Determination of Iatrogenic Hyperinsulinemia's Contribution to Insulin Resistance and Endothelial Dysfunction in Type 1 Diabetes

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1.0 Background

Macrovascular disease is the leading cause of mortality in T1DM and is evident early in the condition's progression. Despite numerous advances(1) to reduce hyperglycemia in T1DM over the past 25 years, coronary arterial disease (CAD) remains a profound contributor to excess mortality in T1DM.(2) 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.(3) Ischemic heart disease contributes the greatest percentage in estimated loss of life expectancy (36% in men, 31% in women).(3) Even contemporary T1DM patients with glycemic control at target (i.e. HbA1c ≤ 6.9%) remain at a 3-fold increased risk from cardiovascular disease death.(4) The atherosclerotic changes of macrovascular disease begin in childhood and adolescence(5-9) and younger adults carry an extremely marked mortality risk.(10-13) Patients with disease onset before age 10 have a 30-fold higher risk for CAD above matched controls without T1DM.(14) Thus, pathophysiologic mechanisms that contribute to macrovascular disease appear early in the course of T1DM even when glycemic control is at target, making early therapeutic interventions necessary.

Hyperinsulinemia is an unintended consequence of peripheral insulin delivery and may be the root cause of a significant portion of macrovascular disease burden in T1DM. Because peripheral insulin delivery bypasses first-pass hepatic extraction, from the moment therapy begins patients with T1DM will have basal insulin concentrations that are ≈2.5-fold higher in the peripheral circulation than matched controls with equivalent glycemia.(15-18) In large epidemiologic studies, hyperinsulinemia is a predictor for ischemic heart disease in nondiabetic individuals, independent of concomitant risk factors such as dyslipidemia and obesity.(19) This study will determine how much iatrogenic hyperinsulinemia is pathophysiologically linked with IR (Aim 1) and endothelial dysfunction (Aim 2) early in the course of T1DM. It will also test how much an intervention to lower hyperinsulinemia—a low carbohydrate diet—can improve IR and endothelial dysfunction (Aim 3).

Iatrogenic hyperinsulinemia's contribution to IR early in the course of T1DM is unestablished. IR is independently correlated with macrovascular disease in T1DM. (20-22) My previous human subjects research strongly implicated iatrogenic hyperinsulinemia as the primary driver of IR in T1DM (see preliminary data). This work aligned with that of others who induced chronic hyperinsulinemia using differing approaches: overexpressing insulin gene expression the mouse(23, 24), delivering excess insulin into the hepatic portal vein of dogs(25), and infusing insulin into a peripheral vein of healthy humans for 2-3 days(26, 27). A common theme throughout these studies is that chronic hyperinsulinemia evokes a downregulation of insulin sensitivity. Although Yki-Järvinen et al. found insulin sensitivity improved during the partial clinical remission (PCR, a.k.a. "Honeymoon") phase, the contribution of decreased iatrogenic hyperinsulinemia to the enhanced insulin sensitivity adjusted for other covariates has not been examined.(28) We will determine this relationship in Aim 1.

Iatrogenic hyperinsulinemia's contribution to endothelial dyfsunction early in the course of T1DM is uncharacterized. Impaired endothelial function has been consistently observed in prepubertal,(29) midpubertal,(6) and postpubertal pediatric patients,(8) as well as young(30) and middle-aged(7) adults with T1DM. Several lines of research suggest insulin may exert beneficial vascular effects by increasing endothelial nitric oxide (NO) synthase gene expression(31) and activity(32, 33) and stimulating NO release and vasodilation.(34-36) On the other hand, hyperinsulinemia may exert detrimental vascular effects by amplifying mitogenic activity of growth factors, increasing expression of endothelial adhesion molecules, and enhancing monocyte adhesion to the endothelium.(34, 37, 38) Induction of hyperinsulinemia in healthy subjects to concentrations matching basal insulin levels typically seen in T1DM drastically diminished flow-mediated dilation (FMD), a frequently-used technique to quantify endothelial function.(39) Further study suggested this effect was mediated *independent of insulin sensitivity*.(34) Thus, my primary outcome in Aim 2 will determine how much clinically driven changes in peripherally delivered insulin doses modify brachial artery endothelial function during the early course of T1DM. As a secondary objective of Aim 2, I will use the contrast-enhanced ultrasound (CEUS) technique(40) to measure insulin-induced microvascular recruitment in adult participants.

Therapies to lower hyperinsulinemia-mediated IR and endothelial dysfunction in T1DM are limited. To date, no study has attempted to improve IR or endothelial dysfunction by lowering the total daily dose of insulin ($TDD_{insulin}$). The dearth of these studies is partially related to concern that lowering $TDD_{insulin}$ would worsen hyperglycemia. A diet that is lower in carbohydrates, however, is one approach to safely lower $TDD_{insulin}$ without raising glycemia. Thus, in Aim 3, we will conduct a 2x2 crossover intervention study testing whether the reduction in $TDD_{insulin}$ over a one-week low-carbohydrate diet (LCD) intervention will result in improved insulin sensitivity compared with one-week standard carbohydrate diet (SCD).

2.0 Rationale and Specific Aims

Insulin resistance (IR) is consistently found among patients with type 1 diabetes (T1DM) and strongly links T1DM with atherosclerotic disease.(9, 20-22, 41-46) IR and nascent atherosclerosis, as characterized by endothelial dysfunction, are present early in T1DM.(6-9, 47) Coronary artery disease (CAD) is the leading cause of death in T1DM, (10, 13, 48) but the early contributors to the pathologic processes underlying this morbidity are not well-characterized.

We have shown that IR in T1DM is minimally attributable to hyperglycemia, but is closely related to iatrogenic hyperinsulinemia. From the time of diagnosis, T1DM therapy relies on "unphysiologic" insulin delivery, where insulin injection into subcutaneous tissue rather than more physiologic delivery directly into the hepatic portal circulation results in

chronically elevated peripheral insulin levels. In the physiologic state, the liver clears ≈50% of secreted insulin before it reaches the peripheral circulation. As a result, hepatic insulin levels are normally ≈2-3 fold higher than insulin levels at peripheral tissues. By contrast, in T1DM injected insulin is directly absorbed into the peripheral circulation. Thus, patients with T1DM have basal insulin concentrations that are ≈2.5-fold higher in the peripheral circulation compared to non-diabetic individuals with matched glycemia.(49-51)

Because 1) hyperinsulinemia is closely associated with IR (23-27, 52), 2) exposure to iatrogenic hyperinsulinemia begins at diagnosis, and 3) hyperinsulinemia is an independent risk factor for CAD in the nondiabetic population,(19) the present proposal will test the hypothesis that iatrogenic-induced hyperinsulinemia independently correlates with IR and endothelial function early in the course of T1DM.

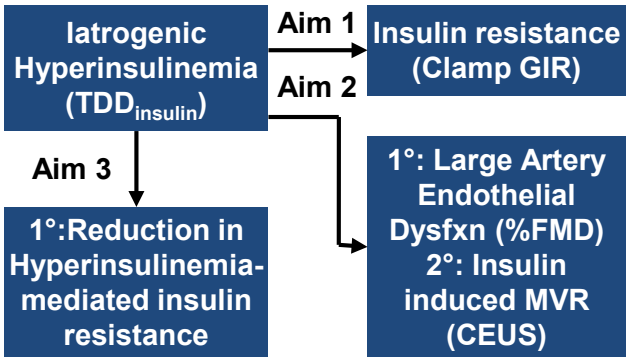


Figure 1: Correlations tested in Specific Aims

GIR=glucose infusion rate; 1°= primary outcome; 2°= secondary outcome; %FMD=% change in flow-mediated vasodilation; MVR=microvascular recruitment

To test this hypothesis, I will analyze the relationship (figure 1) between hyperinsulinemic exposure (quantified by average total daily dose of insulin, $TDD_{insulin}$) and insulin sensitivity (Aim 1) and early endothelial dysfunction (Aim 2) at multiple points in the progression of T1DM (figure 2). In a longitudinal study (T1DM SUBSTUDY in figure 2), I will study patients at three phases over the 12 months following diagnosis, initial diagnosis, honeymoon phase, and post-honeymoon phase. Each phase has distinct insulin exposure: 1) soon after diagnosis (STUDY1: $TDD_{insulin}$≈0.5 units/kg/day), 2) during partial clinical remission (PCR), a.k.a. "Honeymoon phase" (STUDY2: $TDD_{insulin}$<0.4 units/kg/day), and 3) after emergence from PCR (STUDY3: $TDD_{insulin}$>0.6 units/kg/day). Additionally, we will study healthy, matched, euglycemic control participants to assess the contribution of hyperinsulinemia to IR under four experimental conditions (CONTROL SUBSTUDY in figure 2): (i) endogenous insulin only (STUDY 4), (ii) overnight hyperinsulinemia (IV insulin infusion, STUDY 5), (iii) one week of mild hyperinsulinemia (glargine, STUDY 6), and (iv) both overnight insulin and one week of hyperinsulinemia (STUDY 7). To further test this hypothesis, I will conduct a 2x2 crossover intervention study in adults with T1DM testing whether the reduction in $TDD_{insulin}$ associated with a one-week, isocaloric low-carbohydrate diet (LCD) intervention will result in improved insulin sensitivity compared with one-week, isocaloric, standard carbohydrate diet (SCD) (STUDY B-C, figure 3).

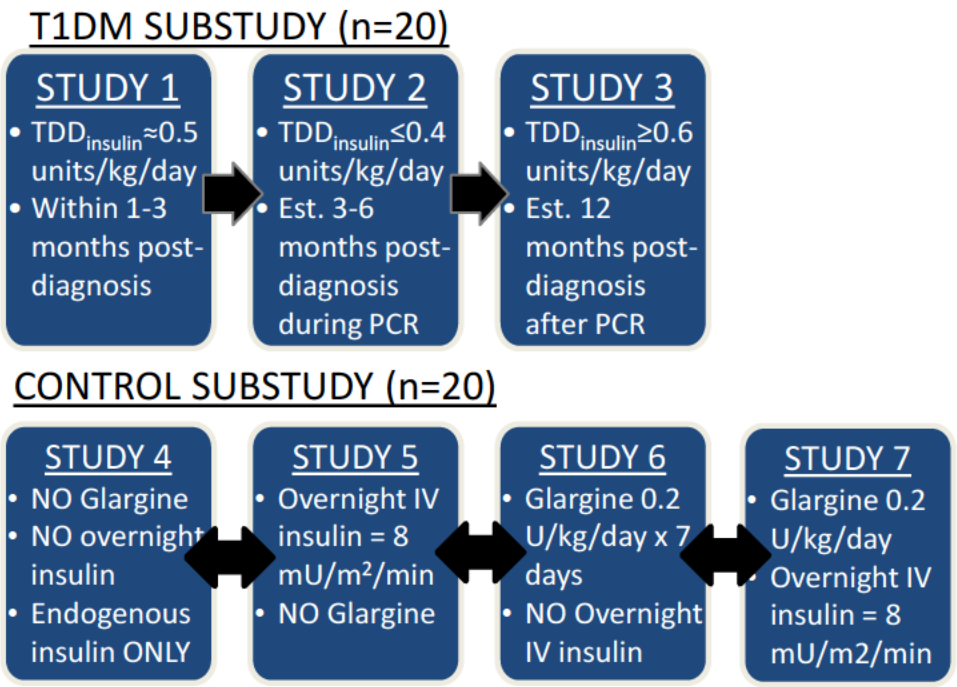


Figure 2: Schematic for STUDY visits. Est. = estimated. PCR=partial clinical remission. TDD$_{insulin}$ = total daily dose of insulin

Specific Aim 1: Quantify the contribution of iatrogenic hyperinsulinemia to IR during the early course of T1DM. The hyperinsulinemic, euglycemic clamp technique will quantify insulin sensitivity at each STUDY visit. Although insulin sensitivity improves during PCR,(28) no study to date has determined how much iatrogenic hyperinsulinemia contributes to reduced insulin sensitivity during this time.

Specific Aim 2: Quantify the contribution of iatrogenic hyperinsulinemia to endothelial dysfunction early in the course of T1DM. During each STUDY visit, I will measure endothelial function in multiple vascular beds. As a primary outcome, I will quantify brachial artery endothelium-dependent flow-mediated vasodilation (%FMD) using high-resolution B-mode ultrasound. As a secondary outcome, I will quantify insulin-induced microvascular recruitment using contrast-enhanced ultrasound (CEUS). Although young people with T1DM have diminished FMD (6), the early time course of functional and structural endothelial abnormalities have not been examined nor has its relationship with iatrogenic hyperinsulinemia been determined.(8)

Specific Aim 3: Determine how much an intervention to reduce hyperinsulinemia can reduce IR in T1DM. Although my previous research showed iatrogenic hyperinsulinemia is the primary driver of IR in T1DM, no investigation to date has

quantified how much an intervention to lower hyperinsulinemia can improve IR. Thus, we will use a LCD as a means to lower hyperinsulinemia and multivariable linear regression analysis to quantify the reduction in IR adjusted for confounding covariables.

