize walking on the day of discharge from the study; activity should gradually be resumed following the procedure. Vigorous activity or sport should be avoided for the first few days and resumed cautiously as discomfort subsides. In addition to contacting the subject's family on the day following the study to review blood glucose numbers, the study team will check on the status of wound healing during the call on the day following the study as well as 5-7 days after the study. The subject will be instructed to notify their primary care physician if they have marked swelling and discomfort of the whole muscle (beyond the anticipated bruising); if the biopsy site is painful more than a week after the biopsy was taken; if the biopsy site appears swollen, purulent, or fluctuant; if there is persistent oozing from the biopsy site; or if a temperature of >= 102 degrees F develops.

6.2.2.5 Rescheduling clamp study after a technical issue

It is possible once a clamp visit has begun a technical issue will prevent the successful completion of the study. For example, IV access may be lost during the study or otherwise uneventful hypoglycemia (glucose < 55 mg/dL) may occur for those participants receiving an overnight IV insulin infusion (section 6.2.2.1). In the event such a technical issue occurs, the PI may elect to reschedule a repeat clamp study with the following stipulations:

A) The PI deems the technical issue poses no serious danger to the participant were it to occur again and the participant wishes to repeat the study
B) The rescheduled clamp occurs within six months of the original screening visit
C) An abbreviated screening procedure has been completed within one month of the repeated clamp visit. This abbreviated screening will re-assess inclusion/exclusion criteria (table 2) and safety to be restudied. The abbreviated screening will include:
    - Fasting blood tests: a complete metabolic panel (screening for hepatorenal disease), hemoglobin (screening for anemia), c-peptide, insulin, HbA1c, lipid panel
    - Urine pregnancy testing: on the night prior to the clamp in females

- History and Physical Exam: the participant's interval clinical history will be reviewed by the PI (or a designated KSP who is an M.D., Nurse Practitioner, or Physician's Assistant) on the evening prior to the clamp study and a physical exam will be performed. Anthropometric measurements will be repeated with the assistance of CRC staff or KSP.

7.0 Risks

During the participant consent/assent process study personnel will address potential discomfort and risks associated with the study protocol. These will be included in the consent/assent form.

The following risks associated with participation in this study are perceived as low:

- venipuncture and intravenous angiocatheter placement: possible hematoma, site infection, nausea, and vasovagal syncope. These risks will be minimized by performing the procedure with the subject seated and cleaning the site with the appropriate antiseptic prior to breaking the skin.

- DEXA scan: studies of the radiation dose to patients from a total body DEXA scan have confirmed that patient radiation exposure is small compared to many other sources of exposure. For the total body fast scan mode that will be employed the average skin entrance dose is 0.2 μSv (51, 52). By comparison a chest x-ray is associated with a patient dose of 50 μSv.

The following risks associated with participation are additionally considered:

7.1 Exercise testing

During the screening visit, subjects will perform an exercise test to measure VO_2 max. Because inclusion/exclusion criteria dictate that the subjects will be young (ages 13-35), not obese (BMI 20-28), and those patients with diabetes will have relatively ideal glycemic control (HbA1c 6.0-8.0%), the risk of a cardiovascular complication of exercise is considered to be low. The greatest risk of the exercise test is hypoglycemia in the T1DM patients who will have been fasting overnight. The patient will be asked to check their capillary glucose using their home meter immediately prior to exercise and if glucose is < 100 mg/dL, they will consume 15 gm of glucose immediately prior to the ~ 10 minute exercise test. Immediately following this, all subjects will be provided with a meal, further minimizing exposure to hypoglycemia subsequent to the brief exercise test.

