The Effect of Pioglitazone on Adiposity, Adiponectin and Carotid Artery Intimal Thickness in Obese but Otherwise Healthy Minority Subjects

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We examined the hypothesis that the PPAR Gamma activator, Pioglitazone, has a fat depot specific effect and aimed at determining the effects of pioglitazone on changes in total and regional fat distribution, serum adiponectin levels, carotid artery intimal thickness, and subcutaneous adipose tissue histology in otherwise healthy obese men and women from a minority population.
Materials and Methods: This is a double-blind, randomized, controlled trial, single site study of the minority population in south central Los Angeles. Thirty-five obese, but otherwise healthy, men and women (waist-to-hip ratio >0.95 for men and >0.85 in women) were randomly assigned into three groups: Group A: placebo; group B, pioglitazone 30 mg/day, and group C, pioglitazone 45 mg/day, for a duration of 6 months. The primary outcome measures were changes in visceral fat as measured by Computerized Tomography scan (CT Scan) of the abdomen and thigh, body composition by Dual energy X-ray Absorptiometry Scan (DXA scan) and carotid artery intima-media thickness as measured by ultrasound. Serum adiponectin levels and the size and number of adipocytes were also measured.
Results: At baseline, mean age, proportion of female participation, race and ethnicity distribu-tion, blood pressure, Body Mass Index (BMI) and anthropometric measures of subjects in the three groups were not statistically different. After 6 months, we observed significant decrease of intramuscular fat and significant increase of subcutaneous thigh fat in groups B and C as compared to group A. The average carotid artery intimal thickness increased in group A (p<0.01), but not in group B, and decreased in group C (p<0.01). Serum adiponectin levels rose sharply (p<0.001) in the treatment groups only. A significant positive correlation was observed between the change of the adiponectin levels and subcutaneous thigh fat.
Conclusion: Results of this study supports the use of pioglitazone in the prevention of stroke and peripheral vascular disease in high-risk populations.

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Introduction

Pioglitazone is a Peroxisome proliferator-activated receptor gamma (PPARγ) agonist and has been shown to exert several key metabolic effects. The activation of PPARγ is associated with a depot-specific adipose tissue redistribution,[1,2] an increase in serum adiponectin levels,[3,4] and an anti-inflammatory

effect by suppression of the Tumor Necrosis Factor Alpha (TNFα).[5] Peroxisome Proliferator Activated Receptor (PPAR) gamma nuclear receptors are mostly expressed in adipose tissue[6] compared to skeletal muscle,[7] and increase glucose uptake when activated.[8]

The visceral accumulation of fat is strongly associated with increased risk for cardiovascular disease and type 2 diabetes mellitus.[9-12] Even in people of nearly normal weight, high levels of central or visceral obesity are associated with increased risk of diabetes, hypertension, and coronary artery disease.[13] More recent data suggest that the risk of cerebrovascular disease also increases with a waist-to-hip ratio >0.95.[12] Accumulation of fat in the intra-abdominal tissue, particularly those drained by the portal circulation, lead to high portal vein free fatty acid concentrations.[14] Free fatty acids interfere and compete with glucose uptake in the liver and muscle and leads to development of hyperinsulinemia, insulin resistance and fatty liver.[14] Evidence confirms that PPAR γ activators not only improve insulin sensitivity, but also increase adiponectin;[15] it also reduces intra-abdominal fat mass by a depot-specific, effect in Zucker rats and in humans,[16,17] and decreases intimal thickness in carotid arteries in patients with type 2 diabetes mellitus.[18]

Although much is known about the effect of pioglitazone in subjects with type 2 diabetes, the data on the effects of pioglitazone on adipose tissue, adipose depot, and on carotid artery intimal thickness in healthy obese subjects is relatively limited. Furthermore minority populations represent the highest percentage of obese people in US and very limited data is available on this specific target population.[19]

In this study, we examined the fat depot specific effect of pioglitazone in an obese, but otherwise healthy, minority population; we also examined the effect of pioglitazone on carotid artery intimal thickness and its relationship with adiponectin.

