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Objective—To determine whether changes in standard and novel risk factors during the Actos Now for Prevention of Diabetes trial explained the slower rate of carotid intima media thickness (CIMT) progression with pioglitazone treatment in persons with prediabetes.

Methods and Results—CIMT was measured in 382 participants at the beginning and up to 3 additional times during follow-up of the Actos Now for Prevention of Diabetes trial. During an average follow-up of 2.3 years, the mean unadjusted annual rate of CIMT progression was significantly (P=0.01) lower with pioglitazone treatment ($4.76×10^{-3}$ mm/year; 95% CI: $2.39×10^{-3}$–$7.14×10^{-3}$ mm/year) compared with placebo ($9.69×10^{-3}$ mm/year; 95% CI: $7.24×10^{-3}$–$12.15×10^{-3}$ mm/year). High-density lipoprotein cholesterol, fasting and 2-hour glucose, HbA_{1c}, fasting insulin, Matsuda insulin sensitivity index, adiponectin, and plasminogen activator inhibitor-1 levels improved significantly with pioglitazone treatment compared with placebo ($P<0.001$). However, the effect of pioglitazone on CIMT progression was not attenuated by multiple methods of adjustment for traditional, metabolic, and inflammatory risk factors and concomitant medications, and was independent of changes in risk factors during pioglitazone treatment.

Conclusion—Pioglitazone slowed progression of CIMT, independent of improvement in hyperglycemia, insulin resistance, dyslipidemia, and systemic inflammation in prediabetes. These results suggest a possible direct vascular benefit of pioglitazone. (***Arterioscler Thromb Vasc Biol.*** 2013;33:393-399.)

Key Words: carotid atherosclerosis progression ■ impaired glucose tolerance ■ insulin resistance ■ inflammation ■ pioglitazone

Individuals with prediabetes are at increased risk for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). They are excellent candidates for early interventions to reduce development of T2DM, atherosclerosis, and CVD events. As large-vessel atherosclerosis frequently accompanies the development of diabetes mellitus, and both these conditions have many common genetic and environmental risk factors, they may share a common soil.[1] Trials of lifestyle modification and several diabetes mellitus medications in individuals with prediabetes have effectively reduced the development of T2DM.[2–4] However, few studies have addressed whether these same medications can slow progression of atherosclerosis or reduce CVD events in prediabetes.

In clinical studies, thiazolidinedione drugs (TZDs) not only reduce hyperglycemia and insulin resistance and conversion to T2DM, but also lower blood pressure, increase high-density lipoproteins, and adiponectin concentrations, and reduce plasma levels of a wide range (but not all) of inflammatory markers,[4–8] changes that may reduce atherogenesis. Furthermore, data from in vitro and animal models suggest that TZDs may slow development and progression of atherosclerosis through direct vascular effects.[9–12]

Despite these observations, TZDs have not uniformly reduced clinical CVD events in people with T2DM and established atherosclerosis.[13,14] However, potential for a greater disease-modifying role before the onset of advanced

atherosclerosis and clinical CVD events is suggested by evidence that TZDs, and pioglitazone in particular, consistently slow preclinical atherogenesis in T2DM patients.[15–17] Secondary analyses of these latter studies demonstrated that this effect was, in part, explained by improved plasma high-density lipoprotein cholesterol (HDL-C) levels[18,19] and triglyceride/HDL-C ratio.[18,19] However, many other previously demonstrated effects of pioglitazone that could potentially explain antiatherosclerotic effects of pioglitazone were not assessed. Clarifying the antiatherogenic mechanisms of TZDs would not only provide insight into the pleotropic actions of TZDs, but may also help identify new targets for future drug development.

The Actos Now for Prevention of Diabetes (ACT NOW) study was designed to test the effects of pioglitazone on both conversion to T2DM and progression of atherosclerosis in persons with prediabetes.[20] A specific goal of the study design was the measurement of multiple metabolic parameters and CVD risk factors to investigate potential mechanisms underlying the development of T2DM and carotid atherosclerosis. We recently reported that pioglitazone was associated with slower progression of carotid intima media thickness (CIMT) at the end of the study, as compared with placebo.[5] Here, we sought to gain insight into the mechanisms by which pioglitazone reduced progression of atherosclerosis.

Materials and Methods
Study Design and Participants
The ACT NOW study design, along with exclusion or inclusion criteria, has been previously described.[5,20] In brief, ACT NOW was a prospective, randomized, double-blind, placebo-controlled trial to examine the effectiveness of pioglitazone in prevention of T2DM and progression of CIMT. A separate major goal was to investigate mechanisms underlying these outcomes. Participants included adults (≥18 years) with impaired glucose tolerance (IGT), as defined by a 2-hour plasma glucose concentration of 140 to 199 mg/dL during a 75 g, 2-hour oral glucose tolerance test (OGTT). In addition, all IGT subjects had fasting plasma glucose (FPG) of 92 to 125 mg/dL, were overweight, and had at least 1 other risk factor for T2DM. Participants were followed on trial for up to 48 months, with a mean and median follow-up time of 2.3 years. Atherosclerosis measures were performed at 7 of the 8 academic medical centers that had the capacity to conduct carotid studies, and was approved by the institutional review boards at each site. All participants gave written informed consent.