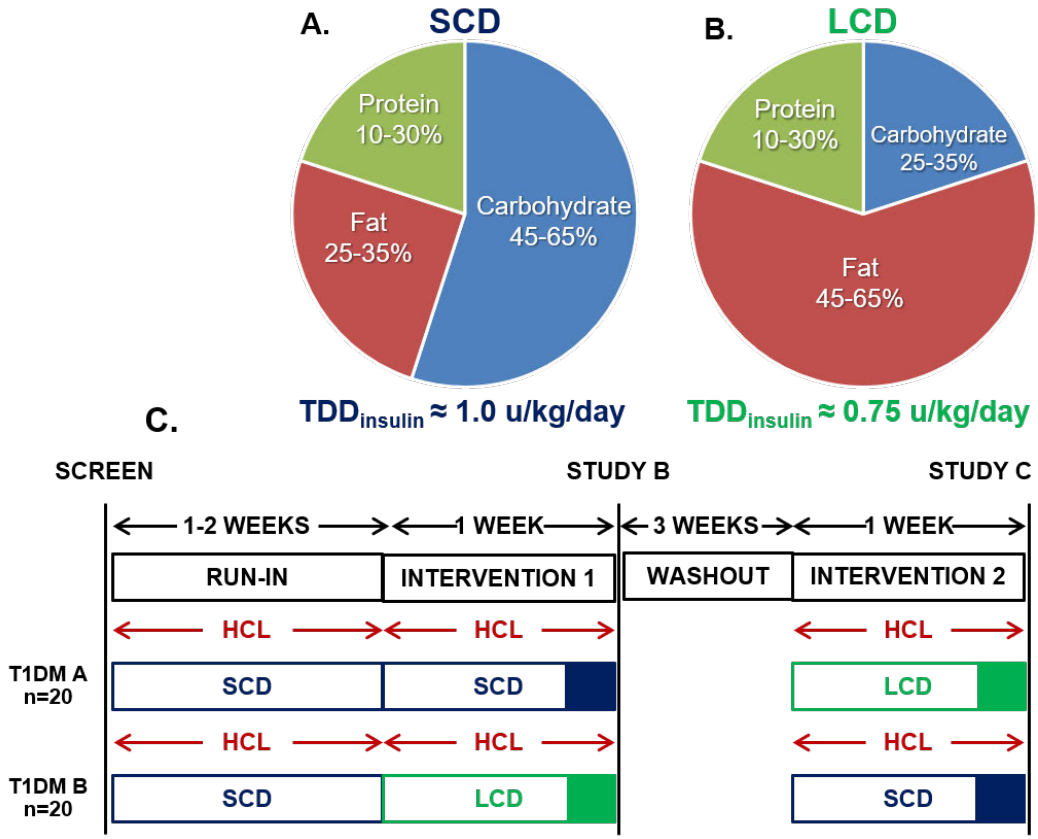


Figure 4: Crossover study design. A) Macronutrient composition of isocaloric, standard carbohydrate diet (SCD) B) Macronutrient composition of isocaloric low carbohydrate diet (LCD). The total daily dose of insulin ($TDD_{insulin}$) on LCD will be 1/3 lower than on SCD. Percentages indicate percent of caloric intake from each macronutrient. C) Schematic of crossover study design. HCL = hybrid closed loop. Solid shaded square at the end of each diet intervention indicates the study team will supply all food in the last 24 hours prior to each study.

3.0 Animal Studies and Previous Human Studies

My previous research aimed to determine the relative contributions of hyperglycemia and iatrogenic hyperinsulinemia to insulin resistance in T1DM. The study quantified tissue-specific insulin sensitivity using a two-step, hyperinsulinemic, euglycemic, clamp in three cohorts (n=10/group) with differing insulinemia and glycemia: healthy controls (with euinsulinemia and euglycemia), MODY2 (with euinsulinemia and hyperglycemia), and T1DM (with hyperinsulinemia and hyperglycemia matching the MODY2 group). I assessed the relative contribution of hyperglycemia to insulin resistance by comparing insulin sensitivity between control and MODY2 groups. Likewise, the contribution of hyperinsulinemia was revealed by comparing insulin sensitivity between MODY2 and T1DM groups.

HbA1c for control, MODY2, and T1DM groups was 4.8 ± 0.4%, 6.2 ± 0.3%, and 6.6 ± 0.5%, respectively. Each group had similar characteristics for additional factors that could influence insulin sensitivity (e.g. age, BMI, etc.). On the night prior to the clamp study, all participants began fasting and glucose was monitored overnight. T1DM subjects additionally discontinued their home insulin regimen and received a variable IV insulin infusion to achieve a plasma glucose concentration between 90-120 mg/dL by morning. Whereas fasting plasma insulin concentrations in the MODY2 and control groups were virtually the same, peripheral insulin delivery in the T1DM group resulted in a ≈2.5-fold higher fasting insulin level (figure 4A) to reach fasting plasma glucose concentrations similar to the other groups (figure 4B). In the first step of the clamp, we infused insulin at 12 mU/m^2/min, a rate chosen to partially suppress glucose production (R_a) and lipolysis. As figure 4C suggests, all groups saw a very similar degree of R_a suppression during first-step insulin infusion. Because the majority of fasting glucose R_a is hepatic in origin (>90%), this finding suggested hepatic insulin sensitivity was no different between cohorts with their differing glycemic statuses.

A different pattern was seen in peripheral tissues (fat and muscle), however. When we assessed insulin's ability to suppress lipolysis by quantifying the decrease in non-esterified fatty acid (NEFA) during the first insulin infusion step, it was evident that control and MODY2 cohorts had nearly total NEFA suppression while a much smaller decrease occurred in the T1DM group (figure 4D). Likewise, when muscle tissue was stimulated by a high insulin infusion (40 mU/m^2/min) in the second step, control and MODY2 cohorts had similarly large increases in glucose disposal (R_d) above baseline, while a smaller increase was seen in T1DM (figure 4E). Multivariable linear regression showed insulinemia, but not glycemia, was significantly associated with muscle insulin sensitivity.

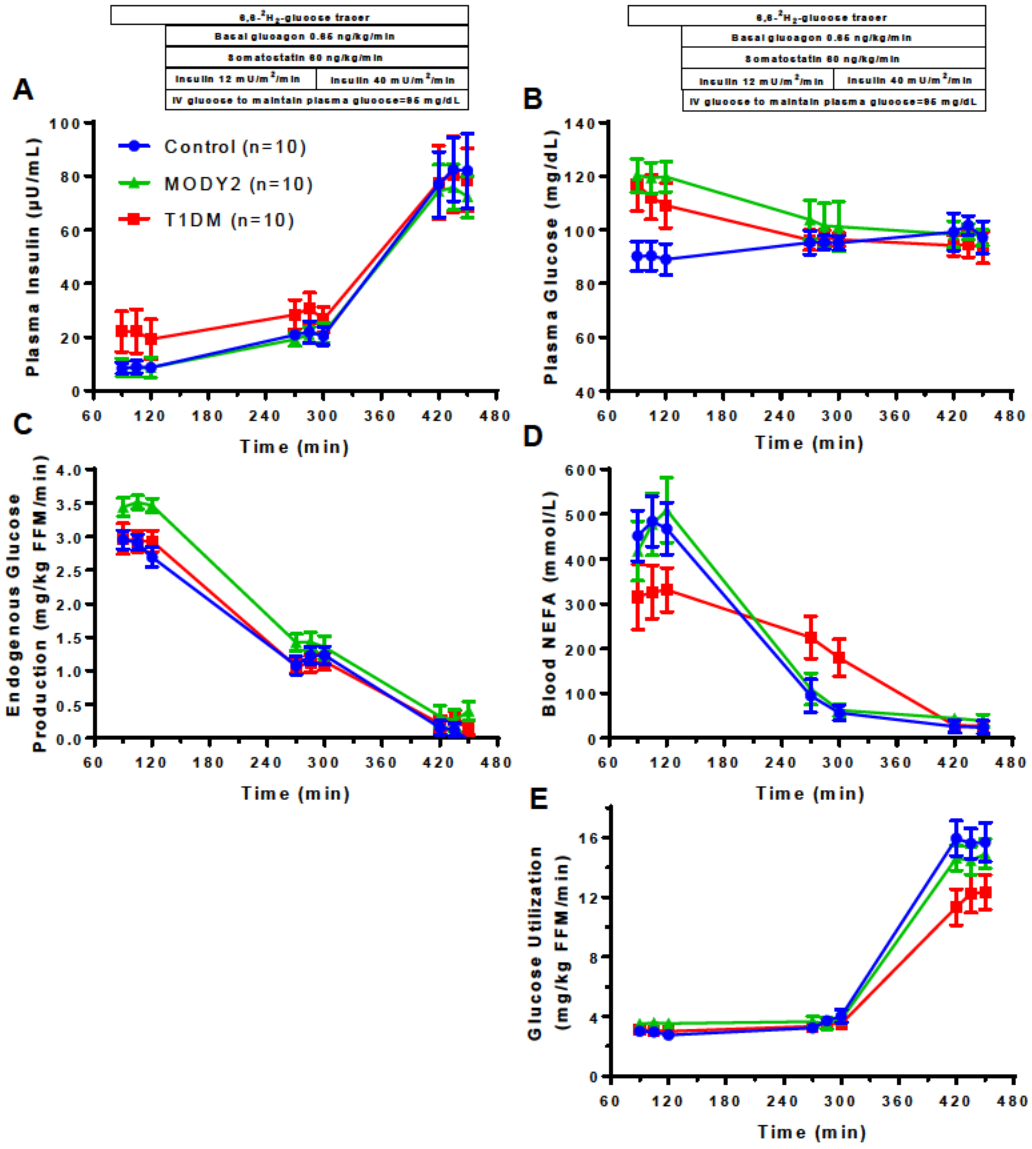


Figure 4: Preliminary Data

These data support the proposed concept that local tissue hyperinsulinemia, as occurs at muscle and fat in T1DM, but not in MODY2 nor control, nor any group at liver, is associated with tissue-specific IR. Despite having hyperglycemia, MODY2 participants had no more IR than control at any tissue. On the other hand, the addition of chronic peripheral hyperinsulinemia onto hyperglycemia in the T1DM group was associated with IR in muscle and fat tissue. The key conclusion is that local tissue hyperinsulinemia, rather than hyperglycemia, is the dominant contributor to tissue-specific IR in T1DM.

4.0 Inclusion/Exclusion Criteria

STUDY 1-7 (Aims 1-2)

Table 1 lists inclusion and exclusion criteria for STUDY1-7 (Aims 1-2), which will be determined at an initial screening visit. If a complete metabolic panel (CMP) or complete blood count (CBC) was completed at Vanderbilt University Medical Center within 6 months prior the screening visit that lab study will be used in lieu of a new CMP or CBC.

Table 1: Study Inclusion and Exclusion Criteria, STUDY 1-7 (Aims 1-2)

ALL GROUPS	T1DM subjects
Inclusion Criteria - BMI: 18-28 kg/m^2	Inclusion criteria - Age: 15-35
Exclusion Criteria - Severe hypoglycemia: ≥1 episode in the past 3 months - Diabetes comorbidities: - Any hospital admissions for diabetic ketoacidosis in the past 6 months. - SBP > 140 mmHg and DBP > 100 mmHg - eGFR by MDRD equation of < 60 mL/min/1.73m^2 - AST or ALT > 2.5 times the upper limit of normal - Hct < 35% - Medications: Any antioxidant vitamin supplement (<2 weeks before a STUDY visit), any systemic glucocorticoid, antipsychotic, atenolol, metoprolol, propranolol, niacin, any thiazide diuretic, any OCP with > 35 mcg ethinyl estradiol, growth hormone, any immunosuppressant, antihypertensive, any antihyperlipidemic - Other: pregnancy, Tanner stage < 5, peri- or post-menopausal women, active smoker	Exclusion criteria: - Medications: any diabetes medication except insulin **Control subjects** Inclusion criteria - Age: 18-35 - HbA1c: <5.5%

Rationale for inclusion and exclusion criteria: To minimize potential confounding effects of increased age,(53) intermittent ketoacidosis,(54) and increased BMI(55) on insulin sensitivity, the study will target relatively young patients with glycemic control within or near target and with BMIs at or below what would be expected in T1DM and the general population. Further, because patients who have diabetic ketoacidosis (DKA) at initial presentation are less likely to experience partial clinical remission (PCR) than patients who do not have DKA at presentation, DKA at diagnosis is an exclusion criterion for the longitudinal study.

STUDY B-C (Aim 3)

Table 2 lists inclusion and exclusion criteria for STUDY B-C (Aim 3), which will be determined at an initial screening visit.

