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COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD
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Project Title: Effect of Metformin on Vascular and Mitochondrial Function in Type 1 Diabetes

Version date: 6/10/2015

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I. Hypotheses and Specific Aims:

Underlying Hypotheses: (1) IR is an important, clinically unaddressed contributor to increased CVD risk in T1D. (2) Treatment of insulin resistance with metformin will improve insulin action, endothelial function, arterial compliance, and mitochondrial function in T1D.

The proposed pilot study is a mechanistic study addressing the hypothesis that the clinically available agent, metformin, widely used as first line therapy in type 2 diabetes, **will improve insulin action and vascular function and have the previously unstudied benefit of reducing mitochondrial oxidant generation in T1D.** Targeting IR is a novel approach to T1D treatment that may significantly improve current management of TID and of CVD risk in this high risk population.

Specific Aims:

SA#1: Determine the effect of metformin on insulin sensitivity in T1D.

Hypothesis 1: Metformin will improve insulin sensitivity and insulin signaling in T1D subjects similarly to type 2 diabetic subjects. Approach: T1D subjects (n=30) will have placebo then metformin added to their usual insulin regimen for six weeks in a single-blinded, cross-over design study. Continuous glucose monitoring (CGM) will be performed throughout to allow statistical adjustment for changes in glycemic control and to facilitate insulin dose adjustment to avoid hypoglycemia. Insulin sensitivity will be measured by two-stage (8, 40 mU/m^2/min) hyperinsulinemic euglycemic clamp with glucose tracer and insulin signaling will be measured in muscle biopsy samples.

SA#2: Determine the effect of metformin on vascular and mitochondrial sequelae of insulin resistance

Hypothesis 2: Metformin will improve vascular and mitochondrial measures in T1D subjects supporting a role for IR in vascular disease in T1D. Approach: T1D subjects (n=30) will have placebo and then metformin added to their usual insulin regimen for six weeks as in SA#1. Vascular function will be measured by flow-mediated dilation, overall vascular reactivity by venous plethysmography, and arterial compliance by applanation tonometry (Sphygmacor Vx device) and brachial artery distensibility (DynaPulse). Heart rate variability will be assessed as a measure of cardiac sympathetic nervous system balance. Mitochondrial function will be assayed by respirometry (Oroboros Oxygraph O2K) and oxidant generation by Amplex Red (invitrogen) in muscle biopsy samples. A subset of subjects (n=10) will also have mitochondrial function assessed by 31P-MRS to measure ATP production.

The results of the above study may support an important new therapeutic approach to T1D and thus have the potential to significantly alter the future of cardiovascular disease risk management and mortality in T1D.

II. Background and Significance:

The prevalence of cardiovascular disease (CVD) is increased in type 1 diabetes (T1D) at least as much as in T2D, and CVD remains the leading cause of death in T1D despite improvement in glycemic control and aggressive management of standard CVD risk factors[1-5]. Recent studies suggest that we have reached the limit of our ability to safely control glucose levels with currently available therapy[6-8]. In addition, we and

others have found that existing CVD risk prediction models do not accurately identify T1D individuals at risk for CVD[9], suggesting that management of standard CVD risk factors may be inadequate in T1D. Clearly there is a significant gap in our therapeutic approach to CVD risk in T1D. Insulin resistance (IR) is a potential candidate for a novel therapeutic target to fill that gap. IR, a known correlate of, and possible contributor to, CVD disease in type2 diabetes (T2D)[6, 7, 10-19] and in the general population[11, 20-26], is also present in T1D but is less well understood in this setting[27-30]. For example, IR is present even in T1D individuals who lack all features of the metabolic syndrome phenotype that characterizes IR in other populations[31-33]. In the Pittsburgh EDIC study and the DCCT, estimated IR predicted CVD events[34, 35], suggesting that IR is a correlate of CVD in T1D as well[35, 36]. We have found that a direct measure of IR correlates with coronary artery calcification (CAC), a surrogate marker for CVD, in T1D. IR thus represents a novel therapeutic target with the potential to impact CVD risk in T1D. However, the best approach to intervention and possible cardiovascular benefits of such intervention are unknown.

Treatment targeted at improving insulin resistance is standard of care for type 2 diabetes and has been shown to improve cardiovascular mortality independent of glucose control and standard cardiovascular risk factors. In type 2 diabetes, metabolic syndrome, and polycystic ovarian syndrome metformin improves measures of blood vessel function and stiffness, measures that correlate with cardiovascular disease risk. Insulin resistance in type 2 diabetes is also associated with mitochondrial dysfunction and considerable recent evidence supports a synergistic relationship between endothelial function and mitochondrial health. Metformin appears to exert its beneficial effects tissues and blood vessels at least in part through decreased mitochondrial reactive oxygen species (ROS) generation [37-42]. A number of small studies have explored metformin's effects on glycemic control, lipids, and insulin dosing in T1D [43]. These studies have found metformin to be safe and well tolerated in T1D. Decreases in insulin requirement seen in these studies support an insulin sensitizing or other glucose utilization promoting effect of metformin. However, the effect of metformin on other measures in T1D is largely unstudied. The primary goal of this proposal is to measure the effect of metformin on insulin sensitivity, vascular function and compliance, and mitochondrial function in T1D. The long term goal is to identify novel non-glycemic approaches to managing cardiovascular disease risk in T1D. The results of this study may validate a novel approach to T1D treatment that could significantly improve current management of cardiovascular disease risk in this high risk population.

Overall Hypothesis: Treatment of insulin resistance with metformin will improve insulin action, endothelial function, arterial compliance, and mitochondrial function in T1D.

MODEL: We propose that IR contributes to CVD risk in T1D through acute effects on vascular function and on mitochondrial function and ROS generation (Figure 1). These studies test the ability of insulin sensitization with metformin to improve vascular and mitochondrial function and challenge the dogma that glycemic control is the only diabetes-specific clinical intervention needed for optimal management of T1D and the associated CVD risk. The results of these clinical translational studies may identify new pharmacological approaches to CVD risk in T1D and could alter the future of T1D management.

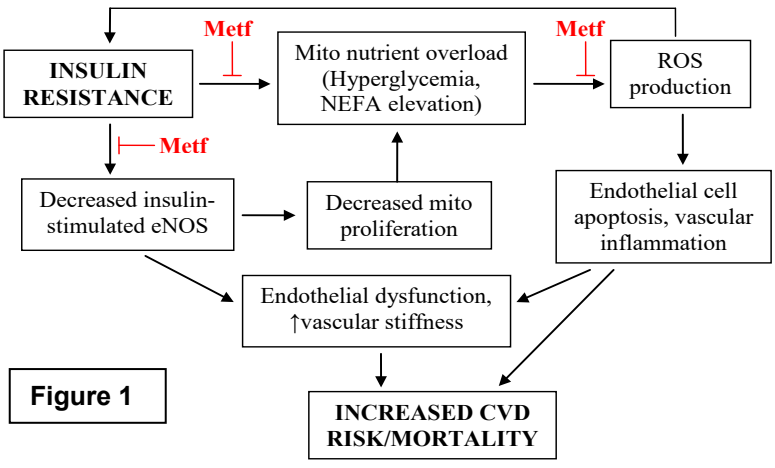


Figure 1

III. Preliminary Studies/Progress Report:

Introduction: The preliminary data presented below provide support for the proposed hypotheses and demonstrate my ability to perform the proposed studies with the mentorship and training described in the career development plan. The data fall into three main categories: (1) data collected in a sub-study of the NIH-CACTI study (PI Dr. Marian Rewers) measuring insulin action in a subset of T1D and non-DM subjects from the CACTI cohort in which I was extensively involved, (2) analyses of data from the overall CACTI study generated specifically in response to this sub-study, and (3) data from Dr. McCurdy demonstrating our ability to measure insulin signaling in tissue biopsy samples.