Materials and Methods
Study Design

This is a double-blind, randomized, controlled trial, single site study of a minority population in south central Los Angeles. Eligible subjects were randomly assigned in a 1:1:1 ratio to receive, placebo, 30 and 45 mg pioglitazone respectively, once a day, for a 6-month period. The randomization was made by random number (between 1 to 100) generated for each treatment group (e.g. random numbers of 1, 3 7, 9, 34 etc were allotted for enrollees 1, 3, 7, 9 and 34 and they received treatment A; similarly for groups B and C). Each subject, based on their enrollment number, was matched for the treatment number and no attempt was made to match between groups at enrollment. Subjects were between 18 to 55 years old and free of chronic diseases such as diabetes, Chronic Obstructive Pulmonay Diseases (COPD), coronary artery disease, and cancer. Obesity was defined as BMI above ≥ 30 (body weight per kg / height in m2), waist-to-hip ratio >0.95 in male and >0.85 in females. Subjects with uncontrolled hypertension (blood pressure>159/99 mmHg) were excluded; however, those with stage I hypertension (140-159/90-99 mm Hg) or those with controlled hypertension (<140/90 mmHg) and on a single antihypertensive medication were eligible to enter into the study. Monthly tablet counts were used to confirm adherence to medications. Research subjects were seen every month and side effects were recorded.

Subjects received diet counseling and were requested to not engage in heavy exercise or sudden change of diet for the 6 month study period. A 3-day food record was obtained at baseline and every 2 months till the end of the study to ensure there were no drastic changes in diet. At baseline and at the end of the 6 month study period, body composition was measured by anthropometry and DXA scan. Subcutaneous and visceral fat accumulations were measured by CAT scan of the abdomen obtained transversally, at levels of L1-L2, L3-4, and L5-S1, and at mid-thigh using standard proto-

col. All scans were read by the same person using slice-o-matic software. The display field was used to scale the image pixels for analysis and demarcate regions of interest. Carotid artery intimal thickness was measured at the common carotid artery 2 cm below bifurcation with use of the Holog ultrasound. The average and maximum thickness over the 2 cm length was measured. Average thickness was calculated, based on the 20 measured areas of the lateral wall of each carotid artery. Serum adiponectin levels were measured by ELISA method at baseline and at monthly intervals until the end of the study (AdipoGen), with a coefficient of variation of 4.8%. We calculated the homeostasis model assessment of insulin resistance (HOMA-IR) according to the following equation: insulin (U/ml) x fasting glucose (mmol/liter)÷22.5.[20] Whole body and regional body composition were evaluated by DXA scanning. The scanners were calibrated by using a soft tissue standard.

Subcutaneous fat biopsies were taken from the abdominal wall and thigh, prior to, and at the end of, the study. Biopsies were performed using 2% lidocaine, with a 0.5 cm incision and use of a skin punch biopsy (medium size), through the incised skin and obtaining fat by scooping from the tissue. A portion of the biopsy was immediately stored at -80C; the remaining tissue was placed in 4.5% formaldehyde for four days, then in concentrated sucrose, followed by embedding in a polyacryl block and sectioned. Cell counts were performed on each sample by a single person (VA). All cells in all available fields were counted in each subject and cell number was adjusted for the square microns. The data from abdomen and thigh were combined.

The study was approved by Institutional Review Board committee at Charles R. Drew University.

Outcome Measures

The primary outcome measurements were the change in the ratios of visceral-to-subcutaneous fat in the abdomen and in the thigh, and common carotid artery intima-media thickness, 2 cm below carotid bifurcation. The visceral and subcutaneous fat in abdomen and thigh was adjusted for each subject's body mass index (BMI), by dividing the sum of the surface of the target adipose tissue (visceral abdomen, intramuscular thigh, subcutaneous thigh etc..) to the subject's BMI. The secondary outcome measure was changes in serum adiponectin levels.

Statistical Analysis

All data are presented as mean±standard error of mean. Comparison within groups A, B and C was performed by analysis of variance at baseline and after the end of the study period. The comparison between each group (before and at the end of the study period) was performed by the student paired t-test. Data were examined for normal distribution, prior to any statistical comparison. Multiple regression analysis at baseline, and during the study, was used to examine correlation between serum adiponectin levels and changes of fat depots and carotid artery intimal thickness. Adipose tissue surface area, both visceral and subcutaneous, was measured and adjusted for the individual's BMI (surface divided by BMI). For non-parametric variables, Chi square test was used, with $p<0.05$ being considered significant. Excel and SPSS software was used for graphs and statistical analysis. The sample size was based on the percent change in visceral fat cross sectional area, the primary outcome during the study. A sample size of 56 subjects (including 25% drop rate) provided 85% power to detect a 10% change in visceral fat measured.

Results
Characteristics of Patients

Out of the 47 subjects that entered the study, 7 subjects withdrew after the first

screening, and an additional 3 were lost at one week, one month, and two months after the first follow-up respectively. One patient was excluded from the study due to engaging in heavy exercise and losing over 20 pounds, and another patient because of heavy alcohol abuse and interruptive behavior.