7.2 Hyperinsulinemia

Subjects will be exposed to hyperinsulinemia during the clamp study, which could lead to hypoglycemia and hypokalemia. We will implement the following to minimize these risks:

- As shown in figure 2, the highest insulin rate used will be 40 mU/m^2/min. This is the most frequently applied insulin infusion rate in human hyperinsulinemic, euglycemic clamp studies.(53) This infusion rate is anticipated to result in plasma insulin concentrations between 50 and 130 μU/mL, (54) levels that are within the range of physiological hyperinsulinemia and comparable to concentrations seen after meal ingestion. (53, 55, 56) By comparison, the most commonly used insulin infusion rate in the treatment of diabetic ketoacidosis is 0.1 U/kg/hr.(57) In a 70 kg, 1.73 m^2 individual, this infusion rate would equal 67 mU/m^2/min, over 50% higher than the 40 mU/m^2/min rate used in this study.
- Glucose will be monitored every hour during the overnight basal insulin infusion as outlined in 6.2.2.1. Glucose will be monitored every 5-10 minutes throughout the hyperinsulinemic clamp to guide the variable dextrose infusion and ensure euglycemia. Because glucose is monitored so frequently, a trend toward hypoglycemia would most likely be detected before hypoglycemia actually occurred. If hypoglycemia did occur, however, this frequent monitoring would allow for rapid detection of the low glucose concentration and rapid corrective treatment with an infusion of IV glucose (dextrose).
- Concentrated dextrose will be at bedside to treat iatrogenic hypoglycemia. If hypoglycemia occurred it could rapidly be corrected. For example, a 75 mL IV bolus of 20% dextrose (15 gm of glucose) would be expected to bring glucose up above the hypoglycemic range in less than 5 minutes. This dextrose could be given into either of the two IV angiocatheters available during the study.
- A licensed MD, NP, or PA will be on call throughout overnight CRC stay and bedside during clamp procedure
- Potassium will be monitored using bedside istat monitor (and oral potassium replacement will be given as needed)
- Glucose will continue to be monitored following the study for at least 1 hour
- Patients will be required to consume mixed meal immediately following clamp procedure. T1DM subjects will not be discharged unless blood glucose > 120.
- Vital signs will be monitored throughout clamp procedure
- Subjects will be counseled on the risk of late hypoglycemia the evening and night following the clamp. They will be advised of the importance of checking their blood glucose at bedtime and during the night to maintain a minimum of 120 mg/dL

7.3 Muscle biopsy

Having a muscle biopsy may cause pain, soreness, bruising, or infection at the biopsy site. Every effort will be made to prevent or minimize these side effects of the muscle biopsy:

- the biopsy will be of a large muscle that is easily identified on physical exam: the vastus lateralus of each lower extremity
- sterile technique will be used to minimize risk of infection
- local anesthesia with lidocaine (0.5-1.0%) will be used prior to the incision
- after local anesthesia wears off, the subject may experience soreness for less than a week; during this time the study team will recommend subjects use acetaminophen 650 mg every four hours as needed for pain or discomfort, with no more than 2,000 mg in a 24 hour period.
- the study team will recommend that subjects minimize walking on the day of discharge from the study; activity should gradually be resumed following the procedure. Vigorous activity or sport should be avoided for the first few days and resumed cautiously as discomfort subsides.
- The subject will be instructed to notify their primary care physician if they have marked swelling and discomfort of the whole muscle (beyond the anticipated bruising); if the biopsy site is painful more than a week after the biopsy was taken; if the biopsy site appears swollen, purulent, or fluctuant; if there is persistent oozing from the biopsy site; or if a temperature of >= 102 degrees F develops.

7.4 Infusion of somatostatin

The following studies will utilize an IV infusion of the peptide hormone somatostatin, a hormone not approved by the FDA for use in humans, but commonly and safely used in human glucose metabolism research in the United States (30-36) and Europe (37-41). A modification of the glucose clamp, commonly referred to as the "pancreatic clamp," employs a short-term somatostatin infusion to suppress endogenous insulin and glucagon release while these hormones are simultaneously replaced at a prescribed infusion rate as shown in figure 2. Importantly, this approach ensures that insulin and glucagon, the key hormones influencing glucose metabolism, are equal between cohorts. This key aspect of the pancreatic clamp is essential for these metabolic studies as it allows for equal plasma levels of both insulin and glucagon between T1DM patients who are not capable of insulin secretion and non-T1DM subjects who are. Failing to equalize levels of these hormones between test subjects would confound the results considerably.