CACTI study: We are prospectively following CAC in a large cohort of T1D and non-DM subjects. The study enrolled 1,416 individuals between 19 and 56 years of age with no known history of CVD. Subjects with T1D (n=652) had longstanding T1D, were insulin-dependent within a year of diagnosis, and were either diagnosed at <30 years of age, antibody positive, or had a clinical course consistent with T1D. Visits for measurement of CAC by electron beam CT occurred at baseline, year 3, and year 6. The overall CACTI study has been described elsewhere[44, 45].

IR sub-study: 87 subjects (40 T1D, 47 non-DM) were recruited from the CACTI cohort to undergo 3-stage hyperinsulinemic euglycemic clamps for measurement of insulin action. Subjects were excluded for HbA1c >9.5 or macroalbuminuria. Baseline characteristics are shown in Table 1. T1D and non-DM groups were well matched for age, BMI, body fat, and habitual physical activity. T1D subjects had higher HbA1c and fasting glucose values as expected. In addition, significant differences were found in LDL, total cholesterol, triglycerides, and adiponectin, with T1D subjects having "better" (i.e. lower CVD risk) values in all categories. Though liver fat has been found to be a strong indicator of IR in T2D, we observed no differences in a measure of liver fat content, liver:spleen density ratio. *These data show that the T1D subjects in our sub-study cohort do not exhibit a metabolic phenotype by criteria of HDL, TG, waist circumference, blood pressure, or BMI. In fact, by lipid criteria, our non-DM cohort appears more IR than our T1D cohort.*

Table 1: Baseline characteristics for clamp sub-study

	T1DM (n=40)	Control (n=47)	p-value
Age (years)	45.2 ± 9.2	45.9 ± 7.2	0.7
M/F	19/21	20/27	--
Duration of DM (years)	22.6 ± 7.8	--	--
HbA1c (%)	7.5 ± 0.9	5.4 ± 0.3	<0.0001
Fasting preclamp glucose (mg/dl)	116 ± 39	96 ± 8	0.0024
Fasting preclamp insulin (μU/ml)	32 ± 28	8.7 ± 3.9	<0.0001
Body fat (%)	28.6 ± 7.5	29.6 ± 7.1	0.53
BMI (kg/m^2)	27.0 ± 4.4	26.0 ± 4.1	0.31
Visceral fat area (cm^2)	72.7 ± 50.5	80.5 ± 50.5	0.49
Subcutaneous fat area (cm^2)	271 ± 126	273 ± 112	0.97
Sagittal diameter (mm)	214 ± 36	214 ± 31	0.96
Waist circumference (cm)	88.5 ± 12.3	86.3 ± 12.4	0.42
waist:hip ratio	0.84 ± 0.08	0.83 ± 0.09	0.44
Liver/spleen density ratio	1.26 ± 0.08	1.24 ± 0.24	0.58
Total cholesterol (mg/dl)	139.9 ± 32.4	171.1 ± 28.8	<0.0001
HDL-cholesterol (mg/dl)	58.0 ± 22.4	53.4 ± 15.0	0.27
Triglycerides (mg/dl)	69.7 ± 33.8	108.0 ± 57.2	0.0002
LDL-cholesterol	67.6 ± 24.8	96.1 ± 26.0	<0.0001
Systolic blood pressure (mmHg)	114 ± 11	113 ± 10	0.61
Diastolic blood pressure (mmHg)	73 ± 7	76 ± 8	0.09
On Htn meds (%)	45	13	0.0005
Habitual physical activity (logkcal)	7.2 ± 1.2	7.4 ± 7.1	0.42
Adiponectin (geomtric mean±SD)	10.9 ± 1.7	8.2 ± 1.8	0.02
Cortisol	11.8 ± 4.3	11.5 ± 4.4	0.8

IR in T1D: Despite the lack of metabolic syndrome characteristics, hyperinsulinemic euglycemic clamps reveal that our T1D subjects are significantly more insulin resistant than our non-DM subjects [glucose infusion rate (mg/kgFFM/min) 4.46±0.48 vs. 8.58 ±0.45, p<0.0001] (Table 2). Multiple adjustments shown in Table 2, including age, fasting glucose, final clamp glucose and insulin, and BMI or visceral fat area attenuate the difference in IR only very slightly[46]. *These data raise the possibility of IR as a novel therapeutic target in T1D.*

Glycemic control and IR: Linear regression analysis shows no correlation between either HbA1c or continuous glucose monitoring (CGM) measures of glycemic variability, hyperglycemia, or hypoglycemia and GIR (Table 3) or failure of NEFA suppression (not shown). Furthermore, when analyzed by HbA1c quartile, no significant differences in GIR are found across the quartiles (not shown). Thus within these moderately controlled T1D subjects glycemic control does not appear to mediate or affect IR.

Table 2: Hyperinsulinemic-euglycemic clamp results

	T1D	non-DM	p-value
Stage 3 clamp parameters:			
Final clamp glucose (mg/dl)	89.1 ± 3.2	89.9 ± 3.4	0.28
Final clamp insulin	106 ± 35	99 ± 30	0.36
Glucose Infusion Rate (mg/min/kgFFM)			
Unadjusted Mean ± SD	5.8 ± 3.5	13.2 ± 5.7	<0.0001
LS MEAN ± SE, adjusted for age, sex, BMI, final glucose and insulin, fasting glucose	6.19 ± 0.72	12.71 ± 0.66	<0.0001
LS MEAN ± SE, adjusted for age, sex, VF, final glucose and insulin, fasting glucose	6.24 ± 0.68	12.75 ± 0.62	<0.0001

Table 3: Glycemic control and IR

		correlation to GIR	
	Mean ± SD	r-value	p-value
HbA1c	7.5 ± 0.9	0.13	0.5
CGM parameters:			
Mean glucose	141 ± 26	0.2	0.26
% > 180	26.2 ± 12.9	0.25	0.14
% < 70	16.2 ± 11.9	-0.02	0.91
Overall SD	65.9 ± 20.1	0.21	0.22

Adipocyte IR in T1D: Early clamp stages with lower insulin infusion rates reveal the novel finding that adipocyte IR is also greater in T1D than in non-DM subjects (Fig 2). Baseline fasting NEFA levels did not differ by diabetes status, and, as expected, were higher in women (620±26) than in men (463±32), p=0.0004. At 8mU/m2/min of insulin (clamp stage 2), NEFA levels were more suppressed in non-DM (193±24 μM) than T1D (370±26) subjects, p<0.0001. In addition, the sex difference observed at baseline is reversed in non-DM, but not T1D subjects, such that the highest NEFA levels are in T1D women (405±33), while the lowest levels are in non-DM women (137± 29), p<0.0001, indicating greater relative adipose tissue IR in T1D women than men. The observation that fasting NEFA levels are not different by diabetes status despite overnight IV insulin to normalize AM glucose levels and resulting high AM insulin levels in T1D subjects also sup-ports adipose tissue IR in T1D. Glycerol levels show a similar pattern (not shown), *suggesting that failure of NEFA suppression reflects failure to suppress lipolysis, a potential mechanism for a role of IR in CVD.*

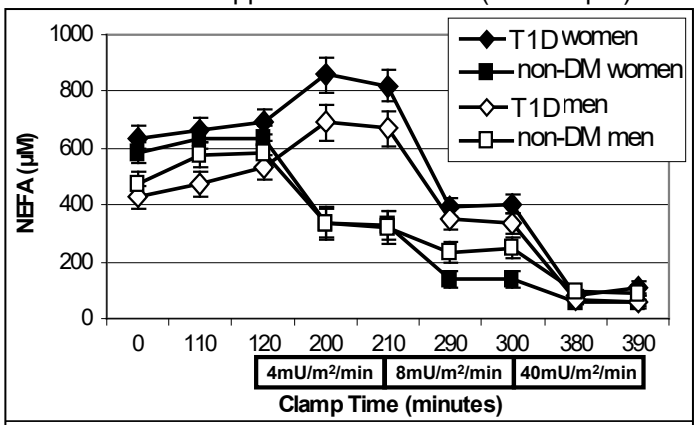


Figure 2 §Least square means adjusted for age, BMI (kg/m2), gender, starting glucose concentration, and time point insulin level. * p < 0.05 for difference by diabetes in men; # by diabetes in women; † p < 0.05 for sex difference in DM; ‡ in nonDM.