Measurement of CIMT
High-resolution B-mode carotid artery ultrasound (Logiq, GE Medical Systems, Milwaukee, WI) was used to image the far wall of the right distal common CIMT at baseline, midstudy (15–18 months after baseline), and at the end of study or at the time of diagnosis of diabetes mellitus to examine the rate of change in CIMT. In a small number of participants, an additional scan was performed at some point during study follow-up. All readable scans were included in the final dataset to increase the ability to assess linearity of CIMT change during the follow-up period. A detailed description of the standardized protocol[21] is provided online (Material and Methods in the online-only Data Supplement).

Other Measurements
Participants underwent baseline and annual 2-hour 75 g OGTT, after an 8-hour overnight fast. A frequently sampled intravenous glucose tolerance test (FSIVGT) was performed at baseline and at the end of the study, at 4 centers that had the capability of performing that test.[5,22] In addition, HbA$_{1c}$ levels were measured every 6 months. Plasma levels of lipids and novel risk factors, including adiponectin, interleukin-6, C-reactive protein, leptin, plasminogen activator inhibitor-1, monocyte chemotactic protein-1, and tumor necrosis factor-α, were measured at baseline and at the end of the study. Insulin sensitivity from OGTTs was calculated as the Matsuda index.[23] Insulin sensitivity from FSIVGTs was calculated using the Minimal Model.[24] Additional detail for other measurements, laboratory methods, definitions of variables, and calculated variables is provided online (Material and Methods in the online-only Data Supplement).

Statistical Analysis
Baseline group comparisons were assessed with unpaired *t* tests for normally distributed variables, Mann–Whitney *U* test for variables with skewed distributions, and χ^2 tests for proportions. General linear mixed-effects models were used to estimate the annualized rate of change in CIMT from sequential measurements of CIMT during the study. We considered random subject effects for intercept and slope of the time variable, which reflected individual subject difference from the group mean for baseline CIMT and rate of CIMT progression, respectively. As indicated by likelihood ratio tests, both of these random subject effects were needed and were included in the models presented. The interaction between treatment effects and risk factors was tested with single 3-way multiplicative interaction (and lower level factors) between follow-up time, treatment, and individual covariate. However, none of these 3-way interaction terms were significant and were not included in the final models. Final models included an interaction term between treatment and follow-up time to test whether treatment modified CIMT progression over follow-up time, after sequential adjustment for increasing numbers of risk factors. Multicolinearity between explanatory variables was reduced by centering the variables by subtracting the mean from individual values. Data for participants who developed diabetes mellitus were censored at the time of diabetes mellitus development. We analyzed the data using several different methods. These included (1) the primary analysis by intention-to-treat to estimate the effect of randomization to treatment, (2) treatment received in successive intervals between CIMT scans to investigate the effect of pioglitazone with optimal adherence, and (3) an analysis by treatment received, after excluding 21 participants who did not adhere to study drug. Reasons for nonadherence were weight gain (n=2), shortness of breath (n=1), edema (n=3), other adverse events (n=9), reasons unrelated to study medication, lack of interest, and work or schedule conflicts (n=6).

Cardiovascular risk factors (explanatory variables) were also examined in several ways. First, they were considered as time-varying covariates. Second, we fitted models with baseline variables and their change during the study. Third, we compared the pioglitazone effect in subgroups defined by extent of change in risk factors (follow-up − baseline values) divided at 0 or at the median change. There was no evidence for heterogeneity of the response according to subgroups.

All models were fitted to same dataset with no missing values for any covariates, and include 324 persons and 818 combinations of persons and CIMT scan times. Corresponding models fitted to this smaller dataset with complete measured or estimated values for covariates and to the larger dataset with some missing covariates (n=1006 combinations of persons and CIMT scan times) provided similar results. Statistical analyses were conducted with SAS version 9.2 (SAS Institute, Cary, NC). Additional detail for handling of missing data is provided online (Material and Methods in the online-only Data Supplement). A 2-sided *P*-value of <0.05 was considered significant. However, to reduce the type-1 error associated with multiple comparisons, we used *P* values adjusted by the Bonferroni method (*P* value/number of comparisons) as our reference for comparison.