Table 2: Study Inclusion and Exclusion Criteria, STUDY B-C (Aim 3)

INCLUSION CRITERIA
- Age: 18-60 - HbA1c: 5.6-9.0% - Insulin delivery: must use an insulin pump - Glucose monitor: must use a CGM. - BMI: 18-33 kg/m^2 - Body mass: ≥ 50 kg
EXCLUSION CRITERIA

- Severe hypoglycemia: ≥1 episode in the past 3 months
- Diabetes comorbidities:
  - Any hospital admissions for diabetic ketoacidosis in the past 6 months.
  - SBP > 140 mmHg and DBP > 100 mmHg
  - eGFR by MDRD equation of < 60 mL/min/1.73m^2
  - AST or ALT > 2.5 times the upper limit of normal
  - Hct < 35%
- Medications: Any diabetes medication besides insulin, antioxidant vitamin supplement (<2 weeks before a STUDY visit), any systemic glucocorticoid, antipsychotic, atenolol, metoprolol, propranolol, niacin, any thiazide diuretic, any OCP with > 35 mcg ethinyl estradiol, growth hormone, any immunosuppressant, antihypertensive, any antihyperlipidemic
- Other: pregnancy, Tanner stage < 5, peri- or post-menopausal women, active smoker, gluten-free diet requirement

Rationale for inclusion and exclusion criteria: To minimize potential confounding effects of intermittent ketoacidosis (54) and increased BMI(55) on insulin sensitivity, the study will target with glycemic control within or near target and with BMIs at or below what would be expected in T1DM and the general population. Since the crossover design minimizes between-intervention variance, inclusion criteria for age are loosened compared with the criteria for STUDY 1-7. Additionally, because the total daily dose of insulin ($TDD_{insulin}$) is the key independent variable of interest in the study, each T1DM participant must use an insulin pump. An insulin pump digitally records all insulin a participant uses. Because the importance of glycemic variability in the data analysis, all T1DM participants must use a

continuous glucose monitor. Finally, STUDY B-C will focus on recruiting adult participants, to avoid excessive recruiting overlap with STUDY1-7.

5.0 Enrollment/Randomization

5.1 Recruitment

5.1.1 Recruitment Resources

We will use multiple tools to identify prospective study participants. The PI will contact intramural and extramural endocrinology colleagues at Vanderbilt and in the region to discuss this study and ask them to refer potential subjects. The Vanderbilt Eskind Diabetes Clinic is a large academic center that sees approximately 4,000 patients with T1DM annually. The study team will also use additional research tools including Research match, Subject locator, and My Research at Vanderbilt. We will post flyers and send an institution-wide mass email through the Research Notifications Distribution List. In addition, we will post our research flyer on social media platforms (e.g. Facebook and Twitter) to target further potential T1DM and control group participants. For example, the Middle TN JDRF Chapter Facebook page has a large number of followers across the state of Tennessee many of whom may participate in our research.

5.1.2 Initial Contact with Potential Participants

Once the research team becomes aware of a potential subject, we will either telephone the participant (or parents in the case of potential pediatric participants) or visit with them in person during an endocrinology clinic visit. The initial discussion will aim to make a preliminary 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 or that the individual is interested in being a control subject
- age
- weight
- height
- most recent HbA1c (if applicable)
- diabetes duration (if applicable),
- medications
- history of severe hypoglycemia in the past 3 months
- history of any episodes of DKA in the past 6 months
- physical activity level

- food preferences and restrictions

Inclusion and exclusion criteria for STUDY1-7 AND STUDY B-C are listed in tables 1 and 2, respectively. We will invite eligible subjects to participate in the initial screening visit. The initial screening visit will occur either in the Vanderbilt Clinical Research Center (CRC) or in the Vanderbilt Eskind Diabetes Center. The participant will receive an electronic or paper copy of the informed consent document to review prior to the initial screening visit.

5.2 Enrollment

A member of the study team (i.e. key study personnel, KSP) will obtain written, informed consent or adolescent ascent with parental consent at the beginning of the screening visit as detailed in section 6.1.1.1. Once consent/assent is obtained, the participant will be enrolled in the study. A detailed description of special protection for adolescents as research subjects follows in section 7.3 below.

The Vanderbilt CRC is located on the 2rd floor of Medical Center North on the Vanderbilt University Medical Center (VUMC) campus (1211 Medical Center Dr, Nashville, TN 37232; telephone number: [redacted] The Vanderbilt Eskind Diabetes Center is on the 8th floor of Medical Center East on the VUMC campus (1215 21st Ave S 8th floor, Nashville, TN 37232; telephone number: [redacted]

5.3 Randomization

STUDY1-7 (Aims 1-2), include a longitudinal study of newly diagnosed T1DM patients during the first twelve months after diagnosis (T1DM SUBSTUDY, STUDY1-3) Because of the longitudinal nature of the T1DM substudy, no randomization process is needed for STUDY 1-3. The CONTROL SUBSTUDY investigates the effect of small doses of insulin in healthy, nondiabetic individuals over differing durations: one week (STUDY 5), one night (STUDY 6), both one week and one night (STUDY 7), and with no exogenous insulin at all (STUDY 4). Control participants will go through STUDY 4-7 in a random order. The study team will randomize participant into a treatment order group using a web-based randomization tool (http://www.sealedenvelope.com), a Microsoft Excel macro, or a randomization tool within Redcap.

STUDY B-C (Aim 3), is a random-order, 2x2 crossover study. On one arm, participants will consume a low-carbohydrate diet (LCD). On the other arm, participants will consume a standard carbohydrate diet (SCD). As shown in figure 3, T1DM and control participants will be randomized into one of two treatment orders (“A” vs. “B”).

Random permuted blocks with a fixed size of 4 will be used to randomize patients into treatment order A (SCD then LCD) vs. treatment order B (LCD then SCD). The study team will randomize participant into a treatment order group using a web-based randomization tool (http://www.sealedenvelope.com), a Microsoft Excel macro, or a randomization tool within Redcap.

6.0 Study Procedures

6.1 Research visits: STUDY1-7 (Aims 1-2)

All subjects will participate in a screening visit (section 6.1.1) then will participate in one of two substudies based on whether they are a participant recently diagnosed with T1DM (T1DM SUBSTUDY) or if they are a healthy control participant without T1DM (CONTROL SUBSTUDY). Figure 5 summaries the T1DM and CONTROL substudies.

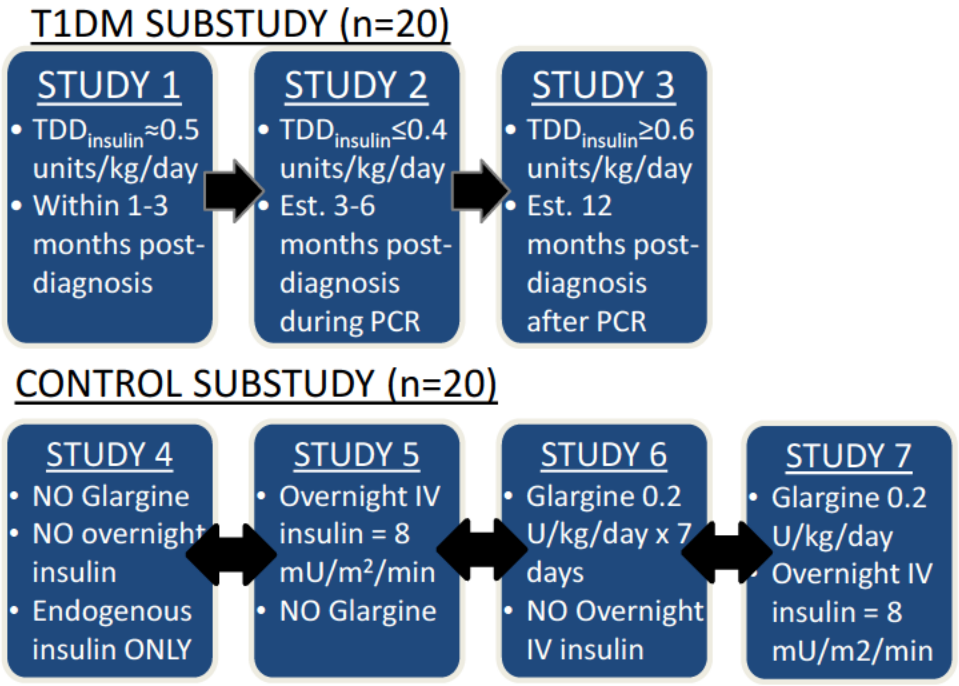


Figure 5: Schematic for STUDY visits. Est. = estimated. PCR=partial clinical remission. TDD$_{insulin}$ = total daily dose of insulin

1. Participants selected for the T1DM SUBSTUDY (n=20) will have a T1DM duration of less than one year. They will participate in three identical STUDY visits (STUDY1-3).
   - STUDY1 will occur within 1-3 months of diagnosis in most instances. In our practice, the initial starting total daily dose of insulin (TDD$_{insulin}$) upon diagnosis is usually 0.5 units/kg/day.(56) STUDY1 will require each participant's TDD$_{insulin}$ averaged over 5 consecutive days fall between 0.4-

0.6 units/kg/day, which is a common dosing range during the first 2-3 months after diagnosis.

- For participants to enter STUDY2, their mean $TDD_{insulin}$ must be ≤ 0.4 units/kg/day. This usually occurs between 3-6 months post-diagnosis.
- To enter STUDY3, the $TDD_{insulin}$ must exceed or equal 0.6 units/kg/day, which usually occurs by 12 months post-diagnosis.

2. Participants selected for the CONTROL SUBSTUDY (n=20) will not have T1DM and have no chronic illnesses. They will participate in STUDY 4-7 (each CRC study visit follows the same protocol).

6.1.1 Initial Screening Visit

Screening visits will take place in either the Vanderbilt CRC or a room designated for research in the Eskind Diabetes Center, based on the convenience of the participant (e.g. they are already coming to a diabetes clinic visit) and availability of the room in the Eskind Diabetes Center.

The purpose of the screening visit is to obtain informed, written consent/assent, conduct a history and physical exam, instruct the participant in the use of a continuous glucose monitor and a digital insulin pen device, calculate the estimated energy requirement to be consumed before STUDY1-7 visits, and show participants how to log their food intake on their digital device.

Each screening visit will consist of the following:

6.1.1.1 Consent/Assent

The PI or designated KSP will obtain consent/assent from all participants. Consent/assent will be obtained in a private room in the CRC or Eskind Diabetes Center 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 discussing any questions. The study team will provide a copy of the consent/assent form to the participant and parents if applicable.

6.1.1.2 History and Physical Exam

The PI or designee will review each subject's clinical history, perform a physical exam, and take anthropometric measurements.

6.1.1.3 Blood draw and urine collection

If the participant has not had a complete metabolic panel (CMP), complete blood count (CBC) drawn at Vanderbilt University Medical Center within six months prior to the screening visit, blood will be drawn to determine eGFR, LFTs, and hematocrit meet inclusion and exclusion criteria for study. If a CMP or CBC was drawn within six months prior to the screening, those results can be used at the discretion of the PI. HbA1c will be drawn at the screening visit.

Female participants will provide a urine sample to exclude pregnancy.

6.1.1.4 Continuous Glucose Monitor Instructions

The study team will instruct T1DM participants in the use of a continuous glucose monitor (CGM) such as Dexcom G6 or Medtronic Guardian for the monitoring of glycemia during the study. The team will then show T1DM participants how to upload both CGM data and insulin pump data (if using an insulin pump) into a HIPAA and FDA-compliant cloud-based, data-integration platform such as Dexcom Clarity, Medtronic Carelink, or Tidepool.

Although the insulin glargine dose taken by nondiabetic participants in the CONTROL SUBSTUDY before STUDY 6 and 7 is low (0.2 units/kg/day), this medication incrementally increases the risk of iatrogenic hypoglycemia. Accordingly, control participants will monitor their glucose at home during the week prior to each of the STUDY 4-7 visits. The PI may elect to use either CGMs or conventional capillary blood glucose monitors on control participants. As part of the initial screening visit, KSP will teach CONTROL SUBSTUDY to identify symptoms of hypoglycemia (e.g. shakiness, weakness, etc.), to check their glucose to confirm hypoglycemia (glucose < 70 mg/dL), and to use the commonly-taught “rule of 15” to treat a hypoglycemic episode. If a conventional capillary glucose monitor is used, KSP will instruct CONTROL SUBSTUDY participants to check their glucose each morning prior to eating and each time he or she has symptoms of hypoglycemia. If a CGM is used, the team will show the control participants how to use the CGM device. CONTROL SUBSTUDY participants using a CGM will wear the device for 7-10 days prior to each STUDY 4-7 visit.

6.1.1.5 Digital Insulin Pen Instructions for T1DM SUBSTUDY participants

The study team will provide a digital insulin pen such as the InPen (Companion Medical) or Echo (Novo Nordisk) to subjects with

T1DM who do have an insulin pump. Digital insulin pens use removable insulin cartridges and record each dose of insulin. The study team will show participants how to upload their insulin pen data using their HIPAA-compliant, FDA-approved smart phone app.