At the end of the study period, we had complete pairs of tests for 35 subjects; 11 patients in group A, 15 patients in group B, and 9 patients in group C.

At baseline, there were no significant differences among the three groups in terms of age, the proportion of female participation, race and ethnicity distribution, (except other races participation), their blood pressure, fat distribution measured by DXA and CT scan, carotid artery intimal thickness, serum adiponectin levels and adipocyte number per field measured.

Table 1. Characteristics of patients at randomization according to treatment group

Characteristic	Group A	Group B	Group C	P Value
Number	11	15	9	
Age (YearM±SEM)	41.9±3	41.8± 2.5	41.5± 3.7	0.75
Female sex (%)	8 (88.8)	11(73)	6(66)	0.25
AA race (%)	45	40	33	0.25
Latina (o)s (%)	55	33	55	0.25
Other races (%)	0	27	12	0.01
BMI (kg/m^2) (18-25)	38.2±1.2	39.9± 1.7	40.61± 1.7	0.82
Abdominal circumference (cm)	114.4±2.3	120.9± 3	124.3±5.4	0.29
In females (< 80 cm)	115.8±3.1	118.4± 3.7	121.6± 6.7	0.73
In males (<94 cm)	110±1.2	127.7±3.3	130.5± 9.7	0.35
Waist to Hip ratio	0.93±0.01	0.95± 0.01	0.96± 0.02	0.61
In females (<0.95)	0.92±0.02	0.94± 0.01	0.93± 0.03	0.96
In males (<100 cm)	0.96±0.02	1.01± 0.01	1.0± 0.02	0.49
Right arm A circumference (cm)	38.9±0.9	39.3± 0.7	42.2± 1.86	0.11
Left arm Circumference (cm)	39±1.04	39.3± 0.7	41.8± 2.1	0.28
Left anterior arm fat thickness (mm)	25±3.3	33.7± 2.1	29.7± 2.3	0.08
Left posterior arm fat thickness (mm)	32.25±4.2	38.25± 1.7	35.06± 3.0	0.33
Suprailiac fat thickness (mm)	30.6±2.8	36± 2.1	37.5± 3.1	0.19
Fasting blood glucose (70-110 mg/dL)	78.3±2.68	90.4±2.2	84.4±3.21	0.01
				between A &B
HOMA Index (IR)	3.52±1.97	4.5±1.7	3.35±1.3	0.42
HOMA Beta cells index (%)	48±9.89	45.8±6	64.3±13	0.30
Total body fat by DXA (%)	43±2.4	41.2±1.25	41±2.7	0.76
Trunk fat by DXA (%)	40.8±1.7	39.1±0.9	38.5±2.6	0.63
Lower extremities fat by DXA (%)	44±1.9	41.8±1.3	43.3±2.5	0.70
Visceral BMI adjusted abdominal fat (mm^2/BMI)	2058±74.8	2330±205	2375±264	0.58
Intramuscular BMI adjusted thigh fat (mm^2/BMI)	521±40	445±37	437±56	0.40
SubQ BMI adjusted thigh fat (mm^2/BMI)	332±22	401±15	470±38	0.25
Visceral / total subQ fat ratio (adjusted)	0.51±0.02	0.56±0.02	0.54±0.02	0.46
IM/subQ fat ratio	1.35±0.15	1.06±0.09	0.99±0.16	0.19
Adiponectin levels (µg/mL)	6.0±1.2	6.8±1.1	9.1±4.1	0.73
Carotid artery intimal thickness (mm)	0.73±0.01	0.79±0.02	0.80± 0.02	0.32
Adipocyte numbers	62±7.7	60±4.5	57±5.6	0.81

A; For all reference range please refer to http://www.kidney.org/PROFESSIONALS/kdoqi/guidelines-updates/nut-appx07a.html and AR Frisancho New standards of weight and body composition by frame size and height for assessment of nutritional status of adults and the elderly. American Journal of Clinical Nutrition, Vol 40, 808-819, 1984; Group A received placebo, Group B received pioglitazone 30 mg once a day, and Group C received pioglitazone 45 mg a day.

Table 1 summarizes characteristics of the 35 subjects who completed the study. The mean age of subjects was 42±1.8 years; 75% were female, 45% were self-reported black and 42% Latinos and 12% were Asian or Caucasians. There was no significant difference between groups A, B, and C among variables measured (Table 1).