Results
The current analysis included 382 individuals of the total of 602 ACT NOW participants, with baseline and follow-up

Table 1. Baseline Characteristics

	Placebo (n=194)	Pioglitazone (n=188)	P Value
Age, y	53 (12)	54 (11)	0.185
Men	85 (44)	90 (48)	0.426
Race/ethnicity			
White vs. others	112 (58)	96 (51)	0.191
Black	21	24	
Hispanic	56	59	
Mixed	5	9	
Ever smoker	93 (48)	95 (50)	0.522
Current smoker	17 (9)	20 (11)	0.643
Prior CVD	17 (9)	23 (13)	0.268
Antihypertensive agents	72 (37)	82 (44)	0.195
ACE, ARB	21	22	
Diuretic	10	21	
β-blocker	15	11	
Calcium channel blocker	16	16	
Other	10	12	
Lipid-lowering agents	57 (29)	68 (36)	0.157
Statin	49	61	
Fibrate	7	3	
Niacin	0	2	
Other	1	2	
Aspirin	35 (18)	31 (16)	0.688
CIMT, mm ($\times 10^3$)	7.59 (1.57)	7.58 (1.54)	0.959

CIMT indicates carotid intima media thickness; ACE, anigotensin-converting enzyme inhibitors; and ARB, angiotensin II receptor blocker.

Means (SD) and numbers (%) are presented.

measurements of CIMT. Nonparticipants (220 individuals) were significantly younger, more obese, and had higher blood pressure, fasting insulin, and 2-hour OGTT glucose values than participants (Table I in the online-only Data Supplement). However, as shown in Tables 1 and 2, among CIMT participants, there were no significant differences at baseline between the 188 individuals randomized to pioglitazone compared with the 194 in the placebo group.

As shown in Table 2, a large number of variables were increased or decreased at the end of the study in the pioglitazone group compared with the placebo group. However, after Bonferroni adjustment, among the changes in the 23 variables shown in Table 2, only the change in body mass index, waist circumference, HDL-C, fasting and 2-hour glucose, fasting insulin levels, HbA_{1c}, Matsuda index, adiponectin, and plasminogen activator inhibitor-1 are considered significantly different ($P<0.002$) between pioglitazone and placebo groups. Furthermore, the percentage of participants with actual improvements in fasting glucose and insulin values, Matsuda index, adiponectin, and plasminogen activator inhibitor-1 was significantly higher in the pioglitazone group compared with placebo treatment (Figure I in the online-only Data Supplement). These changes in risk factors did not appear related to use of concomitant medications (eg, antihypertensive, lipid-lowering, and aspirin

agents), as use of these medications increased similarly in both groups (data not shown).

To determine whether the favorable effect of pioglitazone on CIMT progression was mediated by beneficial changes in potential mediators during the study, we performed a series of multivariable mixed models. As shown in the Figure, in the unadjusted model (Model 1), the mean annualized rate of CIMT progression in individuals randomized to pioglitazone was 4.76×10^{-3} mm/year (95% CI, 2.39×10^{-3}–7.14×10^{-3} mm/year), only 49% of the rate observed in individuals randomized to placebo (9.69×10^{-3} mm/year; 95% CI, 7.24×10^{-3}–12.15×10^{-3} mm/year; $P<0.01$). After adjustment for all of the putative mediators of this difference in CIMT from Table 2 (Models 2–4), and for concomitant medications (Model 5), the annualized rate of CIMT progression during pioglitazone treatment remained 53% of the rate in the absence of pioglitazone (5.86×10^{-3} versus 10.96×10^{-3} mm/year; $P<0.01$). Similar results were obtained in a parsimonious model (Model 6) that was fitted, including only predictors with $P<0.1$ in the full model (Model 5). Parameter estimates for all variables included in the final parsimonious Model 6 are provided online (Table II in the online-only Data Supplement). The numerical differences between pioglitazone treatment and placebo were similar in the unadjusted ($9.69–4.76=4.93\times10^{-3}$ mm/year), fully adjusted ($10.96–5.86=5.10\times10^{-3}$ mm/year), and parsimonious ($10.88–5.64=5.24\times10^{-3}$ mm/year) models. The models fitted with baseline CVD risk factors and their change during the study yielded similar results. To evaluate the potential antiatherosclerosis mechanisms of pioglitazone under conditions of optimal adherence, we also analyzed the data based on treatment actually received with study drug, included in the models as a time-dependent variable. We also examined the data after excluding the 21 subjects, who did not fully comply with the pioglitazone treatment protocol. The results did not change with either of these additional analyses, demonstrating the robustness of the results. Furthermore, we explored whether the effect of pioglitazone on CIMT progression was modified by the direction of change in risk factors. After adjustment for age, sex, race and ethnicity, study site, and history of CVD at baseline, pioglitazone reduced CIMT-progression rates to similar degrees, regardless of whether risk factors improved or worsened (Figure II in the online-only Data Supplement). Thus, we were unable to identify attenuation of the effects of pioglitazone on CIMT progression by any of the putative mediators that we examined.

