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Title: COMBINED INFLUENCE OF PUBERTY AND OBESITY ON INSULIN RESISTANCE IN ADOLESCENTS

A. STATEMENT OF HYPOTHESES AND SPECIFIC AIMS:

Pediatric type 2 diabetes mellitus (T2DM) is increasing in prevalence, and leads to significant morbidity and mortality. Although the exact incidence in youth is largely unknown, it has been increasing over time. Studies suggest that the prevalence of T2DM in adolescents is somewhere between 3 and 7/1000, and that it represents approximately one-third of new onset cases of diabetes in adolescents. Thus, measures aimed at understanding its causes and preventing its onset are in critical need. The physiologic decrease in insulin sensitivity (IS) in all adolescents during puberty is well-established. It is also known that obese adolescents start out less insulin sensitive at the onset of puberty than lean adolescents, and that their insulin sensitivity decreases further as puberty progresses. While there are both longitudinal and cross-sectional data confirming the natural recovery of pre-pubertal insulin sensitivity in normal weight adolescents after puberty is completed, it is unknown whether obese adolescents follow the same pattern. Furthermore, the characteristics that differentiate the natural decrease in insulin sensitivity during puberty from the pathologic decrease in insulin sensitivity seen in obese children are poorly understood. If obese youth fail to regain their pre-pubertal insulin sensitivity at the end of puberty, the resulting stress on the pancreatic β-cell required to compensate with increased insulin secretion may contribute to the progression from obesity to insulin resistance to T2DM in at-risk youth. Our long-term goal is to better understand the metabolic changes that occur during puberty, their underlying mechanisms, and their potential contribution to adult disease. In particular, we hypothesize that insulin sensitivity and its correlates will change adversely in obese adolescents during puberty, but will not recover after puberty is complete. Furthermore, we hypothesize that treatment with metformin during puberty will help obese adolescents improve pubertal insulin sensitivity, will help preserve insulin secretion, and that such changes will be sustained after cessation of metformin.

Our unique combination of pediatric and adult endocrinology skills, laboratory expertise in insulin signaling, and clinical research experience in insulin sensitivity testing in obese youth make us perfectly poised to address these hypotheses. In addition, the use of a longitudinal study design will allow us to examine differences in percent body fat, body fat distribution and changes in BMI over time in the observational arm of the study. It also allows to examine whether treatment of obese children during this time can have beneficial effects on insulin sensitivity and secretion in the treatment arm. Enhancing our understanding of longitudinal changes of insulin sensitivity in obese adolescents and the response to metformin will help us identify potential interventions aimed at the prevention of T2DM.

The studies in this proposal will test the following hypotheses:

HYPOTHESES:

1. Obese adolescents, unlike normal weight controls, fail to improve insulin sensitivity at the end of puberty.

2. Treatment of obese adolescents with metformin during puberty will help these individuals result in sustained improvement in insulin sensitivity at the end of puberty, decrease compensatory hyperinsulinemia, and preserve insulin secretion, thereby improving their disposition index (DI).

To test these hypotheses, we propose to address the following Specific Aims:

SPECIFIC AIM 1 (Observational Arm):

1. **To compare longitudinal changes in insulin sensitivity and secretion and their correlates in obese and normal weight adolescents during puberty.**
    a. **To compare changes in insulin sensitivity and secretion as measured by frequently sampled intravenous glucose tolerance test (IVGTT) from early puberty to puberty completion between obese and normal weight adolescents.**

b. **To compare changes in body composition, fat distribution, and inflammatory markers over time between these groups.**

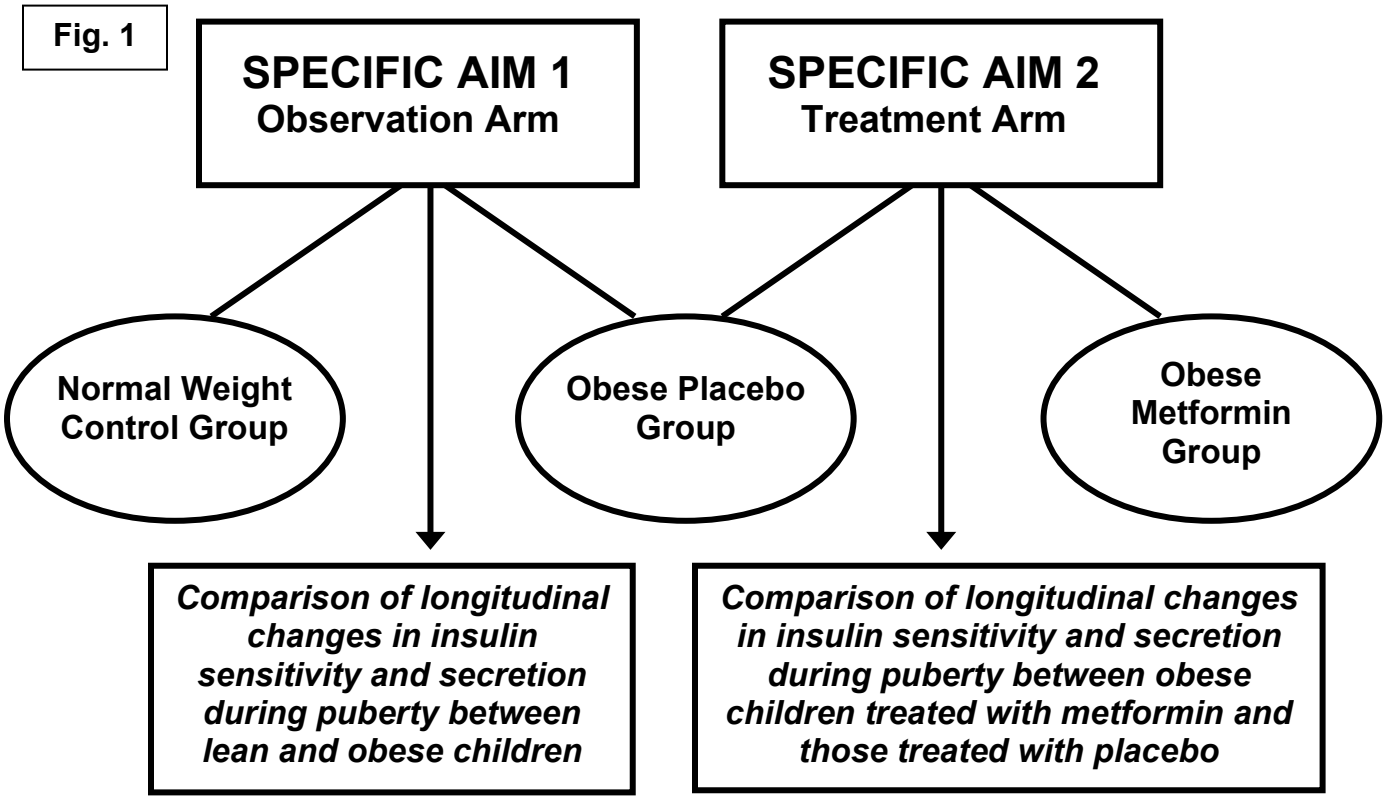
Under this Specific Aim, insulin sensitivity and secretion will be measured by frequently sampled IVGTT in early puberty and at puberty completion in obese and normal weight adolescents. Changes in insulin sensitivity will be correlated with changes in inflammatory markers in all participants and body composition in a subset of these participants.