Changes during the Study Period

No significant changes was observed in BMI, anthropometric measures, total adiposity and percent adiposity (total and regional) as measured by DXA scan in the trunks or lower extremities in groups B and C, as com-

pared to group A. Visceral fat, measured by CAT scan, decreased in groups B and C, compared to group A, but not at a statistically significant level. However, in the thigh, there were significant increases in subcutaneous fat in group C compared to group A (Fig.1) and significant decrease in the intramuscular fat (Fig. 2). The ratio of intramuscular fat to subcutaneous fat was significantly different in group C compared to group A (Fig. 3). We did not observe any statistically significant change in the ratio of visceral to subcutaneous fat after 6 months of therapy between groups.
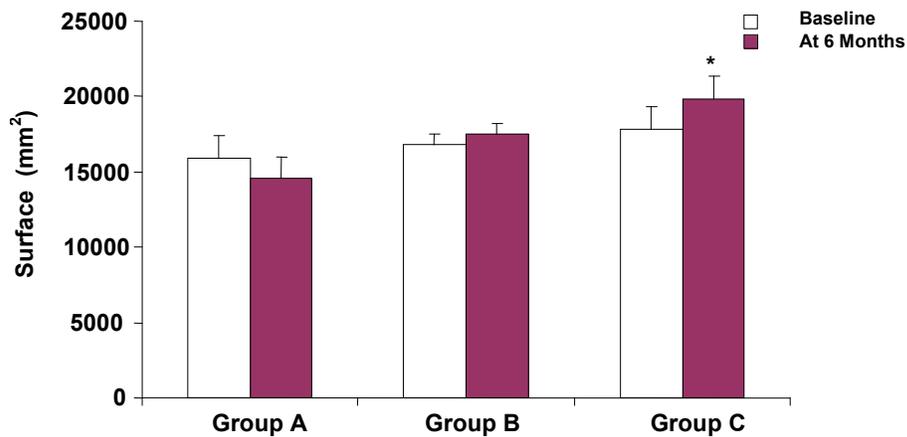

Fig. 1. Comparison of subcutaneous fat surface in the thigh as measured by square millimeters, obtained by mid-tight CAT scan at baseline and after 6 months treatment with placebo (group A), with pioglitazone 30 mg daily (group B), and with pioglitazone 45 mg daily (group C)
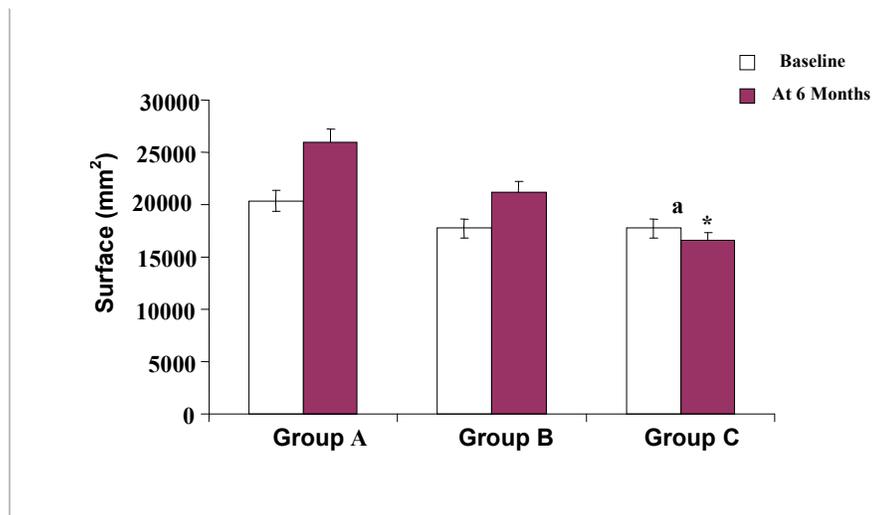* Denotes P<0.05 within groups by Analysis of Variance