Discussion

In this study, the annualized rate of CIMT progression in individuals with IGT was reduced by nearly half during pioglitazone treatment. Pioglitazone also improved metabolic and inflammatory parameters that are known risk factors for atherosclerosis and, thus, potential mediators of the effect of pioglitazone on CIMT. However, none of these beneficial changes in risk factors, individually or in combination, accounted for the slower rate of CIMT progression in the pioglitazone group. These findings suggest that the effects of pioglitazone on CIMT progression are mediated either directly on the arterial wall or possibly through less

Table 2. Risk Factors at Baseline and End of the Study

	Placebo		Pioglitazone		P Values for Change Between Groups
	Baseline	Absolute Change	Baseline	Absolute Change	
BMI, kg/m^2	33.40 (5.60)	0.09 (1.88)	32.80 (4.80)	1.56 (2.03)	<0.001*
Waist, cm	106 (13)	1 (6)	105 (12)	4 (6)	<0.001*
Systolic BP, mm Hg	127 (17)	−2.33 (15.20)	125 (16)	0.07 (14.98)	0.121
Diastolic BP, mm Hg	73 (11)	−0.52 (9.17)	73 (9)	−1.88 (9.47)	0.165
Total cholesterol, mg/dL	173 (33)	1 (33)	167 (33)	2 (31)	0.771
LDL-C, mg/dL	107 (30)	−1 (30)	103 (30)	−2 (29)	0.627
HDL-C, mg/dL	41 (10)	3 (8)	40 (10)	7 (9)	<0.001*
Triglycerides, mg/dL	112 (84–154)	−4 (57)	107 (84–146)	−10 (52)	0.12
Total cholesterol/HDL-C	4.4 (1.2)	−0.29 (0.99)	4.4 (1.2)	−0.59 (1.06)	0.004
Triglyceride/HDL-C	3.3 (2.0)	−0.34 (1.88)	3.3 (2.1)	−0.67 (1.54)	0.057
Fasting glucose, mg/dL	105 (8)	−5 (13)	105 (12)	−10 (11)	<0.001*
2-hour glucose, mg/dL	169 (19)	−7 (37)	170 (22)	−26 (37)	<0.001*
HbA$_{1c}$, %	5.4 (0.4)	0.19 (0.36)	5.5 (0.4)	0.08 (0.32)	<0.001*
Fasting insulin, mU/L	8.1 (4.2–13.5)	0.96 (6.80)	7.8 (4.1–12.7)	−3.67 (8.85)	<0.001*
Matsuda index	3.3 (2.1–5.5)	1.52 (3.90)	3.0 (2.1–5.1)	3.98 (4.56)	<0.001*
SI	2.4 (1.7–3.3)	0.68 (2.70)	2.3 (1.6–3.4)	2.22 (6.42)	0.019
Adiponectin, μg/mL	10 (7–15)	2 (9)	10 (6–14)	29 (35)	<0.001*
Leptin, pg/mL	29 (14–47)	2 (12)	27 (14–43)	6 (17)	0.005
CRP, mg/L	2.0 (0.9–4.2)	0.06 (5.88)	1.9 (0.6–4.6)	−0.38 (6.56)	0.508
IL-6, ng/mL	1.5 (0.9–2.1)	0.46 (3.08)	1.3 (0.9–2.2)	−0.77 (7.57)	0.057
PAI-1, ng/mL	14 (10–20)	1.83 (10.38)	13 (9–19)	−2.19 (8.18)	<0.001*
MCP-1, pg/mL	128 (97–165)	9 (38)	136 (100–173)	12 (44)	0.556
TNF-α, pg/mL	3.8 (2.9–4.7)	0.52 (1.24)	3.8 (2.9–5.1)	0.16 (1.61)	0.024

BMI indicates body mass index, calculated as weight in kilogram divided by height in m^2; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA$_{1c}$, glycosylated hemoglobin; SI, insulin sensitivity index determined from a frequently sampled intravenous glucose tolerance test; CRP, C-reactive protein; IL-6, interleukin 6; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocyte chemotactic protein-1; and TNF-α, tumor necrosis factor-α. Values are mean (SD) or median (25th–75th percentiles). No statistically significant differences were identified between the groups at baseline. Paired and unpaired *t* tests were used to compare differences of log-transformed values of absolute change ([follow-up−baseline]+a constant number to eliminate negatives and 0's) within and between groups, respectively. Absolute change was statistically significant for all variables within each group. *After Bonferroni adjustment, P values ≤0.002 are considered significant. Fifty to 60 subjects had missing values for cytokines and SI.

well-established or yet to be identified mediators that were not measured in this study.

Several treatment-induced changes in measured risk factors in our study were in line with pioglitazone-induced changes reported in the CHICAGO[18] and PERISCOPE[19] trials. These studies reported higher HDL-C, and lower triglyceride/HDL-C ratio, C-reactive protein, and insulin levels, as well as slower rates of CIMT or coronary atherosclerosis progression with pioglitazone therapy, respectively. In these trials, the increase in HDL-C and decrease in triglyceride/HDL-C ratio largely explained the effect of pioglitazone on atherosclerosis progression. In contrast, accounting for changes in these and other lipids did not attenuate the estimated effect of pioglitazone in our study. This was also the case in the TRIPOD study of an earlier TZD, troglitazone.[25]