6.1.1.6 Basal insulin instructions for CONTROL SUBSTUDY participants

CONTROL SUBSTUDY participants (who do not have diabetes) will inject low doses of subcutaneous insulin glargine (0.2 u/kg/day) over 7 days preceding STUDY 6 and 7 and placebo over 7 days preceding STUDY 4 and 5. The study team will show these participants how to properly perform these injections at home. Please see section 7 regarding risk mitigation measures to prevent serious hypoglycemia.

6.1.1.7 Diet instructions

During the week preceding each STUDY visit, research participants will consume a weight-maintaining diet designed by the research team. The research team will discuss food preferences with each participant to design diet plan with standard macronutrient contents. The Mifflin-St. Jeor formula (57) will be used to calculate the estimated energy requirement (EER).

EER for males (kcal/day) = PA (10 x weight [kg] + 6.25 x height [cm] – 5 x age [y] + 5)

EER for females (kcal/day) = PA (10 x weight (kg) + 6.25 x height (cm) – 5 x age (y) – 161)

Where PA is the physical activity coefficient:

PA = 1.20 for little-to-no exercise

PA = 1.38 for exercising 3 times weekly

PA = 1.42 for exercising 4 times weekly

PA = 1.46 for exercising 5 times weekly

PA = 1.55 for daily exercise

PA = 1.72 for daily intense exercise or exercising twice a day every day

PA = 1.90 for daily exercise plus physical job

Where exercise is 15-30 mins of elevated heart rate and intense exercise is 45+ mins of elevated heart rate.

Macronutrient content will reflect the acceptable macronutrient distribution range of the Institute of Medicine's Recommendations for Nutrition: carbohydrate (45%–65% of energy), protein (10%–35% of energy), and fat (20%–35% of energy).(58)

The study team will use the Nutrihand and/or the MyFitnessPal software program to design meal plans and participants will log their daily caloric intake.

Nutrihand uses web-based or Smartphone app interfaces. Nutrihand creates a unique encrypted key whenever a healthcare professional adds a client to their account. That key can be used only once and by the specific email address used when the account was created. The participant will receive an automated email instructing them to log into the application for the first time. When he/she logs in, the first screen will ask the participant to agree to let the study team view their information and work with them. The participant can break the linkage with the study team at any time by removing the HIPAA key from their account.

MyFitnessPal is a smartphone app and website that tracks diet and exercise to determine optimal caloric intake and nutrients for the users' goals. Users can either scan the barcodes of various food items or manually add them in the database of over five million different foods. Users can export their nutrient and exercise log as a de-identified CSV file (an example is shown in Appendix 2). Participants will email this de-identified nutrition log to the study's email address. KSP will then upload the CSV file into the RedCAP database for subsequent data analysis.

6.1.2 Management Before and In Between STUDY 1-7 visits

Each T1DM participant's endocrinologist will manage his or her glycemic control and insulin doses before and between T1DM SUBSTUDY visits. The study team will review each T1DM SUBSTUDY study participant's insulin doses on at least a monthly basis between study visits.

Each CONTROL SUBSTUDY participant will take insulin glargine 0.2 u/kg/dose each day for seven days preceding STUDY 6 and 7 and an equal volume of a placebo injection (saline) prior to STUDY 4 and 5. The participant will be blinded to whether they are taking placebo or insulin glargine.

Although the study takes several measures to mitigate the possibility of hypoglycemia in these participants (section 7.1), the investigator will not be blinded to whether or not control participants are taking insulin prior to CONTROL SUBSTUDY participants. This enables the investigator to rapidly alter the course of the intervention in the event of hypoglycemia.

In the unlikely event severe hypoglycemia occurs (i.e. loss of consciousness or seizure due to hypoglycemia) blinding will be broken to ensure the patient's hypoglycemia is treated appropriately.

Approximately 10-14 days before STUDY visits 1-7, a study team member will contact the participant to remind them to:

1. Wear their CGM for at least the 7 days leading up to the STUDY visit for T1DM SUBSTUDY participants CONTROL SUBSTUDY participants will be reminded to wear either their study-supplied CGM or conventional capillary blood glucose monitor.
2. Record food and drink consumed for the 7 days leading up to the STUDY visit in their digital food log.

6.1.3 STUDY1-7 visits (Aims 1-2)

We will measure insulin sensitivity and endothelial dysfunction at each STUDY visit. Figure 6 summarizes the protocol of each STUDY visit. The following subsections describe the protocol in detail.

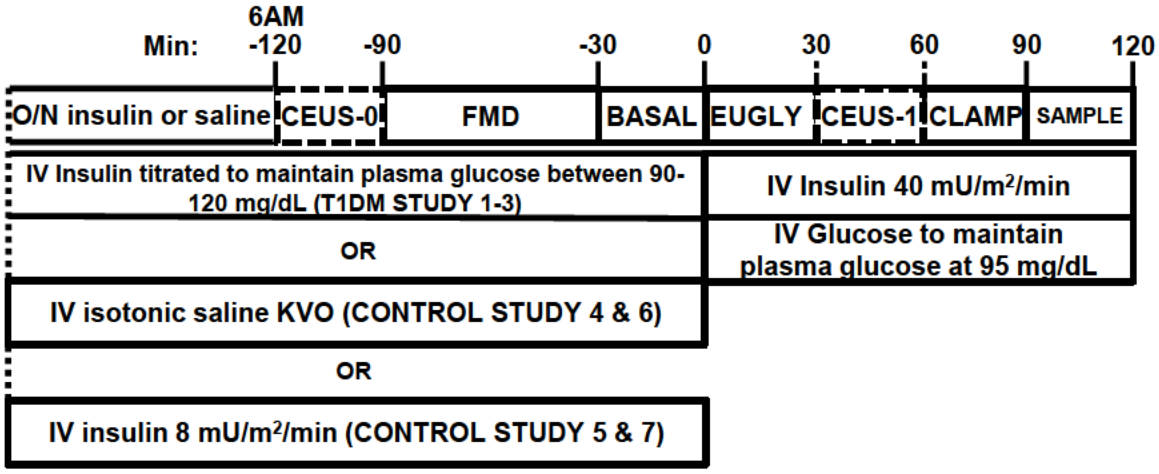


Figure 6: Schematic for STUDY visits. KVO = keep vein open

6.1.3.1 Arrival and Preparation

Preparation: To ensure an accurate measurement of key study outcomes with minimal confounding, participants will take the following precautions before each STUDY visit:

- Hold vitamin supplementation for 72 hours
- Avoid NSAIDs and aspirin for 24 and 72 hours, respectively, if feasible

- Refrain from smoking and avoid secondhand smoke exposure for 12 hours
- Consume no caffeine for 12 hours
- Abstain from exercise for 12 hours

Study participants will arrive to the CRC between 1700-2100 on the evening prior to STUDY visits.

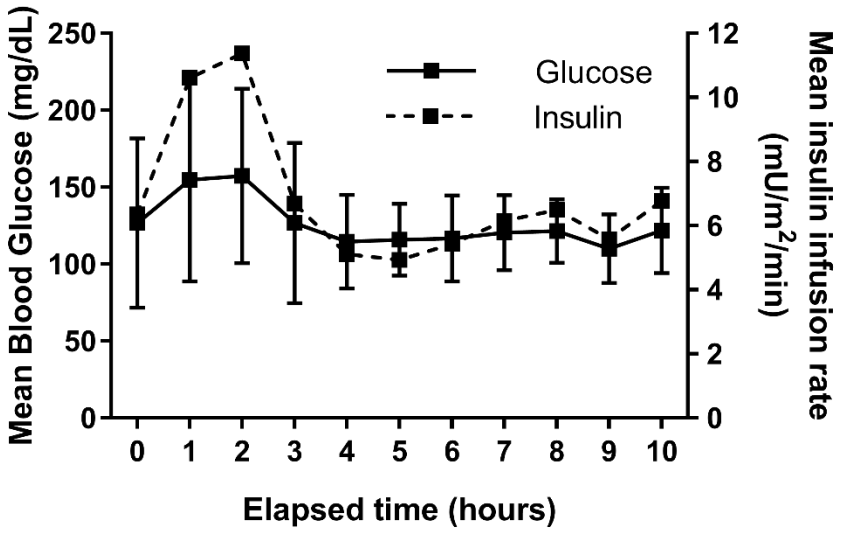
CRC staff will draw the following labs prior to conducting the clamp study:

- Pregnancy test (urine or blood) on female participants to rule out pregnancy.
- I-STAT potassium: to screen for hypokalemia

T1DM participants who use an insulin pump will discontinue and detach their insulin pump at 2130 on the night of CRC admission. T1DM participants who take multiple daily insulin injections take their last basal insulin analog dose on the evening prior CRC admission and hold their basal insulin dose on the evening of admission. CRC staff or KSP will place an intravenous angiocathers in each arm.

6.1.3.2 Overnight insulin infusion (T1DM SUBSTUDY participants only)

At 2200 a continuous, IV infusion of regular human insulin (recombinant DNA origin) will be given to T1DM participants according to the protocol of Goldberg et al.(59), modified for use in healthy patients with T1DM (Appendix 1). This protocol targets a glucose of 90-120 mg/dL by the next morning. CRC staff will check blood glucose of T1DM participants on an hourly basis using a calibrated, hospital bedside glucose monitor. This protocol was implemented in a previous study (IRB # 161504) and achieved ideal glycemic control as shown in the figure below (n=9 T1DM participants, figure shows means ± SD for glucose and means for insulin infusion rate).



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 experiences 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 PI will postpone the following morning's clamp study to a later date because of the confounding influence of the counter-regulatory response to hypoglycemia.

The PI will be readily available to CRC nursing staff via telephone and [redacted]

6.1.3.3 Overnight IV saline infusion (CONTROL SUBSTUDY 4 and 6)

Non-diabetic control subjects participating in the CONTROL SUBSTUDY will receive an IV infusion of isotonic saline at a rate of sufficient to keep the vein patent. Blood glucose will be checked every 1-2 hours overnight.

6.1.3.4 Overnight IV insulin fusion (CONTROL SUBSTUDY 5 and 7)

Non-diabetic control subjects will receive an IV infusion of regular human insulin at a rate of 8 mU/m^2/min. Blood glucose will be checked every hour overnight. If the blood glucose level falls to below 75 mg/dL, IV glucose will be infused to maintain blood glucose between 75 and 100 mg/dL.

6.1.3.5 Contrast-enhanced ultrasound (CEUS)

Beginning at approximately 6 AM, baseline vital signs will be measured. We will then measure microvascular blood volume

(MBV) using CEUS twice as shown in figure 6. The study team will measure MBV during basal conditions (CEUS-0) and insulin stimulated conditions (CEUS-1). Because perflutren is not FDA approved for use in the pediatric population, at present we will only use this technique in adult participants.

A secondary objective of the study is to determine the relationship between hyperinsulinemia and the magnitude of insulin-induced microvascular recruitment (MVR, i.e. MBV_1-MVB_0). The CEUS technique employed is modeled after the protocol of Sjøberg et al.(40)

We will fix a linear-array transducer connected to an ultrasound system to the forearm, allowing for cross-sectional imaging of the forearm muscle bed.

Perflutren lipid microspheres (Definity, Lantheus Medical Imaging) will be activated by a vial mixer at 4,500 oscillations per minute for 45 seconds. Perflutren lipid microspheres in suspension in a 1.5 mL will be diluted at bedside to 20 mL with isotonic saline in a sterile fashion. The diluted microsphere solution will then be infused intravenously at 1.2-1.5 mL/min using a rotating syringe pump to ensure a homogenous microsphere solution. After beginning the perflutren lipid microsphere infusion, there will be a 10-minute equilibrium period to allow the acoustic intensity signal to reach a steady plateau before measuring MBV. A region of interest (ROI) will be marked on a cross-section of the forearm avoiding areas of connective tissues or large blood vessels. The same ROI will be used in subsequent MBV measurements.

Real-time imaging will be conducted using a low ultrasound mechanical index of 0.08. At this mechanical index, microspheres resonate without destruction. Thus, the acoustic index, generated by the resonating bubbles, is proportional to the microsphere concentration (and by extension blood volume) in the ROI interest.

Once the 10-minute equilibrium period is complete, a high (1.20) mechanical index pulse will be delivered by the transducer to completely destroy the microspheres. This allows the replenishment of the microspheres within the microvasculature inside the ultrasound beam's ROI to be measured. Following the high mechanical index pulse, the microspheres will be replenished within the microvasculature and the acoustic intensity across the ROI will be recorded. These data will then be exported to quantification software to determine MBV, calculated in accordance with Wei et al.(60)

6.1.3.6 Flow-mediated dilation (FMD)

After CEUS-0, we will determine FMD. Originally developed in 1992, the FMD technique has become the most commonly utilized non-invasive method for assessing vascular endothelial dysfunction.(61) The study team will use the Phillips EPIQ 7C ultrasound machine with an L12-3 transducer. Ms. JoAnn Gottlieb, an ultrasonographer with extensive experience using this technique, will assist in these studies.

Baseline Measurements: The baseline radial artery diameter and velocity will be measured. The brachial artery will be scanned beginning at the insertion of the biceps and proceeding proximally. Color flow imaging will verify the brachial artery and to locate collateral vessels to serve as landmarks in sequent studies. Once a suitable position is found, brachial artery diameter will be measured over at least 10 cardiac cycles. Velocity will be averaged over at least a 10-20 second period.