SPECIFIC AIM 2 (Treatment Arm):

2. **To compare changes in insulin sensitivity and secretion and their correlates, in obese adolescents treated with metformin or placebo during puberty.**
    a. **To compare changes in insulin sensitivity and secretion from early puberty to puberty completion between obese controls and obese adolescents treated with metformin.**
    b. **To compare changes in body composition, fat distribution, and inflammatory markers over time between these groups.**

    Under this Specific Aim, obese participants from Specific Aim 1 will be randomized to receive placebo or metformin treatment during puberty. Frequently sample sampled IVGTT will be performed to measure insulin sensitivity and secretion in early puberty, at the end of puberty when treatment is stopped, as well as six months after treatment completion. Changes in body composition will also be measured in a subset of participants and changes in inflammatory markers will be measured in all participants at these time points.

The nested study design (Fig 1) will efficiently address four important knowledge deficits with a single recruited cohort: 1) Whether obese adolescents recover their pre-pubertal insulin sensitivity at the end of puberty; 2) The relationships between longitudinal changes in insulin sensitivity and changes in body composition in obese and normal weight youth; 3) Whether treatment with metformin during puberty can improve insulin sensitivity in obese adolescents to the level seen in normal weight pubertal adolescents; and 4) Whether treatment of obese adolescents during puberty will result in prolonged improvement of insulin sensitivity and secretion.



B. BACKGROUND AND SIGNIFICANCE
Insulin Resistance and T2DM in Youth

The proportion of adolescents who are obese has been increasing markedly in developed countries in the past 20 years (1), with a parallel increase in the incidence of type 2 diabetes mellitus (T2DM), so much so that it has been dubbed an "epidemic" by the American Diabetes Association (ADA)(2). Obesity is thought to promote the development of T2DM and related disorders at least partly through the development of decreased insulin sensitivity, a condition in which maintenance of normal blood glucose requires higher than normal insulin concentrations (3;4). This results in hyperinsulinemia as the pancreas compensates by increasing insulin production. Overt T2DM develops when the pancreas is unable to compensate for IR by increasing insulin production to the levels necessary to maintain normal carbohydrate metabolism. This results in relative insulin deficiency, while circulating insulin levels may still be normal or elevated. Thus, progressive β-cell failure in insulin-resistant, primarily obese youth is proposed to be a critical factor in the pathophysiology of childhood T2DM. IR correlates with increasing body weight and decreasing physical activity, as well as increased deposition of fat in the visceral cavity and skeletal muscle (5;6).

Insulin Resistance in puberty

The vast majority of youth who develop T2DM are in mid-puberty at the time of clinical presentation (7;8) Puberty, similarly to pregnancy, is known to be a period of physiologic decrease in insulin sensitivity (9). Amiel et al (9), reported a 36% reduction in insulin sensitivity in pubertal non-diabetics when compared with pre-pubertal non-diabetics, using the hyperinsulinemic euglycemic clamp technique. Interestingly, insulin sensitivity of adult subjects does not differ from pre-pubertal children, suggesting that insulin sensitivity improves again after puberty. Multiple cross-sectional and limited longitudinal studies have verified this phenomenon (10-17). The largest study involved 357 hyperinsulinemic-euglycemic clamps performed on children in Tanner stages I (pre-pubertal) through V (fully pubertal) and was cross-sectional in nature (16).

This study found that insulin sensitivity decreases during puberty independently of changes in body mass index (BMI) and body fat (as determined by skinfold thickness), but that obese participants were more insulin resistant at all points before and during puberty. This study did not report comparison data between obese children in Tanner V and in Tanner I, did not measure insulin secretion, and did not follow the same subjects over time.

Longitudinal studies allow investigators to control for intra-individual changes in body fat distribution, BMI and hormonal changes throughout puberty. Several longitudinal trials have confirmed a decrease in insulin sensitivity during puberty, which seems to be most impaired at Tanner stage III and returns to baseline after puberty is completed (11;13-15).

Multiple studies investigating insulin sensitivity in puberty have used the frequently sampled IVGTT (11;13;15;18-20), including one sentinel study performed at our institution (17). This method allows for calculation of both insulin sensitivity and insulin secretion using the minimal model method (21). The IVGTT has been used extensively in both obese and normal weight children both by our group and others and has been shown to correlate well with the gold-standard measures of insulin sensitivity and insulin secretion: the hyperinsulinemic-euglycemic clamp and the hyperglycemic clamp techniques, respectively (22;23).

Although it is clear that insulin sensitivity decreases during puberty, the majority of adolescents maintain normal glucose homeostasis. Studies show almost universally that insulin secretion increases during puberty and DI, a measure of glucose homeostasis, calculated by the ratio of insulin sensitivity to insulin secretion (21), is maintained, therefore pubertal adolescents preserve normal glucose tolerance. That is, in normal subjects, if there is a physiologic decrease in insulin sensitivity, such as occurs during puberty or pregnancy, insulin secretion increases to compensate and DI is maintained, leading to maintenance of normoglycemia. Conversely, DI decreases as patients progress from normal insulin sensitivity to insulin resistance with normoglycemia, to T2DM.

In contrast, insulin secretion and/or DI decreases as puberty progresses in adolescents who have risk factors for developing diabetes, such as in African-Americans or Hispanics (7). Ball et al analyzed glucose metabolism longitudinally in 46 Caucasians and 46 African-Americans using the frequently sampled IVGTT and reported a steeper decline in insulin secretion in blacks than in whites as puberty progresses (11). Both ethnic groups showed a decline in DI between Tanner stages I and V, however the only significant predictor of impaired DI in Caucasians, as determined by linear regression, was fat mass, whereas Tanner stage impacted DI independently in African-Americans. In another cross-sectional study performed by the same group, 214 overweight Hispanic children with a family history of T2DM were examined by IVGTT across all stages of puberty (18). These patients had multiple risk factors for diabetes including obesity, ethnicity and family history. insulin sensitivity did not differ significantly according to Tanner stage, but subjects did tend to have a reduced DI by Tanner stage, suggesting declining β-cell function in these at-risk children. These results suggest that there is some element of β-cell failure occurring as puberty progresses in at-risk youth. However, obesity as a risk factor has not been specifically investigated in any of these studies.