Other effects of somatostatin infusion include the inhibition of growth hormone secretion and the inhibition of gastric emptying. However, growth hormone is not known to be an acute modulator of glucose metabolism, thereby making this off-target effect of no meaningful consequence. Likewise, all subjects will be fasted while receiving the somatostatin infusion so its effect on gastric emptying will be of little consequence. We are not aware of somatostatin having been "withdrawn from the market" for any reason related to safety or effectiveness. Finally, somatostatin's short half-life (1-3 minutes, (58) the primary factor driving the development and clinical application of longer-acting synthetic

analogs such as octreotide) ensures that these suppressive effects are of very short duration after the infusion is discontinued.
Somatostatin used in these studies will be manufactured by Bachem Americas, Inc, in conformity with the requirements for Current Good Manufacturing Practice as specified in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Testing for determination of purity and uniformity was performed by HPLC. The final drug product will be composed of 2.5 mg of somatostatin dissolved in 50 mL NS. The drug substance will be received by Vanderbilt's Investigational Drug Service (IDS) as a powder and will be compounded to obtain the IV formulation. Vanderbilt's IDS has extensive experience managing and compounding investigational agents for FDA-regulated studies and follows stringent procedures for aseptic compounding that adhere to USP<797> guidelines. The final IV formulation will be compounded by dissolving somatostatin in NS to achieve a 50 µg/mL solution. The IV formulation will be prepared sterile by utilizing a sterile, 0.2 micron filter, transferred into either sterile syringes or IV bags, and labeled as an investigational product. Filter integrity will be verified via bubble point testing as described in USP<797>. Beyond use dating for the final product will be 24 hours at controlled room temperature. A representative batch of somatostatin 4.5 mg in 50 mL NS was formulated as specified and demonstrated to be sterile. A 1 mL sample from each drug product will be sent for endotoxin testing utilizing the Kinetic Turbidimetric Assay in compliance with USP<85>. An Investigational New Drug (IND) application for the use of somatostatin in this protocol is presently under review by the FDA (# 132209).

7.5 Infusion of glucagon

Glucagon is a peptide hormone secreted by the alpha cells of the endocrine pancreas. Its effect is generally opposite that of insulin as it raises hepatic glucose production by stimulating glycogenolysis, (59) a process that raises plasma glucose levels. It is FDA approved and commonly used in the treatment of hypoglycemia.

As noted previously, glucagon will be infused into a peripheral vein at a rate chosen to approximate basal concentrations of glucagon at the liver, preventing a fall in glucagon that would otherwise occur secondary to the hyperinsulinemia and somatostatin. This allows for insulin and glucagon concentrations to be identical between test subjects, enabling a direct assessment of hepatic insulin sensitivity.

Although rapid infusions of glucagon are commonly associated with nausea and headache, our infusion rate of 0.65 ng/kg/min has been shown to maintain glucagon at a consistent, basal concentration throughout pancreatic clamp studies. (33, 38, 60) Because glucagon will

remain at fasting levels throughout the study, these adverse effects are not anticipated.

While glucagon potently raises plasma glucose in the hypoglycemic state, its glucose-raising affect is attenuated in the euglycemic and hyperglycemic state. (61) For this reason and because of IV glucagon's short half-life (≈ 10 minutes), glucagon is not expected to cause iatrogenic hyperglycemia during or after the clamp study.

7.6 Special Protections for Adolescents as Research Subjects

Because adolescent subjects between ages 13-18 are included in the T1DM and MODY2 cohorts, special consideration has been given to provisions designed to protect children as outlined in 45 CFR 46, Subpart D.