Coronary artery calcification in T1D: 6 year CAC data for the entire T1D cohort were examined in linear regression models. Adjustment for standard CVD risk factors attenuated the increased risk of CAC progression, but did not eliminate it. In a forward selection multivariate analysis after adjustment for all standard cardiac risk factors that affect the model, including presence of CAC at baseline, the OR of progression of CAC from baseline to 6 years in T1D relative to non-DM was maximally decreased by 49.7% to 1.7 (1.1-2.6). *Thus only half of the increase in 6 year progression of CAC in T1D is explained by known CVD risk factors.*

Correlation of CAC to IR: Presence of CAC and progression of CAC were found to correlate inversely with GIR and directly with NEFA levels during clamp stage 2 for both T1D and non-DM groups after adjustment for age (Table 4)[47]. By logistic regression analysis the odds ratio for existence of CAC is 0.45 for every 1 SD increase in GIR and 2.4 for every 1 SD increase in stage 2 NEFA levels. *These data support the underlying rationale for studying IR in T1D, indicating that as in other populations, IR is a correlate and predictor of CVD.*

	Spearman correlation coefficient IR vs CAC volume for total cohort (n=87)		
	V3 CAC volume	Baseline to V3 change in CAC	V2 to V3 change in CAC
GIR (mg/kg FFM/min)	-0.42 (p<0.0001)	-0.41 (p<0.0001)	-0.24 (p=0.028)
Stage 2 NEFA level (μM)	0.41 (p<0.0001)	0.40 (p=0.0001)	0.27 (p=0.01)

	Logistic regression analysis Odds ratio for any CAC at visit 3 (n=87)	
	OR per 1 SD change	p-value
GIR (mg/kg FFM/min)	0.45 (0.22-0.93)	0.03
Stage 2 NEFA level(μM)	2.40 (1.08-5.32)	0.032

Table 4 : IR predicts CAC and correlates with CAC volume

Increased vascular stiffness in T1D correlates with an estimate of IR: Measurement of pulse wave augmentation index (AI), a measure of arterial stiffness, has been performed in a large subset of the CACTI population (n=385). After adjustment for age and gender, AI remains significantly greater in T1D than non-DM subjects (mean, 95% confidence interval: 19.5, 18.2 - 20.7 vs. 16.6, 15.4 - 17.7; p=0.0011). When estimated glucose disposal calculated by the Pittsburgh prediction equation[30] is added to the model the effect of T1D is completely lost, *suggesting that the increased arterial stiffness seen in T1D may be explained by their increased IR*.

IV. Research Methods

A. Outcome Measure(s):

- Primary outcomes (post each intervention period (placebo vs Metformin): **(1)** Glucose infusion rate (GIR) during high insulin infusion in hyperinsulinemic euglycemic clamps, **(2)** Endothelial function measured as percent flow-mediated brachial artery dilation (FMD),

- Secondary (confirmatory and exploratory) outcomes (post each intervention period (placebo vs metformin): **(1)** NEFA suppression during clamp stage 2, **(2)** arterial stiffness (PWV and AI), **(3)** CGM measures of hyperglycemia, hypoglycemia, and glycemic variability for one week prior to each clamp**, (4)** Metabolic (NEFA, lipids, insulin, glucose), inflammatory (IL-6, TNF-α, hsCRP, adiponectin), thrombotic (PAI-1), oxidative stress (urinary isoprostane, TBARs), and endothelial (ICAM, endothelin-1) markers at baseline each study day, **(5)** Counter-regulatory hormones (cortisol, growth hormone, glucagon, and catecholamines prior to each clamp**, (6)** Respiratory quotient at baseline and after low dose insulin infusion, **(7)** Heart rate variability, **(8)** Mitochondrial content (DNA and electron transport chain complexes), **(9)** Mitochondrial function (carbohydrate and lipid oxidation, max state 3 O_2 consumption, uncoupled O_2 consumption), **(10)** Mitochondrial state 3 O_2 consumption, **(11)** Mitochondrial oxidant generation (H_2O_2 production, glutathione redox state), and (12) Mitochondrial ATP production/ADP depletion by 31P-MRS.

B. Description of Population to be Enrolled: Study Design and Research Methods

Hypotheses: Metformin will improve **(1)** insulin action, **(2)** vascular function and compliance, and **(3)** mitochondrial function and oxidant generation in T1D subjects supporting a therapeutic role for metformin in T1D management, including CVD risk management**.**

Specific Aims: Specific Aim 1: Determine the effect of metformin on IR in T1D. **Specific Aim 2:** Determine the effect of metformin on vascular correlates of IR. **Specific Aim 3:** Determine the effect of metformin on mitochondrial function and oxidant generation.

Overall research plan: I propose to address these hypotheses and aims with a single-blinded, fixed-order , placebo-controlled cross-over design study in T1D subjects. The intervention will consist of 6 weeks of treatment with metformin, separated from the placebo period by a ≥ 2 weeks washout period. The fixed order is needed because we and others have found persistent residual effects from metformin treatment, and the necessary washout period is not known. Metformin has been studied to a limited degree in T1D, primarily in terms of glycemic control. It has been found to be safe and well-tolerated in these studies (Vella) and the proposed dose and duration have precedent in the literature. Dr. Phillipe Walravens at this institution has performed a study using metformin in T1D youth. From his contact with the FDA, an IND is not required for this study.

Surrogate measures of CVD risk: The proposed study uses known sequelae of IR as surrogate markers for longterm cardiovascular damage (Figure 3)[48]. The proposed markers are well supported in the literature as sequelae of IR, as mediators and/or predictors of CVD outcomes or CVD mortality, and as measures capable of response to short-term interventions.