Treatment of Insulin Resistance

If T2DM results from a progressive decrease in insulin sensitivity, leading to compensatory insulin hypersecretion and eventual β-cell failure, improvements in insulin sensitivity through pharmacologic intervention should reduce the need for insulin secretion, prevent β-cell fatigue and prevent or delay diabetes onset. Evidence to support this hypothesis was first demonstrated in adults by the Diabetes Prevention Program, where subjects with impaired glucose tolerance were randomized to placebo, lifestyle intervention or metformin (27). After an average 2.8 years of treatment, both lifestyle and metformin were able to prevent conversion to diabetes compared to placebo. In participants older than 45 years, lifestyle was more effective than metformin in preventing conversion to T2DM, whereas, in younger participants, the two treatments were equivalent. Although lifestyle changes and weight loss are preferable treatments for obesity and decreased insulin sensitivity, this intensive intervention is expensive and success has been limited in a real world setting, especially among adolescents who already struggle with the developmental challenges inherent to the teenage years.

Many pediatric providers have responded to this evidence by prescribing metformin to pediatric patients with obesity and/or hyperinsulinism. There are, however, several problems with extrapolating adult data to

the pediatric population. First, mechanisms underlying the insulin sensitivity changes during puberty may be different and, therefore, metformin may not have the same benefit in adolescents as in adults. Second, beginning metformin in adolescence would be potentially committing pediatric patients to a much longer treatment period without any definable endpoint. It is much more difficult to study diabetes prevention in adolescents because conversion rates to diabetes are much lower than in adults with impaired glucose tolerance (IGT). Thus, conversion to diabetes is an unrealistic research endpoint.

Metformin acts by decreasing endogenous hepatic glucose production, thereby lowering insulin requirements (28). Several studies of metformin have been performed on insulin resistant children, with variable outcomes. Three of these studies were controlled, randomized, double-blind and specifically targeted obese, insulin resistant adolescents with normal glucose homeostasis (29-31). Srinivasan et al treated 28 adolescents aged 9-18 years in a one-year double-blind crossover trial (6 mos. metformin at a dose of 1g BID, 6 mos. placebo). Fasting insulin, glucose and BMI standard deviation scores (SDS) were significantly decreased after the treatment period compared with the placebo period. However, insulin sensitivity, insulin secretion and DI, as measured by Bergman's minimal model method, did not change (31). Similarly, 32 obese adolescents aged 12 to 19 years with a family history of T2DM had a significant decrease in BMI SDS, fasting glucose and fasting insulin after 6 months of 500 mg metformin BID compared with placebo (29). Again there was no change in insulin sensitivity as measured by the minimal model. Data on insulin secretion and DI were not reported. Finally, Kay et al assessed effects of 850 mg metformin BID on obesity and insulin sensitivity in 24 obese adolescents in a randomized, placebo controlled trial. Those in the treatment group had greater weight and body fat loss than the controls and had a decrease in area under the curve (AUC) for insulin (as measured by OGTT), but no change in glucose AUC. They also had a significant decrease in triglycerides. Insulin secretion was not measured. In all of these studies the treatment period was relatively short and none of them targeted the most resistant time of puberty. Furthermore, the Freemark study used a metformin dose that is approximately one half the standard dose used to treat diabetes or PCOS.

Ibanez et al, in Spain, have used metformin in multiple clinical trials of female children and adolescents with premature pubarche (PP) associated with a history of low birth weight. Being small for gestational age is thought to predispose certain infants to developing decreased insulin sensitivity later in life. The girls participating in these studies had evidence for reduced insulin sensitivity, as assessed by elevated insulin levels, but were not obese (32-37). Treatment length and doses were variable, as well as timing of treatment in relation to puberty. Furthermore, none of these trials was randomized or double-blinded, and measures of insulin sensitivity were not consistent. Regardless, treatment with metformin in all these trials resulted in improvements in insulin levels and triglycerides and in several, a reduction in BMI. Most interestingly, one trial showed a persistence of beneficial effects 12-18 months after the metformin was stopped (32). In this trial, 22 girls with a history of PP and low birth weight were randomized to receive metformin treatment (850 mg daily) or no treatment within one year of starting breast development and treated for 36 months (32;35). At the end of the treatment period, change in BMI, IR as measured by HOMA, triglycerides, abdominal fat mass, leptin and total fat mass were all improved in the metformin group compared with the untreated group. Furthermore, these differences between the groups persisted for 12 months after the treatment was stopped. Despite some design flaws, this study suggests that short-term treatment with metformin during puberty can result in sustained metabolic benefit in adolescents at increased risk for developing T2DM.

Inflammatory markers and cytokines: relation to IR

There is a well-established association in adults between obesity and a low-grade inflammatory state, as evidenced by elevated C-reactive protein (CRP) and tumor necrosis factor- α (TNF-α). Furthermore, obesity is associated with dysfunctional adipocytes that secrete higher levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and decreased levels of protective cytokines, such as adiponectin (38;39). Adiponectin levels have been demonstrated to be inversely related to IR, obesity, CRP and TNF-α (40-42). Adiponectin levels strongly inversely correlate with degree of insulin sensitivity in adults, even after adjustment for other risk factors for T2DM such as BMI and fat mass. In fact, adiponectin may even have a stronger relationship with insulin sensitivity than adiposity (42).

An increasing number of studies have shown that these relationships also exist in children of all ages (43-47). One recent study paired obese subjects with IR (as demonstrated by hyperinsulinemic euglycemic clamp technique), but normal glucose tolerance, with obese, insulin-sensitive adolescents of similar gender, pubertal status, weight and percent body fat (48). The IR subjects had lower adiponectin levels than their insulin-sensitive counterparts, indicating that adipocyte dysregulation may precede development of IR and may be a marker of increased risk for IR and eventual development of T2DM. Other studies have confirmed a positive correlation between adiponectin and insulin sensitivity (49;50). Furthermore, BMI was found to be the best predictor of CRP levels according to the National Health and Nutrition Examination Survey (51-53) and fasting insulin levels have been shown to correlate with CRP levels (54;55). In summary, it is well-documented that obesity-related IR is associated with an inflammatory response in both adults and children. However, it is not know if physiologic pubertal IR is also associated with inflammation.