7.6.1 Justification and rationale for inclusion of adolescent subjects with T1DM or MODY2

T1DM

Despite numerous advancements to reduce hyperglycemia in pediatric T1DM(62) since the publication of the groundbreaking Diabetes Control and Complications Trial in 1993,(63) macrovascular disease remains a profound contributor to excess mortality for the nearly 20,000 pediatric patients who are diagnosed with T1DM in the United States each year.(64) Among present-day adolescents with T1DM, the estimated loss in life expectancy is 11 years for males and 13 years for females compared to their non-diabetic counterparts.(65) Overall, the largest percentage in estimated loss of life expectancy was related to ischemic heart disease (36% in men, 31% in women).(65) Even contemporary T1DM patients with ideal glycemic control (indicated by an average HbA1c ≤ 6.9%) remain at a 3-fold increased risk from cardiovascular disease death.(66) Thus, despite major advancements in the treatment of T1DM over the past 2 decades, the risk of death from macrovascular disease remains profoundly high.

The atherosclerotic changes of macrovascular disease (e.g. increasing carotid intima media thickness) appear to begin in childhood and adolescence(67, 68) and morbidity and mortality risk is staggeringly high in younger adults.(69) As an example, among individuals ages 1-39, the standardized mortality risk (i.e. ratio between the observed number of deaths in a study population and the number of deaths that would be expected) for ischemic heart disease death is 8.9 (95% CI 6.2–12.9) in males

and an astounding 41.7 (95% CI 28.9–58.2) in females. Thus, it is clear that pathophysiologic mechanisms that contribute to macrovascular disease *are operative early in the natural history of T1DM*. These same early pathophysiologic mechanisms have not been identified, however.

IR is a consistent(8-12) and under-recognized finding in T1DM, even among patients who lack traditional IR risk factors (such as those commonly seen in type 2 diabetes). Because IR has been strongly and independently correlated with macrovascular disease in T1DM,(14-16) an increased understanding of its root cause is needed. While earlier investigations attributed T1DM IR to hyperglycemia, (17-22) more recent studies have shown little to no correlation between hyperglycemia and insulin sensitivity.(10, 11, 13, 23) Thus, the degree to which hyperglycemia contributes to IR in T1DM is unclear, and this uncertainty suggests that other factors contribute prominently. This study tests the hypothesis that *iatrogenic hyperinsulinemia* rather than *hyperglycemia* is the primary contributor to T1DM IR. To date *only one study* has examined pediatric T1DM patients with typical glycemic control, which found that adolescents were 37% less insulin sensitive than matched control subjects,(11) *suggesting that exposure to IR begins early in the natural history of T1DM.*

To determine the root cause of T1DM IR and translate this information into therapies that would ameliorate IR in T1DM and its contribution to macrovascular disease, the mechanisms causing IR must be assessed *early in the disease duration*. This is because:

1) T1DM IR appears to begin early in the disease process,(11, 17)
2) the incidence of T1DM onset is highest between ages 10-14,(70) and
3) at younger ages and shorter disease durations there are minimal confounding covariables that would contribute to IR at a molecular level. These confounding covariables include co-morbidities associated with T1DM such as diabetic nephropathy,(71) prolonged exposure to hypertriglyceridema,(72, 73) excessive BMI(74, 75) and visceral adiposity,(76, 77), deleterious effects of aging and progressive inflammation on extracellular matrix remodeling,(78, 79) and increasing age per se.(80, 81)

By studying young patients early in the disease duration, this investigation will not only more precisely determine whether *iatrogenic hyperinsulinemia* or *hyperglycemia* is the key modifiable risk factor to address in alleviating

T1DM IR, but will simultaneously define the mechanisms causing the IR at a molecular signaling level when confounding covariables are very minimal. This knowledge is critical towards guiding subsequent therapy to mitigate IR which will considerably reduce early mortality risk from macrovascular disease in the same subjects tested in the research.