This apparent difference between our results and prior work relating changes in risk factors because of TZDs and CIMT progression may be related to differences in participant characteristics. Although PERISCOPE and CHICAGO trials studied patients with overt T2DM, both ACT NOW and TRIPOD study enrolled nondiabetic individuals. Not surprisingly, given the earlier stage of disease, ACT NOW participants had higher HDL-C levels and lower triglyceride levels at baseline, than did subjects in the PERISCOPE and CHICAGO studies.[18,19] Moreover, subjects in the ACT NOW study appeared to respond relatively well to the lifestyle changes recommended to all subjects. For example, over the course of the study, participants in the placebo group demonstrated no increase in body mass index, a trend for lower blood pressure, a 10% increase in HDL-C, a modest decline in triglyceride levels, and a trend for improved insulin sensitivity.[5] These favorable changes in many of the standard risk factors in the placebo group, particularly the increase in HDL-C, presumably helped reduce the difference in changes in several risk factors between pioglitazone and placebo groups. As shown in Figure I in the online-only Data Supplement, there were relatively similar numbers of participants with improved lipid profiles in both groups. This may have diminished the ability to detect a pioglitazone effect on CIMT that could be attributed to improvements in these lipid risk factors.

Pioglitazone is an agonist of the peroxisome–proliferator-activated receptor (PPAR-γ) and causes marked improvement
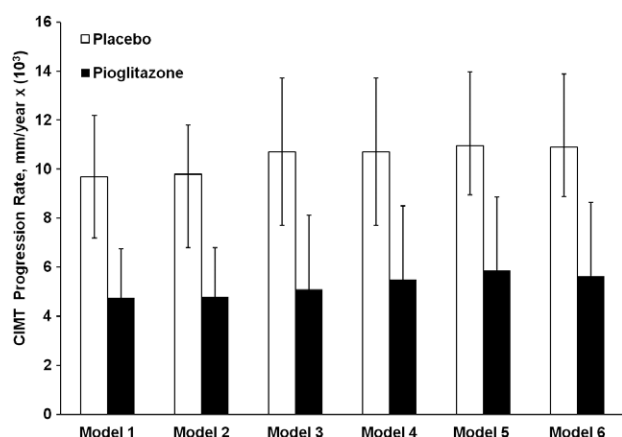
Figure. Annual carotid intima media thickness (CIMT) progression rates, mm/year ×10³ by intention to treat groups. White bars represent those randomized to placebo. Black bars represent those randomized to piogitazone treatment. Error bars represent 95% CI. The difference in progression rate between treatment groups was significant in all models ($P < 0.01$). All models include random subject effects representing individual differences from the group mean for baseline CIMT and rate of CIMT progression. Model 1: Unadjusted. Model 2: Adjusted for age, study center, sex, race/ethnicity (non-Hispanic Whites vs others) and prior cardiovascular disease (CVD). Model 3: Adjusted for Model 2+HbA$_{1c}$, systolic and diastolic blood pressure, body mass index, triglyceride/high-density lipoprotein cholesterol (HDL-C) ratio, and Matsuda index. The results were not different when adjusted for other lipid values, such as total cholesterol/HDL-C ratio, or total cholesterol, triglycerides, low-density lipoprotein cholesterol, and HDL-C, individually or in combination. Model 4: Adjusted for Model 3+adiponectin, leptin, C-reactive protein, tumor necrosis factor-α, interleukin-6, monocyte chemotactic protein-1, plasminogen activator inhibitor-1. Model 5: Adjusted for Model 4+concomitant medications (lipid-lowering agents, antihypertensives, and drugs containing salicylic acid; all parameterized as yes vs no). Model 6: Parsimonious model fitted, including all covariates from Model 5 that were $P<0.1$ (age, study center, prior CVD, HbA$_{1c}$, and total cholesterol/HDL-C ratio).

in glucose homeostasis and insulin sensitivity in patients with T2DM and IGT.[5,26] However, PPAR-γ is also abundantly expressed in endothelial cells, vascular smooth muscle cells, and monocytes/macrophages, providing a pathway for direct antiinflammatory, antioxidant, and other protective actions of pioglitazone on the vasculature.[9,27–32] In vitro and in vivo studies demonstrate that TZD-mediated PPAR-γ receptor activation improves insulin signaling in vascular cells that favors antiatherogenic properties in these cells.[6,33] Improvements in insulin signaling increases endothelial cell nitric oxide generation, decreases smooth muscle cell migration,[34] and reduces macrophage uptake of modified low-density lipoprotein cholesterol and formation of foam cells.[33,35] Data from animal models have demonstrated that TZDs may slow development and progression of atherosclerosis, and this was not accounted for by improvements in metabolic factors.[10–12,36] Thus, there is substantial evidence that TZDs may have direct antiatherogenic effects on the vasculature.

