Pharmacogenomics of Thiazolidinediones

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RESEARCH PLAN

A. SPECIFIC AIMS

The Thiazolidinediones (TZDs) are a class of insulin sensitizing drugs used in the treatment of type 2 diabetes (T2DM). In addition, trials suggest that the TZDs may be useful in the prevention of progression from impaired glucose tolerance to T2DM and have salutary effects on other aspects of the metabolic syndrome. However, clinical and mechanistic studies indicate that not all patients exhibit the expected response to TZDs. Approximately 1/3 of those treated show little or no improvement in insulin sensitivity or HbA1c.

The TZDs are ligands for the nuclear receptor peroxisome proliferator activated receptor-gamma (PPARγ). When this receptor is activated by TZDs, many genes involved in adipogenesis and insulin signaling are up- or down-regulated.

The overall objective of this proposal is to define the molecular and genetic basis of TZD responsiveness using a phenotype to genotype approach and state-of-the-art functional genomics and high-throughput genetics. Our ***hypotheses*** are:

(1) Differences in clinical response (primarily improvement in insulin sensitivity) can be defined at the molecular level as differences in expression of genes involved in TZD-activated pathways.

(2) Genes whose expression change is correlated to clinical response can be identified through cDNA microarrays of muscle and fat, the two target tissues for TZDs.

(3) Sequence variants in these candidate genes will determine TZD response, particularly sequence variants within regulatory regions.

To directly test these hypotheses, the ***Specific Aims*** of this proposal are to:

1. Perform comprehensive testing in 75 individuals before and after a 3-month rosiglitazone intervention and characterize the variability in TZD response at the physiological, cellular, and molecular levels. The primary TZD response trait will be changes in insulin sensitivity as measured by an intravenous glucose tolerance test (IVGTT). We will also define secondary response traits, which will include body fat composition and distribution (DXA and abdominal CT), serum lipid levels (HDL, LDL, triglycerides, lipoprotein particle sizes), inflammatory markers (hsCRP, SAA, IL1β, IL6, TNFα), prothrombotic factors (plasminogen activator inhibitor 1; PAI-1), adipokines (leptin, adiponectin), and adipocyte metabolism (glucose uptake, lipolysis). From these data, we will:
   a. Identify the baseline factors (e.g., age, gender, body mass index, etc.) that are predictive of TZD response to the measured traits.
   b. Determine if the response of the primary trait (i.e., insulin sensitivity) to TZDs is correlated with the response of each of the secondary traits to TZDs.
   c. Perform cDNA microarray analysis of muscle and fat before and after 3 months of rosiglitazone therapy to identify the genes and their corresponding pathways that are correlated to TZD response.
   d. Perform quantitative real-time PCR to accurately quantify changes observed in the microarray analysis and to study expression of other genes of interest.
2. Define genes whose regulation correlates to TZD response. One-hundred of these TZD-response candidate genes will be extensively studied by:
   a. Identifying common sequence variants through DNA sequencing and *in silico* SNP discovery.
   b. Validating these SNPs and defining allele frequencies through genotype analysis of approximately 2000 SNPs in the 100 candidate genes in DNA samples from Caucasian and African American subjects.
3. Identify the SNPs and haplotypes in these 100 candidate genes that influence TZD response by performing association analysis in (1) the 75 subjects recruited for this study, (2) a subset of 597 individuals from the Diabetes Prevention Program study who were treated with the TZD,

troglitazone, and (3) 97 gestational diabetic Mexican American women who were treated with troglitazone in TRIPOD (and more recently with pioglitazone in PIPOD).

In summary, this project will seek to determine the genetic underpinnings of inter-individual variation in TZD response. Since TZDs are agonist for the nuclear receptor, PPARγ, we will use a functional genomics approach to identify the molecular phenotype of TZD response from which candidate genes will be selected and studied in several populations. This proposal combines powerful functional genomics and high-throughput genetic approaches in a unique and innovative fashion to study physiologically well-characterized subjects in whom a controlled TZD intervention is performed.

B. BACKGROUND AND SIGNFICANCE

1. Thiazolidinediones (TZDs), a Novel Class of Insulin Sensitizers

a. PPARγ and TZD mechanism of action

The thiazolidinediones (TZDs) are synthetic ligands for the nuclear receptor PPARγ (peroxisome proliferator activated receptor-gamma). Agents approved in the United States include rosiglitazone, pioglitazone, and troglitazone (the latter is no longer marketed due to liver toxicity). PPARγ is an important regulator of adipocyte differentiation and insulin signaling (22, 33, 46, 64, 72, 78), which forms heterodimers with RXR. When a ligand binds to the PPARγ-RXR heterodimer, this event promotes association of the heterodimer to specific DNA sequences within regulatory regions of target genes (so-called PPREs) to activate or repress transcription, thereby promoting adipogenesis and increasing insulin sensitivity. The expression of many (hundreds of) genes is affected by PPARγ activation (35, 40, 47, 58).

Human trials have shown that TZDs decrease insulin resistance, improve glycemic control and decrease hypertriglyceridemia, and they are now a mainstay of treatment for people with T2DM (10, 32, 54, 56, 57, 69). The mechanisms whereby TZDs increase insulin sensitivity in humans likely include up-regulation of cellular pathways involved in lipid oxidation, glucose uptake, and glucose utilization (reviewed in 22, 43, 64). Improved insulin sensitivity induced by TZDs also appears related to the ability of TZDs to increase adiponectin expression as well as to decrease the expression and insulin-antagonistic effects of tumor necrosis factor-α (TNFα)(34, 44, 50, 59), and possibly other inflammatory factors. TZDs are also known to promote changes in body fat distribution from visceral to subcutaneous fat, which may increase insulin sensitivity (2, 3, 41, 53).

b. Anti-inflammatory and other actions of TZDs

Insulin resistance is thought to be a mainstay of the metabolic syndrome, which is clinically defined by the NCEP ATP-III guidelines based on specific cut points for waist circumference, triglycerides, HDL-cholesterol, fasting glucose, and blood pressure (21). In addition to insulin resistance, it is becoming increasingly evident that the syndrome also involves chronic inflammation and a prothrombotic state manifest in elevations of factors such as C-reactive protein (CRP) and PAI-1. CRP is an acute phase reactant that has been associated with all the traditional components of the metabolic syndrome (60, 79, 86). In addition to insulin resistance, CRP levels are correlated to the number of elements of the metabolic syndrome present (23).

TZDs have anti-inflammatory effects that include the suppression of CRP levels (63), PAI-1, serum amyloid A (SAA), and complement C (20). When TZDs are used in obese subjects with and without diabetes, it results in reduced NFκB-binding activity in mononuclear cell nuclear extract and reduced plasma monocyte chemoattractant protein-1 (MCP-1) and CRP. Plasma TNF-α and SAA concentrations are additionally inhibited in those without diabetes (52). In addition to these anti-inflammatory effects, TZDs reduce blood pressure (63), and possibly have direct effects on the vascular endothelium, which expresses PPARγ. Lastly, PPARγ is also expressed in pancreatic β-cells and appears to play a role in maintenance of β-cell function and insulin secretion. Although much remains to be learned about the relation between insulin resistance and chronic inflammation, it is likely that TZD treatment, offering a combination of insulin sensitization and anti-inflammatory properties, may be especially promising for the large group of individuals predisposed to the metabolic syndrome.

2. TZD Response is Variable and Influenced by Genetics

TZDs induce clinically significant improvements in insulin sensitivity, glucose tolerance, lipid profile, and blood pressure compared to placebo (7, 10, 29, 32, 36, 54, 56, 69, 82). However, the efficacy of TZD therapy is highly variable among subjects as shown by x lines of evidence. First, premarketing studies sponsored by drug companies to prepare for FDA approval. In these studies, as much as 25% to 50% of TZD-treated subjects were considered non-responders (1, 6, 29, 45, 66, 68, 76). That the estimates differed between the drugs is likely explained by differences in definitions of non-response and the eligibility criteria of the studies, because when rosiglitazone, pioglitazone, and troglitazone were compared side-by-side they did not differ in efficacy (42). Second, in the TRIPOD study, nondiabetic Hispanic women with previous gestational diabetes were randomly assigned to troglitazone (400 mg/day) or placebo to test the hypothesis that troglitazone treatment would protect the subjects against the development of T2DM. Intravenous glucose tolerance tests were performed at randomization and after 3 months of treatment. Troglitazone/placebo treatment continued for a median duration of 30 months. As the investigators had hypothesized, assignment to troglitazone was associated with a reduced incidence of diabetes compared to placebo for the duration of the study. The change in insulin sensitivity between baseline and the 3 month treatment point was highly variable among TZD treated subjects. Indeed, in the bottom third of the TZD treated subjects it was no different from placebo-treated controls (60; Fig. 1). Moreover, as a group, this stratum did not enjoy the protection from T2DM otherwise associated with troglitazone treatment.

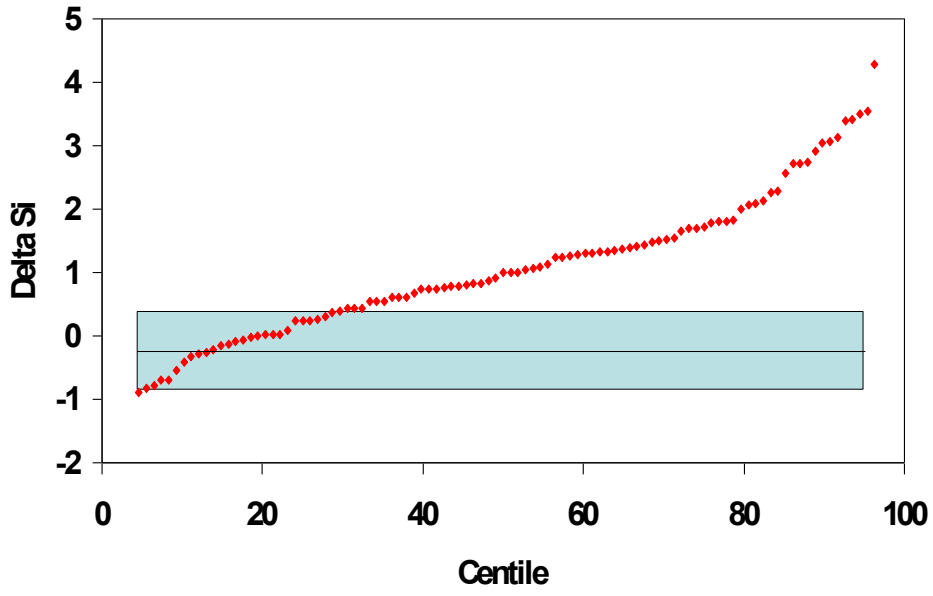


Fig. 1. rank ordered delta Si (post minus pre value) among troglitazone-treated TRIPOD subjects (n=108). In the interest of visual resolution on the vertical axis, only the central 95% of the distribution (n=102) is shown (lowest measured value was -2.1, highest measured value was 7.7). The box shows the mean and SD among the placebo-treated subjects. Unit for delta Si is 10^{-4} min^{-1} per μU/ml (with permission from TA Buchanan).

In the Tripod study, the change in insulin sensitivity and the 3 month time point was normally distributed in the placebo group Physical and biochemical patient characteristics that predict who will respond to TZD treatment have not yet been recognized. Variation in drug response is conceptually divided into pharmacokinetic (metabolic) and pharmacodynamic (action on receptors and pathways) variability. Pharmacokinetic variability is unlikely to play a great role because, as stated in the FDA-approved prescription information, response (or lack thereof) was not associated with plasma drug concentrations in premarketing trials (66). On the other hand, there is evidence that more than one pharmacodynamic aspect of TZD response is genetically determined. Two candidate genes have been examined for putative associations with clinical TZD response, the PPARγ2 receptor and adiponectin (a.k.a. ACDC or APM1). Despite the fact that the Pro12Ala variant in the PPARγ2 receptor is inactivating in vitro, several studies have failed to demonstrate a relation between this variant and TZD effects on insulin action (1, 71 – see Preliminary Studies). However, consistent with biological predictions, one report indicates that

this variant is protective against fluid retention with ensuing edema and congestive heart failure (31), a serious adverse effect that limits the usefulness of the TZDs and is caused by PPARγ–induced sodium reuptake in the distal nephron (87). Expansion of this knowledge may enable physicians to genetically stratify patients into categories of risk for fluid retention. Dr. Watanabe (consultant on this proposal) and collaborators have demonstrated association between other variants in the gene encoding the PPARγ2 receptor and TZD response in the TRIPOD study (81), but these findings await biological confirmation. In the adiponectin gene, the two variants SNP45 and SNP276 both confer a blunted reduction in fasting glucose in response to a TZD (38). Further implications of the above findings are that variants that determine drug response may be located not in a drug's immediate target molecule (i.e., PPAR) but in numerous downstream mediators (e.g., adiponectin). Especially when studying drugs that operate at the transcription level (as TZDs do), the multitude of putative pharmacogenetic candidates supports the use of gene expression arrays to determine genes that are up- or down-regulated in response to treatment, as we do in this proposal.