In summary, the temporary fall in insulin sensitivity that occurs during puberty is well-recognized. This is typically overcome by an increase in insulin secretion. However, certain risk factors for T2DM seem to affect the ability of the pancreas to compensate for this change in insulin sensitivity. The mechanism of pubertal IR remains poorly understood, with suggestions that GH/IGF-1 and sex steroids might play a causative role. Furthermore, little is known about the affect of obesity on decreased pubertal insulin sensitivity. If obesity is superimposed on the already IR state of puberty, the resulting amplified IR may stress the beta cells and lead to the development of T2DM. Thus, puberty may be an ideal time to treat IR, with the hope of preventing or at least delaying onset beta cell failure. In the current proposal we will examine the longitudinal changes in insulin sensitivity, body composition and inflammatory markers of normal weight and obese children during puberty. Furthermore, we will compare these changes in obese children with and without metformin treatment during this time. These studies will increase our understanding of the contribution of puberty to the progression of obesity to IR and to DM in adolescents. This knowledge will, in turn, allow to better target our preventive efforts.

Liver fat, Body Composition and IR

In a normally insulin sensitive subject, excess calories are stored in adipose tissue as a result of insulin release, or burned for fuel in skeletal muscle. Fat is stored preferentially in the adipose depot, not in the viscera, liver or skeletal muscle tissue. However, in humans with insulin resistance, an abnormal pattern of fat distribution is seen, with ectopic deposition of lipid in the liver, viscera and skeletal muscle, specifically as intramyocellular lipid (IMCL) (38), and appears to play an important role in the pathogenesis of reduced insulin sensitivity. IMCL by muscle biopsy (39) and by nuclear magnetic resonance (NMR) (40) are strongly correlated with insulin resistance, including in adolescents (41). Resistance is strongly associated with visceral adiposity as demonstrated by various methods including MRI and waist-to-hip ratio (42-44). Furthermore, data in adults have shown that accumulation of visceral fat poses a greater health risk than accumulation of subcutaneous fat (45). In T2DM, visceral fat content is associated with a decrease in peripheral insulin sensitivity and an increase in gluconeogensis (46;47), and is highly correlated with fatty liver disease in this population (48). Finally, fatty liver disease is thought to be the most common cause of liver pathology in obese children (49) and resistance is thought to play a role in the pathogenesis of fatty liver disease in both children and adults (50;51). Changes in total body fat and fat distribution occur during puberty, and these changes may correlate with changes in insulin sensitivity, however this has not been clearly defined.

C. PRELIMINARY DATA

1. Studies of metformin treatment in obese adolescents

Our previous research using metformin in conjunction with personal goal setting in 85 IR adolescents was the largest trial to date using metformin in obese adolescents without T2DM(52). This study was conducted by our group, including my co-mentor Dr. Phil Zeitler and my consultant, Dr. Kristen Nadeau. Kathy Love-Osborne, M.D., one of the consultants on this application, was the primary investigator. The mean age of subjects was 15.7 years. 76% of participants completed the study. Mean body mass index (BMI) was 39.7 kg/m2. 71% were female, 58% were Hispanic and 34% Black. Overall, there was no

significant difference in weight loss among subjects receiving metformin or placebo. However, 11 (23%) subjects on metformin had a BMI decrease of 5% or more, while no subjects on placebo were able to attain this degree of weight loss. Of 11 subjects decreasing BMI by 5%, 82% were female. 45% were Hispanic, 27% Black, 18% Caucasian and 9% Asian.

All subjects had elevated fasting insulin levels at baseline, with an average level of 39.8 μIU/ml. At the end of 6 months, the metformin group had a significant decrease in fasting insulin compared with the placebo group (-9.8 ±3.4 μIU/ml vs. -3.1 ±2.6 μIU/ml, $p<0.05$). Furthermore, the metformin group had significantly decreased fasting triglycerides at the 6 month follow-up compared with the placebo group (-26.2 vs. -0.2, $p<0.05$) (unpublished data) and triglycerides were shown to be a significant predictor of IGT in this cohort (53). Finally, the metformin group had a significantly improved fatty liver score, as measured by ultrasound (mean change -0.4 ±0.1 vs. +0.25 ±0.28, $p<0.05$) (54).

Subjects on metformin were slightly less likely to have good pill compliance (68% vs. 78% on placebo). 29% of subjects on metformin reported side effects: nausea or vomiting (14%), diarrhea (14%), or abdominal pain (11%) vs. 22% of subjects on placebo: nausea (11%) or diarrhea (11%) but no subjects required dose reductions. There were no significant differences in attrition between the groups.

Thus, metformin may be a candidate for primary prevention of T2DM in high-risk adolescents. Extended release metformin is available as a generic medication; it is relatively inexpensive and accessible. Furthermore, this trial shows that long-term treatment with metformin is safe and feasible in our adolescent population.

2. Studies of Intramyocellular lipid deposition in relation to Insulin Sensitivity

In a study designed to evaluate the association of exercise dysfunction with insulin sensitivity, my consultant, Dr. Nadeau, examined 37 adolescents (13 T2DM, 11 obese, and 11 lean) of similar age, sex, Tanner stage and activity level. Muscle lipid content was assessed by muscle biopsy as well as NMR, a noninvasive imaging technique that allows separation of intramyocellular lipid, which has been negatively correlated with insulin sensitivity, and extramyocelluar lipid, and was well tolerated by adolescents. In addition, insulin sensitivity was measured by hyperinsulinemic euglycemic clamp. In this study, obese adolescents had significantly decreased insulin sensitivity when compared with normal weight controls, had significantly increased soleus extramyocellular lipid (EMCL) as measured by NMR ($p=0.017$) and had a trend toward increased soleus IMCL (2014 ±947 in obese vs. 1166 ±458 in lean controls). Maximal exercise capacity (VO2max/kg) significantly positively correlated with insulin sensitivity (mg/kg/min) ($r=0.82$, $p<0.0001$) and negatively with soleus IMCL ($r=-0.645$, $p<0.0001$) (55). These data demonstrate the experience of this institution at using NMR to detect soleus IMCL and EMCL. The NMR imaging in Dr. Nadeau's protocol was performed by Dr. Mark Brown, who is consulted in this proposal, and the muscle biopsies and insulin clamps were performed by Dr. Nadeau at our pediatric Clinical Translational Research Center.

These preliminary data show the feasibility and tolerability of these procedures in adolescents. These studies also demonstrate our group's ability to recruit and maintain study participation in a group of obese adolescents receiving treatment with metformin.

Although I was not actively involved in these projects, I am a co-investigator on Dr. Love-Osborne's current study, which is an expansion of the previous metformin project and I am involved in the data analysis for Dr. Nadeau's study.