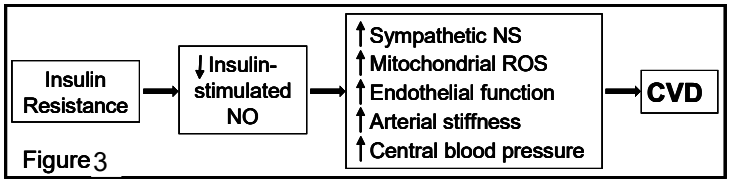


Figure 3

Endothelial function: It is generally accepted and widely supported in the literature that IR states are accompanied by impaired endothelial function[49, 50] and that endothelial dysfunction responds to acute manipulations[51] and correlates with CVD risk and outcomes[52]. Arterial stiffness and central blood pressure: Central artery stiffness is increased in obesity and diabetes, including T1D, and correlates with IR in T2D and non-DM populations[53]. Arterial stiffness[54] correlates with CVD mortality and central arterial blood pressure measured by applanation tonometry appears to be a stronger predictor of CVD than overall blood pressure[55]. Arterial stiffness is increased with just two hours of physiological-range NEFA elevation by lipid infusion and is thus a dynamic vascular measure[56].Mitochondrial function and oxidant generation: Though proposed as a mechanism for IR, most data supports mitochondrial dysfunction and resulting oxidant generation as a sequela of IR and a contributor to complications thereof[57, 58]. NO has recently been shown to play a key role in mitochondrial biogenesis and function suggesting that vascular IR may be a contributor to this mitochondrial dysfunction[59]. Sympathetic nervous system (SNS) activation: IR states are accompanied by SNS activation and considerable evidence supports this as a contributor to CVD risk[60]. NO plays a key role in modulating SNS activity suggesting again that vascular IR may play a role in SNS activation[61, 62].

Recruitment: 100 eligible T1D subjects (50% male) will be enrolled for this study with the goal of starting 40 subjects in order to complete both arms in 30 T1D subjects (see power analysis). Recruitment will be by IRB approved flyers and email advertisement, and through clinical providers at the University Hospital and Barbara Davis Institute Endocrinology clinics. Subjects will also be recruited from the CACTI cohort (>1100

active members) by mass mailing to the full cohort of an IRB-approved letter describing the study, followed by phone calls to invite questions. Although this cohort is an epidemiological outcome cohort, and the initial opinion was that this duration of intervention was inappropriate for such a study, funding pressures have influenced the CACTI PI to consider mechanistic studies and to agree that a short term metformin intervention is acceptable. In fact, a large grant to expand upon this pilot study is currently pending with the NIH. A strength of this study is thus access to the existing NIH-CACTI cohort that has been prospectively studied for ≥6 years. This is an active cohort with a great interest in diabetes and its management, and recruitment of 90 subjects for our recent clamp sub-study was easily accomplished.

Study population: The goal is to complete 30 T1D subjects (50% male in each group) for the pilot study. We expect to screen up to 100 subjects to enroll 40 and complete 30. Inclusion criteria:(1)age 20-59 years of age, (2) type 1 diabetes based on antibody-positivity, rapid persistent conversion to insulin requirement after diagnosis, absent C-peptide, or DKA at diagnosis, or a clinical course consistent with T1D, (3) HbA1c 6.0-9.5 (4) willing/able to commit to two 6 week-long study phases, each consisting of blinded medication with blinded CGM, frequent phone follow-up, and 3 days of prescribed diet followed by hyperinsulinemic euglycemic clamp, vascular testing, and 2 muscle biopsies. Exclusion criteria include:

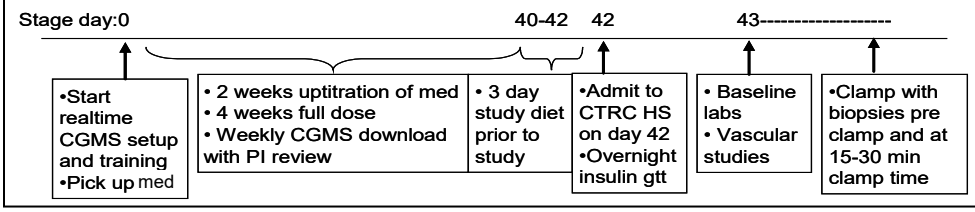
- Any comorbid condition associated with inflammation, IR, or dyslipidemia
- Tobacco use
- Pregnancy
- Steroid use
- Scheduled vigorous physical activity >3 days a week
- Angina, known CAD, or any other cardiovascular or pulmonary disease
- A history of COPD or asthma
- Presence of systolic blood pressure >190 at rest or >250 with exercise or diastolic pressure >95 at rest or >105 with exercise
- Untreated thyroid disease
- Proteinuria (urine protein >200 mg/dl) or a creatinine $\geq$ 1.5 mg/dl(males) or 1.4 mg/dL (females), suggestive of severe renal disease
- Proliferative retinopathy.
- Niacin treatment
- Administration of experimental agent for T1D within 30 days prior to screening
- Recent (prior 6 months) or current metformin or thiazolidenedione use
- Hypoglycemia unawareness or recurrent severe hypoglycemia (no symptoms of hypoglycemia with FSBS<40 and episodes of this severity >1 per week)
- Weight instability (weight change >5% in last 6 months)
- History of any organ transplant, including islet cell transplant
- Current or prior infection with HIV, hepatitis B or hepatitis C, or transaminases >2x ULN
- Any condition, medical or otherwise that would, in the opinion of the investigator, prevent complete participation in the study, or that would pose a significant hazard to the subject
- History of substance abuse within the 12 months prior to screening

Screening: A phone screen will be followed by provision of the consent form and a screening/enrollment visit at the outpatient CTRC. This visit will include the consent process, history, physical exam, non-fasting screening labs [ß-HCG, HbA1c, metabolic panel, TSH, urine microalbumin, and CBC], detailed menstrual history for women, and a DEXA scan for body composition and estimation of caloric needs.

Study design:
T1D subjects (n=30) will have a fixed order of treatment (placebo-metformin) and will be given their first medication after screening is complete and eligibility confirmed. Medications (metformin 500, 1000mg and identical placebo) will be dispensed by the pharmacy and the subject will be blinded to the fixed order of the medications to avoid biased treatment effects throughout the intervention period. As in previous Barbara Davis Center (BDC) metformin studies, this study will use the CTRC pharmacy to dispense the active drug and placebo. Both are provided by Caraco manufacturer. My colleagues at the BDC already purchased tooling for the Caraco brand placebo tablets and the company has agreed to make more placebo tablets for this study as necessary to cut out the upfront cost of tooling.

Insulin dosing during treatment will be per the subjects' usual insulin regimens with continuous glucose monitoring (CGM) throughout. CGM for the first four weeks will be real-time and monitored to allow insulin dose adjustments if needed to avoid hypoglycemia. The last two weeks of CGM will be blinded and the last week of data will be used to allow detailed statistical adjustment for any changes in glycemic control that occur during treatment. In order to minimize risk of hypoglycemia and changes in glycemic control, subjects' CGM reports or glucometer readings will be downloaded and monitored weekly by the PI and insulin doses adjusted appropriately to maintain approximate prior levels of control. This adjustment will be at an in-person outpatient visit at the end of the first study week to ensure patient is able to reinsert CGM properly. All others will involve electronic access to CGM data and phone contact. The PI will also be available by phone all working hours for advice on insulin dosing.