Despite demonstration of the many improvements in CVD risk factors in trials of TZDs, their use has not consistently translated into improved cardiovascular outcomes in clinical studies in T2DM. For example, compared with pioglitazone, rosiglitazone was associated with increased risk of CVD

events and all-cause mortality.[37] Discordant results between TZD studies may be explained by differential effects of individual TZD medications on cardiovascular risk profiles,[8,38] or may reflect greater benefit of these agents in earlier stages of atherosclerosis. However, although not all TZDs are cardioprotective, pioglitazone has been shown to slow the progression of carotid and coronary atherosclerosis in CHICAGO, PERISCOPE, and PIPOD studies,[15,19,39] and to effectively reduce the secondary composite cardiovascular end point in the PROactive study, although the reduction in risk of primary composite cardiovascular end point was statistically not significant.[13]

However, given the known side effects of TZDs (eg, weight gain, edema and congestive heart failure, and atypical fractures) and potential for other adverse events (eg, bladder cancer), the risk/benefit ratio should be thoughtfully evaluated before considering off-label use of pioglitazone in individuals with prediabetes. Importantly, identifying the direct vascular mechanisms by which pioglitazone reduces atherosclerosis may help refine targets for new therapeutic approaches with TZD-like, or other, medications. This is particularly relevant, as recent work has revealed that many PPARγ-based drugs have a separate biochemical activity. These agents lack the classical transportation agonism, but have potent anti-diabetic activity by blocking the obesity-linked phosphorylation of PPARγ by cyclin-dependent kinase 5.[40] This has stimulated development of newer compounds that appear to retain many of the beneficial effects of TZDs, but may not have the side effects also associated with these agents.

Strengths of this study include a careful (and adequately powered) assessment within a randomized trial of a broad array of standard and novel CVD risk factors that could mediate the favorable effect of pioglitazone on CIMT progression. In addition, results were robust and consistent, regardless of the different statistical methods we used to analyze the data. Furthermore, studying individuals with prediabetes, who are at an earlier stage in the spectrum of diabetes mellitus and vascular disease, may have facilitated discerning the metabolically independent effects of pioglitazone. Conversely, there are some limitations. There were several statistically significant differences in characteristics between CIMT study participants and nonparticipants. There were more morbidly obese individuals among nonparticipants, which contributed to the few metabolic differences seen between participants and nonparticipants. We therefore cannot exclude the possibility that the magnitude of the pioglitazone effect on CIMT progression may have been slightly different, if it had been possible to include all ACT NOW participants. However, most importantly, there were no significant differences at baseline between the 2 treatment arms in our study. Second, there are undoubtedly other potential individual or combinations of mediators that we did not measure. Yet, given the magnitude of the pioglitazone effect on CIMT progression and the modest role for many of the best-characterized risk factors currently recognized, it seems unlikely that any currently known plasma risk factor would account for the observed differences in CIMT progression. Finally, the use of surrogate markers, such as CIMT, as a measure of cardiovascular benefit of piogltazone

remains uncertain. Importantly, inhibition of atherosclerosis by pioglitazone has been demonstrated in multiple studies that assessed atherosclerosis by a variety of methods and support the notion that the favorable effects of pioglitazone on risk factors and atherosclerosis may translate into reduced CVD events. Particularly important in this instance is the PERISCOPE study which, as discussed above, found very similar beneficial effects on atherosclerosis progression, with direct measurement of the coronary arteries.[16,19]

In summary, the current analysis of the ACT NOW study examines changes in standard and novel risk factors as potential mediators of vascular effects of pioglitazone in IGT. The results indicate that in this sample of individuals with prediabetes, pioglitazone-induced changes in lipids, glucose metabolism, and inflammatory markers do not account for its effective inhibition of atherosclerosis progression. These findings provide additional support for the concept that pioglitazone may have direct vascular-protective effect.

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Online supplemental material

Methods

Other Measurements

As part of the baseline examination, demographic information (age, gender, race/ethnicity) and presence of traditional risk factors including prior CVD, tobacco use and information for concomitant medications were collected. Blood pressure, waist circumference, weight, and height were measured as previously described.[5, 20] Participants returned every 2 months during the first year, and every three months thereafter. At each visit, information about concomitant medications and heath status was updated and weight, blood pressure, and fasting plasma glucose were measured.

Carotid Intima-Media Thickness standardized protocol

A standardized protocol was followed by all centers to assure quality control for CIMT measurements. Prior to initiating the study, all ultrasound technologists were trained at the coordinating center, directed by Dr. Howard Hodis at the University of Southern California, to ensure uniformity of measurement amongst centers. An image of the far wall of the common carotid was obtained and CIMT scans were sent to the coordinating center, where they were read without knowledge of drug assignment by two readers, as previously described.[20,21] In a subset of subjects, a second measurement was performed within 2 weeks of the initial scan to determine reproducibility and technologist performance. The coefficient of variation of these two measurements was 0.72% (range = 0.14%–0.90%). CIMT measurements were performed at baseline, mid-study (15 to 18

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months after baseline) and at the end of study or at the time of diagnosis of diabetes to examine the rate of change in CIMT.