Fig. 2. Comparison of intramuscular fat surface as measured by square millimeters, obtained by mid-thigh CAT scan at baseline and after 6 months treatment with placebo (group A), with pioglitazone 30 mg daily (group B), and with pioglitazone 45 mg daily (group C)
* Denotes P<0.05 between groups by paired t-test and subscript a denotes p=0.05 by analysis of variance within groups A and C after the treatment
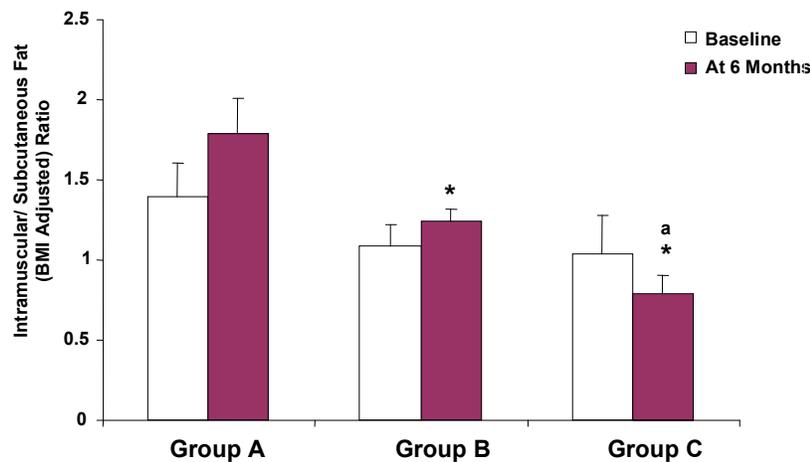

Fig. 3. Comparison of the mean of BMI adjusted intramuscular / subcutaneous fat ratio, obtained by mid-thigh CAT scan at baseline and after 6 months treatment with placebo (group A), with pioglita-zone 30 mg daily (group B), and with pioglitazone 45 mg daily (group C)
* Denotes P<0.05 between Group by paired t-test and subscript a denotes p<0.05 by analysis of variance within Group C compared to group B and A

The serum levels of adiponectin at baseline were not different within groups. At the end of the study period the adiponectin levels in group A was 4.8±0.9 μg/mL (p=NS within

group A), in group B 12.0±2.6 (P<0.03 within group B) and in group C 16.0±6.8 µg/mL (p<0.05 within group C). However, the mean percent changes in serum levels of adiponec-

tin from baseline were statistically significant within groups by analysis of variance (Table 2).

Table 2. Mean serum adiponectin levels at baseline and after 6 months treatment with placebo (group A), with pioglitazone 30 mg daily (group B), and with pioglitazone 45 mg daily (group C)

Group	Adiponectin µg/mL		P between groups after therapy	P within groups
	Baseline	**After Therapy**		
A	6.0±1.2†	4.8±0.9	0.73 (A Vs B&C)	0.2
B	6.8±1.1	12.0±2.6	0.10 (B Vs A)	0.03
C	9.1±4.1	16.0±6.8	0.3 (C Vs A)	0.04

† mean±SD

Multiple linear regression analysis showed statistically significant correlation between the levels of serum adiponectin at baseline and subcutaneous fat in the thigh, the intramuscular fat, and the ratio of intramuscular to subcutaneous fat (Multiple r=0.97, P<0.01) but not with visceral fat.

A positive correlation was also observed with the change in subcutaneous fat in thigh

and change in serum adiponectin levels (r=0.61; P=0.032) during the study period.

Carotid artery intimal thickness increased by an average of 30 micron in group A, remained unchanged in group B, and decreased by 30 microns in group C. Within group analysis using paired Student's t-test, indicated significant changes in group C only (Fig. 4).
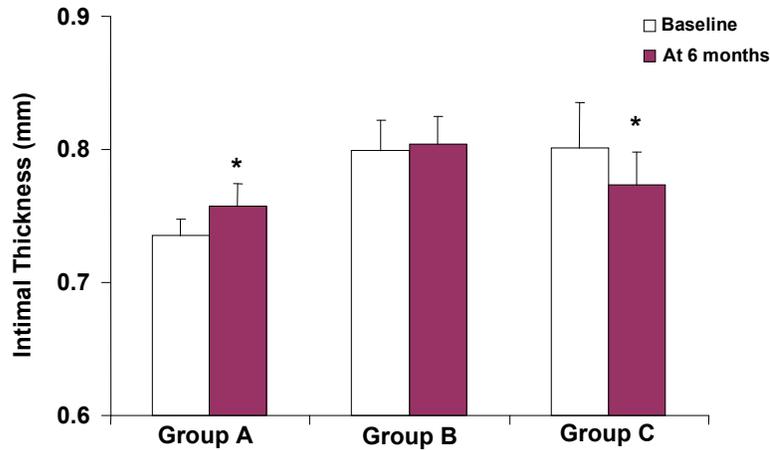