Figure 4: Study timeline



Subjects will be instructed to take study drug at 500mg at bedtime for the first week, 500 mg twice daily for the second week, 500/1000mg daily for one week and 1000mg twice daily for the remainder of the 6 week period. Subjects who fail to tolerate an uptitration will be held at the prior dose for an additional 3 days and then retried. Subjects who have not reached at least 500 mg BID dosing by the end of week 3 will be withdrawn from the study. Thus, final metformin dosing will be 500 BID or 1000 BID. During the last three days of each treatment phase, subjects will be placed on a standardized typical diabetic diet (40% carbohydrate, 35% fat, 25% protein; based on CACTI food records) for three days prior to late evening admission on day 3 to the CTRC. Overnight admission is critical to ensure that all subjects are in the same state prior to each testing session. Remaining pills will be turned in to allow a pill count as a measure of compliance. Subjects will be transitioned to an insulin drip for overnight glucose control using the protocol previously used for CTRC protocol 1499 (see appendices for starting doses and titration schedule). Inpatient studies (baseline labs, vascular studies, biopsies, and hyperinsulinemic euglycemic clamp) will be performed the following day (Figure 4). After a two week to two month washout period, the full cycle will be repeated with the second intervention. Women will be tested at the same time in their menstrual cycle (day 4-9) after each intervention period. Allowing up to 2 months between interventions is necessary to allow this type of scheduling with the possibility of a missed cycle due to scheduling conflicts. The study timeline and detailed visit schedule are as follows:

Screening:

- Visit 1: Screening visit - consent process, H&P, non-fasting labs, DEXA, dietary questionnaire

Stage 1 (Study drug A, Placebo):

- Visit 2: Stage 1 start visit (day A1) – Insert CGM and receive instruction on use and download, pick up Placebo, Instructions from PI re med dosing and insulin dose self-adjustment
- Visit 3: Stage 1 CGM follow-up visit (day A8) – in person CGM download and reinsertion of sensor for the next week. Review glucoses with PI.
- Visit 4: Stage 1 prestudy day visit (day A39) – pick up three day diet and receive instructions on insulin dosing and times for the pre-study day. Accelerometer set-up
- Visit 5: Admission for Placebo study day (Days A42, 43) – see below

2 weeks- 2 months wash-out

Stage 2 (Study drug B, Metformin):

- Visit 6: Stage 2 start visit (Day B1) - Insert CGM and receive instruction on use and download, pick up Metformin, Instructions from PI re med dosing and insulin dose self-adjustment
- Option for one week visit 6b if desired for first repeat CGM insertion
- Visit 7: Stage 2 prestudy visit (Day B39) - pick up three day diet and receive instructions on insulin dosing and times for the pre-study day. Accelerometer set-up
- Visit 8: Admission for Metformin study day (Days B42,43) - see below

During the study visits procedures will include the following: baseline blood draws for inflammatory, metabolic, endothelial, and thrombotic markers; heart rate variability; vascular function and stiffness by flow-mediated dilation (FMD), plethysmography, brachial artery distensibility, and pulse wave velocity (PWV), and augmentation index (AI); insulin sensitivity by 2-stage hyperinsulinemic clamp (8, 40 mU/m^2/min) with glucose tracer; muscle biopsies preclamp If blood sugar at start of the tracer infusion and of the clamp is <60 or >180, the study visit will be concluded without the clamp.
^{31}P-MRS optional substudy: DRI pilot funding has been obtained to add a ^{31}P-MRS study (method described further below) to the late afternoon preceding each admission to the CTRC for 10 of the MeT1 subjects. On recruitment into MeT1, subjects will be given the option to enroll in this additional sub-study. Specific exclusion criteria for this sub-study will include presence of any metal or other contraindications to MRS, including significant claustrophobia. A separate opt-in section in the consent form will be prepared and submitted to the IRB for this sub-study. Once 10 subjects have opted in and completed, the sub-study will be considered complete and closed to further enrollment. At that point, an interim analysis, including unblinding of these 10 subjects, will be performed. Those who opt in to the sub-study will follow the original study design exactly, with the exception that on day 3 of their study diet, they will be asked to present to the UCD Brain Imaging Center (behind the Leprino Building) in the late afternoon (fasting since lunch; 4-6 hours) for the MRS scan and then proceed to the CTRC for admission for the full study day the following day.

Subject discontinuation criteria: (1) Failure to tolerate uptitration to a dose of metformin of at least 500 mg BID within 4 weeks, (2) Patient report of increased hypoglycemia not resolved with insulin dose adjustments within 2 days, (3) Other events or adverse reactions that are possibly related to study drug, that are contraindications to study drug, or that interfere with study measures.
Protocol stopping rules: Occurrence of any severe adverse reaction including but not limited to hypoglycemia leading to unconsciousness or requiring external assistance will immediately be discussed with the data safety officer to evaluate need for protocol termination. Occurrence of more than one such event will lead to an immediate discontinuation of the protocol.

D. Description, Risks and Justification of Procedures and Data Collection Tools:

Methods: All measures will be assessed post 6 weeks of each of two blinded study drugs (metformin and placebo).

Continuous glucose monitoring: Subjects will have continuous glucose monitoring (CGM, (DexCom 7-plus® system)) through both intervention periods. This will be real-time during the first four weeks (insulin dose adjustment and increased safety) and blinded during the last two weeks (statistical adjustment for changes in glycemic control).

Hyperinsulinemic euglycemic clamp with tracer infusion: In order to determine insulin sensitivity, subjects will be admitted to the CTRC and fasted for ≥12 hr for a hyperinsulinemic euglycemic clamp by the method of deFronzo et al[63] omitting the lowest insulin stage (4mU/m^2/min) with 6,6 ^{2}H-glucose infusion (4 minute prime at 3mg/kg/min, then 0.04 mg/kg/min) for 2 hours preceding the clamp to allow differentiation of hepatic and muscle insulin sensitivity. Two intravenous cannulae will be established: one for infusions, and the second in retrograde fashion in a dorsal vein of the opposite arm for blood sampling. To obtain arterialized venous blood samples, this hand will be kept warm with a heating pad. Tracers will be initiated at T=0 (about 7AM) and continue for 2 hours. Blood samples will be drawn at T=100, 110, and 120 for tracer and metabolite analysis. A descending primed continuous infusion of insulin (final 8 mU/m^2/min) will be initiated at T=120 and continued until T=240 min, then increased with a second descending prime to 40 mU/m^2/min for an additional 120 minutes for determination of insulin sensitivity. Plasma glucose will be maintained at 90 mg/dl by variable infusion of 20% dextrose (spiked with tracer). An infusion of potassium will also be given to maintain normal potassium concentration. The rate of glucose infusion will be adjusted based on arterialized blood specimens drawn every 5 minutes, with plasma glucose quantified using a ™Analox glucose analyzer at the bedside. Blood samples will also be obtained for determinations of free fatty acids, triglycerides, insulin, and glycerol at 60 min. intervals and three times over the last half hour of each stage (as above). After insulin infusion is stopped, glucose infusion will be continued until the subject has completed a standard meal, and plasma glucose levels measured every 15-30 min. for an hour to check for possible hypoglycemia. The subject will then be discharged.

Blood collection and preparation: During the screening visit 1, non-fasting blood and urine sampling (chemistry-7, complete blood count, HbA1c, liver function tests, TSH, pregnancy test, and urine

microalbumin) will be performed to screen for exclusionary medical conditions. During study visits blood sampling will be done at baseline (AM of study day) for measurement of metabolic markers (NEFA, triglycerides, glycerol, glucose, lactate, and insulin levels), endothelial and inflammatory markers (endothelin 1, interleukin 6, TNF- α, and hsCRP) and a prothrombotic marker (PAI-1); and during the clamp as specified above.

Endothelial function: By brachial artery flow-mediated dilation (FMD) methodology described by Celermajer[64, 65]. FMD is measured using B-mode ultrasound images, with the use of a GE VingMed Vivid Five ultrasound system and a 10.0-mHz linear-array transducer. The brachial artery is scanned in longitudinal sections 2-15 cm above the elbow. When a satisfactory transducer position is found, the skin is marked and the arm left in the same position throughout the study. Increased flow is then induced by inflation of a pneumatic tourniquet around the forearm proximal to the scan site to a pressure of 200mmHg for 5 minutes, followed by release. Doppler measures of blood velocity and diameter will be done at baseline and the first 10 seconds post cuff release. Hyperemic response is then measured for two minutes after cuff deflation. Analysis of recordings will be performed using the CTRC vascular software package available in the Leprino Building. FMD is calculated and adjusted for hyperemic flow velocity.