Laboratory Methods

The following analyses were performed in the Central Laboratory (Texas Diabetes Institute, San Antonio): plasma glucose by the glucose oxidase method (Beckman, Fullerton, CA); HbA_{1c} by ion-exchange HPLC (Bayer DCA 2000, Leverkusen, Germany); and plasma cholesterol and triglycerides by a combination of CHOD-DAOS method (Wako, Richmond, VA) and an enzymatic assay (Stanbio Laboratory, Boerne, TX). HDL-cholesterol was measured after precipitation of apolipoprotein B-containing lipoproteins (Wako, Richmond, VA). Levels of high sensitivity CRP were determined with ELISA kit (ALPCO Diagnostics, Salem, NH), with intra-assay and inter-assay CVs ranging from 5.5 to 6% and from 11.6 to 13.8% respectively. Novel risk factors, including PAI-1, total adiponectin, IL-6, TNF-α, leptin, and MCP-1 were measured by Luminex xMAP technology, ELISA based immunoassays conducted on the surface of fluorescent-coded beads that yield an inter-assay coefficient of variation (CV) of < 21% and intra-assay CV% ranging between 1.4% to 7.9% (Millipore, Billerica, MA).

Handling of Missing Data

In order to take advantage of the mid-study scans in the analyses of time-dependent covariates as mediators, mid-study values of lipids were estimated as the average of baseline and end-of-study lipids. Because we expected changes in inflammatory markers and measures of insulin sensitivity to be achieved relatively early after treatment onset, we estimated mid-study values of these biomarkers with the end-of

2

study values. For 57 persons, baseline values of HbA_{1c} were missing, for these persons we estimated baseline HbA_{1c} with the first available measurement of HbA_{1c}. In parallel models we estimated missing covariates by carrying prior measurements forward, by carrying later measurements backwards, or by averaging available measurements.

Variable Definitions and Calculated Values

Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. LDL-C was calculated by Friedewald equation. Due to smaller sample sizes of non-Hispanic White groups and similarities in their response to pioglitazone, race and ethnicity were described by a variable that contrasted non-Hispanic Whites versus all others (Hispanic Whites, African Americans, Asians or those of mixed races).

3

Supplemental Figure Legends

Supplemental Figure I

Frequency of participants with improved risk factors at the end of the study.

Placebo (white bars), Pioglitazone (black bars); *After Bonferroni adjustment, *P*-values

< 0.003 for differences between groups are considered significant. Abbreviations: BMI,

body mass index; HDL-C, high density lipoprotein cholesterol; TC/HDL-C, total

cholesterol/HDL-C ratio; TG/HDL-C, triglyceride/HDL-C ratio; G0, fasting glucose; G120,

2-hour glucose; HbA_{1c}, glycosylated hemoglobin; SI, insulin sensitivity index by

frequently sampled intravenous glucose tolerance test; IL-6, interleukin 6; PAI-1,

plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor-α.

Supplemental Figure II

Annual CIMT progression rate during pioglitazone treatment as compared with placebo

and stratified by change (decrease or increase) in risk factors. Placebo is set at zero

CIMT progression. All models are adjusted for fixed effects age, gender, race/ethnicity

(non- Hispanic Whites vs. Others), study site and prior CVD. Models also included

random subject effects representing subject differences in baseline CIMT and rate of

CIMT progression. Abbreviations: BMI, body mass index ; HDL-C, high density

lipoprotein cholesterol; G0, fasting glucose; G120, 2-hour glucose; HbA_{1c}, glycosylated

hemoglobin; SI, insulin sensitivity index by frequently sampled intravenous glucose

tolerance test; IL-6, interleukin 6 ; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumor

necrosis factor-α.

O Decrease in risk factor during treatment   ▲Increase in risk factor during treatment

4

Supplemental Table I. Baseline Characteristics for CIMT Cohort and other ACT Now Participants without CIMT Scans

	CIMT Cohort (n=382)	Others without CIMT (n=220)	*P*-value
Age, years	53 (12)	50 (12)	<0.001*
Men	175 (46)	78 (35)	0.013
Race / Ethnicity			
White vs. Others	208 (54)	119 (54)	0.932
Black	45	55	
Hispanic	115	38	
Mixed	14	8	
Ever smoker	188 (49)	108 (49)	0.420
Current smoker	37 (10)	23 (13)	0.944
Prior CVD	40 (10)	17 (8)	0.268
BMI, kg/m^2	33.1 (5.2)	36.5 (7.8)	< 0.001*
Waist circumference, cm	105 (13)	109 (16)	0.002
Systolic BP, mmHg	126 (16)	130 (16)	0.004
Diastolic BP, mmHg	73 (10)	76 (9)	0.001*
Total cholesterol, mg/dL	170 (33)	171 (37)	0.346
LDL-C, mg/dL	105 (30)	107 (31)	0.812
HDL-C, mg/dL	40 (10)	40 (11)	0.267
Triglycerides, mg/dL	110 (84-150)	101 (79-149)	0.219
HbA$_{1c}$, %	5.5 (0.4)	5.5 (0.4)	0.178

Fasting glucose, mg/dL	104 (10)	103 (8)	0.392
2-hour glucose, mg/dL	169 (19)	164 (19)	0.001*
Fasting insulin, mU/L	7 (4-13)	9 (5-16)	<0.001*