3. Significance

TZDs are insulin-sensitizing drugs that are useful in the treatment of T2DM, potentially having a future application in prophylaxis. Moreover, they appear to have salutary effects on a host of other medical conditions. Unfortunately, response to TZDs is very variable. The proposed studies will define the molecular and genetic underpinnings of TZD response. This information will have important implications for patient care. Physicians will be able to identify patients who are more likely to respond to TZDs for the treatment of insulin resistance and the prevention of CVD. In addition, some of the gene variants we identify may turn out to be determinants of T2DM risk.

C. PRELIMINARY STUDIES

Over the years, Dr. Snitker, Dr. Shuldiner, and other members of Dr. Shuldiner's group have worked intensively to understand the genetic and molecular mechanisms of insulin resistance and the complex relationship between obesity and insulin resistance, applying state-of-the art physiological, molecular and cellular, genetic, pharmacogenetic, and functional genomic approaches to the questions at hand. As an experienced member of Dr. Shuldiner's multidisciplinary group, Dr. Snitker is well suited to perform the proposed pharmacogenomic study. Below are highlights of previous work by the group leading up to this proposal, including some multi-center network studies.

1. Identification of a Common Amino Acid Variant in the Human PPARγ2 Gene

Because PPARγ is an important mediator of insulin signaling and adipogenesis, we hypothesized that mutations in the PPARγ gene might predispose to obesity and/or type 2 diabetes. Therefore, we isolated and characterized the human PPARγ gene and determined its chromosomal localization (8). We found that the coding region of PPARγ is encoded by seven exons. The downstream six exons are common to both the γ1 and γ2 isoforms, while the γ2 isoform has an additional upstream exon that encodes the additional 28 amino acids (8). We screened *PPARG* for mutations in obese subjects and were the first to identify a C→G nucleotide change in the γ2-specific exon that predicted the substitution of proline at position 12 to alanine (Pro12Ala PPARγ2)(85). This genetic variant is common in diverse populations, especially Caucasians and Mexican Americans with a prevalence of 20% to 25% (14).

Because proline residues are known to disrupt α-helix formation whereas alanine easily participates in α-helix formation, this alanine substitution could have profound effects on this protein's structure and function. Proline at position 12 of PPARγ2 is invariant between human, rhesus, mouse, and rat sequences, which further suggests that it may be structurally and functionally important (85). Indeed, others have shown that the Ala^{12} allele leads to a decrease in protein function; Deeb and coworkers (16) found that Ala^{12} PPARγ2 had 1.6 to 2.5-fold lower affinity for the PPARγ response element (PPRE) than Pro^{12} PPARγ2. Similar defects in signaling of Ala^{12} PPARγ2 were reported by Masugi and coworkers (49).

2. Pro12Ala PPARγ2 Is Associated with Increased Insulin Sensitivity

a. African Americans of the Atherosclerosis Risk in Communities (ARIC) Study

To investigate the relevance of the Pro12Ala PPARγ2 variant in African Americans, we characterized

this variant in 1,441 middle-aged African American individuals with respect to diabetes, obesity, and other cardiovascular-related traits, both cross-sectionally and prospectively. Subjects were participants in the ARIC Study, a prospective, population-based study investigating the etiology and natural history of atherosclerosis and its clinical manifestations (5). Approximately 16,000 persons aged 45-64 at baseline (Visit 1: 1987-89) from four U.S. communities: Forsyth County, NC (12% African Americans); Jackson, MS (all African Americans); suburban Minneapolis, MN (mostly white); and Washington County, MD (mostly white); were enrolled in ARIC. During baseline exam, data on health status, selected risk factors, family medical history, employment and educational status, diet, and physical activity were collected in an interviewer-administered questionnaire. Participants completed 4 clinic examinations, conducted every 3 years. The follow-up for each visit ranged from 80% to 90%. The overall frequency of the Ala^{12} allele in African Americans was 1.9% (95% confidence interval: 1.5 - 2.5%). Consistent with previous findings in Caucasians (6), African American carriers of the Ala^{12} allele tended to be protected from the development of T2DM (odds ratio [OR] 0.64, 95% CI 0.34-1.20); however, this OR was not statistically significant. Among non-obese individuals, the Pro/Ala genotype was associated with significantly lower (insulin) ($p = 0.001$), lower (HOMA-IR) (homeostasis model assessment of insulin resistance) ($p = 0.002$), higher fasting glucose-to-insulin ratio ($p = 0.005$), and lower diastolic blood pressure ($p = 0.02$). Among overweight individuals (BMI 25-29.9 kg/m^2), the Pro/Ala genotype was associated with greater BMI ($p = 0.02$), waist-to-hip ratio ($p = 0.01$), and waist circumference ($p = 0.04$). These studies support the notion that the Pro12Ala PPARγ2 variant is associated with markers of insulin sensitivity and may be protective against risk of T2DM, and is consistent with several other studies in humans (14, 74, 75). Although the mechanism of increased insulin sensitivity with an allele that has decreased activity is unclear, these findings are consistent with studies in mice in which heterozygous PPARγ knockout animals have increased insulin sensitivity relative to their wild type littermates (51, 83).

b. Pro12Ala PPARγ2 Associates with Improved Insulin Sensitivity after Weight Loss and Predicts Weight Regain in Obese Postmenopausal Caucasian Women

We performed retrospective PPARγ2 genotype analysis in 49 obese postmenopausal women who had undergone metabolic testing and weight loss (55). Forty-one subjects were Pro^{12} homozygotes (Pro/Pro), 7 subjects were heterozygotes (Pro/Ala), and 1 subject was an Ala^{12} homozygote (Ala/Ala). Before weight loss, there were trends toward increased BMI and lower fasting glucose in those with the Ala variant as reported in other populations (data not shown). Subjects underwent a weight loss program, which included a hypocaloric diet (deficit = 250 - 350 calories per day), low intensity exercise (walking 45 min per day three times per week), and psychobehavioral treatment. The intervention similarly decreased body weight by 8 ± 1% in women homozygous for the Pro^{12} allele and by 7 ± 1% in women with the Ala^{12} allele ($p < 0.0001$), but women with the Ala^{12} allele decreased their insulin area during the OGTT more than women homozygous for the Pro^{12} allele ($p < 0.05$). Fat oxidation did not change in Pro/Pro women but decreased 19 ± 9% in women with the Ala^{12} allele ($p < 0.05$). Reciprocally, carbohydrate oxidation increased to a greater extent in Ala^{12} carriers ($p < 0.05$). Lower fat oxidation and increased insulin sensitivity in weight reduced individuals is a well-known metabolic milieu for weight gain. Indeed, weight regain during follow-up was significantly greater in women with the Ala^{12} allele than women homozygous for the Pro^{12} allele (5.4 ± 0.9 vs. 2.8 ± 0.4 kg, $p < 0.01$). PPARγ2 genotype was the best predictor of weight regain ($r = 0.50$, $p < 0.01$), followed by the change in fat oxidation (partial $r = 0.35$, $p < 0.05$; cumulative $r = 0.58$). These studies support the notion that the Pro12Ala variant in PPARγ2 has clinical significance in influencing insulin sensitivity and in predicting recidivism after weight loss. It also provides an example of an interventional study in which careful phenotyping was performed to dissect significant differences in response to the intervention between genotypes, similar to the study design of this proposal.

3. <u>Pharmacogenetics of Pro12Ala PPARγ2 (TRIPOD Cohort)</u>

Because Pro12Ala PPAR γ 2 is an inactivating variant, we hypothesized that the Ala^{12} allele would explain TZD non-response. In collaboration with Drs. Tom Buchanan and Richard Watanabe of the University of Southern California, Dr. Snitker explored this question in participants in the TRIPOD (Troglitazone in the Prevention of Diabetes) study (71).

Nondiabetic Hispanic women with previous gestational diabetes were randomly assigned to

troglitazone (400 mg/day) or placebo for a median duration of 30 months. Assignment to troglitazone was associated with a reduced incidence of diabetes for the duration of the study compared to placebo. To explore the mechanism of action, intravenous glucose tolerance tests were performed at randomization and after 3 months of treatment. Troglitazone-treated subjects were divided into thirds based on their change in minimal model insulin sensitivity (ΔS_i) during these 3 months. The mean change in insulin sensitivity in the top, middle, and bottom third of ΔS_i was 2.58 ± 1.32, 0.91 ± 0.26, and - 0.21 ± 0.57 $\times$ 10^{-4} min^{-1} per µU/ml, respectively. The ΔS_i of those in the bottom tertile was not statistically different from that of placebo-treated subjects whose mean ΔS_i was - 0.17 $\times$ 10^{-4} min^{-1} per µU/ml (Fig. 1; see Background and Significance, Section 2). Therefore, the bottom third of treated subjects was considered non-responders. The non-responders also did not differ from placebo in terms of T2DM incidence, suggesting that troglitazone's diabetes-preventive effect is mediated by improved insulin sensitivity.

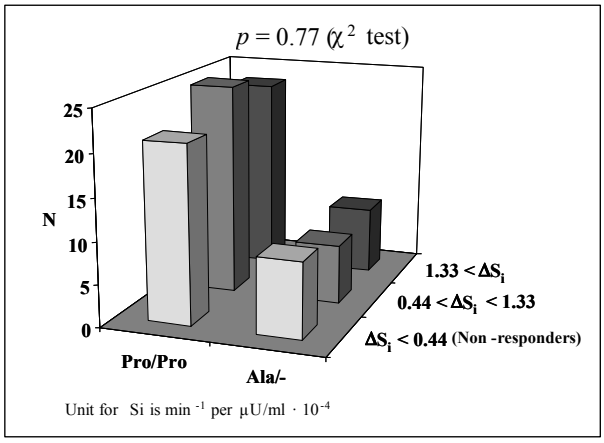


Figure 2. Change in insulin sensitivity (ΔS_i) in response to troglitazone by Pro12Ala PPARγ2 genotype in women of the TRIPOD study.

In the TRIPOD population, 30% were Ala^{12} carriers. This frequency coincides with the 1/3 of the troglitazone-treated participants who failed to improve insulin sensitivity. In combination with the in vitro and clinical studies of others, this observation suggested the validity of the hypothesis that the Ala^{12} variant would explain non-response. However, In contrast with the hypothesis, the distribution of ΔS_i in response to troglitazone (by thirds) did not differ by Pro12 Ala PPARγ2 genotype (n=93, p = 0.77; see Figure 2). Of note, the sole Ala/Ala subject was in the middle third. Thus, despite convincing in vitro data, the Pro12Ala PPARγ2 variant did not explain non-response. These results support the view that a much broader approach must be applied to dissect the genetic underpinnings of TZD response. Consequently, we applied a pharmacogenomic (as opposed to pharmacogenetic) approach in our ensuing studies and the present proposal.

4. The Pharmacogenomics of PPARγ Study

The TRIPOD analysis taught us that we would need to pursue a broader approach to understand the genetic underpinnings of TZD response. We therefore initiated the Pharmacogenomics of PPARγ Study at the University of Maryland. This project, which uses a phenotype-to-genotype approach, began recently and is ongoing. Non-diabetic, insulin resistant subjects are being treated with rosiglitazone for 12 weeks. We chose rosiglitazone since this agent is a "pure" PPARγ agonist, not cross-reacting with other PPARs. Insulin sensitivity and other clinical parameters are measured before and after the 12-week treatment period. As a novel aspect, we obtained fat and muscle biopsies before and after treatment to quantify changes in gene expression on a genome-wide level and to perform *in vitro* functional assays. At present, 11 subjects have completed the protocol. Additional details of the study design and preliminary results from these 11 subjects are provided below.

a. Ascertainment and phenotyping

Subjects are recruited without regard to genotype. Eligible subjects are age 35-65 years with a BMI ≥ 25 and < 40 kg/m^2. This group of overweight and obese subjects is likely to have some degree of insulin resistance. Subjects with frank diabetes are excluded due to the potential confounding effects of metabolic instability, medications, and diabetic complications. All subjects are treated with rosiglitazone for 12 weeks; there is no placebo group or blinding. Genetic material is obtained at the beginning of the study. Before and after the rosiglitazone intervention subjects undergo extensive testing, including:

- Frequently sampled intravenous glucose tolerance test to determine insulin sensitivity (using minimal modeling) and acute insulin response.
- Vital signs, including rigorous measures of blood pressure
- Blood for lipid profile, inflammatory factors, adipokines, and PAI-1

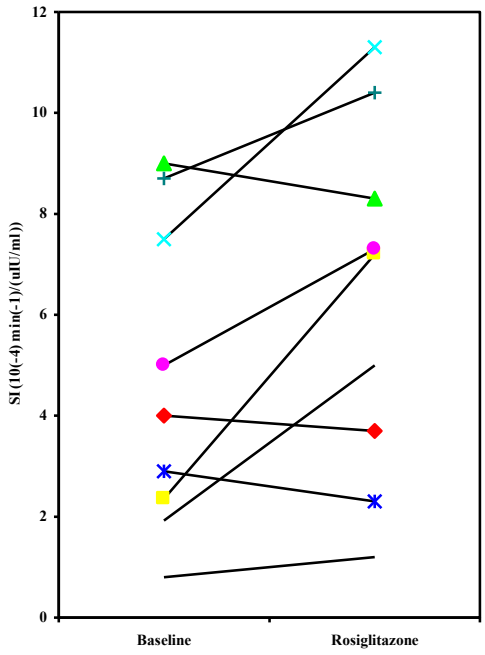
- Imaging tests to determine total body fat and its distribution
  - Total body fat by dual energy X-ray absorptiometry (DEXA).
  - Two single slice CT scans (abdomen and thigh) for intraabdominal and subcutaneous fat area
- Muscle biopsies (vastus lateralis)
  - mRNA expression profile of 47,400 genes and ESTs using the Affymetrix U-133 Plus 2.0 chip.
  - Confirmation of the above findings by Q-RT-PCR
- Fat biopsies (abdomen)
  - *In vitro* functional studies performed by Dr. Susan Fried
    - Tissue studies: secretion of TNF and IL-6
    - Cellular studies: lipolysis, glucose transport, lipoprotein lipase activity
    - Fat cell sizing
  - mRNA expression profile of 47,400 genes and ESTs using the Affymetrix U-133 Plus 2.0 chip
  - Confirmation of the above findings by Q-RT-PCR

b. Preliminary results of the Pharmacogenomics of PPAR Study

To date, 11 subjects (8 female and 3 male) have completed the protocol.