Arterial stiffness: Arterial stiffness will be assessed non-invasively by ™Sphygmocor BPAS 1device (Atcor Medical) applanation tonometry measurement of augmentation index (AI) and pulse wave velocity (PWV)[66, 67 68]. Three electrocardiography leads are placed with removable adhesive on the chest wall, for the purpose of timing systole. A small metal tonometer is sequentially placed on the skin overlying the radial, femoral and carotid arteries. The anterior wall of the artery is flattened, as with palpation of a pulse. The tonometer non-invasively measures the pressure pulse waveform at each of these sites and correlates this with ECG data and the distance between the femoral artery and carotid artery sites to determine pulse wave velocity.

Echocardiogram: Standard 2-D and Doppler echocardiography with images adequate for speckle tracking analysis will be performed during the inpatient study visits to assess cardiac function.

Sympathetic/parasympathetic balance: Respiratory heart rate variability by standard methods[60]. Study participants will undergo supine resting electrocardiogram (ECG) recording for 5 minutes early in visits in a quiet room, with a stable room temperature. Participants will be asked to relax but not fall asleep and to breathe normally and remain still without talking during the ECG recording. Following the resting ECG, a second supine ECG will be recorded while study partici-pants take in a deep breath for 10s. The ECG recordings will be transmitted electronically to the Epidemiological Cardiology Research Center (EPICARE) Center (Wake Forest University School of Medicine, Winston-Salem, NC) for analysis, interpretation, and calculation of heart rate variability. The SD of consecutive RR intervals of the entire record in Lead II will be used as a measure of overall HRV. In addition, autoregressive power spectral analysis will be used to determine the spectral power in the following bands: high frequency (HF, 0.15–0.45 Hz, reflects parasympathetic outflow), low frequency (LF, 0.04–0.15 Hz, reflects both sympathetic and parasympathetic outflow), and very low frequency (0.01–0.04 Hz). LF/HF and HF normalized to total power will be analyzed as measures of sympathetic and parasympathetic outflow, respectively.

Indirect calorimetry: For measurement of RQ fasting and during low infusion rate to assess insulin response of shift from fat to carbohydrate metabolism.

Muscle biopsy: Vastus lateralis muscle biopsies by the Bergstrom technique[69]. Samples will be analyzed fresh for respirometry and H_2O_2 production; others will be flash frozen in liquid N_2 and stored at -80^o for PCR, glutathione assays, and immunoblotting. Dr. Leigh Perreault does these biopsies regularly and will assist initially and train the PI in performance of these biopsies.

Mitochondrial isolation, content, function, and oxidative stress: Respirometry of muscle tissue will be performed in isolated mitochondria by the method of Anderson et al [70] using the Oroboros Oxygraph O2K respirometer. Portions of muscle tissue will be immediately homogenized and fresh mitochondrial preparations will be used to determine carbohydrate and lipid oxidation rates. Remaining tissue will be flash frozen in liquid nitrogen for appropriate analyses. Viable mitochondria will be isolated from fresh skeletal muscle tissue by trypsin collagenase digestion, homogenization and differential centrifugation by the methods developed by K Boyle, consulting mentor. Mitochondrial protein concentrations will be determined using a bicinchoninic acid (BCA) assay. For mitochondrial assays, viable mitochondria will be introduced into the Oxygraph O2K chambers in duplicate with sequential addition of a combination of lipid and carbohydrate

substrates as well as physiological and chemical uncouplers, activators and inhibitors to gain a comprehensive view of metabolic and mitochondrial function in the physiological and supraphysiological energy states. A portion of the viable mitochondria isolated from the fresh tissue samples will also be used to assess hydrogen peroxide (H_2O_2) emission in the presence of the same substrates that were used in the mitochondrial respiration experiments. H_2O_2 is a common product of superoxide degradation and will be measured using a standard kit assay (Amplex Red®, Invitrogen), modified for mitochondria. A portion of the frozen tissue sample will be used to measure glutathione levels using a standard kit (Bioxytech® GSH/GSSG- 412TM, OxisResearch). The ratio of reduced to oxidized glutathione is a standard indicator of oxidative stress. Mitochondri-al content is measured by QRT-PCR and blue native gel electrophoresis of isolated mitochondria.

Study drug: Metformin and placebo will be provided by Caraco Pharmaceutical Labs as indicated above. They will be given in fixed order single-blinded fashion.To minimize GI side effects, study drug will be given in a forced uptitration with 500 mg once daily for one week, twice daily for one week, and then the higher dose (1000 mg) for the remainder of the 6 week intervention. Subjects who fail to tolerate an uptitration will be held at the prior dose for an additional 3 days and then retried. Subjects who have not reached at least 500 mg BID dosing by the end of week 3 will be withdrawn from the study. Thus, final metformin dosing will be 500 BID or 1000 BID.

DEXA (Dual-energy X-Ray Absorptiometry): Body composition and bone mineral density will be measured using the DEXA technique[71].This technique relies on the absorption of dual electron wavelengths for the assessment of body fat, lean tissue, and bone mineral density. During the procedure, the subject will be supine on the measurement table, and the arm of the machine will slowly pass over their body. Body fat distribution will be determined using the waist-to-hip ratio where the waist circumference is measured 1/2 the distance from the lowest point of the ribs to the iliac crest and the hip circumference is measured at the level of the greater trochanter.

Diet Instruction and diet lead-in: Customary macronutrient pattern and diet preferences will be ascertained by an online diet questionnaire provided by the CCTSI Nutrition Core team. A three day diet will be provided by the CTRC prior to both study visits. A CTRC dietician will prescribe a standardized nutrient breakdown including 25% protein, 35% fat and 40% carbohydrate for the three days prior to visits 5 and 8. The sodium in the diet will be limited to ≤2300 mg/day.

Immunoblot analysis: Frozen muscle biopsies are weighed, cut into 30mg sections, homogenized, solubilized, centrifuged, assayed for protein concentration, subjected to electrophoresis (50 µg), and transferred to membranes (Bio-Rad). Blots are exposed to appropriate antibodies. Immunoreactive products are detected using enhanced chemiluminescence and exposure to film, quantified, and normalized to an internal standard.

Quantitative Real Time PCR: Quantitative real-time PCR is performed with the Roche LightCycler 480 instrument and 480 SYBR Master mix for total mitochondrial DNA. Primer specificity is confirmed by melt curves. Relative abundance of mitochondrial DNA is quantified by standard curve method and normalized to the 18S rRNA gene as a chromosomal DNA marker.

^{31}P-MRS: Methods: Calf MRS123-126: a 3.0 T whole–body MRI scanner (GE Medical Systems, Waukesha, WI), with a dual frequency ^{31}P/^{1}H Flex Coil (Clinical MR Solutions) creates scout images of the dominant leg gastrocnemious and soleus muscles. The widest cross sectional area is fitted in the equation described in our preliminary results to determine expected force. A resistance strap is affixed to the hips, dominant leg, ankle and foot, on a specialized portable, in-MRI exercise bench to allow isolated static plantar flexion against force. Flexion is done at 70% of the subject's predicted maximal force for 90 seconds, monitored by a Single Point Load Cell (Tedea Model 1250), connected via an A/D board to a laptop for analysis on LabView software. After scout images and shimming using the ^{1}H portion of the flex coil, ^{31}P measurements are made at rest, during calf exercise, and for at least 15 min immediately following the exercise bout (figure 5). Total study time is <1 hour. Output is analyzed using JmRUI and Sigma Stat to calculate PCr, phosphate (Pi), ADP, and ATP, which are used to calculate pH, ADP $t_{1/2}$, PCr $t_{1/2}$, Pi $t_{1/2}$, and substrate dependent (VPCr, Qmax, the apparent mitochondrial capacity i.e. maximal oxidative ATP production rate; anaerobic glycolysis; oxidative phosphorylation; CK, the non-mitochondrial dependent ATP production from creatinine kinase; muscle efficiency) and oxygen-dependent components (time constant for PCr, Pi & ADP recovery), allowing differentiation of substrate vs. oxygen-dependent causes of mitochondrial dysfunction. Primary outcomes for this portion of this sub-study will be QMax (max oxidative ATP production rate) and ADP depletion half-time.