By studying younger patients early in the disease process, this investigation will minimize the confounding effect of these comorbidities and allow a more precise determination of whether *iatrogenic hyperinsulinemia* or *hyperglycemia* is the key modifiable risk factor to address in alleviating T1DM IR. With this knowledge, subsequent therapy can be directed towards mitigating IR and in so doing considerably reduce early mortality from macrovascular disease. Additionally, it will establish the presence or absence of other risk factors for macrovascular disease (e.g. dyslipidemia, endothelial dysfunction measured by peripheral artery tonometry, reduced exercise capacity) at an early point in disease duration. These data would inform subsequent longitudinal studies of macrovascular disease progression as a future direction.

MODY2

MODY2 is a less prevalent(82) form of diabetes seen in pediatrics, characterized by mild fasting hyperglycemia (110-145 mg/dL)(83) and mild chronic hyperglycemia (mean HbA1c 6.4-7.6%).(82, 84) An assessment of IR using the hyperinsulinemic, euglycemic clamp technique in this patient group has only been attempted once, was with technical limitations, and studied only one patient younger than age 18.(85) The proposed study will therefore considerably increase our knowledge of IR in MODY2 and represents the first-ever study of IR in this condition in the adolescent age group.

As with adolescent T1DM subjects, this investigation of adolescents MODY2 will determine if IR is present when other, age-related confounding covariables that might influence IR are minimized. It will also provide valuable information about the presence or absence of other macrovascular disease risk factors at a relatively early point in the natural history of the condition.

With these considerations in mind, this study proposes to test diabetic subjects in **adolescence** (ages 13-18, Tanner stage 5) and young adulthood (age 18-35).

7.6.2 Assessment of risk to adolescent participants relative to 45 CFR part 46, subpart D

It is our impression that T1DM and MODY2 subjects would fall under 45 CFR 46.406, as outlined in Vanderbilt's Human Research Protections Program Policy Number IX.A, section II.C. As discussed in section 7.6.1, the proposed research is likely to yield generalizable knowledge about these adolescents' condition. Specifically,

1. The risk represents a minor increase over minimal risk.
    - The hyperinsulinemic, euglycemic clamp has been used safely and extensively in children and adolescents elsewhere. The technique has been performed over one thousand times in healthy adolescent subjects at other institutions such as the University of Minnesota,(86-89) hundreds of times in overweight adolescent(90-92) and normal weight prepubertal subjects(93, 94) at the University of Pittsburgh, and a similar number of times in normal weight children and adolescents at the National Institutes of Health.(95, 96) Our insulin maximal infusion rate of 40 mU/m^2/min is approximately equal to the rate used in studies of healthy subjects at each of these institutions and half that used in overweight subjects studied at the University of Pittsburgh.
    - As outlined in section 7.2, extensive efforts have been employed to minimize risk of hypoglycemia and hypokalemia associated with hyperinsulinemia.
    - Although use of the "pancreatic clamp technique" employed here is not used in adolescent studies at these institutions, it is commonly in adult studies elsewhere with no reported adverse effects.(30-41) Finally, as outlined in sections 7.4 and 7.5, every effort has been made to minimize any risks associated with the infusion of somatostatin and glucagon.
    - The study will infuse a stable isotope (deuterium) instead of a radioactive isotope (tritium) as a glucose tracer.
    - The PI has personally performed the hyperinsulinemic, euglycemic clamp over 50 times in human subjects with T2DM and a similar number of times in canine studies.
2. The procedure presents experiences to participants that are reasonably commensurate with those inherent in their actual or expected medical situation.
    - Hypoglycemia is a fact of life for most patients with insulin-treated diabetes. The average patient with T1DM experiences two episodes of hypoglycemia per week.(87) Every effort will be made to prevent

hypoglycemia from occurring during the clamp study at all, however.