Physical characteristics of completed subjects are shown in Table 1. During the 3-month intervention, body weight did not change, but there may have been a shift in fat distribution, although further enrollment is needed for the observed differences to reach statistical significance. Rosiglitazone treatment resulted in marked decreases in fasting insulin levels, consistent with an overall improvement in insulin sensitivity. There was a slight reduction in fasting glucose levels and no change in HbA1c, as one would expect in this non-diabetic cohort. Notably, there was a marked decrease in both systolic and diastolic blood pressure as well as the inflammatory markers CRP and SAA (serum amyloid A, an adipokine). As with changes in insulin sensitivity, there was great individual variation in the magnitude of change in each of these traits to rosiglitazone therapy.

Table 1. Phys. Characteristics/ Results from Pharmacogenomics of PPARγ	Pre	Post
Age (y)	45.8 ± 9.0	
BMI	30.8 ± 3.1	
Body weight (kg)	88.0 ± 14.4	88.0 ± 15.1
CT Intraabdominal fat (cm^2)	140.0 ± 59.4	134.6 ± 51.3
CT Subcutaneous fat (cm^2)	396.2 ± 168.0	408.7 ± 180.6
DXA total body fat (%)	37.4 ± 4.5	37.5 ± 6.1
SBP (mm Hg)	128.6 ± 13.4	122.9 ± 9.6
DBP (mm Hg)	76.3 ± 7.1	71.6 ± 6.1
Fasting plasma glucose (mg/dl)	98.5 ± 3.8	94.8 ± 6.5
Fasting plasma insulin (μU/ml)	16.9 ± 5.4	10.1 ± 2.2
HbA1c (%)	5.2 ± 0.5	5.3 ± 0.4
Plasma hsCRP (mg/L)	4.2 ± 3.5	1.6 ± 1.1
Plasma Serum Amyloid A (SAA) (μg/ml)	14.0 ± 10.4	10.2 ± 7.7



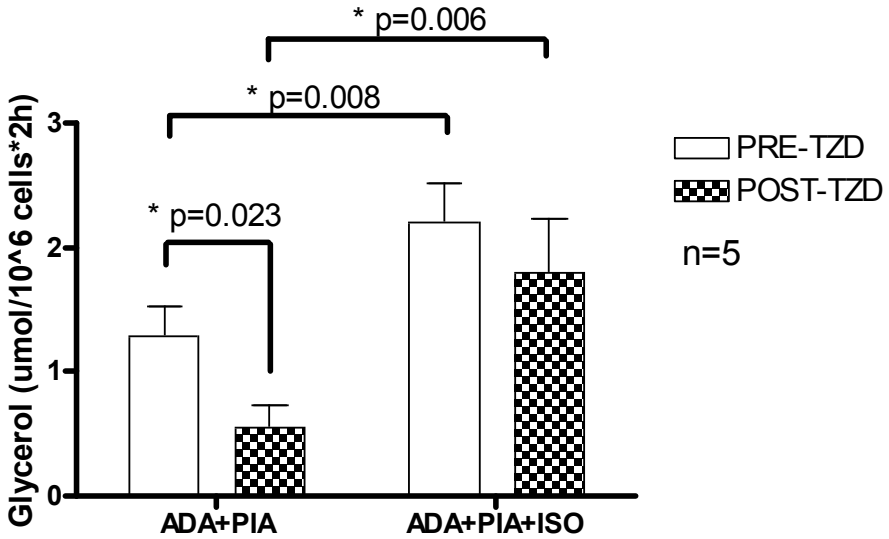
Insulin sensitivity data are available for 9 of the 11 subjects and appears consistent with the reported non-response rates in previous clinical trials (Figure 3).

← Figure 3. Insulin sensitivity (S_i) before and after rosiglitazone treatment in 9 subjects. Note that in 3 subjects, S_i did not improve.

Studies of muscle and fat tissue including mRNA expression. In addition to studies of whole-body metabolism, tissue samples were obtained and studied because it is thought that individual response is better understood if it is characterized at lower levels. Fig. 4 shows results of our *in vitro* lipolysis studies. Again, although the number of subjects is small, on average, rosiglitazone increased insulin's ability to inhibit lipolysis, consistent with increased insulin sensitivity in adipocytes.

Microarray studies

To date, we have performed a preliminary microarray analysis of two pairs of pre/post fat and 3 pairs of pre/post muscle samples from our subjects. These experiments were conducted using Affymetrix U-133 Plus 2.0 chips to measure the expression of ~47,400 transcripts. Comparisons were made pre- versus post-rosiglitazone in the same subject, and changes in each gene were averaged. Fat tissue was analyzed separate from muscle tissue. At threshold p-values of $p < 0.05$ and 1.5 fold-change, we found 167 genes in subcutaneous fat and 141 genes in muscle that were significantly up- or down-regulated. Table 3 shows the 10 genes that had the largest changes in expression in fat (left) and muscle (right). In addition to our human data, the table shows the changes in same genes in mice fed a high-fat diet with or without rosiglitazone (5 mice/group, 30 mmol rosiglitazone/kg diet, 4 weeks). A comparison between the human genes responsive to rosiglitazone and their mouse HomoloGenes in Table 3 shows that the genes generally change in the same direction despite differences in magnitude. We acknowledge that with the small number of samples examined, these findings must be interpreted with great caution. Nevertheless, the comparison of genes responsive to rosiglitazone between humans and mice will disclose the genes that are co-regulated in the two species in a same manner. These genes are likely important for TZD-



mediated insulin sensitivity. These studies demonstrate that our group has the expertise and infrastructure to perform the proposed studies. They also show that these studies are likely to provide valuable information regarding genes and pathways that are associated with TZD response, which will be very useful in our choice and prioritization of candidate genes of importance to drug response.

Table 3. Five genes most up-regulated and five genes most down-regulated in fat and muscle tissue pre- versus post-rosiglitizaone in humans. Mouse column shows expression changes in mice (see text). N/A, not on chip.

Fat				**Muscle**			
Gene Name	Fragment Name	Fold change		Gene Name	Fragment Name	Fold change	
		Human	Mouse			Human	Mouse
Mitochondrial solute carrier protein	222529_at	20.39	3.8	ATPase, Class I, type 8B, member 1	241534_at	8.7	4.52
Leukocyte specific transcript 1	211582_x_at	9.84	N/A	Similar to protein sp:P39188	222375_at	5.31	N/A
T cell receptor beta chain BV20S1 BJ1-5 BC1	211796_s_at	8.09	3.09	FGF 18	206986_at	3.64	1.58
Amylase, alpha 2B; pancreatic, hypothetical protein FLJ25070	226999_at	7.27	N/A	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	244368_x_at	3.61	1.22
Neurogranin (protein kinase C substrate, RC3)	204081_at	6.09	2.01	Paired-like homeodomain transcription factor 1	209587_at	3.43	1.38
RasGEF domain family, member 1A	230563_at	-4.31	-1.59	FLJ20533	217220_at	-4.73	N/A
Stathmin-like 2	203001_s_at	-4.54	-1.29	DKFZP586K1520	233415_at	-5.08	1.2
AAA-type ATPase family protein	233039_at	-4.83	-2.11	IMAGE Clone 246773	241863_x_at	-5.17	N/A
hypothetical protein FLJ20712	237678_at	-5.89	N/A	FLJ20890	216155_at	-5.24	-1.47
G protein-coupled receptor 115	237690_at	-10.94	1.4	Dachshund homolog 2 (Drosophila)	239738_at	-12.05	-1.30

Q-RT-PCR studies of gene expression. Steady state mRNA levels of selected genes were determined by quantitative real time PCR (Q-RT-PCR) using a Lightcycler (Roche Diagnostics). Total RNA extracted from omental and subcutaneous adipose tissue was converted into cDNA using 1st Strand cDNA Synthesis Kit for RT-PCR from Roche. cDNA was quantified for CD68 levels using an ASSAYS ON DEMAND Taqman primer/probe set purchased from ABI and the LightCycler Fast Start DNA Master Hybridization Probe Kit from Roche Diagnostics (Fig. 5). These studies show that we have the ability to confirm microarray results and study other genes of interest by Q-RT-PCR.

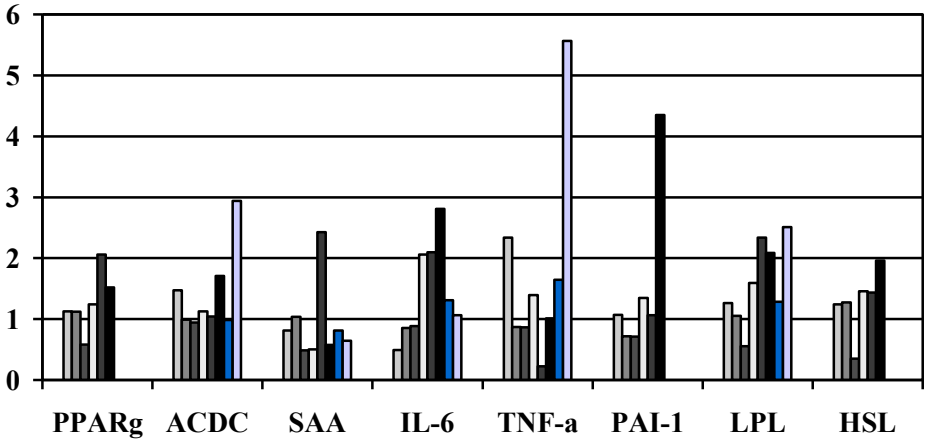


Fig. 5. mRNA expression in adipocytes before and after 12 weeks of rosiglitazone treatment. Each vertical bar represents one subject. Expression after 12 wk. rosiglitazone treatment is expressed relative to baseline (subject's baseline = 1.00). In some, rosiglitazone stimulated expression of its receptor, PPAR-gamma. Also adiponectin (ACDC) was often stimulated. Expression of the inflammatory adipocytokine, SAA, was generally decreased consistent with the drop in plasma SAA levels. The enzymes lipoprotein lipase (LPL) and hormone sensitive lipase (HSL) were both upregulated in response to treatment. Adding interest to this proposal, changes in expression levels of all genes were widely variable between individuals.

Genotyping of candidate genes

To test hypotheses regarding the putative effect of selected candidate genes, we have genotyped our subjects for these genes using a pyrosequencer. As we have only 11 completed subjects, the statistical power to test hypotheses is limited, but as recruitment progresses power will increase. These results demonstrate that we have the ability to genotype our subjects for genes of interest.

Table 4. Sex, race, age, and selected genotypes of enrolled subjects

ID	Sex	Race	Age	PPAR-gamma Pro12Ala	ACDC +2019	ACDC SNP45	ACDC SNP276
PPAR001	F	Caucasian	45	Pro/Pro	ins/ins	T/T	G/G
PPAR008	F	Caucasian	36	Pro/Pro	ins/del	T/T	G/T
PPAR009	F	Caucasian	35	Pro/Pro	ins/del	G/T	G/G
PPAR011	F	Caucasian	37	Pro/Pro	del/del	T/T	T/T
PPAR012	M	Caucasian	59	Pro/Pro	ins/ins	T/T	G/G
PPAR013	F	Caucasian	43	Pro/Pro	ins/del	T/T	G/T
PPAR014	F	Caucasian	57	Pro/Pro	ins/del	G/T	G/G
PPAR016	M	Asian	45	Pro/Ala	ins/del	T/T	G/T
PPAR018	M	Caucasian	60	Pro/Pro	del/del	G/T	G/T
PPAR022	F	Caucasian	40	Pro/Ala	del/del	G/T	G/T
PPAR023	F	African American	36	Pro/Pro	ins/del	T/T	G/T

5. Functional Genomic Studies of Adipose Tissue: Defining the Micro-Architecture of Adipose Tissue

As mentioned, an intense ongoing interest of several members of our research team is the link between obesity (increased adipose mass) and insulin resistance. A number of studies are now underway to elucidate the molecular pathways of this link. It is increasingly recognized that adipose tissue is a dynamic endocrine organ that participates actively in energy homeostasis. In addition to adipocytes, adipose tissue contains other cell types whose function is unknown. To further define the micro-architecture of adipose tissue, Dr. Da-Wei Gong, a co-investigator on this proposal, separated human adipocytes from supporting stromal vascular cells by collagenase treatment followed by gravity separation. Cells were isolated, RNA prepared, and microarray analyses performed using the Affymetrix U133 chips described above. Figure 6 shows an example of the chip analysis revealing marked differences in gene expression between adipocytes and stromal vascular cells (SVCs). Table 5 shows summary statistics of this experiment. For example, the table shows that 592 genes are differentially regulated in fat cells compared to SVCs with fold changes (FC) > 3 and p-values < 0.001. These studies again demonstrate our team's ability to perform state-of-the-art functional genomics studies. Although we do not plan to separate fat cells from stromal vascular cells in this project, knowledge of gene expression profiles of the different adipose tissue cell types will aid in the interpretation of our pharmacogenomic results and may aid in our selection of candidate genes.