Risks:

Continuous glucose monitoring: The continuous glucose monitor involves a small catheter placed under the skin in the area to the side of your belly. Potential risks of this procedure include bleeding, infection, pain, and catheter placement into a muscle causing pain and bruising.

Blood Draws: Pain may be felt when the needle goes into the vein. A bruise may form at the site. There is a risk of infection. Standard precautions will be used to minimize these risks and only CTRC nurses will perform blood draws.

Fasting: Subjects who are taking insulin run a risk of hypoglycemia during fasting. Fasting will only be required during the inpatient stays. Symptoms listed above will be described in advance and subjects will be monitored closely.

DEXA: During DEXA testing the amount of radiation received is less than 25mRem total body equivalent dose per measurement. This is estimated to be less than one-half the amount received from a chest x-ray. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risk is not known. Women who are or could be pregnant should receive no unnecessary radiation, and should not participate in this study.

IV Risks: When the needle carrying the plastic tube goes into a vein, it hurts for a short time. The needle is then removed and only the plastic tube remains in the vein. There will also be the minor discomfort of having the plastic tube taped to the arm. In about one in 10 cases a small amount of bleeding under the skin will produce a bruise. The risk of a blood clot forming in the vein is about one in 100, while the risk of infection or significant blood loss is one in 1000. Standard precautions will be used to minimize risk and only trained CTRC personnel will place IVs.

Arterial stiffness measurement and HRV measurements: There is a very slight chance that fainting or stroke may occur. No actual fainting or stroke have ever been reported. The risk of these happening has been estimated to be less than 1/1,000,000.

Brachial Artery FMD: There may be a slight discomfort while the arm cuff is inflated.

Muscle Biopsies: May be uncomfortable or cause pain. Infection, bleeding, or damage to nerves is reported to be 1/1000. The incision will be closed with a steri-strip or, if necessary, a single suture. A bruise may result temporarily, but a scar may be permanent. Numbing medicine will be used to reduce the pain. Rarely this causes an allergic reaction. A total of 2 muscle biopsies is required. Soreness or cramping in the muscle can occur during or after the procedure. Sometimes people faint.

Indirect calorimetry: Some people experience feelings of claustrophobia under the plexiglass hood used for these measurements.

Insulin Clamp: With the insulin clamp there is the risk of hypoglycemia. The symptoms of hypoglycemia are excessive sweating, faintness, headache, pounding heart, trembling, and impaired vision. Under very extreme degrees of hypoglycemia, a person may lose consciousness. Blood glucose levels will be monitored throughout this test to prevent hypoglycemia from occurring. I have performed >50 clamps and have caused no episodes of symptomatic hypoglycemia.

Echocardiography: This is a noninvasive ultrasound tests with no significant risk or discomfort. There may be slight pressure from the wand over the chest area and women may feel uncomfortable about the having the wand placed and held near the breasts.

Metformin: Though metformin is generally well tolerated, there is the potential for side effects/adverse reactions. With metformin there is a small risk of a serious allergic reaction with anaphylaxis (difficulty breathing, swelling of lips, face or tongue). Milder allergic reactions with rash also occur. Metformin is contraindicated in anyone with a history of lactic acidosis or renal insufficiency (Cr >1.5(males) or 1.4 mg/dL (females)) because of the risk of acidosis. This would present as shortness of breath, muscle or bone pain, dark urine, and/or fever. The most common side effects with metformin are headache and GI side effects including abdominal pain, nausea, vomiting, diarrhea, loss of appetite, abdominal bloating/discomfort, and flatulence. These are reported in some literature in up to 50% of patients. However, with a low mealtime starting dose and slow uptitration, this risk is much lower and can be largely avoided. The PI has considerable experience with metformin and has found that with the planned upitration <10% of patients do not tolerate metformin. Some attrition due to GI side effects is expected in this study.

For these subjects with diabetes and on insulin there is also a risk of hypoglycemia, especially through the start and uptitration of metformin. Subjects will continue to do their normal blood sugar checks. In

addition, their CGM monitors will be downloaded weekly for review of blood sugars and insulin dose adjustment if needed by the PI. Subjects are well aware of the symptoms of hypoglycemia and will be advised to check their blood sugar for any symptoms.

Though metformin is frequently taken during pregnancy, it is not fully proven safe in pregnancy and we will be excluding subjects who are pregnant or planning to get pregnant. Therefore, a pregnancy test will be done with the initial screening labs, and if a subject is pregnant or breastfeeding, she will be excluded from this study. If a subject becomes pregnant during the study she will be instructed to notify the study coordinator and withdraw from the study.

^{31}P-MRS: Subjects may develop claustrophobia in the magnet or mild muscle cramping during the exercise portion

Unforeseen Risks: This study may include risks that are unknown at this time.

E. Potential Scientific Problems:

Interpretation of results/pitfalls and limitations: This study addresses the question of whether metformin affects insulin action and vascular and mitochondrial sequelae of IR in T1D. Based on the literature, I expect that GIR will be increased by this intervention, though it is unclear whether this will be mediated through changes in classical insulin signaling. An improvement in GIR would support the hypothesis that metformin is a viable approach to the treatment of IR in T1D that may have CVD benefits similar to those seen in T2D. If cardiovascular and/or mitochondrial measures improve (with or without changes in IR), this also lends support to metformin as a potentially CVD protective intervention and provides data to support a longer study looking at clinical outcomes. If no change in GIR or vascular measures occurs, this would suggest that IR and CVD risk in T1D are mechanistically different from other populations, an exciting result in itself, and future efforts would explore other mechanisms such as inflammation, sympathetic nervous system activation, and counter-regulatory hormones. Other outcomes will provide hypothesis-generating data to support and direct exploration of these other mechanisms of IR in T1D.

Other potential limitations of this study are:

(1) Small size: it is possible that a smaller effect will occur in T1D than in other populations and this may therefore be missed in this small sample size.

(2) The optimal length of time for this intervention is open to debate. Glycemic control changes in type 2 diabetes with metformin occur fairly rapidly (within a few weeks) suggesting that metformin effects on insulin action and glucose metabolism at least, are quite rapid. Most metformin studies in the literature use 3 months of treatment. To minimize subject risk and burden, as well as study costs, I plan to use a 6-week intervention. Evidence certainly supports the expectation that many changes induced by metformin do occur within this time span.

(3) Limited tracer use: due to cost considerations, only glucose tracer will be used. Ideally glycerol and palmitate tracers would also be used to fully characterize insulin sensitivity of metabolic shifts. Again, these will be considered if a larger study is justified by the preliminary data obtained here.

(4) Surrogate measures: Outcomes are surrogates that are believed to correlate with vascular disease outcomes. However, a larger and longer study will be required if this study is promising to demonstrate that changes observed with metformin do translate to improved clinical outcomes.