Mean (SD), median (25%-75%) and numbers (%) are presented. Abbreviations: CVD, cardiovascular disease; BMI, body mass index, calculated as weight in kilograms divided by height in meters squared; BP, blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; HbA_{1c}, glycosylated hemoglobin; *After Bonferroni adjustment, P-values < 0.002 are considered significant.
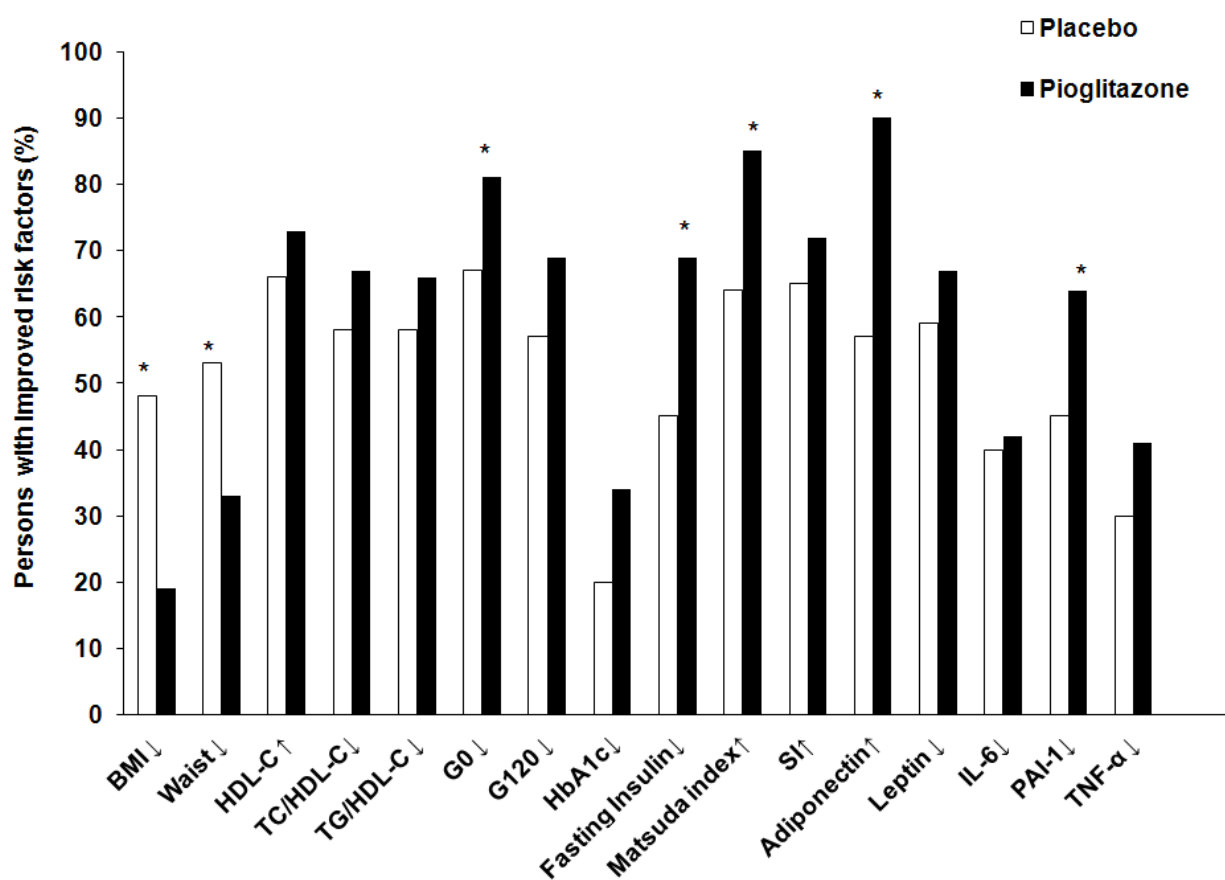
Supplemental Table II. Coefficients, Standard Errors, Confidence Intervals and *P*-values for the Final Parsimonious Linear Mixed Model Estimating Annual Rate of CIMT Progression, mm/year x (10^3)

Effect	Coefficient	SE	95% CI Lower	95% CI Upper	*P*-value
Time (years)					
In pioglitazone group	5.64	1.31	3.06	8.23	< 0.001
In placebo group	10.88	1.29	8.33	13.43	< 0.001
Age, years	6.44	0.75	4.97	7.90	<0.001
Prior CVD	42.44	21.65	-0.15	85.03	0.051
BMI, kg/m^2	0.47	0.58	-0.68	1.62	0.421
Total cholesterol / HDL-C	2.99	1.42	0.19	5.78	0.036
HBA$_{1c}$, %	-7.88	3.69	-15.15	-0.62	0.033

Models also include random subject effects representing subject differences in baseline CIMT and rate of CIMT progression, study center as a categorical variable (*P* =0.033), and interaction of follow-up time and treatment (*P* = 0.003). Abbreviations: CVD, cardiovascular disease; BMI, body mass index; HDL-C, High density lipoprotein cholesterol; HbA$_{1c}$, glycosylated hemoglobin.

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Supplemental Figure I

Supplemental Figure II

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