Fig. 4. Comparison of the mean of carotid artery intimal thickness at 2 cm below carotid bifurcation measured by use of ultrasound at baseline and after 6 months treatment with placebo (group A) pioglitazone 30 mg daily (group B), and pioglitazone 45 mg daily (group C)
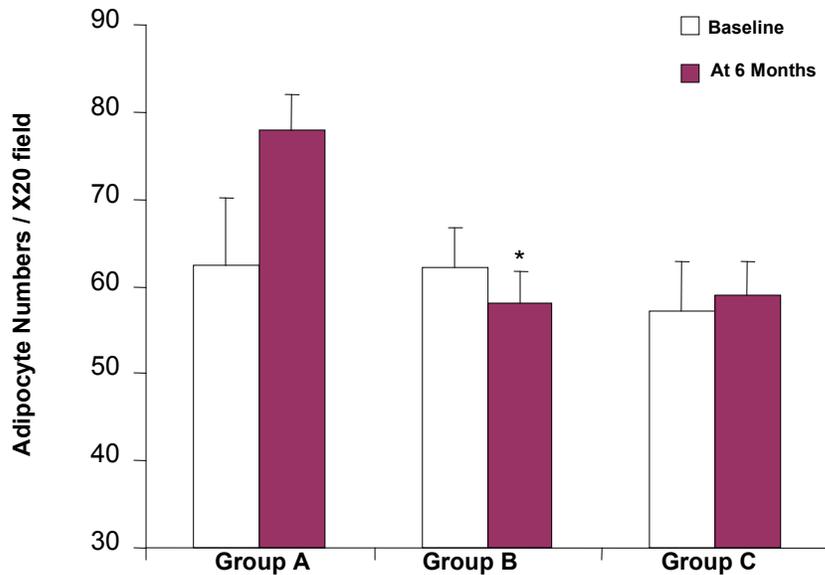* Denotes P<0.05 within groups by paired t-test

Fig. 5. Comparison of the number of adipocytes at baseline from subcutaneous adipose tissue from abdomen and thigh (A1-T1) counted per each field with X20 magnification and after 6 months treatment with placebo(group A), with 30 mg pioglitazone a day (group B), and with 45 mg pioglitazone (group C) a day.
* Denotes significant difference between groups by analysis of variance. No significant difference was observed before treatment and was only observed after the treatment.

The change in subcutaneous fat was concomitant with the histological changes seen in groups B and C. The number of adipocytes increased in group A, with no changes in groups B and C. Analysis of variance demonstrated significant difference in terms of the numbers of adipocytes in groups B and C, compared to group A (Fig.5). From a histological point of view, the adipose tissue membranes became straighter and less convoluted in groups B and C, but not in group A, where no morphological changes were seen (Fig. 6). Also, in groups B and C, the large adipocytes became larger on an average, possibly masking the number/field of any new, small adipocytes.

Discussion

In the present study of minority obese, but otherwise healthy, subjects (primarily African Americans and Latinos), we observed that 45 mg of pioglitazone daily, for 6 months, increases subcutaneous fat in the thigh, while it concomitantly decreases the intramuscular fat in this region; it also decreases carotid artery intimal thickness and increases adiponectin levels. Changes in intramuscular fat, subcutaneous fat, and visceral fat were also correlated with adiponectin levels.