(5) Changes in glycemic control may occur with metformin despite efforts to avoid this confounder. Separation of effects of insulin sensitization from those of altered glycemic control may be difficult. The study design attempts to minimize this effect by using a relatively low carbohydrate diet, minimizing prandial insulin dosing and thus overall insulin dosing. Subjects will be advised that they may need to decrease insulin dosing on metformin and CGM will be used to monitor glycemic control. This will allow for insulin dose adjustments to maintain prior glycemic control and to avoid hypoglycemia, and for statistical adjustment for any changes in glycemic control that do occur. If improvements in outcomes occur, this will support further exploration of metformin use in T1D regardless of the mechanism.

(6) Multiple confounders of an analysis of effect on IR also exist, and every effort will be made to minimize the effect of these confounders including emphasis on a stable activity level throughout the intervention periods and exclusion of highly active subjects. Physical stress will be avoided by rescheduling study days as needed for any illness. Efforts to avoid hypoglycemia will include CGM available to the patient and the PI with insulin dose adjustments as needed. In addition, careful statistical analysis of CGM data will be performed to look for and adjust for any effect of hypoglycemia. If present, this underscores the importance of identifying non-glycemic mechanisms involved in IR as complete normalization of glycemia without

hypoglycemia is unrealistic in T1D, especially in an outpatient, lifelong diabetes regimen. Sex hormones also affect insulin sensitivity in women. The age range of particular clinical interest for this study is primarily middle age, including perimenopausal years in women. I have chosen to include perimenopausal women, but not post-menopausal women in this study. One of my future interests is in the disproportionate increase in CVD risk on premenopausal women with T1D. I have powered the current studies adequately to compare responses to the interventions between genders. I am therefore concentrating on women in which I expect to see sex-based differences, that is those in the pre/perimenopausal stages. Hormonal variation will be dealt with in the usual fashion of scheduling visits during the early follicular phase. Estradiol and FSH levels will be checked with baseline labs on all clamp study days to confirm similar hormone levels at all visits. Other confounders such as emotional stress will be difficult to avoid and are present in any study of this type.

F. Data Analysis Plan:

Data collected from each subject will be verified and entered into a computer database. Confirmation of data quality will be performed using computer programs designed to detect errors using accepted ranges, logic checks, and missing values. Outliers will be checked against the original record, and correct values entered into the final data. Data will be transferred into the SAS System Version 8.0 (SAS Institute, NC) for analysis. We plan to use Redcap for data management and SAS for all statistical analysis. Descriptive statistics of all variables at all time points will be derived and reported.

Statistical Analysis Plan: In order to test the effects of metformin on insulin sensitivity, vascular function and compliance (FMD, PWV, AI, and post-occlusion blood flow) in T1D, we will measure insulin sensitivity and vascular parameters in 30 T1D (50% men) after six weeks of metformin and six weeks of placebo in a fixed-order, single-blind cross-over design study. The effect of metformin treatment on insulin sensitivity and vascular function will be assessed by comparing fasting measures at post each period of treatment within study participants using paired t-tests.

CGM data for the final week of the intervention will be analyzed for mean glucose, %>180, %<60, and SD (measure of glycemic variability). Initial analysis will be by paired t-test to assess for differences between the two intervention periods. The impact on insulin sensitivity of CGM parameters that differ between intervention periods will be investigated using a repeated measures mixed effects model with CGM parameters included as time-varying covariates.

Secondary measures (inflammatory, thrombotic, oxidative stress, and endothelial markers, metabolic parameters, autonomic function, and biopsy assays) will be similarly compared. Further, the association of secondary outcomes with vascular function will be analyzed using Pearson correlation coefficients and multivariate linear regression. I plan to use all of the variables that appear to correlate individually in the multiple regression analysis as an exploratory exercise. I recognize that an n of 30 is inadequate to power this model, but will perform this analysis as an exploratory, hypothesis generating exercise. As a further exploratory approach, I plan to use “best 3” and “best 4” best subset regression analyses to identify the variables that are most strongly associated with insulin sensitivity and endothelial function. Subgroup analyses will also be conducted to examine whether metformin affects vascular function in men and women to the same degree.

Power and Sample Size: Power analysis and sample size are based on expected changes from the literature for the primary outcomes FMD and insulin sensitivity.

Insulin sensitivity: The only existing literature on metformin effects on insulin action in type 1 diabetes is a report by Sarnblad et al [74]. They report on high dose hyperinsulinemic euglycemic clamp in 11 subjects pre and post metformin therapy for 3 months. They report that the increase in glucose uptake (M/I) was just significant in this sample. However, I am unable to duplicate their p-value from the data shown. Values for M/I were measured from the presented graph (Figure 2A) pre and post metformin treatment for n=10. For the measured data shown below, mean M/I pre metformin was 1.845 +/- 1.012 and the mean M/I post metformin was 2.275 +/- 0.784. The mean pre/post difference was 0.48 +/- 1.09. Using the means and the SD of the difference, a paired t-test was performed in SAS 9.2 and generated a p value of 0.295 for n=10 (one hidden data point). Using rounded number from this same data (Means of 1.8 and 2.3 with a SD of the difference of 1) a power analysis yielded 75% power to detect this difference with a sample size of 30 subjects.

Subject*	Pre-Metformin M/I	Post-Metformin M/I	difference
1	4.2	1.7	-2.5

2	2.5	2.6	0.1
3	2.35	2.9	0.55
4	2.2	2.9	0.7
5	1.5	2.2	0.7
6	1.45	2.15	0.7
7	1.2	3.7	2.5
8	1.2	2.1	0.9
9	1.1	1.5	0.4
10	0.75	1.0	0.25
mean	1.845	2.275	0.48
SD	1.012	0.784	1.09

* Top of graph to bottom

Flow-mediated dilation (FMD): Published data indicate that FMD in individuals with metabolic syndrome is normally distributed with a mean of 7.4% ± 2.1% at baseline. Following 3 months of metformin treatment this was increased to 12.4 ± 1.9% [72]. With 30 subjects in a cross-over design and paired analysis, we will have >99% power to detect a comparable difference in T1D, and 80% power to detect a true difference in mean of 1.5%. Should subjects complete only one arm, a two sample t-test of placebo vs metformin groups with n=10 in each group would still have 97% power to detect the published change above. This degree of power will allow additional subgroup analysis for sex-based differences.

The study is powered based entirely on the two primary outcomes. Since these are completely unrelated independent outcomes, no correction for multiple comparisons was used. The secondary outcomes clearly constitute multiple comparisons, but these are all either confirmatory of the primary outcomes or hypothesis generating. Thus, no multiple measures corrections are planned.

Timeline:

Task	**Year 1**	*Total*	**Year 2**	*Total*	**Year 3**	*Total*
Screening & enrollment		*10*		*25*		*40*
Data collection						
Data entry & analysis						
Manuscript preparation						

The first 3-6 months of the grant period is anticipated to be needed for completion of IRB and CTRC approval processes. Screening and enrollment will start within the first half year and enrollment will proceed at a rate of 1-2 subjects per month. This will allow completion of enrollment by early in the third year and completion of the study by late in the third year.

G. Summarize Knowledge to be gained:

Summary: The above cross-over design study will yield important results regarding the mechanism and vascular consequences of IR in T1D and will help guide future research and clinical care of T1D regardless of the results. If NEFA lowering does improve IR and its vascular correlates, this may become a new therapeutic target in the management of T1D. Further exploration of insulin sensitizing agents vs. NEFA lowering agents will be indicated to determine the optimal intervention for CVD risk management.

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