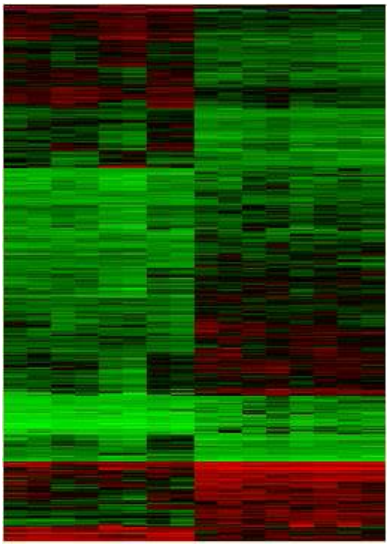


Figure 6. Example of microarray comparison of fat cells and adipose tissue stromal vascular cells isolated from the same person

Table 5. Summary statistics for Fat Cells vs SVC

	p All	*p* < 0.05	*p* < 0.01	*p* < 0.005	*p* < 0.001
FC all	4988	2319	1693	1477	1116
FC >1.1	4473	2319	1693	1477	1116
FC >1.5	2728	2244	1689	1476	1116
FC >2	1613	1571	1398	1286	1049
FC >3	666	665	644	632	592

6. Omentin, a Novel Candidate Gene for Insulin Resistance and the Metabolic Syndrome

Because of these and other high-throughput functional genomics analyses, Dr. Gong and his colleagues identified a gene that was preferentially expressed in visceral compared to subcutaneous fat. He named this gene omentin (84). Immunohistochemistry revealed that omentin is expressed in stromal vascular cells of visceral, but not subcutaneous fat. Immunoprecipitation experiments showed that omentin circulates in blood, thus defining it as a novel adipokine produced by visceral fat tissue.

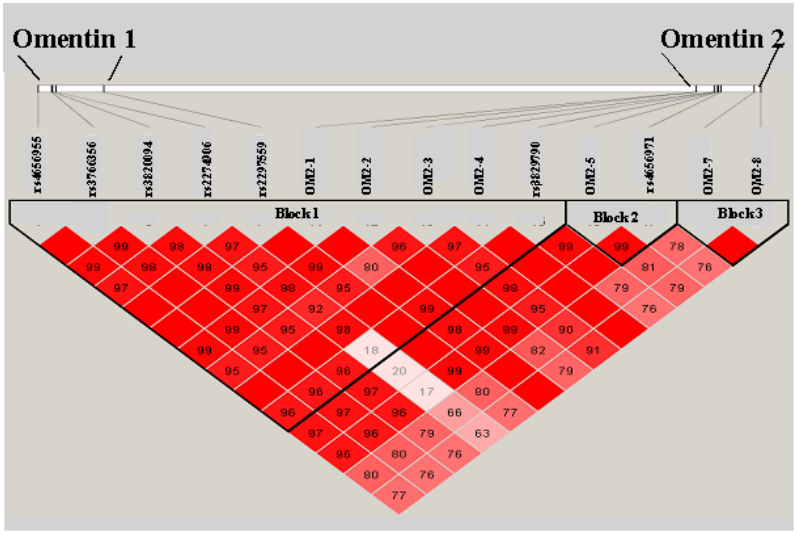


Figure 7. Haplotype structure of omentins 1 and 2 as imaged by Haploview.

Given the important role that visceral fat plays in the development of insulin resistance and metabolic syndrome, Dr. Gong expressed omentin to study its biological properties. Like adiponectin, omentin enhanced glucose uptake in mouse 3T3-L1 adipocytes *in vitro* and increased glucose disposal when injected into mice (84). A bioinformatics search revealed that there are two omentin genes (that we named omentin 1 and 2). Both are located within 100 kb on chromosome 1q21, a region that we and others have found linked to T2DM in genome-wide scans (26).

Since omentins 1 and 2 are excellent positional candidate genes for T2DM and insulin resistance, we sequenced 20.3 kb of DNA encompassing the promoter regions, exons and introns of omentin 1 and omentin 2 (26). For omentin 1, we identified 25 polymorphisms (4 novel polymorphisms), including 1 SNP that changed the amino acid sequence (D109V). In omentin 2, we identified 23 polymorphisms (8 novel polymorphisms), including 2 SNP that changed the amino acid sequence (R103H in exon 4 and S168P in exon 5). Twenty-three haplotype tagging SNPs (Figure 7) were typed in Amish subjects with T2DM (n = 145), impaired glucose tolerance (IGT)(n = 148), and normal glucose tolerance (NGT)(n = 358). Om-74938 and rs1556519, both in intron 6 of omentin 2, were strongly associated with IGT (p = 0.003, 0.005) and IGT/T2DM (p =0.031, 0.048). Om-81237 and om-81873 in the omentin 2 promoter and om-79443 in intron 2 of omentin 2 were also associated with IGT (p = 0.001, 0.012). Haplotype analysis revealed a six-marker haplotype within omentin 2 was associated with both IGT and IGT/T2DM (p = 0.004, 0.018). In nondiabetic subjects (n = 754), the haplotype and T allele of om-74938 associated with higher glucose area under the curve during an OGTT, leptin levels, and BMI. We conclude that these variants in omentin 2 may play a role in susceptibility to IGT and T2DM in the Old Order Amish (26). These studies illustrate our ability to perform state-of-the art SNP discovery and high-throughput genotype and association analysis of candidate genes, approaches that will be key to the successful execution of the Aims of this proposal.

7. The Effects of Olmesartan Medoxomil, Losartan Potassium, and Atenolol on Insulin Sensitivity in Overweight and Obese Subjects with Hypertension

Dr. Snitker was the site principal investigator of this multi-center study. The purpose of the study was to quantify the insulin-sensitizing effects of olmesartan medoxomil, a drug approved for the treatment of hypertension. This question has merit given the large number of individuals in whom hypertension coincides with impaired glucose tolerance or diabetes. Insulin sensitivity was measured with a hyperinsulinemic, euglycemic clamp twice in each subject, before and after treatment with blinded study drug. Consistent with his leading role in the pharmacogenomics of PPAR-gamma study, this study demonstrates that Dr. Snitker is qualified to oversee clinical investigation of a complicated nature.

D. RESEARCH DESIGN AND METHODS

1. Study Overview and Rationale

The overall objective of this proposal is to define the molecular and genetic basis of the inter-individual variation in response to TZDs. We will test the hypothesis that inter-individual variability in TZD response is due, in part, to genetic influences, more specifically sequence variants in one or more candidate genes. The general approach we will use is a phenotype to genotype approach. We will not only be looking at phenotypes on the whole-body level, but also obtain muscle and fat tissue samples to study more immediate phenotypes all the way down to the gene expression level. Because TZDs act by binding to the nuclear receptor PPARγ to elicit direct and indirect changes in gene expression that ultimately exert their phenotypic effects, we reason that a phenotype to molecular (gene expression) profile to genotype approach would have the highest likelihood of identifying the responsible genes and their variants.

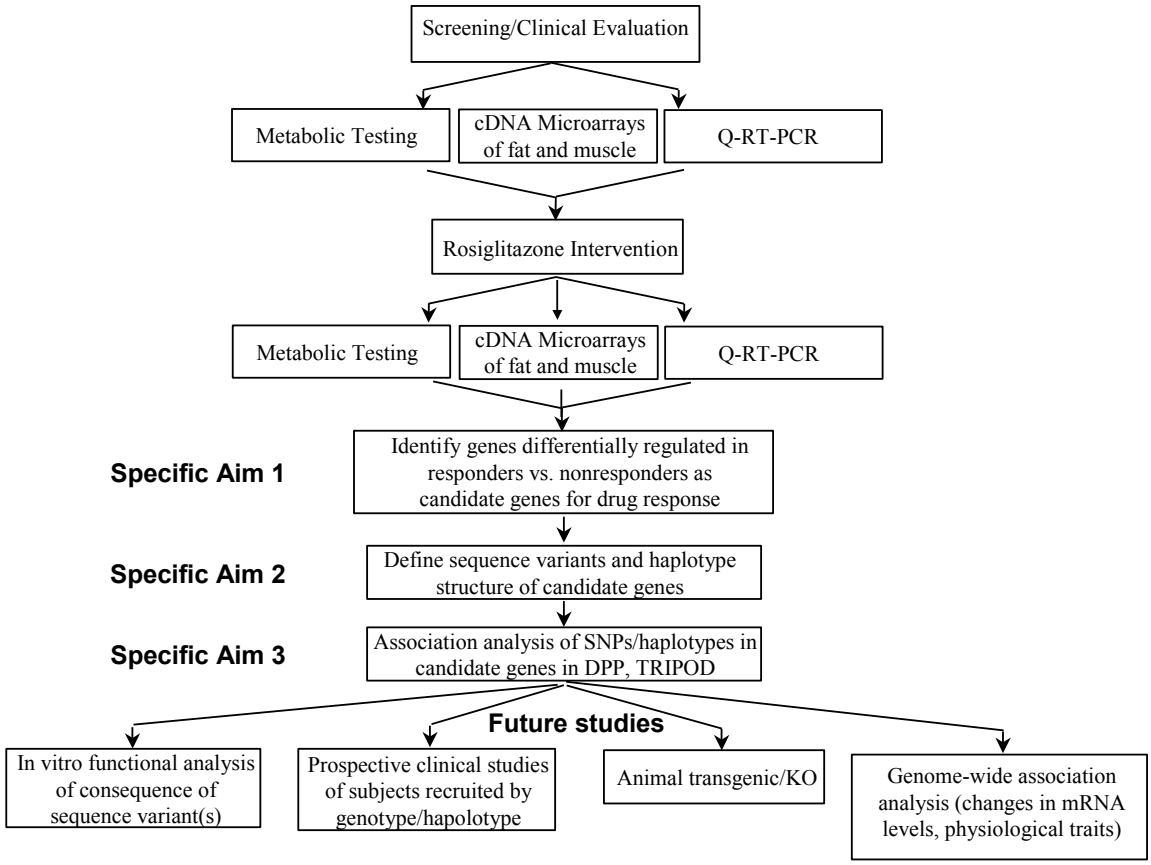


Fig. 8. Schematic of overall study design

The proposed study will expand upon an ongoing study of the pharmacogenomics of PPARγ in which sophisticated phenotyping and microarray studies of fat and muscle are already well underway (see Preliminary Studies). A schematic of the study design and timeline are shown in Figures 8 and 9. We will define the genes whose regulation correlates to TZD response. Comparisons of gene expression profiles of the same tissue in the same person pre- and post- intervention should minimize many of the problems associated with most microarray experimental designs and should minimize the number of false positive (and false negative results).

Furthermore, we will confirm these differences by real-time PCR on RNA from the same sample in which the microarray studies were performed. Confirmed genes whose up- or down-regulation is

correlated to TZD response will be defined as candidate genes and will be pursued using state-of-the-art molecular genetic approaches to define all common sequence variation and haplotype structure of the gene. We will then genotype these SNPs in our subjects to determine if these SNPs or haplotypes associate with TZD response. Although the 75 subjects we plan to recruit for this study will provide very rich phenotype and microarray information for defining TZD response and for selecting candidate genes to study, they will not be particularly powerful to discern the effect of specific gene variants on clinical response to TZDs.

However, they may be useful in discerning associations of gene variants with mRNA levels encoded by that gene (or within the same regulatory pathway). Thus, in the next stage of our study, we will take advantage of established collaborations with investigators of large prospective studies which involved a TZD intervention and for which relevant response traits have been collected, the DPP Study, and the TRIPOD Study (now continued as PIPOD).