D. RESEARCH DESIGN AND METHODS:

HYPOTHESIS 1:

1. Obese adolescents, unlike normal weight controls, fail to improve insulin sensitivity at the end of puberty.

SPECIFIC AIM 1 (Observational Arm):

2. To compare longitudinal changes in insulin sensitivity and secretion and their correlates in obese and normal weight adolescents during puberty.

a. **To compare changes in insulin sensitivity and secretion as measured by frequently sampled intravenous glucose tolerance test (IVGTT) from early puberty to puberty completion between obese and normal weight adolescents.**
b. **To compare changes in body composition, fat distribution, and inflammatory markers over time between these groups.**

RATIONALE

In normal weight adolescents there is a physiologic decrease in insulin sensitivity, beginning at Tanner stage I (T1), as well as changes in body composition. Insulin sensitivity progressively decreases to a nadir at T3 and then returns to pre-pubertal levels after puberty is completed (T5) (9;11;13;14). Obese adolescents are more insulin resistant going into puberty, and also show a pubertal decrease in insulin sensitivity that is independent of BMI and body composition (16). It is still unclear whether obese, insulin resistant adolescents recover insulin sensitivity when puberty is completed.

DESIGN

Participants:

1. 104 Obese participants:
   a.) BMI ≥ 95th percentile
   b.) Tanner stage II – III at baseline
   c.) Age ≥ 9 years
   d.) Absence of IGT, impaired fasting glucose (IFG) or T2DM, as determined by oral glucose tolerance test (OGTT)
2. 52 Normal weight participants:
   a.) BMI ≥ 5th percentile and ≤ 85th percentile
   b.) Tanner stage II – III at baseline
   c.) Age ≥ 9 years
   d.) Absence of IGT, IFG or T2DM, as determined by OGTT

Other inclusion criteria: Every effort will be made to recruit study participants that are representative of the racial and ethnic make-up in Colorado. This means that a significant number of Spanish-speaking only participants that may be recruited. The methods of appropriately recruiting and consenting these subjects in a manner that will ensure that we are appropriately communicating the study protocol and risks and benefits of participating in this study.

Exclusion criteria for both groups:

a). Specific genetic syndrome or disorder known to affect glucose tolerance other than diabetes
b). Inhaled steroids at dose above 1000 mcg daily, inhaled fluticasone or equivalent.
c). Oral steroids within last 60 days or oral steroids more than 20 days during the past year
d). Medication(s) which are known to cause weight gain or weight-loss within the last 30 days, including atypical antipsychotics.
e). Refractory hypertension: average systolic blood pressure > 150 mmHg or average diastolic blood pressure > 95 mmHg despite appropriate medical therapy.
f). Refractory hyperlipidemia: total cholesterol > 300 mg/dL or LDL > 190 mg/dL or triglycerides > 800 mg/dL, despite appropriate medical therapy.
g). Evidence of proteinuria
h). Patients on any form of insulin sensitizer within the last year.
i). Admitted use of anabolic steroids within the past 60 days.
j). Anemia: Hematocrit < 30.0%, a hemoglobin < 11 gm%/dL
k). Weight >300 lbs., due to limits of the NMR and DEXA tables
l). Other significant organ system illness or condition (including psychiatric or developmental disorder) that would prevent participation in the opinion of the investigator.

Approach:

We will recruit obese and normal weight subjects who are in early puberty as determined by Tanner staging of breast development and pubic hair in girls and by Tanner staging of pubic hair and estimation of testicular size in the boys. Adolescents whose pubertal status is at least Tanner II (T2), but no more than Tanner III (T3) will be asked to participate. In two studies that assessed insulin sensitivity across all stages of puberty, it was already significantly decreased from prepubertal insulin sensitivity at T2 and, while it had not yet completely recovered in early T5, it was still significantly increased from T4 insulin sensitivity (11;16). We will recruit through our metabolic syndrome and Good Life clinics, through advertisements, and from local pediatric clinics, including Denver Health Medical Center. Because of the ethnic make-up of our clinics, there will be Spanish-speaking only participants included in the study. The study coordinator is a native Spanish-speaker and, therefore, has the ability to consent the participants in Spanish using our consent and HIPAA forms that have been translated into Spanish. Lifestyle intervention materials are also available in Spanish. When the study coordinator is not available, an official Spanish interpreter will be used for the consent process and all study visits. Because of the anticipated high screen-failure rates due to failure to meet physical examination criteria (most notable Tanner staging criteria and BMI criteria), we will need to consent at least 40% more participants than we ultimately intend to enroll. Therefore, we anticipate that 225 participants will need to be consented for screening to be able to enroll 156 participants. The timeline of our experimental approach is shown below (Fig 2). A fasting baseline screening visit, to be performed at the pediatric Clinical Translational Research Center (CTRC), will include assessment of obesity status as determined by height, weight, BMI, waist circumference and hip circumference. An OGTT will be performed in all obese subjects to rule out IGT, IFG and diabetes in all subjects. The following screening labs will also be performed to examine for any significant underlying illness: AST, ALT, electrolytes, CBC, BUN, creatinine, lipids, and urinalysis.

After determination of eligibility, participants will have a one-day CTRC admission for baseline laboratory studies, IVGTT, and body composition assessment (Study Visit 1) following an overnight fast. Upon arrival they will have an IV placed for blood draws and injections. Fasting blood glucose and insulin will be drawn at this time. Sex steroids and inflammatory markers will also be drawn at this time. (After the blood draw, they will undergo a frequently sampled IVGTT (specific methods for above described after Specific Aim 2). Up to 10 obese subjects will participate in the arm, as pilot subjects, who **are not** part of the treatment arm (i.e. they will not be taking a placebo).

In the afternoon of the Study Visit 1, a dual-energy x-ray absorptiometry (DEXA) scan will be performed to determine body composition. In a subset of participants (13 per group), soleus muscle nuclear magnetic resonance (NMR) and abdominal fast magnetic resonance imaging (MRI) sequence will also be performed to measure body fat distribution in terms of IMCL, liver fat content and visceral adipose content.

The next study visit (Study Visit 2) will occur when the participant has reached Tanner stage IV, approximately one year after the baseline visit. At this time the baseline laboratory draw, imaging studies and IVGTT will be repeated. For those subjects randomized to the MRI, it will not be repeated at this visit.

The next study visit (Study Visit 3) will occur when the participant has reached Tanner stage V, approximately one year after Study Visit 2. At this time the baseline laboratory draw, imaging studies and IVGTT will be repeated. This is the last long visit for the lean subjects. The obese subjects will stop taking the study medication at this time point.