Vascular occlusion: A pillow will support each participant's arm with the palm facing forward. We will position an appropriately sized blood pressure cuff 5 cm distal to the elbow. A rapid cuff inflator will to occlude radial artery blood flow. The cuff will be inflated to at least 25-50 mm Hg above systolic blood pressure for five minutes to elicit a reactive hyperemic stimulus for endothelium mediated vasodilation.

Reactive Hyperemia (Post-cuff release) Measurements: Post-cuff measurements of arterial diameters and blood velocities will be initiated ten seconds prior to cuff release and continued for three minutes following cuff release.

FMD Analysis: The primary outcome of the FMD study is %FMD, defined as:

$$\%FMD = \frac{diameter_{hyperemia} - diameter_{baseline}}{diameter_{baseline}}$$

6.1.3.7 Endothelium independent vasodilation: After the participant rests for another 10 min, we will measure nitroglycerin-induced vasodilation as an index of endothelium-independent vasodilation. We will administer a 400 µg sublingual dose of nitroglycerin and assess the brachial artery response by imaging the artery continuously for 3 min. We will quantify endothelium-independent vasodilation as the percent increase in brachial artery diameter 3 min after the nitroglycerin dose.

6.1.3.8 Basal blood measurements

Following FMD, we will draw blood levels of the hormones, metabolites, and cytokines listed in table 3 up to three times over a 30 min baseline sampling period.

Table 3: Labs drawn during BASAL period

Hormones	Metabolites	Cytokines
Insulin C-peptide Glucagon Cortisol Catecholamines Estradiol Testosterone Adiponectin	Glucose NEFAs Glycerol Lactate Alanine Lipoprotein subclass sizes, concentrations Urine microalbumin	TNF-α IL-1 and 6 hsCRP VEGF ICAM VCAM E-selectin endothelin-1 Fibrinogen PAI-1

6.1.3.9 Hyperinsulinemic-euglycemic clamp (EUGLY CLAMP)

We will conduct a 120 min hyperinsulinemic, euglycemic clamp after drawing basal labs. Regular human insulin will be infused into a peripheral angiocatheter at 40 mU/m^2/min. Plasma glucose will be monitored every 5-10 minutes using a YSI Glucose Analyzer (Yellow Springs, OH), the gold standard instrument for glucose concentration analysis. IV glucose will be infused to maintain plasma glucose at approximately 90-100 mg/dL throughout the study.

6.1.3.10 CLAMP sampling period (SAMPLE)

A second sampling period will measure the blood parameters listed in table 3 under insulin-stimulated conditions during the last 30 minutes of the clamp.

The total blood volume from each study is calculated to equal 124 mL.

6.1.3.11 DEXA scan

Prior to discharge, 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).

6.1.3.12 STUDY visit completion

Upon completion of the study, we will stop the insulin infusion and allow the study subject to eat. The study team will continue monitoring plasma glucose for at least an additional hour and the steadily decrease the glucose infusion rate (GIR) to maintain

glucose > 120 mg/dL. We will instruct T1DM participants to resume their home regimen, however they will be instructed to check glucose before dinner, bedtime, and at 2 AM following the study. 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.

6.1.3.13 Rescheduling STUDY visit 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. In the event such a technical issue occurs, the PI may elect to reschedule a new, repeat STUDY visit provided he deems the technical issue poses no serious danger to the participant were it to occur again and the participant wishes to repeat the study.

6.2 Research visits: STUDY B-C (Aim 3)

Each subject will participate in three research visits to complete participation as depicted in figure 7. The first visit ("SCREEN" in figure 7C) will determine the potential participant's eligibility and allow the study team to provide instructions to the participants. If the participant meets eligibility criteria he/she will begin a 1-2-week "run-in" period within six weeks of the screening visit. After the run-in period, the participant will be randomized to consume either 1 week (+/- 1 day) of a standard carbohydrate diet (SCD) or 1 week (+/- 1 day) of consuming a low carbohydrate diet (LCD) as shown in figure 7. On the final day of the intervention, the participant will consume a standardized 2,000 kilocalorie SCD or LCD provided by the study team. After a week (+/- 1 day) of being on either intervention, participants will report to the CRC for the first of two "STUDY" visits, where insulin sensitivity and endothelial dysfunction will be assessed (STUDY B). Following STUDY B, participants will begin a washout period of approximately 3 weeks where they will resume their usual diabetes management. The study team will schedule STUDY C to follow STUDY B by no more than one month in male participants. For female participants, STUDY B and C will occur during the follicular stage of consecutive menstrual cycles (i.e., day 2-10 of the menstrual cycle). For 1 week (+/- 1 day) preceding STUDY C, each participant's intervention will cross-over to either LCD or SCD. On the final day of the second intervention, the participant will again consume a standardized 2,000 kilocalorie SCD or LCD provided by the study team. Upon completion of the second 1-week (+/- 1 day)

treatment period, each participant will return to the CRC for a second and final assessment of insulin sensitivity and endothelial dysfunction (STUDY C).

Each research visit and intervention period is discussed in the following subsections.

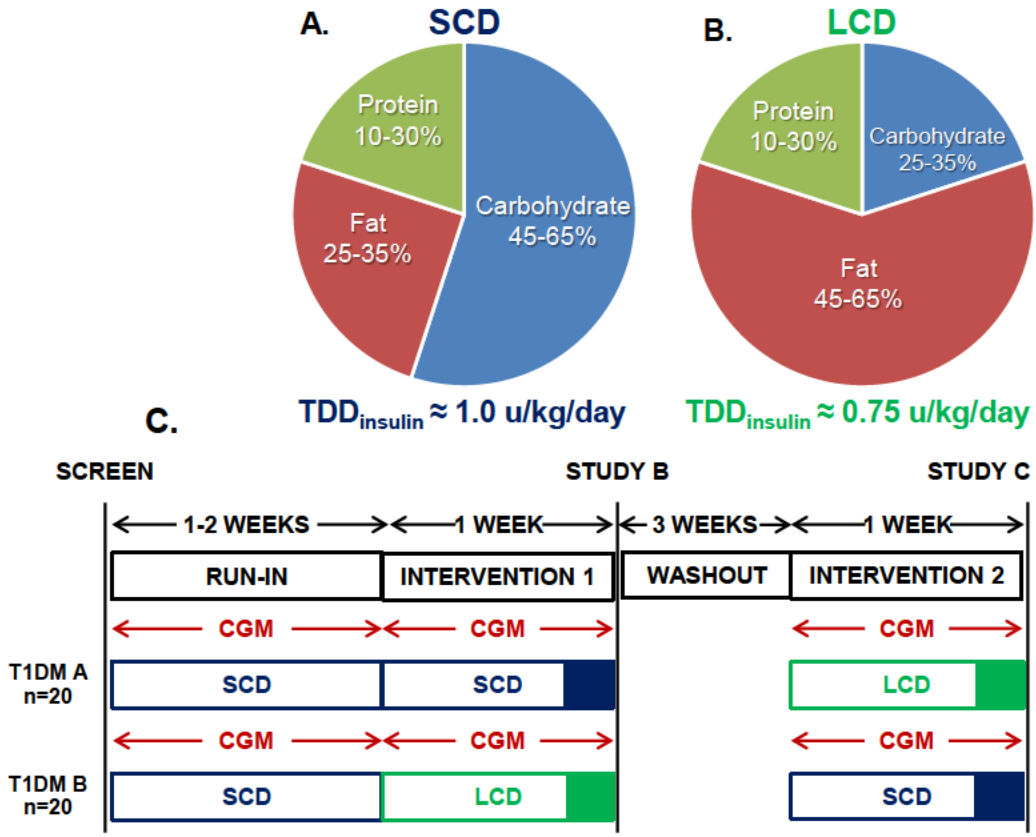


Figure 7: Crossover study design. A) Macronutrient composition of isocaloric, standard carbohydrate diet (SCD) B) Macronutrient composition of isocaloric low carbohydrate diet (LCD). The total daily dose of insulin ($TDD_{insulin}$) on LCD will be 1/3 lower than on SCD. Percentages indicate percent of caloric intake from each macronutrient. C) Schematic of crossover study design. CGM = continuous glucose monitoring. Solid shaded square at the end of each diet intervention indicates the study team will supply all food in the last 24 hours prior to each study.

6.2.1 Initial Screening Visit

Screening visits will take place in either the Vanderbilt CRC or a room designated for research in the Eskind Diabetes Center, based on the convenience of the participant (e.g. they are already coming to a diabetes clinic visit) and availability of the room in the Eskind Diabetes Center.

The purpose of the screening visit is to

- obtain informed, written consent/assent,
- conduct a history and physical exam,
- confirm each participant is able to properly use their insulin pump and continuous glucose monitor (CGM),
- calculate the estimated energy requirement to be consumed before during the study period,
- obtain a blood sample to ensure inclusion/exclusion criteria are met,
- provide the participants with their meal plans and provide standardized meals for the day prior to STUDY visits,
- show participants how to log their food intake on their digital device, and
- perform a DEXA scan to quantify fat-free mass

Each screening visit will consist of the following:

6.2.1.1 Consent

The PI or designated KSP will obtain consent from all participants. Consent will be obtained in a private room in the CRC or Eskind Diabetes Center 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 forms with the participant in detail and provide time for discussing any questions. The study team will provide a copy of the consent form to the participant.

6.2.1.2 History and Physical Exam

The PI or designee will review each subject's clinical history, perform a physical exam, and take anthropometric measurements.

6.2.1.3 Continuous Glucose Monitor and Insulin Pump Instructions

The study team will confirm T1DM participants are able to appropriately use their home continuous glucose monitors (CGMs) such as Dexcom G6 or Medtronic Guardian for the monitoring of glycemia during the study. The team will then show T1DM participants how to upload both CGM data and insulin pump data into a HIPAA and FDA-compliant cloud-based, data-integration platform such as Dexcom Clarity, Medtronic Carelink, or Tidepool. The team will then show T1DM participants how to upload both CGM data and insulin pump data into a HIPAA and FDA-compliant cloud-based, data-integration platform such as Dexcom Clarity, Medtronic Carelink, or Tidepool.

6.2.1.4 Blood draw and urine collection

If the participant has not had a complete metabolic panel drawn and a complete blood count at Vanderbilt University Medical Center within six months prior to the screening visit, blood will be drawn to determine whether the eGFR, AST, ALT, and hematocrit meet inclusion and exclusion criteria for study. If a complete metabolic panel was drawn within six months prior to the screening, those results can be used at the discretion of the PI.

Female participants will provide a urine sample to exclude pregnancy.

6.2.1.5 Diet instructions

The research team will discuss food preferences with each participant to design a weight-maintaining diet plan. The Mifflin-St. Jeor formula (57) will be used to calculate the estimated energy requirement (EER).

EER for males (kcal/day) = PA (10 x weight [kg] + 6.25 x height [cm] – 5 x age [y] + 5)

EER for females (kcal/day) = PA (10 x weight (kg) + 6.25 x height (cm) – 5 x age (y) – 161)

Where PA is the physical activity coefficient:

PA = 1.20 for little-to-no exercise

PA = 1.38 for exercising 3 times weekly

PA = 1.42 for exercising 4 times weekly

PA = 1.46 for exercising 5 times weekly

PA = 1.55 for daily exercise

PA = 1.72 for daily intense exercise or exercising twice a day every day

PA = 1.90 for daily exercise plus physical job

Where exercise is 15-30 mins of elevated heart rate and intense exercise is 45+ mins of elevated heart rate.

The study team will devise two isocaloric diet plans for each participant. One plan, the standard carbohydrate diet (SCD), will reflect the acceptable macronutrient distribution range of the Institute of Medicine's Recommendations for Nutrition: carbohydrate (45%–65% of energy), protein (10%–30% of energy), and fat (25%–35% of energy) (figure 7A).(58) The other plan, the low carbohydrate diet (LCD), will reduce the percent caloric intake

from carbohydrates and replace that amount with calories from dietary fat. Thus with the LCD, 45%–65% of energy will come from fat, 10-30% of energy will come from protein, and 25%–35% of energy will come from carbohydrates (figure 7B). The study team will provide standardized, 2,000 kcal/day diets for participants to consume on the day preceding STUDY B and C visits. The macronutrient content of these standardized meals will match the LCD or SCD intervention.

As with STUDY1-5, the study team will use the Nutrihand and/or MyFitnessPal software program to design meal plans and participants will log their daily caloric intake.

Nutrihand uses web-based or Smartphone app interfaces. Nutrihand creates a unique encrypted key whenever a healthcare professional adds a client to their account. That key can be used only once and by the specific email address used when the account was created. The participant will receive an automated email instructing them to log into the application for the first time. When he/she logs in, the first screen will ask the participant to agree to let the study team view their information and work with them. The participant can break the linkage with the study team at any time by removing the HIPAA key from their account.