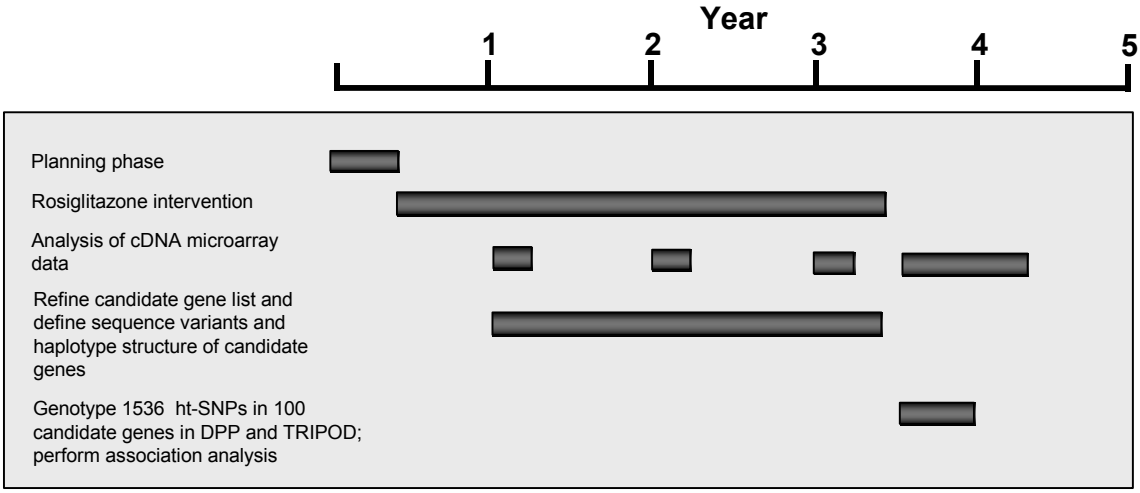


Fig. 9. Timeline of the study

High throughput genotyping of 1536 SNPs in 100 candidate genes will be performed in these cohorts and association of SNPs and haplotypes with TZD response will be examined. It is our hope that in a subset of these 100 candidate genes we will find consistent evidence for a role in TZD response, e.g., altered mRNA levels in those with the variant SNP/haplotype and association with TZD response in both populations.

2. Specific Aim 1. Defining interindividual variability in TZD response

a. Subject recruitment and screening

Seventy-five subjects will be recruited for this study from the following sources:

- A large database belonging to the Division comprising over 500 subjects recruited at health fairs. These subjects have provided basic health information, given blood for genotyping, and indicated that we may contact them to inform them about future genetic studies. Additionally, some subjects have had oral glucose tests. We have previously successfully recruited subjects from this collection. This collection will also allow rapid identification of potential volunteers for our study if the focus should later change to a particular genotype or clinical profile.
- Database of the Center for Clinical Trials (CCT) at the University of Maryland School of Medicine. Our CCT maintains a database of several thousand individuals who have expressed an interest in participating as research volunteers into clinical trials and other clinical research studies.
- Flyers on the University of Maryland Baltimore campus and notices in the campus newsletter. In the past, this approach has been very successful.

- The website of the University of Maryland Medical Center and the Division of Endocrinology, Diabetes and Nutrition.
- Newspaper advertisements. In addition, volunteers for this study will be recruited by advertisements in local and regional newspapers.

Initial screening for eligibility will be assessed by brief communication (telephone or in person) and include the following:

Inclusion criteria:

- Subjects will be overweight (25 kg/m^2 $\leq$ BMI < 40 kg/m^2) but otherwise healthy men and women between the ages of 35 and 65 years who self-identify as (in NIH classification terms) White. As shown in a similar population in Section C4, the ability to show an improved insulin sensitivity in response to TZD treatment does not appear influenced by initial insulin sensitivity. Thus, no insulin sensitivity criteria will be applied.

Exclusion criteria:

- Diabetes (history of diabetes or as per screening tests)
- Medications that the investigator judges will make interpretation of the results difficult or increase the risk of participation (e.g., anticoagulants).
- Medical history that the investigator judges will make interpretation of the results difficult or increase the risk of participation (e.g., anemia, cardiac decompensation, intolerance to rosiglitazone, lidocaine or other agents used).
- Pregnancy
- Physical dimensions exceeding the limits of any equipment used

We will obtain written informed consent from all subjects. Further screening will include history, vital signs, physical exam, and blood tests. ***Please see Section E for detailed screening procedures and exclusion criteria.***

b. Metabolic testing:

Subjects who qualify for the study will then enter into the metabolic testing phase of the study. The identical metabolic testing will be performed before and after the 12-week rosiglitazone intervention. Methods for performing these tests have been standardized and are essentially identical to those being performed in our ongoing Pharmacogenetics of PPARγ study. All testing will be performed at the University of Maryland General Clinical Research Center (see Letter from Dr. Carol Tacket, GCRC Program Director).

Metabolic testing will include:

- Blood for fasting lipid profile and lipoprotein particle size, C-peptide, leptin, adiponectin, IL1β, IL6, TNFα, hsCRP, SAA, adiponectin, and rosiglitazone concentrations.
- Imaging tests to determine total body fat and its distribution
  - Total body fat by dual energy x-ray absorptiometry (DEXA). We will use a Hologic QDR 4500 W scanner with Windows XP-based software allowing for region-of-interest analysis. The scans will be performed and analyzed by trained operators.
  - A single slice CT scan will be taken mid-way between the L4 and L5 lumbar vertebrae to determine intraabdominal fat area, subcutaneous fat area, and sagittal diameter. Another single slice CT scan will taken at the thigh (halfway between the femoral head and the top of the patella) to assess limb fat and muscle area.
- Frequently sampled intravenous glucose tolerance test (IVGTT) to determine insulin sensitivity (using minimal modeling) and acute insulin response. As in our previous studies, data will be analyzed to provide parameters of insulin sensitivity and glucose effectiveness by the minimal model using a modified version of the computer program MINMOD.
- Muscle biopsy (100-250 mg) will be obtained under local anesthesia from the vastus lateralis. Connective tissue will be removed. A representative portion of the tissue will be formalin fixed,

paraffin embedded, sectioned, and stained with hematoxylin and eosin using standard histological methods. Slides will be examined visually to assess cell populations/tissue composition in order to ensure that morphologically similar tissue biopsies were sampled before and after rosiglitazone therapy. The remainder of the tissue will be snap frozen in liquid nitrogen for subsequent RNA extraction and microarray analyses (see below).

- Subcutaneous adipose tissue (3-5 g) will be removed from the abdominal region by aspiration with a 16-gauge needle under local anesthesia. A representative portion of the tissue will be handled as noted above. A portion of the tissue will be snap frozen in liquid nitrogen for subsequent RNA extraction and microarray analyses (see below). An aliquot of fresh adipose tissue will be transported immediately to Dr. Susan Fried's laboratory for the *in vitro* adipocyte studies (see below).

c. Rosiglitazone intervention:

Following the initial metabolic testing and sample acquisition, the subjects will initiate rosiglitazone therapy at 4 mg/day. At 4 weeks following the initiation, subjects will be evaluated by Dr. Stuart Haines, Pharm.D., in order to ensure that there are no adverse effects from rosiglitazone therapy. Although liver dysfunction is much less common with rosiglitazone than troglitazone, we will measure serum ALT concentrations monthly. Medication compliance will be monitored using Trackcap (Medication Event Management Systems; Aprex Corp.), and by pill counting at each monthly visit. Frequent telephone contact will be maintained. If rosiglitazone is tolerated well after 4 weeks of treatment, the dose will be increased to the maximum dose of 8 mg (4 mg twice per day). We have chosen the maximum dose for rosiglitazone since to maximize the differences between responders and nonresponders. At each monthly visit, rosiglitazone drug levels will be measured to control for differences in drug when analyzing responsiveness.

d. Metabolic testing after rosiglitazone therapy:

After 12 weeks of rosiglitazone therapy, subjects will repeat metabolic testing identical to that described above taking place before the rosiglitazone intervention. Three months of therapy was chosen as the end point for the metabolic studies since by this time, rosiglitazone will have had its maximal effects on insulin sensitivity, the primary response outcome. Results of metabolic testing and clinical outcomes will be compared to the identical tests performed 12 weeks earlier (prior to the initiation to rosiglitazone) in the same subject.

e. Analysis plan:

We will define the response variable for each of the 75 individuals studied to be the relative change in the primary (insulin sensitivity) and secondary (lipid, body fat composition, inflammatory markers, etc.) endpoints, expressed as the (post-intervention value minus the pre-intervention value) divided by the pre-intervention value. We will then begin with a series of descriptive analyses to identify the correlates of response to TZD therapy.

Specific Aim 1a. We will use conventional regression analysis and analysis of variance approaches to determine if baseline subject characteristics, including age, gender, ethnicity, and body mass index, are correlated with response. We will perform separate analyses for the primary and each of the secondary response variables. All analyses will also be adjusted for the baseline (pre-TZD treatment) value.

Specific Aim 1b. After identifying baseline variables that are significantly associated with response, we will then will evaluate, as Specific Aim 1b, the pairwise relationships between the insulin sensitivity response and each of the secondary response variables. We predict that the insulin sensitivity responses will be correlated with the secondary trait responses, although the degree of correlation will not necessarily be equal in magnitude across all pairs (e.g., the correlation between the insulin sensitivity response and HDL cholesterol response may be greater than the correlation between the insulin sensitivity response and SBP response). We will estimate these correlations while adjusting for the effects of baseline variables identified in Specific Aim 1.

These correlation analyses will provide important descriptive information on this novel phenotype. For example, we predict that response in the secondary traits will generally be driven by the

insulin sensitivity response. Hence, we would expect there to be very limited little mean change in the secondary responses among those with limited insulin sensitivity response. Our proposed sample of 75 subjects should provide us with 80% power to detect correlations of size (population r) = 0.31.

Specific Aim 1c. We will perform cDNA microarray analysis of muscle and fat before and after 3 months of rosiglitazone therapy for the purpose of identifying genes (and their corresponding pathways) whose changes in expression correlate with the change in insulin sensitivity in response to TZDs. This proposed experiment extends our ongoing work from the Pharmacogenetics of PPARγ Study (see Preliminary Data).

We will initially compute the change in expression levels for each of the 40,000 examined genes. We will then use correlation analysis to determine the genes whose changes in expression levels best correlate with change in insulin sensitivity and the other clinical outcomes. We anticipate that some genes will be up-regulated in response to TZD therapy and others will be down-regulated, and the magnitude of these changes will correlate with clinical response. Our goal will be to identify the 100 genes whose differential in up (or down) regulation most closely correlate with clinical response.

We recognize that the results of microarray experiments must be interpreted cautiously, especially in light of the multiple comparisons issue. However, the major goal of our experiment is to identify genes that might mediate the response to TZDs and which hence might be candidates for further studies. Our selection of genes will therefore be guided by several factors, including the strength of the statistical evidence, and our best understanding of the likely function of the differentially expressed genes. Please see the Microarrays section below for a description of the statistical analysis of the microarray data.

f. Laboratory methods:

Serum/plasma assays. Cytokines will be measured by the Cytokine Core Laboratory at the University of Maryland by ELISA (see Letter from Dr. Jeffrey Hasday). C-peptide, leptin and adiponectin will also be measured by ELISA at the GCRC Biochemistry Core Laboratory. Lipid profile will be measured by Quest and lipoprotein particle sizes will be measured by NMR (Liposciences, Raleigh, NC).

Adipocyte assays. Secretion of TNF and IL6 from adipose tissue fragments will be determined after 3-hour incubations of tissue fragments as we have previously described (67). Total tissue TNFα and IL6 content will be measured from tissue extracts after lysis in buffer containing 1% Triton-X-100 and protease inhibitors. TNFα and IL6 in the media and in lysates will be measured by ELISA (R&D Systems, Minneapolis, MN). TNFα and IL6 mRNA levels will be determined by quantitative real time PCR using the Lightcycler (Roche Diagnostics). Data will be normalized to expression of 18S RNA or cyclophilin. Lipolysis will be measured in isolated fat cells under four conditions: 1) adenosine deaminase (ADA); 2) 20, and 100 nM phenylisopropyl adenosine (PIA); 3) ADA+ 20 nM PIA + 1000 nM isoproterenol; 4) ADA + PIA (20 nM) + 8-bromocyclic AMP + insulin (0, 25, 50, 100, 200, 400, 2000 pM). Glycerol concentrations will be determined as a measure of lipolysis as previously described (48, 80). To measure glucose transport, fat cells will be incubated with tracer U-^{14}C-glucose and varying insulin concentrations as described by Kashiwagi et al. (39). Fat cell size, number of fat cells per ml of incubation, fat cell surface, and fat cell diameter will be determined using standard methods. All fat cell metabolic data will be expressed relative to the number and surface area of the fat cells. Within-individual comparisons (pre- versus post-rosiglitazone) will be made for all adipose biology traits. Absolute and percent changes will be used as quantitative traits for the proposed analyses (See Specific Aims 2 and 3).

Microarrays. The Biopolymer/Genomics Core and its Scientific Director Dr. Stine have the capacity (see Resources and Environment) and the expertise (see letter of collaboration) to perform all of the microarray experiments described in this proposal. The reproducibility of microarray data from the Affymetrix system depends in part on the use of standardized methods developed by the manufacturer. Our laboratory follows the manufacturer guidelines for each of the protocols.