3. The procedure is likely to yield generalizable knowledge about the participants' disorder or condition which is of vital importance for the understanding or amelioration of the participants' disorder or condition.
    - As previously discussed, the proposed research will provide very important information about the root cause of IR in T1DM and possibly in MODY2, which in turn contributes considerably to macrovascular disease mortality.
4. Adequate provisions are made for soliciting assent of the children and permission of their parents or legal guardians as detailed in the present IRB application.

The study team will discuss all procedures, risks, and benefits with potential study participants, as part of the consent/assent process. An IRB approved written informed consent/assent document will be required for participation in this study. It is understood that consent/assent is a process and not a discrete event. A participant's decision to withdraw consent/assent will be respected throughout the duration of each subject's participation in this study. It is also understood that there may be as-yet unknown or unanticipated adverse effects of this study. The study team will continually monitor for these effects and consider altering the protocol as needed to ensure patient safety. Changes in the procedures of the study, as well as any change(s) in the risks and/or benefits will be presented to and discussed with the subjects upon approval from the IRB for implementation of such revision(s), and any IRB revised written consent will be signed, as appropriate.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

An AE will be considered to be any untoward medical occurrence in a subject, not necessarily having a causal relationship with the study. The PI will continually monitor for any AEs.

AEs will be graded according to following scale:

0 = No adverse events
1 = Mild: no limitation of usual activities, did not require treatment
2 = Moderate: some limitation of usual activities, resolved with or without treatment,
3 = Severe: inability to carry out usual activities, requiring medical intervention
4 = Life-threatening or disabling
5 = Death

Hypoglycemia and hyperglycemia will be reported as adverse events only in the case of requiring the assistance of others due to loss of consciousness or DKA. These will be considered serious adverse events (SAEs).

A member of the study team will contact each subject on the afternoon following the clamp study. Subjects who underwent a muscle biopsy will be asked to grade their pain on a 1-10 scale. Subjects taking insulin will be asked to report their 24-hour post-study glucose log numbers. The PI will continuously monitor these post-study data for hypoglycemia and alter the procedure for preventing hypoglycemia described in section 6.2.2.4 if needed.

Consistent with FDA guidelines, SAEs will be defined as any untoward medical occurrence that:

- requires inpatient hospitalization
- results in persistent or significant disability
- is suspected to cause a congenital anomaly or birth defect in a subject's unborn child
- is life-threatening
- results in death
- is considered to be an important medical event based on appropriate medical judgement (e.g. bronchospsasm requiring emergency department referral, seizures that might not result in hospitalization).

Additionally, an SAE's relationship to the study procedures will be assessed and graded as either: not related, unlikely, possible, probable, or definite.

Any AE will be assessed for whether or not it was an anticipated problem. In accordance with DHHS guidance and consistent with 45 CFR part 46, an "unanticipated problem" will include any incident, experience, or outcome that meets all of the following criteria:

1. unexpected (in terms of nature, severity, or frequency) given
    a. the research procedures that are described in the protocol-related documents, including the IRB-approved research protocol and informed consent document; and
    b. the characteristics of the subject population being studied; related or possibly related to participation in the research; and
2. suggests that the research places subjects or others at a greater risk of harm.

3. related or possibly related to participation in the research;

4. suggests that the research places subjects or others at a greater risk of harm than was previously known or recognized.

All unanticipated, non-serious AEs and the study team’s response to the non-serious AE will be included in a report at the time of annual continuing review. The PI will review the AEs and notify the Data Safety Monitor (DSM), Dr. Kevin Niswender, and the IRB of any changes needed to the protocol. If needed, appropriate changes will be made to the consent form.

In accordance with IRB policy, any unanticipated SAE that is considered possibly related to participation in the study will be reported within 7 calendar days of the PI's

notification of the event to the IRB and DSM. The study team will continue to follow or obtain documentation of the resolution of any SAE.