MyFitnessPal is a smartphone app and website that tracks diet and exercise to determine optimal caloric intake and nutrients for the users' goals. Users can either scan the barcodes of various food items or manually add them in the database of over five million different foods. Users can export their nutrient and exercise log as a de-identified CSV file (an example is shown in Appendix 2). Participants will email this de-identified nutrition log to the study's email address. KSP will then upload the CSV file into the RedCAP database for subsequent data analysis.

6.2.1.6 DEXA scan

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

6.2.2 Management Before and In Between STUDY B-C visits

Each T1DM participant's will manage his or her glycemic control before and between STUDY B-C visits using insulin as prescribed by their endocrinologist. The study team will monitor adherence by reviewing each participant's dietary log, CGM data, and insulin pump data in an ongoing

fashion. Should the research team detect suboptimal adherence a member of the team will contact the participant to help troubleshoot the reason for the suboptimal adherence. Participants who are not fully vaccinated will obtain a SARS-CoV-2 PCR test within 72 hours prior to STUDY B-C visits at a Vanderbilt associated clinic. Participants who are fully vaccinated or who are within three months of a COVID-19 infection will not be required to have a negative test prior to STUDY visits.

6.2.3 STUDY B-C visits (Aim 3)

We will measure insulin sensitivity and endothelial dysfunction at each STUDY visit. Figure 8 summarizes the protocol of each STUDY visit. The following subsections describe the protocol in detail.

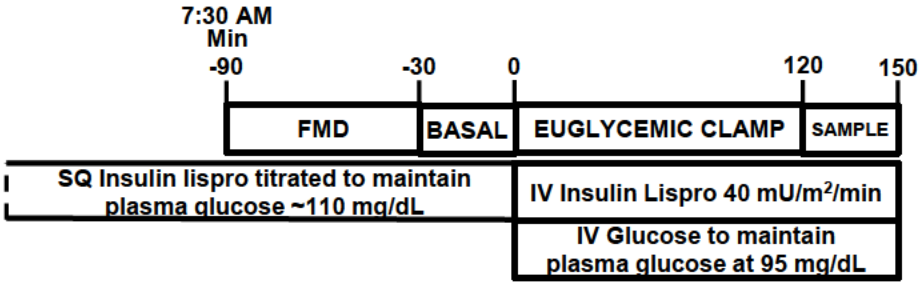


Figure 8: Schematic for STUDY A-C visits. FMD = flow mediated dilation, SQ = subcutaneous

6.2.3.1 Arrival and Preparation

Preparation: To ensure an accurate measurement of key study outcomes with minimal confounding, participants will take the following precautions before each STUDY visit:

- Hold vitamin supplementation for 72 hours
- Avoid NSAIDs and aspirin for 24 and 72 hours, respectively, if feasible
- Refrain from smoking and avoid secondhand smoke exposure for 12 hours
- Consume no caffeine for 12 hours
- Abstain from exercise for 12 hours
- Consume a standardized 2,000 kcal/day diet on the day preceding each STUDY visit.
- Consume the standardized dinner between 1700 and 1900 on the evening prior to STUDY visit. Thereafter, the participant will begin fasting except for drinking sugar free liquids, except if hypoglycemia occurs. If hypoglycemia occurs, participants should treat the hypoglycemia per their

usual treatment routine (e.g., consuming 15 grams of carbohydrates).

Insulin delivery: Participants using an insulin other than insulin lispro will be provided with this medication for use during the 24-48 hours that precede each STUDY visit, thereby standardizing insulin delivery during the 24-48 hours prior to STUDY B and C.

The inclusion criteria stipulate participants must already use an insulin pump and continuous glucose monitor. Based on which pump the participant uses, these patients can be further categorized by those individuals receiving insulin via hybrid closed loop (HCL) technology and those receiving insulin using sensor-augmented pump therapy. On the night prior to each study visit, individuals will receive insulin based on which modality the participant uses.

- Participants using HCL technology for their routine insulin regimen (e.g., those using the Tandem T:Slim pump and Medtronic pumps connected to a Dexcom CGM) will continue using their insulin pumps per their routine.Participants using sensor-augmented pump therapy will continue their routine treatment with the addition that the study team will ask them to give a corrective dose of insulin as needed for hyperglycemia using their usual insulin dose at bedtime.

Study participants will arrive to the CRC at approximately 0700 on the morning of each STUDY B-C visit. Weight and height will be measured. A urine pregnancy test will be drawn on female participants to rule out pregnancy.

6.2.3.2 Flow-mediated dilation (FMD)

Study staff will instruct each participant to lie comfortably prone in bed. After approximately ten minutes of resting, we will determine FMD. The approach for measuring FMD in STUDY B-C is the same as that described for STUDY 1-5 in section 6.1.3.4.

6.2.3.3 Basal blood measurements

Following FMD, an intravenous angiocather will be placed in each arm; one used for hormone and dextrose infusion and the other

used for sampling blood glucose. An I-STAT machine will be used to screen for hypokalemia upon obtaining IV access. We will draw blood levels of the hormones, metabolites, and cytokines listed in table 4 up to three times over a 30 min baseline sampling period.

Table 4: Labs drawn during BASAL period

Hormones	Metabolites	Cytokines
Insulin C-peptide Glucagon Cortisol Catecholamines Estradiol Testosterone Adiponectin	Glucose NEFAs Glycerol Lactate Alanine Lipoprotein subclass sizes, concentrations Urine microalbumin	TNF-α IL-1 and 6 hsCRP VEGF ICAM VCAM E-selectin endothelin-1 Fibrinogen PAI-1

6.2.3.4 Hyperinsulinemic-euglycemic clamp (EUGLY CLAMP)

In STUDY B-C, we will conduct a 150 min hyperinsulinemic, euglycemic clamp after drawing basal labs in the same manner described for STUDY 1-5 in section 6.1.3.8.

6.2.3.5 CLAMP sampling period (SAMPLE)

A second sampling period will measure the blood parameters listed in table 4 under insulin-stimulated conditions up to three times during the last 30 minutes of the clamp.

The total volume of all blood drawn from each STUDY B-C visit is calculated to equal 127 mL.

6.2.3.6 STUDY visit completion

Upon completion of the study, we will stop the insulin infusion and allow the study subject to eat. The study team will continue monitoring plasma glucose for at least an additional hour and then steadily decrease the glucose infusion rate (GIR) to maintain glucose > 120 mg/dL. We will instruct T1DM participants to resume their home regimen, however they will be instructed to check glucose before dinner, bedtime, and at 2 AM following the study. 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.

6.2.3.7 Rescheduling STUDY visit 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. In the event such a technical issue occurs, the PI may elect to reschedule a new, repeat STUDY visit provided he deems the technical issue poses no serious danger to the participant were it to occur again and the participant wishes to repeat the study.

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 (62, 63). 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 Hyperinsulinemia

The hyperinsulinemic, euglycemic clamp exposes participants to high insulin levels, potentially leading to hypoglycemia and hypokalemia. As shown in figures 6 and 8, the insulin infusion rate during the clamp will be 40 mU/m^2/min. This infusion rate is anticipated to result in plasma insulin concentrations between 50 and 130 μU/mL,(64, 65) levels that are within the range of physiological hyperinsulinemia and comparable to concentrations seen after meal ingestion.(65-67) By comparison, the most commonly used insulin infusion rate in the treatment of diabetic ketoacidosis is 0.1 U/kg/hr.(56) 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.

Nondiabetic CONTROL SUBSTUDY participants will be exposed to low levels of iatrogenic hyperinsulinemia from low doses of insulin glargine (STUDY 6-7)

and overnight IV regular insulin infusions prior to STUDY visits (STUDY 5 and 7). The insulin glargine dose of 0.2 u/kg/day was selected to induce long-term (7 days) iatrogenic hyperinsulinemia while avoiding the confounding effect of hypoglycemia. Based on a previous pharmacokinetic-pharmacodynamic study of glargine, this dose is anticipated to drop fasting plasma glucose by no more than 10 mg/dL.(68) Because insulin glargine is a long-acting insulin analog, we do not anticipate it will cause a rapid drop in plasma glucose concentrations, but rather a slow decrease over the course of hours. For short-term hyperinsulinemia, participants will receive intravenous (IV) regular insulin at 8 $mU/m^2/min$ for 8 hours overnight preceding the STUDY visit. Based on my previous research, I anticipate this dose will raise fasting plasma insulin concentrations in CONTROL SUBSTUDY participants from ≈8 μU/mL to ≈21 μU/mL, matching the average fasting insulin concentration of T1DM SUBSTUDY participants.(69)

We will use the following safety provisions to minimize risk of hypoglycemia and hypokalemia:

7.1.1 Glucose monitoring

Glucose levels will be monitored every hour during the overnight insulin infusion for T1DM participants as outlined in Appendix 1. Because CONTROL SUBSTUDY participants will receive IV insulin overnight prior to STUDY 5 and 7, we will monitor every hour in the CRC on the night preceding these studies. We will monitor plasma glucose concentrations every 5-10 minutes throughout the hyperinsulinemic clamp to guide the variable dextrose infusion and ensure euglycemia. Because of the frequent glucose monitoring, the study team can quickly detect a trend toward hypoglycemia before it actually occurs. If hypoglycemia does occur, however, this frequent monitoring allows for rapid detection of the low glucose concentration and rapid corrective treatment with an infusion of IV glucose (dextrose).

7.1.2 Correction of potential hypoglycemia

A licensed MD, NP, or PA will be on call throughout overnight CRC stay and at bedside during the clamp procedure. Concentrated dextrose will be at bedside to treat iatrogenic hypoglycemia. If hypoglycemia occurred, the study team could rapidly treat the low blood glucose. For example, a 75 mL IV bolus of 20% dextrose (15 gm of glucose) would bring glucose up above the hypoglycemic range in less than 5 minutes. CRC staff could deliver the dextrose into either of the two IV angiocatheters available during the study.

7.1.3 Avoiding post-discharge hypoglycemia

KSP will counsel subjects on the risk of late hypoglycemia the evening and night following the clamp. We will advise them of the importance of

checking their blood glucose at bedtime and during the night to maintain a minimum of 120 mg/dL.

7.1.4 Monitoring CONTROL SUBSTUDY participants for hypoglycemia

CONTROL SUBSTUDY participants will take 0.2 u/kg/day of long-acting insulin glargine during the week prior to STUDY 6-7 and matched saline placebo during the week prior to STUDY 4-5. As discussed at the beginning of section 7.1 this dose is expected to decrease the fasting plasma glucose concentration by 10 mg/dL or less. Even so, the possibility of hypoglycemia in these nondiabetic control participants cannot be entirely avoided. Accordingly, these participants will monitor their glucose closely during the week prior to STUDY visits 4-7. The study will supply CONTROL SUBSTUDY participants with a conventional capillary blood glucose meter or a continuous glucose monitor (CGM). If a conventional capillary blood glucose meter is used, KSP will instruct participants to check their blood glucose each morning prior to breakfast (when they are most likely to have hypoglycemia after fasting overnight) and more often if needed at the discretion of the PI. If a CGM is used, the device will monitor glucose continuously during the week prior to STUDY visits 4-7. At the screening visit, KSP will teach CONTROL SUBSTUDY participants to identify symptoms of hypoglycemia. Should these symptoms occur, CONTROL SUBSTUDY participants will check their glucose level using either their conventional capillary glucose meter or their CGM. If the glucose is less than 70 mg/dL, participants will use the commonly-taught “Rule of 15” (https://www.diabetes.org/diabetes/medication-management/blood-glucose-testing-and-control/hypoglycemia) to treat the hypoglycemia. Participants will contact the study team after such an event occurs. The PI will then consider altering the insulin glargine dose or even withdrawing the participant based on his clinical judgement, in conjunction with the data safety monitor.

7.1.5 Safety provisions to minimize hypokalemic risk

Hyperinsulinemia causes extracellular potassium to shift into the intracellular space, a factor that could contribute to apparent hypokalemia. Vital signs will be monitored throughout the STUDY visit procedure. Potassium will be monitored using bedside istat monitor. Oral potassium replacement will be given as needed at the discretion of the PI or his designated MD, NP, or PA KSP.

7.2 Risks Associated with Perflutren Lipid Microsphere Infusion

7.2.1 Clinical and Research Application of Perflutren

Perflutren is an ultrasound contrast agent indicated for clinical use in patients with suboptimal echocardiograms to opacify the left ventricular chamber and improve the delineation of the left ventricular endocardial

border.(70) This agent was initially FDA approved in 2001 and in Europe indications also include the detection and characterization of liver and breast masses as well as for Doppler enhancement and the assessment of vasculature.(71)

Current ultrasound contrast agents, including perflutren, consist of microbubbles of an inert, relatively insoluble gas encapsulated by an outer lipid shell. Perflutren (octafluoropropane) is a high-density, high-molecular weight gas that exhibits low solubility. The mean diameter of a microsphere ranges between 1.1-3.3 µm,(70) which allows passage from the peripheral injection site into the systemic circulation, but prohibits passage through the endovascular borders. Thus, the microspheres remain as intravascular indicators. When coupled with ultrasound, the microspheres induce a large alteration in the acoustic impedance reflection patterns within tissue or blood. As a result, there is a marked increase in the signal-to-noise ratio, which enhances ultrasound signals and provides a dramatic improvement in the vascular imaging quality.(71)

The use of ultrasound contrast agents in diagnostic imagining extends beyond the currently approved indications as well. Increasingly, perflutren is used in human subjects' research to study changes in microvascular blood volume in muscle and adipose tissue.(40) More recently, contrast-enhanced ultrasound with perflutren quantified insulin-stimulated changes in vasodilation in healthy young adults (72) and individuals with T1DM.(73) Our study will quantify how much decreasing chronic iatrogenic hyperinsulinemia improves insulin-stimulated increases in microvascular blood volume in T1DM using this technique.