The next study visit (Study Visit 4) will occur 6 months after the obese participants have reached Tanner stage V. At this time the baseline laboratory draw, imaging studies and IVGTT will be repeated. This is the last long visit for the obese subjects. For those subjects randomized to the MRI, it will not be repeated at this visit.

Between the visits, participants will be asked to come in every 6 months for anthropometric measurements and Tanner staging. Obese participants in the treatment arm (randomized to either Metformin or placebo) will also be asked to come in quarterly for pill counts to measure medication compliance and to receive a refill on their medication.

Lean subjects will be asked to undergo a total of 3 DEXA scans throughout the course of the study, and for those randomized to the MRI, they will be asked to undergo a total of 2 MRI's. Obese subjects will be asked to undergo a total of 4 DEXA scans through the study, and for those randomized to the MRI, they will be asked to undergo a total of 2 MRI's.

Visit Number	Puberty Exam	Height & Weight	Pill Count	OGTT	IVGTT	DEXA	MRI*	Total Time
Screening Visit	x	x		x				4 hr
Study Visit 1		x			x	x	x	5-8 hr
Short Visits (Every 3 months)	x	x	x					1 hr
Study Visit 2	x	x			x	x		5-8 hr
Study Visit 3		x			x	x	x	5-8 hr
Study Visit 4		x			x	x		5-8 hr

Please see the tables below for a more detailed explanation of the study procedures.

Specific methodology that will be used in this study is described after Specific Aim 2

Treatment table for obese subjects:

*Only those in the group who are asked to have an MRI/MRS

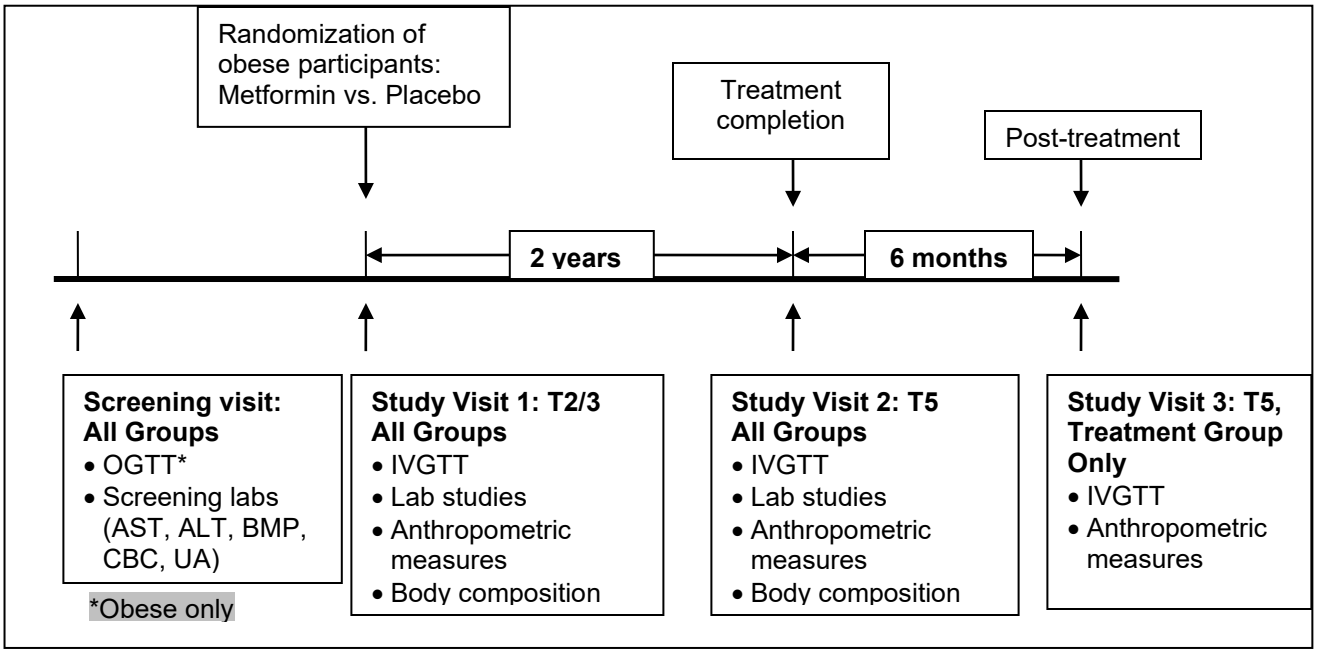
No Treatment table for lean subjects/obese pilot no treatment subjects:

Visit Number	Puberty Exam	Height & Weight	OGTT*	IVGTT	DEXA	MRI/ MRS**	Total Time
Screening Visit	x	x	x				2-4 hr
Study Visit 1		x		x	x	x**	5-8 hr
Short Visits	x	x					1 hr
Study Visit 2	x	x		x	x		5-8 hr
Study Visit 3	x	x		x	x	x**	5-8 hr

*Only participants who are overweight

**Only those in the group who are asked to have an MRI/MRS

Fig. 2 Timeline of Study Participation



HYPOTHESIS 2: 2. Treatment of obese adolescents with metformin during puberty will help these individuals recover insulin sensitivity at the end of puberty, decrease compensatory hyperinsulinemia, and preserve insulin secretion, thereby improving their disposition index (DI).

SPECIFIC AIM 2:

2. **To compare changes in insulin sensitivity and secretion and their correlates in obese adolescents treated with metformin or placebo during puberty.**
    a. **To compare changes in insulin sensitivity, secretion and DI from early puberty to puberty completion between obese controls and obese adolescents treated with metformin.**
    b. **To compare changes in body composition, fat distribution, and inflammatory markers over time between these groups.**

RATIONALE:

The goal of this aim is to see whether treatment with metformin can improve insulin sensitivity and reduce compensatory insulin secretion during puberty. The thought is that, by improving insulin sensitivity and decreasing insulin secretion requirements during this temporary critical period of reduced insulin sensitivity,

β-cell function will be preserved. This would make puberty a potential treatment period to target prevention of T2DM.