The annual summary of all unanticipated adverse events and any audit reports will be sent to the IRB at the time of continuing review. A copy of this report will also be sent to the NIH who currently funds the PI on a K12 grant (and in the future will fund on an individual K-grant) with the Research Performance Progress Report.

Data and safety monitoring activities for this study will continue until all subjects have completed their participation and until a sufficient amount of time has passed beyond which any study-related AEs are unlikely.

This protocol will be reviewed annually (at a minimum) by the Vanderbilt IRB. The goal of this process is to determine the risks and benefits of the study in the actual experience of subjects and that measures taken to minimize risks are adequate.

9.0 Study Withdrawal/Discontinuation

Subjects will be free to withdraw from the study at any time, which will be made clear at enrollment. Subjects will be withdrawn from the study if:

- Pregnancy is detected
- The PI's medical judgement is that participation places the subject at risk for harm

10.0 Statistical Considerations

- Sample size: The sample size in this cross-sectional study design (10 per control group, 16 per T1DM group, and 10 per MODY2 group) was calculated to detect a 40% difference in mean glucose update (R_d) between MODY2 and T1DM subjects during maximal insulin stimulation (sample period 2) with an α-level of 5% and 80% statistical power. The R_d variance and R_d for well controlled T1DM subjects utilized in sample size calculations were taken from Bergmann et al. where a 55% difference in R_d was seen between T1DM and control groups.(10)

- Primary outcome: Differences in R_d during maximal insulin stimulation (sample period #2) between MODY2 and T1DM subjects will be evaluated using the Mann-Whitney U test, presuming a non-parametric distribution in the data. Differences in R_d between MODY2 and control subjects and between T1DM and control subjects will be similarly determined.

- Secondary outcome: The differences in suppression of glucose production (ΔR_a), suppression of NEFA, and suppression of glycerol between each of the 3 subject groups will be assessed using the Mann-Whitney U test as for the primary outcome.

- Timeline and interim analysis: I plan to study 18 subjects in year 1 and 12 additional subjects in a second year of funding. One interim assessment of the

primary outcome (R_d) will be made after 5 studies per group before the end of the year 1 funding period. Obrien-Fleming bounds for a two-sided test with α-level of 5% and a critical z-value of 2.80 (corresponding to a p-value of 0.0051) will be used to assess whether the study can be ended at interim analysis if there were a large difference in R_d between T1DM and MODY2 groups. At this point I will also analyze the variance in R_d and adjust the sample size if needed for subsequent studies in year 2

11.0 Privacy/Confidentiality Issues

A database will be designed for this study using REDCap (Research Electronic Data Capture) tools. REDCap is a secure, web-based application designed to support data capture for research studies, providing validated data entry, audit trails, seamless data downloads to common statistical packages, and mechanisms for importing data from external sources. It will reside on a secure server with access provided exclusively to the research personnel. Subjects will be identified with a study identification number. A key to the subject identification number will be kept in a separate locked file drawer to which only the Principal Investigator and research coordinators have access. Reports will thereby be generated without Protected Health Information (PHI) data, and access will be restricted so that statisticians, etc. don't have access to all data.

Risk of leakage of PHI is minimized by keeping paper records in a locked cabinet and maintaining computerized records in the password protected REDCap data base. The principal investigator and the research staff are trained in HIPAA privacy regulations. The participant's identification is concealed, and a number is used as the identifier instead of the subject's name. Only the principal investigator or members of the research team will have the list of study patient's names as the correlate with the study number.

12.0 Follow-up and Record Retention

The study is anticipated to last for two years. The study results will be maintained indefinitely for research purposes.