7.2.2 Summary of Recent Safety and Efficacy Data

Appis et al. recently reviewed articles reporting safety and efficacy data.(71) Table 5 summaries the key findings from these articles.

Table 5: Summary of Perflutren Lipid Microsphere Safety Data

Citation	Population studied	Analysis Design	Key Safety Findings
(74)	1,513 inpatients with pulmonary hypertension	Retrospective series	3/1513 patients had any SAE and none were directly attributed to perflutren itself
(75)	63,189 patients undergoing 96,705 transthoracic echocardiograms, including 2,518 using perflutren	Retrospective series	24-hour mortality was 0.44% in the definity group and 0.69% in the non-contrast group (p=0.14). Use of perflutren was not associated with increased mortality in multivariate analysis.

(76)	5,576 patients undergoing 5,956 contrast-enhanced echocardiograms	Retrospective series	16 adverse events occurred (0.27%), all mild and transient, most commonly back pain and rash. No SAEs occurred.
(77)	39,020 perflutren-enhanced echocardiograms including in 418 patients with known right to left intracardiac shunts	Retrospective series	No neurological or systemic embolism events occurred. Most common secondary adverse event was back pain (25/39,020)

Package labelling reports adverse reactions from a total of 1,716 subjects studied in pre-market clinical trials.(70) Of these, 144 (8.4%) had at least one adverse reaction. Table 6 summarizes the adverse reactions occurring in at least 0.5% of participants.

Table 6: Summary of Adverse Reactions from Perflutren Lipid Microsphere Package Insert

Adverse Reaction	Number of events (%)
Injection site reaction	11 (0.6%)
Back/renal pain	20 (1.2%)
Chest pain	13 (0.8%)
Pain in body as a whole	41 (2.4%)
Headache	40 (2.3%)
Dizziness	11 (0.6%)
Nausea	17 (1.0%)
Flushing	19 (1.1%)

Extrapolating these safety data—collected from patients with known cardiac disease—to the relatively younger, otherwise-healthy T1DM and control populations of this study suggests the risk of a serious or non-serious adverse event from perflutren infusion is low. Nonetheless, we will take important safety precautions in this elective research study.

7.2.3 Safety Provisions to Mitigate Adverse Events Associated with Perflutren Lipid Microspheres

Because perflutren is not FDA approved for use in the pediatric population, we will only use CEUS in adult participants.

The informed consent document will include the previously mentioned risks associated with perflutren lipid microsphere infusion and discussed during the consent process. The PI will be physically present throughout the

STUDY visits, which include two infusions of perflutren lipid microspheres. Further, the PI will be under the supervision of a board-certified cardiologist (Dr. Beckman) and internist (Dr. Shibao) who have extensive experience using this technique. The PI will discuss the conduct of the study on a routine basis. In rare circumstances, the PI may elect to allow a designed KSP (MD, NP, or PA) to provide medical supervision in his absence. STUDY visits will be conducted in the Vanderbilt Clinical Research Center, a state-of-the-art facility that has cardiac monitoring and resuscitation equipment readily available. Vital signs will be monitored throughout each STUDY visit.

7.3 Anemia

For STUDY B-C we will collect a small blood sample (10 mL) at the screening visit. This blood sample will include a complete blood count (CBC) test to ensure each participant's hematocrit is at least 35%.

Within eight weeks of the screening visit, we will conduct two hyperglycemic, euglycemic clamps on STUDY B-C (Aim 3) participants, with up to one month separating each study (see section 6.2). The total amount of blood drawn in each STUDY B-C visit equals 127 mL. This includes the volume of fluid in the angiocatheter that must be cleared with each blood draw to ensure blood samples are not diluted (41 mL in total). This fluid, often called "waste," is mostly saline running through the angiocatheter to ensure the catheter remains patent but contains some blood. Thus, the maximum amount of total blood drawn in each study is calculated to equal 127 mL. Thus, we calculate a maximum volume from the STUDY B-C visits of 254 mL over one month.

As reviewed in a World Health Organization Bulletin,(78) while several guidelines are available for reference, there is a limited amount of direct evidence on which to base them. We have set 50 kg as the minimum required body mass STUDY B-C participants to align with guidance from the Wayne State University Institutional Review Board for Minimal Risk blood collection which states:

"When the collection of blood samples presents minimal risk to adult subjects they may be one by finger stick, heel stick, ear stick, or venipuncture under the following conditions and guidelines:

a. Blood samples may be collected for research purposes from healthy, nonpregnant adults who weigh at least 110 pounds. The amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week".(79)

Further, our approach aligns NIH policy M95-9(rev.) "Guidelines for Limits of Blood Drawn for Research Purposes in the Clinical Center." For adult patients and volunteers, the policy states, "The amount of blood that may be drawn from adult

patients and volunteers (i.e.,those persons 18 years of age or older) for research purposes shall not exceed 10.5 mL/kg or 550 mL, whichever is smaller, over any eight week period."

STUDY 1-5 poses less risk for anemia. The total blood volume drawn in each STUDY 1-5 visit is 124 mL. Although longitudinal study (STUDY 1-3) participants are studied three times, each study visit is separated by more time. We do not anticipate the time between STUDY1 and STUDY2 will ever be less than 1 month and anticipate the normal interval between STUDY1 and STUDY2 will be between 2-4 months.

In addition to obtaining a complete blood count at screening, the PI will clinically monitor all volunteers for signs and symptoms of anemia throughout their participation. Specifically, the PI will monitor participants for easy fatigability, lassitude, muscle cramps, postural dizziness, or recent onset of dyspnea. If these clinical signs and symptoms are present, a repeat hematocrit will be ordered. If hematocrit is < 35%, the participant will be excluded from further study.

7.4 Hypotension

Nitroglycerin is administered sublingually during the flow mediated dilation studies. This may cause a fall in blood pressure and symptoms of hypotension. To avoid the latter, nitroglycerin will not be administered to any participant whose systolic blood pressure is ≤100 mmHg.

7.5 Special Protections for Adolescents as Research Subjects

Because the study includes adolescent T1DM subjects between ages 15-18, special consideration has been given to provisions designed to protect children as outlined in 45 CFR 46, Subpart D. Because adolescent participants without T1DM would not meet the criteria of 45 CFR 46.406, control participants will only include adult participants.

7.5.1 Justification and rationale for inclusion of adolescent subjects with T1DM

The atherosclerotic changes of macrovascular disease in T1DM (e.g. increasing carotid intima media thickness) appear to begin in childhood and adolescence(80, 81) and morbidity and mortality risk is staggeringly high in younger adults.(10) 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 <u>early</u> in the natural history of T1DM*. These same early pathophysiologic mechanisms have not been identified, however.

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,(9) *suggesting that exposure to insulin resistance begins early in the natural history of T1DM.*

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

1) T1DM insulin resistance appears to begin early in the disease process.(9, 28)
2) The incidence of T1DM onset is highest between ages 10-14.(82)
3) At younger ages and shorter disease durations there are minimal confounding covariables that would contribute to insulin resistance at a molecular level. These confounding covariables include co-morbidities associated with T1DM such as diabetic nephropathy,(83) prolonged exposure to hypertriglyceridemia,(84, 85) excessive BMI(86, 87) and visceral adiposity,(88, 89), deleterious effects of aging and progressive inflammation on extracellular matrix remodeling,(90, 91) and increasing age per se.(92, 93)

By studying adolescent and young adults early in the disease duration, this investigation will more precisely determine iatrogenic hyperinsulinemia's association with insulin resistance and endothelial dysfunction at a very early point in the course of T1DM when confounding covariables are minimal. This knowledge is critical towards guiding subsequent therapy to mitigate insulin resistance and endothelial dysfunction, which will considerably reduce early mortality risk from macrovascular disease in the same subjects tested in the research.

7.5.2 <u>Assessment of risk to adolescent participants relative to 45 CFR part 46, subpart D</u>

It is our impression that T1DM subjects would fall under 45 CFR 46.406, as outlined in Vanderbilt's Human Research Protections Program Policy Number IX.A, section II.C. 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 in adolescents by the PI and elsewhere. The technique has been performed over one thousand times in healthy adolescent subjects at other institutions such as the University of Minnesota,(94-97) hundreds of times in overweight adolescent(98-100) and normal weight prepubertal subjects(101, 102) at the University of Pittsburgh, and a similar number of times in normal weight children and adolescents at the National Institutes of Health.(103, 104) 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 7.1, extensive efforts have been employed to minimize risk of hypoglycemia and hypokalemia associated with hyperinsulinemia.
- The PI has personally performed the hyperinsulinemic, euglycemic clamp over 80 times in human subjects and nearly as many 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.(95) 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.
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.

7.6 Discussion of Risks as Part of the Consent Process

The study team will discuss all procedures, risks, and benefits with potential study participants, as part of the consent process. An IRB approved written informed consent document will be required for participation in this study. It is understood that consent is a process and not a discrete event. A participant's decision to withdraw consent 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

8.1 Adverse Events

8.2 Serious Adverse Events

8.2.1 Defining Serious Adverse Events

Consistent with FDA guidelines, serious adverse events (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. bronchospasms requiring emergency department referral, seizures that might not result in hospitalization).

8.2.2 Assessing relationship between a SAE and relationship to study procedures

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

8.2.3 Assessing whether an AE is an anticipated problem

Any AE will be assessed for whether or not it was an anticipated problem. In accordance with Department of Health and Human Services 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.

8.2.4 Unanticipated, non-serious AEs

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) and the IRB of any changes needed to the protocol. If needed, appropriate changes will be made to the consent form.

8.2.5 Unanticipated SAEs

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.

8.2.6 Adverse Event Reporting

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 whowill fund the PI on a K23 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 (or designated MD, NP, or PA KSP) medical judgement is that participation places the subject at risk for harm

10.0 Statistical Considerations

10.1 STUDY 1-7 (Aims 1-2)

10.1.1 Sample Size Justification

My accrual goal is to obtain an adequate number of participants within both the T1DM SUBSTUDY (STUDY 1-3) and CONTROL SUBSTUDY (STUDY 4-7) to ensure greater than 80% statistical power (1-β) for a two-sided α-level of 5% for each specific aim's primary outcome. The sample size calculations used the best-available previously published descriptions of insulin sensitivity(28, 105) and %FMD(7, 30, 106-108) in T1DM. Table 7 summaries these calculations.

Table 7: Key parameters for sample size calculations for T1DM and CONTROL substudies. n, *δ, 1-β, σ,* and *r* represent the sample size, the expected minimum difference, statistical power, overall standard deviation, and longitudinal correlation, respectively, for each key outcome. * indicates calculations based on preliminary data as previously described. † The detectable differences for GIR of 1.3 to 2.2 mg/kg/min represent a 20% to 21% change in outcomes. ‡ The detectable differences for %FMD of 2.2% to 4.3% represent a 25% to 39% change in outcomes.

	T1DM Substudy						Control Substudy					
Aim	**Key Outcome for Power Calculation**	***n***	***δ***	***1-β***	***σ***	***r***	**Key Outcome for Power Calculation**	***n***	***δ***	***1-β***	***σ***	***r***
1	GIR between STUDY T^{LO} vs. T^{HI} in mg/kg/min	20	1.3	0.88	2.7	0.79	GIR between STUDY C^{BOTH} vs. C^{0} in mg/kg/min	20	2.2	0.92	3.5	0.79
2	%FMD between STUDY T^{LO} vs. T^{HI}	20	2.2%	0.88	4.3%	0.83	%FMD between STUDY C^{BOTH} vs. C^{0}	20	4.3%	0.83	3.7%	0.83

10.1.2 Analysis Plan

The correlation between the independent variable $TDD_{insulin}$ (averaged over 7 days prior to each STUDY visit) and the key dependent variable for each objective will be determined: GIR in Aim 1; %FMD and MVR in Aim 2. Several other explanatory variables could influence each specific aim's key dependent variable, however. For example, confounding variables might include hyperglycemia, hyperlipidemia, or the dependent variables in each aim could affect one another (e.g. insulin resistance could affect endothelial dysfunction). For this reason, I will use multivariable linear regression analysis to account for the effect of potential confounders, which include age, T1DM duration, glycemia (both from both CGM over 7 days and HbA1c), lipidemia (i.e. lipoprotein subclass sizes and particle concentrations), NEFA concentration, hypertension, BMI, body composition, and estrogen level.