DESIGN

Participants:

1. 104 Obese participants:
   a.) BMI ≥ 95th percentile
   b.) Tanner stage II – III at baseline
   c.) Age ≥ 9 years
   d.) Absence of impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or T2DM, as determined by oral glucose tolerance test (OGTT)

Exclusion criteria:

See Specific Aim 1

Approach:

All obese subjects who are recruited will be randomized after Study Visit 1 to receive metformin or placebo. The 52 participants randomized to the placebo group will then also serve as the obese subjects for the longitudinal observational studies of Specific Aim 1 (Please see Fig 2 for further clarification). In other words, 52 obese participants will be included in both Specific Aims 1 and 2, in order to minimize the total number of participants needed. Randomization will be stratified by: 1) Family history in a first degree relative with T2DM or no family history and 2) BMI between the 95th and 99th percentile or BMI greater than the 99th percentile. The extended release metformin will be started at 500 mg daily for one week and then increased by 500 mg a week until the target dose of 2000 mg a day is attained. The extended release preparation allows patients to take the medication once a day to promote improved adherence and is better tolerated. All obese participants will receive standard lifestyle counseling, focusing primarily on dietary improvements and increased physical activity. The total treatment period will be two years to include, on average, the most resistant stages of puberty. In order to monitor metformin compliance closely, the participants will come in for monthly pill-counts. In addition, each participant will be provided with a pill dispenser and a calendar to record each dose that is taken to assist with and track compliance. Furthermore, parents will be encouraged to remind the participant to take his/her medication. Participants will be given a point each time they bring in their pill bottle, whether or not they have been compliant, and will be eligible for an extra gift card for every 6 points they obtain. Our group has extensive experience in maintaining metformin compliance in this population (52). Tanner staging, anthropometric measurements and lifestyle counseling will also be performed at quarterly visits.

The CTRC study visit described previously for Study Visit 1 will be repeated twice more: at the end of this treatment period (Study Visit 2) and six months after the treatment is stopped (Study Visit 3). IVGTT, DEXA, abdominal fast MRI and soleus muscle NMR will also be repeated. We anticipate that the majority of patients will be Tanner stage V (T5) at the end of the 2-year treatment period, as the average time from the onset of breast development until menarche is 2-3 years. If T5 has not been reached after two years, the treatment will continue until the individual is at T5. Follow-up visits for Tanner staging will occur quarterly.

The final visit (Study Visit 3) will be to determine whether insulin sensitizer treatment will result in persistent improvement in insulin senstivity and insulin secretion, even after treatment is stopped. The subjects will again be given a 3-day standard macronutrient diet and will fast overnight prior to admission. Only the IVGTT and anthropometric measurements will be performed at this study visit.

Specific Methods:

Screening laboratory studies: ALT, AST, electrolytes, fasting lipids, CBC, BUN and urinalysis.

Laboratory studies drawn at Visits 1, 2 and (for obese subjects only) 3 : sex steroids, c-reactive protein (CRP), interleukin-6 (IL-6), adiponectin and fasting lipids. Sex steroids (free testosterone, estradiol,

androstenedione and DHEAS) will be drawn in order to confirm or refute previously found associations between sex steroids and IR. Inflammatory markers and other factors that have been associated with IR will also be drawn at this visit, including c-reactive protein, interleukin-6, adiponectin and fasting lipids (LDL, HDL and triglycerides).

Anthropometric measurements: Height, weight, waist-to-hip ratio, and supine abdominal height are measured by standard techniques.

Oral Glucose Tolerance Test: Glucose and insulin drawn at fasting (0), and at 30, 60, 120 and 180 minutes after ingestion of glucola (1.75g/kg, max 75g)

Dual-energy X-ray absorptiometry (DEXA Scan): Body composition will be measured supine using the DEXA technique on the pediatric CRC and will be used to derive fat-free mass (92). This technique relies on the absorption of dual electron wavelengths for the assessment of body fat, lean tissue, and bone mineral density.

Fast Magnetic Resonance Imaging (MRI) sequence: Rapid MRI will be used to assess hepatic fat fraction by the modified Dixon technique, previously described (56). In this technique, in- and out-of phase images of the liver will be obtained using a 1.5 Tesla magnet. The subject will be placed in a supine position on the body coil. The scan parameters include relaxation time = 9.3 ms (in-phase) and 7.3 ms (out-of-phase), echo time = 4.2 ms (in-phase) and 2.1 ms (out-of-phase), flip angle = 30 degrees, slice thickness = 10 mm, field of view = 32 x 16 cm, matrix size = 256 x 128, and number of excitations = 8. Four image slice pairs (in- and out-of-phase) will be obtained through the liver. Fat fraction will be calculated from the mean pixel signal intensity (SI) using the formula: Fat fraction = ($SI_{inphase} - SI_{outofphase}$). A single slice at the level of the umbilicus will be used for determination of abdominal adipose distribution. Fast spin coil with a flip angle of 70 degrees, repetition time = 120 ms, echo time = 1.6 mg, field of view = 36 x 27 cm, slice thickness = 5.0 mm, matrix = 256 x 128, and number of excitations = 1.

Nuclear Magnetic Resonance Imaging (NMR): Dr. Mark Brown, of UCHSC radiology, will perform soleus NMR on a 3.0 T whole–body MRI scanner (GE Medical Systems, Waukesha, WI). The subject is positioned in the MR magnet feet first, prone, with the soleus muscle centered in the extremity coil. Scout images (usually T1-weighted) position the volume-of interest (usually 2cm x 2cm x 2cm), to avoid regions of gross adipose and vascular structures. Spectroscopy acquisition is performed using the PRESS pulse sequence without water suppression (TR/TE = 2000/24 ms, 64 averages, total acquisition time 2 minutes), and analyzed by SAGE spectroscopy analysis (GE). The water peak is fit as a Lorentzian line using Marquardt routine, and subtracted from the spectrum. The remaining peaks (creatinine and choline) are then fit using the Marquardt routine. IMC and EMC triglyceride peaks (1.3 and 1.5 ppm, respectively) are obtained from the fit results, corrected for T1 and T2 relaxation, and expressed as a percentage of the water content (96). Subjects need to hold reasonably still during the scan and cannot weigh >300 lbs.

All of the preceding imaging studies avoid radiation exposure and are regularly available at our institution.

Standard macronutrient diet: 3 days of a diet composed of 55% carbohydrates, 30% fat, and 15% protein will be provided by the CTRC. This diet is the standard used by the UCHSC Center for Human Nutrition prior to insulin clamps or VO2max testing as variations in dietary intake affect insulin sensitivity.

Frequently sampled IVGTT: Prior to admission, they will be given a 3-day standard macronutrient diet by the CTRC and will also be restricted from intense exercise for seven days. They will be asked to fast overnight before admission. An IV catheter will be inserted into each arm. At times -15 and zero, glucose and insulin will be drawn. Participants will then be infused with 0.3g/kg of 25% dextrose over 60 seconds. Glucose and insulin will be sampled from the contralateral arm at times 2, 3, 4, 5, 6, 8,10,12,14,16,19, 22, 25, 30, 35, 40,

50, 60, 70, 80, 90,100,120, 140,160, and 180 minutes. Bergman's minimal model program will be used to calculate insulin sensitivity (S_i), insulin secretion ($AIR_{glucose}$) and DI (DI).