Briefly, total RNA is isolated from frozen tissues using Trizol (Invitrogen). The RNA is cleaned using Qiagen RNeasy mini spin columns and eluted with DEPC treated water. The amount and preliminary estimate of the quality of total RNA is determined using the OD 260/280 ratio. A ratio of 1.2

to 2 is indicative of RNA. A ratio of 1.5 to 1.8 is considered good, while a ratio of 1.8 to 2.0 is considered excellent. We routinely achieve ratios above 1.9.

We further test the quality of the RNA using capillary electrophoresis in our Bioanalyzer (Agilent). This method is comparable to agarose gels, but much more sensitive, i.e. we can test as little as 5 nanograms of total RNA.

The labeling and hybridization is performed following Affymetrix standard protocols. For the following reverse transcription, transcription, labeling and hybridization steps, there are bacterial genes that are added to the sample to serve as controls and are examined as part of the low level dCHIP analyses. 15 micrograms of RNA is reverse transcribed using SuperScript and the other reagents from the One-cycle cDNA Synthesis Kit (Affymetrix). The next step is in vitro transcription and biotin labeling using the IVT Labeling Kit (Affymetrix). The sample is cleaned and hybridization controls are added. After hybridization, the microarrays are washed, stained with streptavidin phycoerythrin and scanned in our Affymetrix scanner. The quality of our hybridizations is very good; our normalization ratios range from 1 to 1.6.

Power considerations for the microarray studies. Although there is no consensus about which sample-size determination procedures for microarray comparison study are best, evidence indicates that a minimum of 5 biological cases per group for a two-group comparison study should be analyzed (4). Therefore, our current microarray comparison (before and after a 3-month rosiglitazone intervention) study with 75 subjects should have an adequate statistical power to detect most differentially expressed genes.

Statistical approach. To study the gene expression profiles that are related to the 3-month rosiglitazone intervention, we will compare the expression profile of the muscle (and fat) tissues from pretreatment subjects to that from post-treatment subjects. We will calculate and normalize gene expression levels by the Robust Multichip Average method (RMA) (37) and quantile normalization (11). The statistical significance of these comparisons will be assessed using LIMMA package software (27) (a linear model approach implemented in Bioconductor) or non-parametric methods (e.g., Kruskal-Wallis- or Mann-Whitney U-tests), using SAS software. A critical issue in microarray analysis is to avoid a high “false discovery rate” (9) (FDR; the probability that a gene is incorrectly identified as differentially expressed), due to the large number of genes compared. We will consider genes to be differentially expressed only if they meet criterion values for fold-change and p-value (for example, >1.5 and <0.05, respectively). For each analysis of differential expression, we will experiment with different combinations of criterion values and calculate the resultant FDR (using MULTTEST package in Bioconductor (61)) for each set of criteria, until we obtain acceptable stringency for fold-change, statistical significance and FDR (we anticipate using conservative FDRs in the range of 1-5%).

To study the association between these differentially expressed (DE) genes and the response of the primary and secondary traits to TZDs, for each DE gene, we will generate linear regression models using the change in the response of the primary (i.e., insulin sensitivity) and secondary responses to TZDs as dependent variables and the gene expression change in response of the treatment and demographic and clinical measures as the independent variables. For the regression models, we will report the adjusted squared correlation coefficient (R^2adj), which corrects for the degrees of freedom. The association between each DE gene and each trait in response to TZDs can be measured by the adjusted squared correlation coefficient. The overall association with primary and secondary traits can be based on the score that is calculated as the weighted sum of the corresponding the adjusted squared correlation coefficients. The weights can be determined by principal component analysis. The importance of each gene can be determined by its score.

Unsupervised clustering techniques will also be used to cluster the subjects according to the similarity of their gene expression profiles (before and after treatment), independently of clinical diagnosis or other investigator-designated categories. To limit noise and increase the strength of our findings, only genes with ample variability are used for cluster analysis (criterion values for variability will be determined empirically). The Hierarchical Ordered Partition and Collapsing Hybrid (HOPAH) (27) algorithm for building a hierarchical tree of clusters will be used for our cluster analysis. The number of clusters will be determined by Median Split Silhouette (MSS) (62) and biological consideration. The non-parametric bootstrap technique will be used for validating the HOPAH clusters. The clustering analyses will be assessed by using HOPAH package software in Bioconductor (27). Clustering of the

subjects based on gene expression would suggest a distinct, common underlying biological basis for disease in these subjects. Such an outcome could be a significant start in achieving one of our goals – a biology-based, rather than a clinical outcome-based classification of TZD response. Comparison of subject symptoms and clinical outcomes within- and across subject clusters based defined by gene expression profiling could be indicative of differences among subjects in response of TZD treatment.

Because the regulation of gene expression is a dynamic process, it is also important to identify and characterize changes in gene expression in response of TZD treatment among subject subgroups defined by expression profiling. Storey, et al. (73) proposed a general statistical method that identifies genes differentially expressed over time among different classes and have developed freely available software called EDGE. It is applicable to detecting differences in the behavior of expression in response to TZD treatment among different subgroups of the subjects. In addition to the pre-post comparison for each subgroup described above, the EDGE program will be used to identify genes that show significant differential expression in response to TZD treatment among different subjects.

We will then analyze the biological significance of the genes which are identified by the above analyses, using GenMAPP (15) (Gene MicroArray Pathway Profiler) and MAPPFinder (19). GenMAPP facilitates the viewing and analysis of gene expression data in the context of biological pathways. The pathways are represented in a special file format called a “MAPP”. The MAPPs are independent of the gene expression data and can be used to group genes and view data by any organizing principle. MAPPFinder uses GenMAPP and the Gene Ontology (GO) database (http://www.geneontology.org) to identify biological trends in gene expression data. MAPPfinder can use the terminology and organizing principles of the GO database, or user-defined MAPPs. The three organizing principles of GO are “molecular function”, “biological process” and “cellular component”, which are attributes of gene products. Each of these terms has sub-terms (“children”) organized in a tree-like structure. The terms are assigned independently, such that each gene can fall into all three groups. Our analysis will identify (on the basis of z-score and permuted p-value) molecular functions, biological processes or cellular components that are potentially influential in the subjects in regard to TZD treatment. Based on microarray and functional genomic analyses and Q-RT-PCR validation, we will select 100 candidate genes that influence TZD response for Aim 2 study.

Software. The statistical software packages SAS, Bioconduct and R (27) are the primary tools for most statistical analysis. SAS software, which is installed on our computers, is a combination of programs originally designed to perform statistical analysis of data. R is a free software environment for statistical computing and graphics. Bioconductor is an open source and open development software project for the analysis and comprehension of genomic data. These approaches as well as others that emerge during the course of this project in the continually evolving field of microarray analysis will be utilized to process the data obtained in our microarray experiments.

Quantitative Real time-PCR. Due to the limited dynamic range of the gene array expression analysis, we will more precisely quantify up- and down-regulated genes by Q-RT-PCR of the transcripts using the Lightcycler (Roche Diagnostics) and standard methods as described under Preliminary Studies. The same RNA samples as were used in the original microarray analysis will be used for Q-RT-PCR.

3. Specific Aim 2. Define and Characterize Genes that are Differentially Expressed between Responders and Non-Responders to TZDs

We will identify sequence variants within the 100 genes targeted for study on the basis of the cDNA microarray experiments and will use this information to characterize the haplotype structure of the candidate gene. We will then identify haplotype-tagging SNPs (htSNPs) to be used for the association analyses described under Specific Aim 3.

a. Specific Aim 2a

We will identify common sequence variants by sequencing 20 individuals (10 European Caucasian and 10 African American) and through in silico SNP discovery. The 20 individuals will be unrelated individuals selected from our existing DNA repository at the Shuldiner laboratory.

We will use a combination of *in silico* and laboratory-based approaches to SNP discovery and

validation. We will choose validated SNPs from the multiple public databases (e.g., dbSNP, CELERA, Hap-Map) as well as from SNPs that have already been converted into Illumina assays for their linkage and fine mapping panels (http://www.illumina.com/snp_linkage.htm). SNPs that appear independently in more than one database or SNPs that have been experimentally validated and have allele frequencies greater than 5%. As these databases become more complete (the next version of dbSNP will have >10 million SNPs; David Altshuler, personal communication), we expect to be able to obtain the great majority of SNPs that we will initially genotype from these databases. In genes that do not have adequate sequence variant information from these sources (i.e., less than a 1 SNP per 3-5 kb density), we will perform DNA sequence analysis to identify new SNPs. The regions of greatest interest will be potentially functional regions, including exons, exon-intron junctions, regulatory regions, and other intragenic regions that are phylogenically conserved. These regions and other regions in which the inter-marker distance exceeds 5 kb will be sequenced in 10 European Caucasians and 10 African Americans (40 alleles). This number of subjects will provide >87% power to detect gene variants with an allele frequency of 10% or greater. When considering the density of SNPs within candidate genes, we will also take into account what is known about the haplotype structure of the gene/region based upon information from Hap-Map and other sources. Indeed, Dr. David Altshuler, consultant on this proposal, is a member of the Hap-Map project and will be able to provide expert advice regarding SNP selection.

b. Specific Aim 2b

We will validate the SNPs identified by sequencing and/or in silico SNP discovery by genotyping a subsample of 96 individuals (48 European Caucasians and 48 African Americans) selected also from our DNA repository. We anticipate that on average, we will have identified 20 SNPs per gene, requiring us to genotype ≈ 2,000 SNPs/person (100 genes × 20 SNPs/gene). Allele frequencies of SNPs and linkage disequilibrium (LD) between intragenic SNPs will be calculated. This information will be used to choose haplotype-tagging SNPs for genotype analysis in the larger sample. With allele frequency and linkage disequilibrium information, we will be able to eliminate some SNPs from subsequent analysis. For example, SNPs with allele frequencies < 5% or SNPs in high LD (e.g., $r^2 > 0.7$) with other SNPs will not be genotyped in the larger cohort. We will use algorithms to select haplotype-tagging SNPs to maximize the likelihood that we have captured most or all of the sequence variation. Haplotype-tagging SNPs (ht-SNPs) will be genotyped in three different population groups as described in the following section. The Illumina platform will be used for this large-scale genotyping effort, which will be performed at the Broad Institute of Harvard and MIT (see Letter from David Altshuler).

4. Specific Aim 3. Association Analysis of Candidate Genes with TZD Response in Three Populations

We will genotype the htSNPs identified in Specific Aim 2 in three separate populations who have undergone a TZD intervention. Approximately 1,500 htSNPs from 100 genes will be genotyped in: (1) 75 subjects recruited for this study; (2) 597 troglitazone-treated individuals from the Diabetes Prevention Program, or DPP; and (3) 97 gestational diabetic Hispanic women who were treated with troglitazone (and more recently pioglitazone) from the TRIPOD/PIPOD study.

Subjects were eligible for DPP if their age was ≥ 25 years, BMI was ≥ 24 (≥ 22 for Asian-Americans), and they had impaired glucose tolerance plus a fasting plasma glucose of 95-124 mg/dl (or ≤ 124 mg/dl for American Indians). Among randomized subjects, 55% were Caucasian, 20% were African-American, 16% were Hispanic, 5% were American Indian, and 4% were Asian-American. Their average age at entry was 51 +/- 10.7 years (mean ± SD), and 67.7% were women. Of the women, 48% were postmenopausal. Overall, BMI averaged 34.0 ± 6.7 at baseline with 57% of the men and 73% of women having a BMI ≥ 30. Average fasting plasma glucose (108 ± 9 mg/dl) and HbA1c (5.9 ± 0.5%) in men were comparable with values in women (106 ± 7 mg/dl and 5.9 ± 0.5%, respectively). For TRIPOD, Hispanic women (of Mexican and Central American origin) in the Los Angeles area were eligible if they were ≥ 18 years of age, had GDM in the previous 4 years, and were willing to use effective contraception. They were offered participation in the study if they had no evidence of chronic disease; a serum alanine aminotransferase concentration < 1.5 times the laboratory upper normal; no diabetes; and a sum of five oral glucose tolerance test (OGTT) plasma glucose concentrations ≥ 625 mg/dl, predicting a 70% risk of diabetes in the next 5 years. Enrollment continued until 266 women had

been randomized. Mean age was 34.9 ± 6.6 yrs, BMI 30.6 ± 6.1, fasting glucose 98.7 ± 10.2 mg/dl, 2-h glucose 154.4 ± 27.0 mg/dl. We have successfully extracted DNA on 217 women who completed the 3-month IVGTT assessment. After termination of the study, women who had not developed diabetes were invited to participate in an extension involving pioglitazone, PIPOD.

Dr. Shuldiner (co-investigator) and Dr. Altshuler (consultant) are co-PIs on an NIH-funded grant to relate candidate genes to DPP data (see letter from Dr. Altshuler), while Dr. Pollin (co-investigator) is PI of the data management and analysis subcontract for the study. The DPP genetics study includes the typing of population markers, which will be used in the proposed replication study. Dr. Snitker has previously preformed a pharmacogenetic study involving the TRIPOD subjects.