The study accrual of 40 participants provides 3 to 4 degrees of freedom for a multivariable linear regression analysis. (i.e. $TDD_{insulin}$ and 3 additional independent variables can be included in an adequately-powered model). To quantify the effect of potential confounders, unadjusted and adjusted linear regression models will be fit and compared for each Aim's primary outcome (GIR and %FMD). I will analyze changes in β-coefficients between unadjusted and adjusted models for scientifically significant differences.

10.1.3 Recruitment goals

Based on Yki-Jarvinen et al., I anticipate a 15% drop out rate.(28) Thus, 23 study participants in each substudy will be enrolled to achieve the recruitment goal of 20 longitudinal study participants.

10.1.4 Interim assessment

I will make one interim assessment after reaching one-half of the target accrual for the longitudinal, cross-sectional, and control studies. Using

Obrien-Fleming bounds for each aim's primary analysis with a two-sided test with α-level of 5%, a critical z-value of 2.80 (corresponding to a p-value of 0.0051) will be used to end the study at interim analysis. We will analyze our study's variance and adjust the sample size if needed.

10.2 STUDY B-C (Aim 3)

10.2.1 Sample size justification

My accrual goal for STUDY B-C is to have a sufficient sample size to conduct multivariable linear regression with 4 degrees of freedom to quantify the effect of the independent variable, mean $TDD_{insulin}$, on the dependent variable, GIR during the last 30 minutes of the clamp study. At a minimum, 10 participants are needed per degree of freedom to conduct this analysis, thus, we will study 30 participants with T1DM and 10 control participants who are otherwise healthy.

Based on Del Prato et al.,(27) I anticipate participants completing the SCD arm will not see a change in GIR during the last 30 minutes of the clamp study compared to baseline, but will see an increase in GIR of at least 0.6 mg/kg/min after completing the LCD arm. This study also suggests the pooled standard deviation of GIR will be 0.8 mg/kg/min. If the true difference in the ΔGIR between LCD and SCD arms for the 30 T1DM participants is 0.6 mg/kg/min, we will be able to reject the null hypothesis that the difference is zero with probability (power) 0.976. The type 1 error probability associated with this test of this null hypothesis is 0.05.

10.2.2 Analysis Plan

The correlation between the independent variable $TDD_{insulin}$ (averaged over 7 days prior to each STUDY B-C visit) and the GIR over the last 30 minutes of the clamp will be determined. Several other explanatory variables could confound this bivariate analysis, however. For example, confounding variables might include treatment order, hyperglycemia, hyperlipidemia, or measures of glycemic variability. For this reason, as with STUDY 1-5, I will use multivariable linear regression analysis to account for the effect of these potential confounders.

10.2.3 Recruitment goals

Based on Yki-Jarvinen et al., I anticipate a 15% drop out rate.(28) Thus, 35 T1DM and 12 control participants will be enrolled to achieve the recruitment goal of 30 T1DM and 10 control participants completing the study.

10.2.4 Interim assessment

I will make one interim assessment after reaching one-half of the target accrual for the T1DM and control participants. Using Obrien-Fleming bounds for each aim's primary analysis with a two-sided test with α-level of

5%, a critical z-value of 2.80 (corresponding to a p-value of 0.0051) will be used to end the study at interim analysis. We will analyze our study's variance and adjust the sample size if needed.

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 up to four years. The study results will be maintained indefinitely for research purposes.

APPENDIX 1: Overnight Insulin Infusion Prior to STUDY 1-3 visits (T1DM Participants only)

Overnight Insulin Infusion Protocol

The following insulin infusion protocol is intended for use in T1DM participants in the setting of the overnight insulin infusion prior to hyperinsulinemic clamp studies. Target blood glucose is **90-119 mg/dL**.

Initiating the Insulin Infusion

1. START TIME: the overnight infusion should begin once IV access is obtained the evening before the clamp study unless directed otherwise.
2. TWO PHASES OF INSULIN INFUSION: The IV insulin infusion will proceed in two phases. First, we will infuse insulin at a constant rate from the time IV access is obtained after admission through midnight. This insulin infusion rate will equal the basal infusion rate provided by the participants' insulin pumps at home. Second, beginning at midnight and continuing through the clamp study in the morning, we will adjust the insulin infusion rate each hour as needed to reach a constant blood glucose concentration of 90-119 mg/dL.
3. PREPARATION FOR INFUSION: insulin will be dispensed as 100 units diluted in 100 mL of 0.9% NaCl.
    a. PRIMING: flush 10 mL of insulin infusion through all IV tubing before insulin infusion begins (to saturate insulin binding sites in the tubing).
    b. CARRIER FLUID: 0.9% NaCl fluid at 20 mL/hr can be run with the IV insulin infusion to keep the vein open.
    c. HYPOGLYCEMIA PREVENTION: 20% dextrose solution should be hung and ready in case of hypoglycemia. Glucagon should be within easy reach.
4. TRANSITIONING FROM HOME INSULIN REGIMEN: T1DM participants will use an insulin pump at home.When IV access is obtained and you are ready to begin the IV insulin infusion, ask the participant to suspend and disconnect his or her insulin pump immediately before starting the overnight insulin infusion
5. INITIAL IV INSULIN INFUSION RATE: initial infusion rate will equal the basal rate being infused by the participant at home using their insulin pump.
    Example: Home insulin pump basal rate = Humalog 0.8 units/hr
    Initial IV insulin infusion rate = 0.8 unit/hr

Blood glucose (BG) Monitoring

1. Check BG hourly on the hour beginning at 2300 and record BG and insulin infusion rate on the task flowsheet on the designated line.

Changing the Insulin Infusion Rate (Beginning at Midnight)

Beginning with the Midnight BG and with each subsequent BG check, change the insulin infusion rate as follows:

IF BG < 55 mg/dL:

STOP INSULIN INFUSION

- Bolus 100 mL of D20 (20 gm of glucose) immediately
- Contact PI (Justin Gregory, mobile: [redacted] pager [redacted]
- Check BG q 15 minutes
- When BG is ≥ 90 mg/dL, wait 1 hour, recheck BG. If still ≥ 90 mg/dL, restart insulin infusion at 50% of the most recent rate

IF BG 55-69 mg/dL:

STOP INSULIN INFUSION

- Contact PI immediately (Justin Gregory, mobile: [redacted] pager [redacted]
- Prepare to give D20 at approximately 3.0 mg/kg/min after discussing with PI.
- Check BG q 15 minutes
- When BG is ≥ 90 mg/dL, wait 1 hour, recheck BG. If still ≥ 90 mg/dL, restart insulin infusion at 75% of the most recent rate

IF BG > 70 mg/dL:

STEP 1: Determine the CURRENT BG LEVEL. This identifies a COLUMN in the table:

BG 70-89 mg/dL	BG 90-119 mg/dL	BG 120-179 mg/dL	BG ≥ 180 mg/dL

STEP 2: Determine the RATE OF CHANGE from the prior BG level. This identifies a CELL in the table. Then move to the right for **INSTRUCTIONS**. *(Note: if the last BG was measured less than an hour before the current BG, calculate the hourly rate of change. Example: if the BG at 0200 was 85 mg/dL and an additional BG is checked early at 0230 was 70 mg/dL, the total change over 30 minutes is -15 mg/dL; however, the hourly rate of change is -15 mg/dL x 2 = -30 mg/dL).*

BG 70-89 mg/dL	BG 90-119 mg/dL	BG 120-179 mg/dL	BG ≥ 180 mg/dL	INSTRUCTIONS*
		BG ↑ by > 40 mg/dL/hr	BG ↑	↑ INFUSION by "2Δ"
	BG ↑ by > 20 mg/dL/hr	BG ↑ by 1-40 mg/dL/hr OR BG UNCHANGED	BG UNCHANGED OR BG ↓ by 1-40 mg/dL/hr	↑ INFUSION by "Δ"
BG ↑	BG ↑ by 1-20 mg/dL/hr, BG UNCHANGED, OR BG ↓ by 1-20 mg/dL/hr	BG ↓ by 1-40 mg/dL/hr	BG ↓ by 41-80 mg/dL/hr	NO INFUSION CHANGE
BG unchanged OR BG↓ by 1-20 mg/dL/hr	BG ↓ by 21-40 mg/dL/hr	BG ↓ by 41-80 mg/dL/hr	BG ↓ by 81-120 mg/dL/hr	↓ INFUSION by "Δ"
BG ↓ by > 20 mg/dL/hr see below†	BG ↓ by > 40 mg/dL/hr	BG ↓ by > 80 mg/dL/hr	BG ↓ by > 120 mg/dL/hr	HOLD x 30 min, then ↓ INFUSION by "2Δ"

***CHANGES IN INSULIN INFUSION RATE** ("Δ") are determined by the current rate:

Current Rate (units/hr)	Δ = Rate Change (units/hr)	2Δ = 2X Rate Change (units/hr)
≤ 1	0.25	0.5
>1-3	0.5	1
> 3	1	2

† **DISCONTINUE** insulin infusion. Check BG q 30 min; when BG is ≥ 90 mg/dL restart insulin infusion at 75% of most recent rate

APPENDIX 2: EXAMPLE OF DE-IDENTIFIED .CSV FILE EXPORTED FROM MYFITNESSPAL

Date	Meal	Time	Calories	Fat (g)	Saturated	Polyunsat	Monounsa	Trans Fat	Cholester	Sodium (n	Potassium	Carbohydı	Fiber	Sugar	Protein (g	Vitamin A	Vitamin C	Calcium	Iron	Note
10/30/2019	Breakfast	7:45 AM	500	28	10	0	0	0	250	1120	0	47	2	15	18	6	0	10	15	
10/30/2019	Lunch	1:00 PM	636	27.8	9	0	0	0	62	1161.2	640	70.8	7.6	1.1	28.2	120	90	8	14	
10/30/2019	Dinner	8:00 PM	1360	53.5	19.3	0	0	0	135	2860	0	154.5	19	15	65	75	61	40	55	
10/30/2019	Snacks	3:50 PM	250	13	5	0	0	0	5	25	0	30	2	25	5	0	0	4	2	
10/30/2019	Snacks	4:00 PM	480	25	4	2.5	3	0	0	600	370	51	7	26	19	10	10	22	18	
10/30/2019	Snacks	10:30 PM	175	8	1.3	0	0	0	0	57.5	160	25	5	17.5	3.5	2	4	0	2	
10/31/2019	Breakfast	7:35 AM	450	20	8	0	0	0	30	1370	0	48	2	6	16	2	0	6	25	
10/31/2019	Lunch		636	27.8	9	0	0	0	62	1161.2	640	70.8	7.6	1.1	28.2	120	90	8	14	
10/31/2019	Dinner	7:05 PM	1060	61	21.5	0	0	0	135	2325	0	79	5	8	48	142	26	70	22	
10/31/2019	Snacks	3:00 PM	450	22	11	0	0	0	124	525	450	54	3	37	13	10	4	36	10	
11/1/2019	Breakfast	9:35 AM	900	72	25.5	0	0	0	865	1910	0	11	0	4	50	0	0	0	0	
11/2/2019	Breakfast	9:05 AM	716.7	65.5	20.8	9	18	2	907.5	1226.7	625	7.7	0.7	5.5	46.2	35	0	20	23	
11/2/2019	Lunch	11:45 AM	1195	64	23	0	0	1	177	1162	0	93	4	0	57	0	20	2	4	
11/3/2019	Breakfast		438.9	23.1	7	0	14	0	65	1571.6	124	81.4	4.1	15.7	9.4	6.2	31	8.8	4.1	
11/3/2019	Breakfast	7:55 AM	210	21	6	4.5	8.1	1.2	507	264	243	0	0	2.4	18	21	0	12	12	
11/3/2019	Snacks	9:55 AM	190	2	0	0	0	0	5	110	0	37	1	31	11	10	0	35	15	
11/4/2019	Breakfast		440	20	8	0	0	0	30	1370	0	48	2	6	16	2	0	6	25	
11/4/2019	Lunch	12:00 PM	510	18	7.5	0	0	0	90	1380	0	54	3	3	36	0	0	0	0	
11/4/2019	Dinner	7:30 PM	740	27	15	2	8	0	190	1218	508	64	4	4	61	16	16	12	22	
11/5/2019	Breakfast	7:20 AM	545	33	14	0	0	0	250	1170	0	45	2	11	18	6	2	10	20	
11/5/2019	Lunch	12:40 PM	636	27.8	9	0	0	0	62	1161.2	640	70.8	7.6	1.1	28.2	120	90	8	14	
11/5/2019	Dinner	5:25 PM	1170	60	21	0	0	1	255	1770	0	112	8	13	43	0	20	2	4	
11/6/2019	Breakfast	7:50 AM	500	28	10	0	0	0	250	1120	0	47	2	15	18	6	0	10	15	
11/6/2019	Lunch	12:30 PM	510	18	7.5	0	0	0	90	1380	0	54	3	3	36	0	0	0	0	

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