Visceral fat differs significantly from peripheral fat depots in its metabolic activity and hormonal regulation. For instance, compared to the peripheral fat compartment, visceral fat has a greater turnover rate, a higher blood flow, higher density of lipolytic beta-adrenergic, glucocorticoid, androgen receptors and has higher expression of GLUT4 than peripheral fat cells.[21] Furthermore PPAR-gamma mRNA levels were significantly
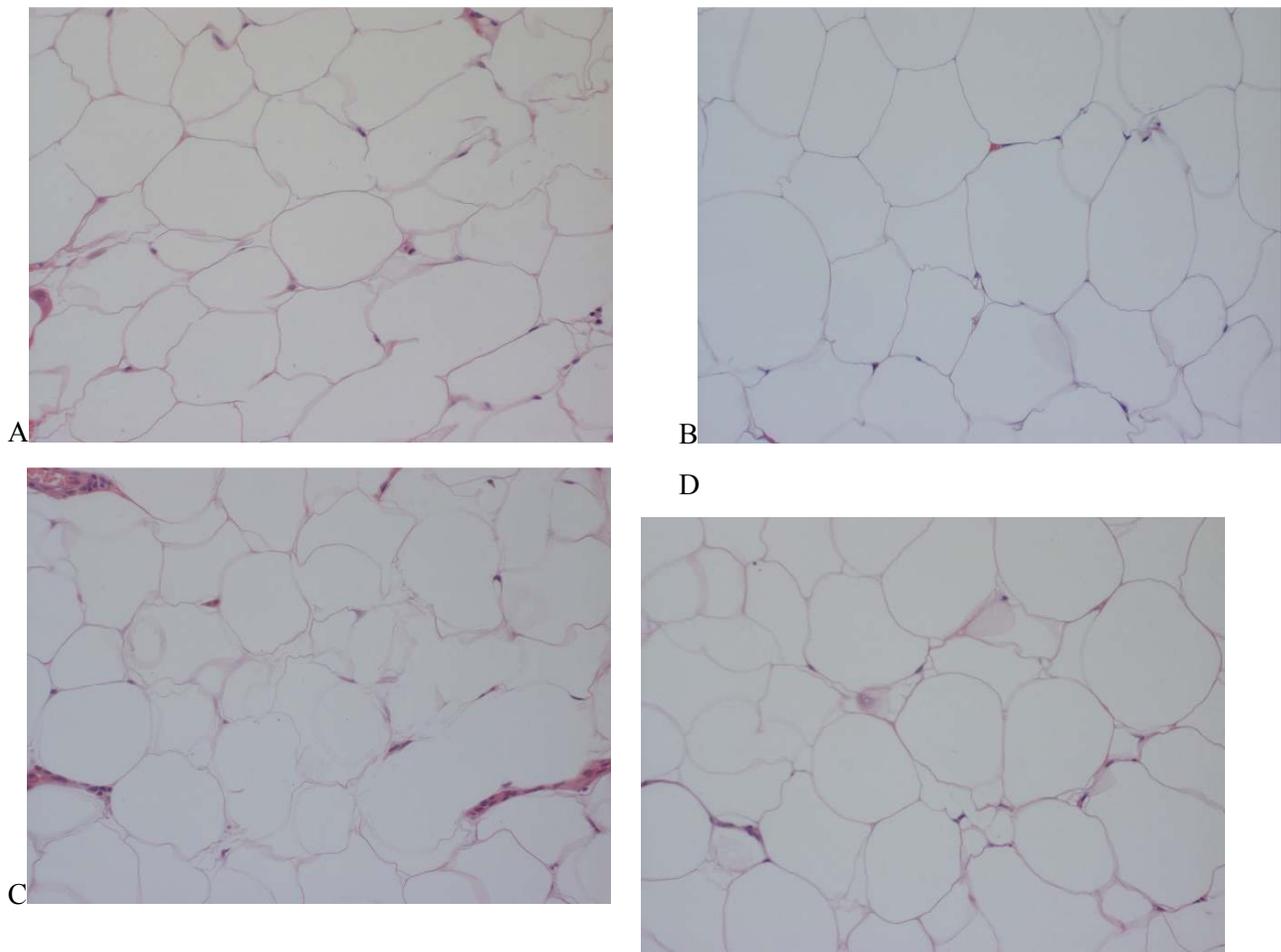
Fig. 6- Comparison of adipose tissue of the thigh in a 45 year old female before (panels A) and after six months therapy with 45 mg/day pioglitazone (B) and from a 42 year old male (panels C before and D after 6 months treatment with 30 mg/day of pioglitazone)

higher in visceral adipose tissue in obese subjects compared to lean subjects, indicating that relative PPAR-gamma expression is increased in omental fat in obesity.[22]

Pioglitazone is known as an insulin sensitizer; while facilitating glucose disposal, it increases hepatic sensitivity to insulin (decreased glucose output by the liver under insulin effect) and decreases free fatty acid in serum, simultaneously facilitating its use/deposition by adipose tissue.[23] The concomitant effects of pioglitazone on intramuscular fat and adiponectin levels, and their

significant correlations, suggest a possible direct role of adiponectin on fat utilization in the muscle.

While facilitating more room for fatty acid deposition[24] in adipose tissue, pioglitazone prevents free fatty acids from competing with glucose at the muscular level. However, as we and others have observed, pioglitazone increases the level of adiponectin,[25-26] which is a hormone with several key effects on glucose and lipid metabolism. Adiponectin activates carnitine palmitoyltransferase-1, malonyl-CoA decarboxylase, and medium-chain