In each population separately, we will compare mean insulin sensitivity according to genotype using analysis of variance. We will include age and sex in the analysis as covariates. In additional models, we will add multiple genotypes from the same or related pathways and test their potential interaction effects in a multiplicative fashion. Prior to analysis, we will perform a series of routine quality control checks on the genotype data that will include a repeatability analysis of a panel of 5% duplicate samples, and chi-square tests to assess compliance of the genotype distributions with Hardy-Weinberg Equilibrium. These procedures are routinely carried out in our laboratory. The primary analysis will be to determine if there is a dose response effect of genotype on the outcome. This analysis is conducted by assigning codes of 0, 1, or 2 to the genotype, with the value of the code reflecting the number of (minor) alleles. In addition, we will also carry out secondary analyses of the dominant and recessive genetic models to evaluate the effects of the respective homozygotes on TZD response.

a. Haplotype-disease association analysis

Haplotypes may potentially provide more information about an unobserved causal variant than single SNPs because of the increased resolution of multiple SNPs considered jointly to tease out the unobserved ancestral haplotype background on which mutation arose and which is shared among affected individuals. We plan to use Helix Tree, a commercially available software package designed for managing and analyzing data from large-scale genome-wide association studies, for our haplotype analysis.

The haplotype trend regression analysis in Helix Tree regresses the response variable using the estimated haplotype probabilities as the regression matrix, thereby properly incorporating the uncertainty in individual haplotype assignment inherent in population-based data. We will compute permutation-derived empirical significance levels for each candidate gene to eliminate bias due to incorrect distributional assumptions.

b. Population stratification

One potential pitfall of association studies in population-based samples is false-positive signals resulting from population stratification. We will use an Illumina panel of 278 ancestry informative markers from the HumanHap 550K chip (Illumina; San Diego, CA) to account for potential effects of population stratification in our analyses either by adjusting the variance of the statistic or by adjusting the populations (88, 89). We attempt to eliminate false positives by assigning higher priority to signals that are replicated in multiple populations either at the same SNP, same haplotype block or same gene. We recognize that these methods represent global adjustments based on genome-wide averages of admixture, thus do not provide information about the chromosomal region containing SNPs of interest. However, newer methods are beginning to appear that attempt to define the ancestral blocks to potentially allow for local admixture adjustment in case-control analyses (90). We plan to evaluate new methods as they become available and integrate those that will add power to our analyses.

c. Power calculation for the candidate gene analyses

We estimated the effect sizes we could reasonably expect to detect at 80% power in these three samples. The calculations were made across a range of allele frequencies, and for sample sizes of 75, 97, and 585, in the current study, TRIPOD, and the Diabetes Prevention Program (DPP), respectively. For purposes of these calculations, we further assumed a dominant genetic model in which mean TZD response levels were to be compared between two genotype groups (e.g., AA vs. Aa/aa). The effect sizes are parameterized to reflect the difference between the group means, expressed in terms of the

standard deviation units. By convention, differences between groups of 0.8 SD, 0.5 SD and 0.2 SD are considered large, medium, and small effects, respectively.

For the current study (n = 75 subjects) and TRIPOD studies (n = 97 subjects), we would have 80% or higher power to detect medium size effects (0.7 SD unit differences between means) across a wide range of allele frequencies (i.e., minor allele frequencies 0.1-0.5). For a putative subgroup of the current study with n=38, we would have 80% or higher power to detect large effect sizes (1.0-1.2) across the same allele frequencies. For the DPP (n = 597 subjects), we would have 80% or higher power to detect small size effects (0.3 SD unit differences between means) also across a wide range of allele frequencies. It is also worth noting that subjects to be recruited from this study will have mRNA levels measured, a very proximal phenotype that can be used as an additional outcome for the genetic association studies. One might expect a very large effect of an associated SNP on this particular phenotype.

E. HUMAN SUBJECTS RESEARCH

Protection of Human Subjects

1. Risks to the Subjects

a. *Human subjects involvement and characteristics*

Inclusion criteria

- Male or female who self-identifies as (in NIH classification terms) White.
- Age 35 - 65 years
- BMI 25 - 40 kg/m²

The age and BMI criteria were chosen to favor subjects with a relatively high likelihood of having insulin resistance and chronic inflammation, which makes intervention clinically meaningful.

Exclusion criteria

- History of diabetes (Doctor told pt. they had diabetes or pt. is taking diabetes medication)
- Fasting glucose value diagnostic for diabetes
- 2-h oral glucose tolerance test diagnostic for diabetes
- Medications that the investigator judges will make interpretation of the results difficult or increase the risk of participation (e.g., anticoagulants)
- Disease that the investigator judges will make interpretation of the results difficult or increase the risk of participation (e.g., anemia, cardiac decompensation, intolerance to rosiglitazone, lidocaine or other agents used)
- Pregnancy
- Physical dimensions exceeding the limits of any equipment used

Screening will consist of medical history, vital signs, physical exam, and blood tests (a comprehensive chemistry profile, CBC, TSH, pregnancy test, 75 gram oral glucose tolerance test). Based upon this testing, subjects will be excluded if they have (1) stage III or greater congestive heart failure, symptomatic peripheral vascular disease, or stroke; (2) severe hypertension (BP>170/100); (3) anemia; (4) thyroid, pituitary, kidney or liver disease; (5) diabetes; (6) weight loss or gain of >5 kg in the previous six months; (6) hormone replacement therapy; (7) excessive use of caffeine (greater than three caffeinated drinks per day), or alcohol (greater than 3 ounces of liquor, or 3 x 4 ounce glasses of wine, or 3 x 12 ounce cans of beer per day); (8) use of medications that may affect glucose or lipid metabolism including beta blockers, thiazide diuretics, hypolipidemic agents, thyroid hormone, or

weight loss medications or formulas; (9) smoking >1/2 pack per day of cigarettes; and (10) pregnancy by urine testing.

Individuals with diabetes will be excluded because their metabolic control may vary, their likely use of multiple of multiple medications, and possible complications. From a statistical point of view, each source of variation can be regarded as an additional error term, requiring a much larger sample size for the same amount of power.

b. Sources of materials

Blood samples, urine samples, tissue samples, and results of physiological tests.

c. Potential risks

We expect no physical, psychological, social, or legal risks from a subject's participation in this protocol. Subjects who are screened, but not selected for participation for any reason will be referred to their primary health care provider for follow-up. Some of the procedures (DXA and CT scans) involve exposure to low doses of radioactivity; so pregnant women will be excluded from the study.

Body composition: Dual energy x-ray absorptiometry (DXA) and CT scan entail exposure to radiation. The cumulative total body dose throughout the study is up to 0.015 rem from the DEXA scans and up to 0.040 rem from the CT scans. However, some organs will receive a larger dose from the CT scans: 0.092 rem to the ovaries (women only), 0.054 rem to the testes (men only), 0.060 rem to the bladder, and 0.058 rem to the rectum. Other organs and tissues will receive lesser radiation doses. These radiation doses are within institutional and federal guidelines for radiation exposure to research subjects and have never been associated with any definite adverse effects.

Phlebotomy and oral glucose tolerance test: The placement of intravenous needles and catheters may cause transient pain, and occasionally infection or bruising at the needle insertion site. The frequency and severity of these potential adverse events will be minimized by using sterile technique, universal precautions, and trained personnel. Blood volumes to be drawn are minimal, and therefore these tests are unlikely to cause anemia. For the oral glucose tolerance test, an indwelling catheter will be placed in an antecubital vein. Normal saline will be infused to maintain venous patency during the 2-h test. A solution containing 75 g of glucose is ingested by the fasting subject, which occasionally causes nausea.

Frequently sampled IV glucose tolerance test: Indwelling catheters will be placed in both antecubital veins. Glucose is given IV at T=0 minutes and regular insulin is given IV at T=20 minutes. The amount of glucose and insulin given are based on the subject's weight (glucose 0.3 g/kg; insulin 0.025 U/kg). Blood will be drawn from one of the IVs for glucose and insulin measurements at -15, -10, -5, -1, 2, 3, 4, 5, 6, 8, 10, 14, 19, 22, 25, 30, 40, 50, 70, 100, 140, and 180 minutes (3 ml/time point x 22 = 66 ml). Additional glucose levels will be drawn at 35, 45, 55, 60, 80, and 90 minutes (1.5 ml/time point x 6 = 9 ml) to monitor for possible hypoglycemia. Proinsulin will be measured at -1, 10 and 60 minutes. The total amount of blood drawn for the FSIGTT will be 255 ml. In addition to the risks described above for phlebotomy, IV administration of glucose may sometimes result in lightheadedness, tingling in the arm or shoulder, and rarely phlebitis. To prevent phlebitis from occurring, a large volume of saline is run in through the IV used for the glucose bolus immediately after the bolus in order to decrease the likelihood of glucose irritating the vein. IV insulin may cause hypoglycemia with symptoms of dizziness, diaphoresis, palpitations and fatigue. Blood glucose will be monitored frequently and the test will be terminated if hypoglycemia develops and a glucose drink will be given.

Adipose and muscle biopsies: There may be slight discomfort and burning when the local anesthetic is injected prior to the needle biopsy, but subjects are not expected to experience discomfort during the biopsy procedure. Following the biopsies, there may be tenderness, bleeding, bruise formation, or infections. Ice will be applied to the site immediately after the procedure to limit bruising,

swelling and tenderness. Aseptic technique will be used to minimize chances of infection.

Rosiglitazone:

(1) *Mechanism of action.* Rosiglitazone is a potent and highly selective agonist of peroxisome proliferator-activator receptor-gamma (PPARγ)(70). As a member of the thiazolidinedione antidiabetic class of medications, rosiglitazone improves glycemic control by increasing the action of insulin in tissues that express PPARγ. In animal studies, rosiglitazone reduced blood glucose concentrations and demonstrated significant insulin-sensitizing activity in skeletal muscle, adipose tissue, and the liver. In addition, rosiglitazone decreased hyperinsulinemia and increased GLUT-4 transporter expression in adipose tissue. While the exact onset of action has not been established in humans, glucose-lowering effects are generally evident 2 to 3 weeks after initiating rosiglitazone (70). Maximal effects are observed after 6 to 12 weeks of therapy. Since rosiglitazone effects gene expression, its pharmacological half-life is much longer and not closely related to its elimination half-life. In vitro data suggests that the pharmacodynamic half-life of rosiglitazone is greater than 24 hours.

(2) *Pharmacokinetics and drug metabolism*

Absorption. Rosiglitazone is well absorbed from the gastrointestinal tract, with an absolute bioavailability of 99% (70). Peak plasma concentrations are seen approximately 1 hour following an oral dose. While food appears to slow the rate of absorption of rosiglitazone (Tmax delayed by 1.75 hours), it does not alter the extent of absorption (AUC unchanged)(25). Rosiglitazone displays linear pharmacokinetics over a wide dose range (0.2 to 20 mg)(70).

Distribution. Rosiglitazone is highly protein bound (99.8%) and its mean volume of distribution (Vss/F) is 17.6 liters (70).

Metabolism. Rosiglitazone is extensively metabolized in the liver by N-demethylation and hydroxylation, primarily through the cytochrome P450 CYP2C8 isoenzyme pathway. The CYP2C9 isoenyzme pathway is a minor route. No unchanged drug is excreted in the urine. None of the metabolites contributes significantly to the insulin-sensitizing activity of rosiglitazone (70).

Excretion. The majority (64%) of labeled rosiglitazone appears in the urine following oral or intravenous administration. A substantial portion (23%) is eliminated in the feces (70).

Half-life/plasma concentrations. The single-dose elimination half-life of rosiglitazone is approximately 3.3 ± 0.7 hours and is independent of dose (70). The approximate rate of clearance is 3 ± 0.7 L/hour. In a dose ranging pharmacokinetic study, peak plasma concentrations of 182 ± 48.3 ng/ml were observed 1 hour following a single 4 mg oral dose of rosiglitazone. The elimination half-life may become significantly shorter following repeated doses of rosiglitazone (70). A reduction in t½ of 0.75 to 1.35 hours was observed in one study after 10 days of therapy in obese patients given rosiglitazone daily. The mechanism for this observed increase in clearance is unknown. Based on these observations, steady state trough plasma concentrations are anticipated to be 5 to 20 ng/ml in most patients given 4 mg twice daily.

Special populations. Based on a population-based analysis from clinical trials, the pharmacokinetics of rosiglitazone appears to be similar in young (<65 years old) and elderly (>65 years old) patients (18, 70). Females clear rosiglitazone at a slightly slower rate than males and, in monotherapy studies, demonstrated a slightly greater therapeutic response. However, in obese patients, differences between genders were less apparent. Neither race nor smoking status influences the pharmacokinetics of rosiglitazone. Furthermore, patients with mild to severe renal impairment or who are dependent on hemodialysis do not exhibit altered pharmacokinetic properties of rosiglitazone (77).