13.0 References

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Appendix A: Pre-Clamp Glucose Log (image smaller to fit page)

PRE-CLAMP GLUCOSE LOG **SUBJECT ID: X** **TOTAL CALORIES PER DAY: 2926**

DATE	BREAKFAST				LUNCH				DINNER				BEDTIME SNACK & OVERNIGHT			
	PRE-BG	TIME	CARBS (G)	CALORIES	PRE-BG	TIME	CARBS (G)	CALORIES	PRE-BG	TIME	CARBS (G)	CALORIES	BEDTIME-BG	TIME	BEDTIME CARBS	CALORIES
	POST-BG	TIME	INSULIN (U)		POST-BG	TIME	INSULIN (U)		POST-BG	TIME	INSULIN (U)		2 AM-BG	TIME	BEDTIME INSULIN	
EXAMPLE	120	7:00 AM	50	1219	150	12:00 PM	60	1126	100	6:00 PM	90	260	150	10:00 PM	0	321
	180	9:00 AM	5		200	2:00 PM	6		130	8:00 PM	9		120	2:00 AM	0	
2 DAYS BEFORE																
DAY BEFORE																
ADMIT DAY																

DATE	ADDITIONAL CALORIES & BG CHECKS				NOTES
	BG	TIME	CALORIES	TIME	
	BG	TIME	CALORIES	TIME	
EXAMPLE	50	11:00 PM	1583	3:00 PM	
			814	11:00 PM	
2 DAYS BEFORE					
DAY BEFORE					
ADMIT DAY					

Abbreviations: BG = Blood Glucose (blood sugar)
G = grams
U = units

Instructions to study participants: For 3 days leading up to your overnight stay in the hospital ("ADMIT DAY") you have been asked to check your blood sugar (glucose) 8 times each 24 hour period. With each of the 3 daily meals, please check your blood sugar before you begin eating and two hours after you begin eating. Please check your sugar before going to bed also. If you have a bedtime snack, please check your blood sugar right before the bedtime snack. Finally, please check your blood sugar at 2 AM. These blood sugar numbers and the time they were checked should be written down in the table above. At the top of the table, you will find an example of how this should be done. Please include your insulin doses and the grams of carbohydrates in each meal.

Please note the "total calories per day" on the top line above. Your goal is to budget your food intake to try and match the number of calories you eat each day to this number.

If you need to check your blood sugar more often (if you have a low blood sugar, for example) please record the time and blood sugar number in the lower table. If you have an additional snack, please note the calories in the lower table. Remember, try to budget your eating to match the number of calories allowed per day.

Appendix B: Post-Clamp Glucose Log (image smaller to fit page)

POST-CLAMP GLUCOSE LOG **SUBJECT ID:** **X**

DATE	BREAKFAST			LUNCH			DINNER			BEDTIME SNACK & OVERNIGHT		
	PRE-BG	TIME	CARBS (G)	PRE-BG	TIME	CARBS (G)	PRE-BG	TIME	CARBS (G)	BEDTIME-BG	TIME	CARBS
			INSULIN (U)			INSULIN (U)			INSULIN (U)	2 AM-BG	TIME	INSULIN
EXAMPLE	120	7:00 AM	50	150	12:00 PM	60	100	6:00 PM	90	150	10:00 PM	0
			5			6			9	120	2:00 AM	0
CLAMP DAY												
DAY AFTER												

HYPOGLYCEMIC EPISODES

DATE	TIME	BG	COMMENTS

Abbreviations:
BG = Blood Glucose (blood sugar)
G = grams
U = units

Instructions to study participants:
After going home, you should resume your routine medication regimen.
In the 24 hours following the completion of the clamp study, we would like for you to monitor closely for low blood sugar.
After going home, at a minimum, please check your sugar at bedtime, at 2 AM, at breakfast, and at lunch. Please check sugar before driving. If your blood sugar is less than 120 mg/dL, eat an additional 15-30 grams of carbs with 5-15 grams of protein. As an example, one Luna bar has 26 grams of carbs and 9 grams of protein. A package of 6 peanut butter and crackers has 24 grams of carbs and 5 grams of protein.

Please write your blood sugar numbers down using the tables above. We will call you approximately 24 hours after you have been discharged. We will ask you to tell us these blood sugar numbers over the phone.