acyl-CoA dehydrogenase in the hepato-cytes,[27] theoretically facilitating beta-oxidation of fatty acids in the liver.[28] It also activates carnitine palmitoyl transferase III and acetyl-CoA carboxykinase in muscle mitochondria,[29] thereby accelerating glucose utilization in the Krebs cycle. In hepato-cytes,[30-31] adiponectin suppresses glucose-6-phosphatase and phosphoenolpyruvate car-boxykinase, as well as inhibiting gluconeo-genic pathways independent of insulin. Hence, on the one hand, adiponectin facili-tates entrance of free fatty acids into both adipocytes and hepatocytes and beta-oxidation of free fatty acids in the mitochon-dria of the muscles and hepatocytes by en-hancing fuel metabolism, and on the other, it also facilitates conversion of excess calories into fat[32] as shown by the larger adipose tis-sues in our biopsy samples. The association observed between adiponectin and intramus-cular fat (or with the ratio of intramuscular fat and subcutaneous fat) suggests the impor-tance of better metabolism of fuel in obese subjects who are in excess of their reserve. It seems that pioglitazone not only increases the reservoir location for the triglycerides, but al-so facilitates their catabolism in the muscle through adiponectin, as observed in our study, with decreases in intramuscular fat. It is known that PPAR gamma agonists in-crease flux of fatty acids into adipocytes in rats by virtue of fatty acid transport protein gene- PPAR gamma response element.[33] Se-questration of lipid in adipose tissue takes place, while whole-body resting or postpran-dial fat oxidation does increase in rats and humans,[34] by increasing the uncoupling pro-tein 2 expression however, not necessarily, causing weight loss but, rather, weight gain may be compensated by eating more; the rea-son for increases in food consumption is not yet understood during obesity.[35]

Pioglitazone is also known to inhibit pro-liferation, hypertrophy, and migration of vas-cular smooth muscle cells (VSMC) induced by growth factors.[36] These processes are cru-cial in the development of vascular remode-ling, atherosclerosis, and diabetic organ complications. One important finding of our study is the decrease of intimal thickness in carotid arteries. Pioglitazone is known to de-crease inflammatory cytokines secreted by macrophages thereby decreasing inflammato-ry processes leading to atherosclerosis.[37] Also the fact that pioglitazone induced an in-creased apoptosis of macrophages suggests that the same phenomena might take place in carotid artery intima thus explaining rapid regression of carotid artery intimal thick-ness.[38-39] The intimal thickness of carotid ar-teries has been shown as one of the best pre-dictors of coronary artery disease and stroke.[40] In our study, we saw an average de-crease of carotid artery intimal thickness of 30 microns in subjects given 45 mg pioglita-zone daily for 6 months. Whether this also implies decrease of intimal thickness, par-ticularly in the lower extremities is not known, but theoretically it could be rapidly effective in alleviating ischemic limbs and be beneficial in terms of macrovasculopathy in diabetic subjects; in the same context, family members of a subject with history of stroke may benefit not only from aspirin but also from pioglitazone in delaying progression of the carotid artery atherosclerosis or cerebro-vascular events.

Surprisingly, a significant inverse rela-tionship was observed between visceral fat measured by CAT scan and carotid artery in-timal thickness. Taking into account the dy-namic nature of adipose deposition, there seems to be a 20 to 30 year lapse between the start of visceral fat accumulation and devel-opment of advanced carotid artery atheros-clerosis. Thus, it could be that at an early phase of metabolic syndrome, the accumula-tion of fat in abdomen is beneficial for pre-venting accelerated atherosclerosis. These vascular effects of pioglitazone provide addi-tional mechanisms by which treatment of pa-tients with visceral obesity using these agents might reduce cardiovascular risk.

Regarding study limitations, our study has a small sample size and hence limited power

analysis. Further studies are clearly needed to address the role of pioglitazone in prevention of peripheral vascular disease in diabetic subjects and also in prevention of stroke in high risk obese subjects.

A study of retroperitoneal fat pads in Zucker rats demonstrated an increase in adipocyte size and number, due to combined differentiating effect and an increase in biosynthesis of triglycerides.[41] We were unable to confirm this observation, since the number of adipocytes per field was decreased and although they were larger, they also had a less convoluted membrane.

The recent observation of the decrease in number of macrophages in the adipose tissue due to increase in apoptosis induced by pioglitazone highlights the role played by adipose tissue in secreting TNF-alpha and its consequences which is insulin resistance.

These data provide a rationale for the use of PPARγ activators combined with physical activity for the treatment of visceral obesity and its hallmark, the insulin resistance.

In conclusion, pioglitazone in healthy obese minority subjects was associated with redistribution of fat from intramuscular to subcutaneous fat depot and decline in carotid artery intimal thickness, all associated and correlated with changes in serum adiponectin levels. This study also suggests the use of pioglitazone, as an adjunct, in prevention of stroke and peripheral vascular disease in high-risk populations.

Acknowledgment

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