(3) Clinical effects

In clinical trials, rosiglitazone resulted in improved glycemic control as measured by hemoglobin A1c and fasting plasma glucose concentrations (24, 30, 70). Improvements in glycemic control were

sustained for 52 weeks in clinical trials. The maximum recommended dose is 8 mg daily. No additional blood glucose lower effects were observed when a total daily dose of 12 mg was used. Twice daily dosing of rosiglitazone produces greater reductions in HgbA1c and fasting plasma glucose when compared to equivalent doses given once a day. When compared to placebo, the reductions in HgbA1c observed with rosiglitazone therapy were 1.2% for once daily administration and 1.5% for twice-daily administration. The percentage of patients treated with rosiglitazone 4 mg BID considered responders, defined as a 0.7% or greater decrease in HgbA1c from baseline, was approximately 50% in clinical trials.

When used in combination with other oral antidiabetic agents or insulin, rosiglitazone demonstrates additive effects. In combination with metformin, rosiglitazone 8 mg daily reduced HgbA1c by 0.8% when compared to baseline and 52% of patients were considered responders (28, 70). Similar reductions in HgbA1c and response rates have been observed in trials combining rosiglitazone and a sulfonylurea (28) or insulin therapy (65).

(4) Adverse effects/precautions

Rosiglitazone therapy is associated with weight gain (70). In clinical trials, the mean increase in weight was 3.5 kg in patients treated with 8 mg per day when given as monotherapy. Metformin attenuates the amount of weight gain associated with rosiglitazone therapy. Some of this weight gain may be attributable to an increased plasma volume and mild to moderate lower extremity edema (incidence 5%). Mild anemia, reported in 1.9% of patients on rosiglitazone monotherapy, may also be attributable to increased plasma volume. The incidence of all other reported side effects were similar to placebo.

In clinical trials and during post-marketing surveillance, rosiglitazone has not been associated with hepatotoxicity. Troglitazone, another member of the thiazolidinedione class of drugs, has been implicated in numerous cases of severe hepatotoxicity, thus requiring meticulous liver enzyme (ALT) monitoring. In clinical trials, significant elevations in serum ALT (greater than 3 times the upper limit of normal) was observed in only 0.2% of patient treated with rosiglitazone compared with a 0.2% incidence with placebo and a 0.5% incidence with metformin, insulin, and sulfonylureas. Evidence to date suggests that rosiglitazone is a safer medication than troglitazone with regard to liver toxicity. However, given their structural similarities, periodic serum ALT monitoring is recommended.

(5) Use in non-diabetic individuals

Rosiglitazone is FDA approved for use in diabetic subjects to improve glycemic control. As demonstrated in TRIPOD (see Preliminary Studies) and other clinical studies it also ameliorates insulin resistance and chronic inflammation in overweight individuals, the population eligible for this study. Thus, the genetic determinants of drug response can be studied in this population. However, because the use of rosiglitazone in non-diabetic subjects is an unlabeled use of a marketed drug, we need to ensure the safety of the intended use. Precedence established by other studies is reassuring. In particular, rosiglitazone does not appear to have the potential to elicit hypoglycemia in a non-diabetic person. The Diabetes Prevention Project (DPP) was a federally funded, multicenter trial to assess various strategies to prevent or delay conversion from impaired glucose tolerance to diabetes. One treatment arm received troglitazone. The troglitazone arm was discontinued two years into the study due to liver toxicity, a problem inherent to troglitazone but not to other TZDs. The exposure of 585 impaired glucose tolerance subjects to troglitazone in the DPP did not lead to study-terminating side effects other than liver toxicity (17). In the TRIPOD study, troglitazone was administered to 133 women with previous gestational diabetes without any reports of hypoglycemia (12, 13). This cohort is now receiving pioglitazone and the trial continued as PIPOD. With the FDA's approval, a multicenter trial, the DREAM study, is being conducted to test the use of rosiglitazone in impaired glucose tolerance to prevent diabetes.

On the occasion of the ongoing PPAR study (see Preliminary Studies), we petitioned the FDA via IND 67,070 to use rosiglitazone in non-diabetic individuals. The FDA informed us that all requirements for an FDA exemption were met. We have not observed any drug-related adverse events

in PPAR.

We will heed the WARNINGS included in the rosiglitazone Product Information. We will monitor every four weeks for adverse events, including those mentioned in the Product Information, i.e., lower extremity edema and anemia. Patients will be questioned and examined at each visit regarding leg swelling. Rosiglitazone therapy will be decreased or discontinued in case of adverse events that are likely to be related to the drug. A complete blood count will be obtained at baseline and after 12 weeks to monitor for a decline in red blood cell concentrations as well as any other hematological derangements. Individuals with preexisting fluid retention or heart failure are ineligible for the study.

Although evidence indicates that rosiglitazone does not share the hepatotoxicity of troglitazone, we will monitor alanine transaminase every four weeks.

Subjects will be cautioned about the possibility of rosiglitazone to produce ovulation in some premenopausal women who might think themselves infertile. Adequate contraception in premenopausal women will be recommended.

Rosiglitazone will not be used in patients in whom it is CONTRAINDICATED (known hypersensitivity to the product or any of its components).

Genetic testing: Since the clinical relevance of the genetic testing is not yet proven, subjects will not be given any genetic information. If, in the future as a result of these studies or studies of others, gene variants that have clinical relevance are detected, all subjects who were genotyped will be contacted and first asked if they wish to know the result of their genotype analysis. If they do wish to be informed of their genotype, they will be informed by the investigators with the assistance of a genetics counselor, and with advice from an ethicist. However, subjects are given the option to decline all future genetic information when signing the consent form.

2. Adequacy of Protection Against Risks

a. Recruitment and informed consent

Written informed consent will be obtained from all subjects. The IRB approved consent form will be given to each subject, and informed written consent will be obtained by one of the investigators or a study coordinator following a personal interview with each subject. Subjects will be encouraged to read the consent form closely and the rationale, methods, potential risks and benefits of the interventions will be explained.

b. Protection against risks

Please see potential risks section above.

3. Potential benefits of the proposed research to the subjects and others

Anticipated benefits to health include screening for anemia, liver and kidney dysfunction and cardiovascular risk factors (lipids and blood pressure). The primary beneficiaries will be future patients in whom treatment with a TZD may be an option. Our study may help determine whether a given patient stands to benefit from treatment based on his/her genetic makeup or clinical presentation.

4. Importance of the knowledge to be gained

The metabolic – or insulin resistance – syndrome is a clustering of cardiovascular risk factors. Although weight reduction and physical activity are first-line treatments for this condition, drug therapy with TZDs may improve not only insulin resistance, but also – directly or indirectly – inflammatory and prothrombotic conditions. Because 1/3 of subjects treated with TZDs typically do not respond to the insulin sensitizing effects of TZDs, it is important to know whether this non-response also extends to other effects of the drug, and to understand the drugs' pharmacogenetic profile.

5. Data and Safety Monitoring Plan

An independent Data Safety and Monitoring Board (DSMB) will be convened on a quarterly basis to insure protection of human subjects. The DSMB will consist of at least five individuals with expertise in clinical investigation, clinical cardiology and research subject advocacy. Dr. Shuldiner and the Steering Committee will prepare accurate and timely data tables and reports for DSMB meetings and will present regular updates on the research projects. The objectives of the DSMB will be:

- To ensure that that research in conducted in a safe and ethical manner
- To ensure those adverse events (AEs) that occur while conducting this trial is adequately reported and investigated
- To conform with UM IRB and NIH policies and procedures
- To assist the UM IRB and the NIH in their mission to protect subjects participating in study protocols under their jurisdiction
- To ensure that any medical-coverage-related problems are addressed if an AE occurs

The PI will uphold the following responsibility to the DSMB:

- The Principal Investigator (PI) and members of the staff will be responsible for reporting all new clinical experiences, exacerbations, and/or deterioration of any existing clinical condition occurring after a study subject has entered the study.
- The PI will be responsible for reporting Serious Adverse Events (SAEs) to the UM IRB and the DSMB.
- The PI will be responsible for determining the causality of all AEs/SAEs subject to review by the UM-IRB and DSMB.
- The PI will be responsible for determining whether test results are “clinically significant” (CS) or “not clinically significant” (NCS). The PI will sign and date all lab reports as “CS” or “NCS”.
- The PI and staff will be responsible for follow-up information on all AEs until resolution or an appropriate endpoint is reached.

The DSMB in concert with UMB-IRB will be responsible for reviewing all reports of SAEs which occur in this clinical trial and for determining whether any corrective actions need to be taken regarding: communication issues between the PI and the DSMB, management decisions of the PI and staff, whether protocol violations are congruous with patient welfare taking precedence over protocol, whether there are any issues among the research staff which need to be addressed, etc. The DSMB will be responsible to determine if the results of its review require a revision/modification of the RPN and/or the consent form.

6. Inclusion of women and minorities

Women are eligible for this research and will be actively recruited. We will halt recruitment of men or women if we reach more than 2/3 of either sex. To avoid analytical problems related to the inclusion of a highly stratified sample, only Caucasians will be eligible for Aim 1. However, because non-response to TZDs has never been associated with a particular race, we believe that the genes identified will be relevant to minorities as well, which we will be able to test in Aim 3, using banked DNA from the DPP and TRIPOD studies. Please see body of proposal for demographic description of the DPP and TRIPOD samples, which do not meet the requirements for listing in the Targeted/Planned Enrollment Table. The analysis strategy will address hypotheses related to sex and ethnicity/race. Please see enrollment table below.

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Sex/Gender**		
	Females	**Males**	**Total**
Hispanic or Latino	0	0	0
Not Hispanic or Latino	37	38	75
Ethnic Category: Total of All Subjects *	37	38	75
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	0	0	0
White	37	38	75
Racial Categories: Total of All Subjects	37	38	75

7. Inclusion of children

NIH policy provides that applicants must include children in research unless there are scientific and ethical reasons not to include them. Children are not included because (1) rosiglitazone treatment in children is still undocumented, and (2) even if it were, the protocol involves risks and discomforts (radiation, tissue biopsies) that are not justified considering that we have little reason to expect that the genetic determinants of rosiglitazone response differ between adults and children.

F. VERTEBRATE ANIMALS – not applicable.

G. SELECT AGENT RESEARCH – not applicable

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I. MULTIPLE PI LEADERSHIP PLAN -Not applicable

J. CONSORTIUM/CONTRACTUAL AGREEMENTS -Not applicable

K. RESOURCE SHARING (This item is gladly addressed although not a formal requirement for investigators that do not seek $500,000 or more in direct costs in any year).

Dr. Snitker's commitment to collaboration with other groups goes back to his appointment at the NIH as demonstrated in his publication record. He has an ongoing relationship with the TRIPOD study group at the University of Southern California. Dr. Watanabe of this group is a collaborator on this project. It is expected that data from this study will enter into the pool of data we share with the TRIPOD group (while observing HIPAA privacy rules).

Dr. Snitker and the other University of Maryland investigators participate in multiple collaborative (U01) projects. These efforts have taught us to collaborate closely with the NIH Program Officers to promote data sharing. We envision that the data may be of interest to the Pharm-GKB database, an NIH effort.

L. LETTERS OF SUPPORT

Consultants (alphabetical; letters attached):

1. **David Altshuler, MD, PhD;** *Consultant***.** Dr. Altshuler is an Associate Professor of Genetics and Medicine at Harvard Medical School and Director of the Medical and Population Genetics at the Broad Institute of Harvard and MIT. Dr. Altshuler is an expert in the area of genetics of complex diseases and traits, high-throughput SNP genotyping and defining haplotype diversity in populations. He is a member of the Hap-Map project and is also collaborating with Dr. Shuldiner and Dr. Pollin (co-investigators) on genetic studies of the Diabetes Prevention Program (DPP) cohort. Dr. Altshuler will provide consultative services regarding the SNP selection and genotype analysis. He will also provide expertise in haplotype construction and in interpretation of association and linkage results. He will meet at least once per year with the University of Maryland group, either in Baltimore and or at the annual meeting of either the American Society of Human Genetics or the American Diabetes Association.

2. **Richard Watanabe, Ph.D.;** *Consultant***.** Dr. Watanabe is an Assistant Professor of Medicine at the University of Southern California Keck School of Medicine. Dr. Watanabe is an expert of genetic empidemiology. He will collaborate with us in regard to candidate gene analyses of the TRIPOD (now PIPOD) sample. He will meet at least once per year with the University of Maryland group, either in Baltimore or at the annual meeting of either the American Society of Human Genetics or the American Diabetes Association.

Other Letters:

1. **Jeffrey Hasday, M.D.;** Professor of Medicine, University of Maryland School of Medicine; Director, University of Maryland Cytokine Core Laboratory

2. **Colin Stine, Ph.D.**; Associate Professor of Epidemiology, University of Maryland School of Medicine; Director, University of Maryland Biopolymer/Genomics Core

3. **Carol Tacket, M.D.**; Professor of Medicine, University of Maryland School of Medicine; Program Director of the University of Maryland General Clinical Research Center

4. **Sonia Anand, M.D., Ph.D, FRCPc;** Chair of Genetics Subcommittee, DREAM

5. Memo of Understanding regarding Dr. Shuldiner's joint VA-University appointment