Sample size calculation: A recruitment goal of 52 participants per group has been set (normal weight controls, obese controls, obese on metformin—a total of 156 participants). Assuming a 30% drop-out rate, this will give a final sample size of 30 per group. Power was run in PASS (NCSS Software; Kayesville, UT) and was calculated using a one-way ANOVA with a two-sided significance level of 0.05. Variability of the estimates was based on data available in an article by Buchanan et al. (57) in which IVGTT insulin sensitivity was measured in high-risk Hispanic women at baseline and 3 months for a placebo (n=109) and a troglitazone group (n=108). The 3-month change was -0.17 and 1.16 min^{-1} per μU/ml x 10^{-4}, for the placebo and treatment groups, respectively. Assuming a correlation of 0.5, the estimated overall standard deviation of the 3-month change for both the placebo and treatment groups was 1.85 min^{-1} per μU/ml x 10^{-4}. For this study, we assume similar variability and expect the 2-year change for the obese control and metformin groups to also be similar to those seen in the Buchanan et al. groups. The 2-year insulin sensitivity change in normal controls is expected to be similar to the change seen in Ball et al. (11) for 46 white adolescents from Tanner II / Tanner III to Tanner V, which was 1.6 min^{-1} per μU/ml x 10^{-4}. Therefore, with a standard deviation of 1.85 and expected 2-year changes of 1.16, -0.17 and 1.6 for the metformin, obese control and normal control groups, respectively, a sample size of 30 per group achieves 93% power to detect an overall difference between the groups.

Statistical analysis: All analyses will assume a two-sided test of hypothesis with an overall significance level of 0.05 and will be performed in either SAS v9.1.3 or above (SAS Institute, Inc., Cary NC) or Splus v7.0 or above (Insightful Corp, Seattle WA).

Simple descriptive statistics will be calculated for all variables (mean, median, standard deviation, minimum and maximum values for continuous variables; number and percentage of subjects for categorical variables). Linear regression will be used to compare 2-year change in insulin senstivity, insulin secretion, and DI between groups for Specific Aims 1 and 2 (normal controls vs. obese controls and obese treated vs. obese controls). Potential confounders such as sex, 2-year change in percent body fat and 2-year change in BMI will be included in each model. In order to determine if the benefit of treatment persists beyond stopping treatment, the above analysis will be repeated on the change from baseline to 6 months post-treatment for the obese treated and obese control groups only. The mean change in each outcome from treatment stoppage to 6 months post treatment will also be calculated with 95% confidence intervals (CI) in order to assess maintenance of the level of each outcome. Although we are not powered for equivalence (to test the hypothesis that the 6-month change is equal to zero), the precision of the estimate and whether the difference is *clinically* significant will be evident from the 95% CI. A CTRC biostatistician will be available to consult on all matters of statistics.

Potential Difficulties and Limitations/Alternatives

In adolescence, the number of routine visits to the pediatrician tends to drop. Therefore, we foresee a potential difficulty in recruiting children who are just starting puberty. For this reason, we are including children who are both Tanner 2 and Tanner 3 at study entry. In both of these stages of puberty, insulin sensitivity has been shown to be significantly decreased when compared with Tanner 1 and Tanner 5. The length of time required for progression through puberty varies in individuals. It is important to measure the post-treatment insulin sensitivity in Tanner stage 5, because insulin sensitivity in Tanner 4 may not be significantly different from earlier in puberty and only at T5 insulin sensitivity returns to its pre-pubertal level. For this reason, if a subject has not reached Tanner 5 in two years, they will not have their Study Visit 3 until Tanner 5 has been attained. This means that some subjects may need to be treated for a longer period of time. However, in order to compare differences in insulin sensitivity after treatment is stopped between subjects, the interval from treatment stop (Study Visit 2) to post-treatment follow-up (Study Visit 3), should be consistent.

The natural recovery of pubertal insulin sensitivity may confound measurement of metformin effects on insulin sensitivity as puberty progresses. In order to control for this, we have included a placebo-controlled group and a group of normal weight controls. This will allow us to compare changes in insulin

sensitivity from baseline to the end of puberty between obese treated subjects and both normal-weight and obese subjects who have not been treated.

The length of this study and potential difficulty with attrition are also limitations of this study. To limit the number of participants lost to follow-up, we will maintain frequent contact with participants with quarterly visits for anthropometric measurements, monthly visits for pill-counts and phone contact at least once between these visits. In addition to these visits, the participants in the treatment group will be contacted by telephone at least monthly to inquire about medication side effects and to review changes in diet and exercise. Due to our involvement in the TODAY study and other trials of metformin, our staff are experienced with working on long-term projects with obese and T2DM adolescents, with excellent study retention out as far as four years.

Adverse effects of metformin include nausea, anorexia, diarrhea and vomiting. These adverse effects could potentially interfere with patient compliance. These effects have been shown to be reduced when the dose is titrated up slowly, as described in the Approach section of Specific Aim 2. Furthermore, in our experience with metformin, adverse effects did not result in decreased dose or significant patient attrition. Participants in the metformin group were only slightly less compliant than the placebo group in our blinded study of metformin in obese adolescents.

Finally, IVGTT is not the gold standard for measurement of insulin sensitivity. However, it has been shown to correlate well with the hyperinsulinemic-euglycemic clamp, which is the gold standard (21). It was chosen for this study as it is slightly less invasive and allows measurement of both insulin sensitivity and insulin secretion. In this population, which is as young as 9 years old and will be undergoing repeated measures, it is necessary to avoid time intensity and invasive procedures as much as possible. Therefore, it was felt that the benefits of IVGTT far outweigh the limitations in this protocol.

Summary

The temporary fall in insulin sensitivity that occurs during puberty is well-recognized. This is typically overcome by an increase in insulin secretion. However, certain risk factors for T2DM appear to affect the ability of the pancreas to compensate for this change in insulin sensitivity. The mechanism of pubertal insulin resistance remains poorly understood, with suggestions that GH/IGF-1 and sex steroids might play a causative role. Furthermore, little is known about the affect of obesity on decreased pubertal insulin sensitivity. If obesity is superimposed on the decreased insulin sensitivity of puberty, the resulting amplified insulin requirements may stress the β cells and lead to the development of T2DM. Thus, puberty may be a critical time to treat resistance, with the hope of preventing or at least delaying onset of β-cell failure. In the current proposal we will examine the longitudinal changes in insulin sensitivity, insulin secretion and body composition of normal weight and obese children during puberty. Furthermore, we will compare these changes in obese children with and without metformin treatment. These studies will increase our understanding of the contribution of puberty to the progression from obesity to IR and DM in adolescents. This knowledge will, in turn, allow to better target our preventive efforts.

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