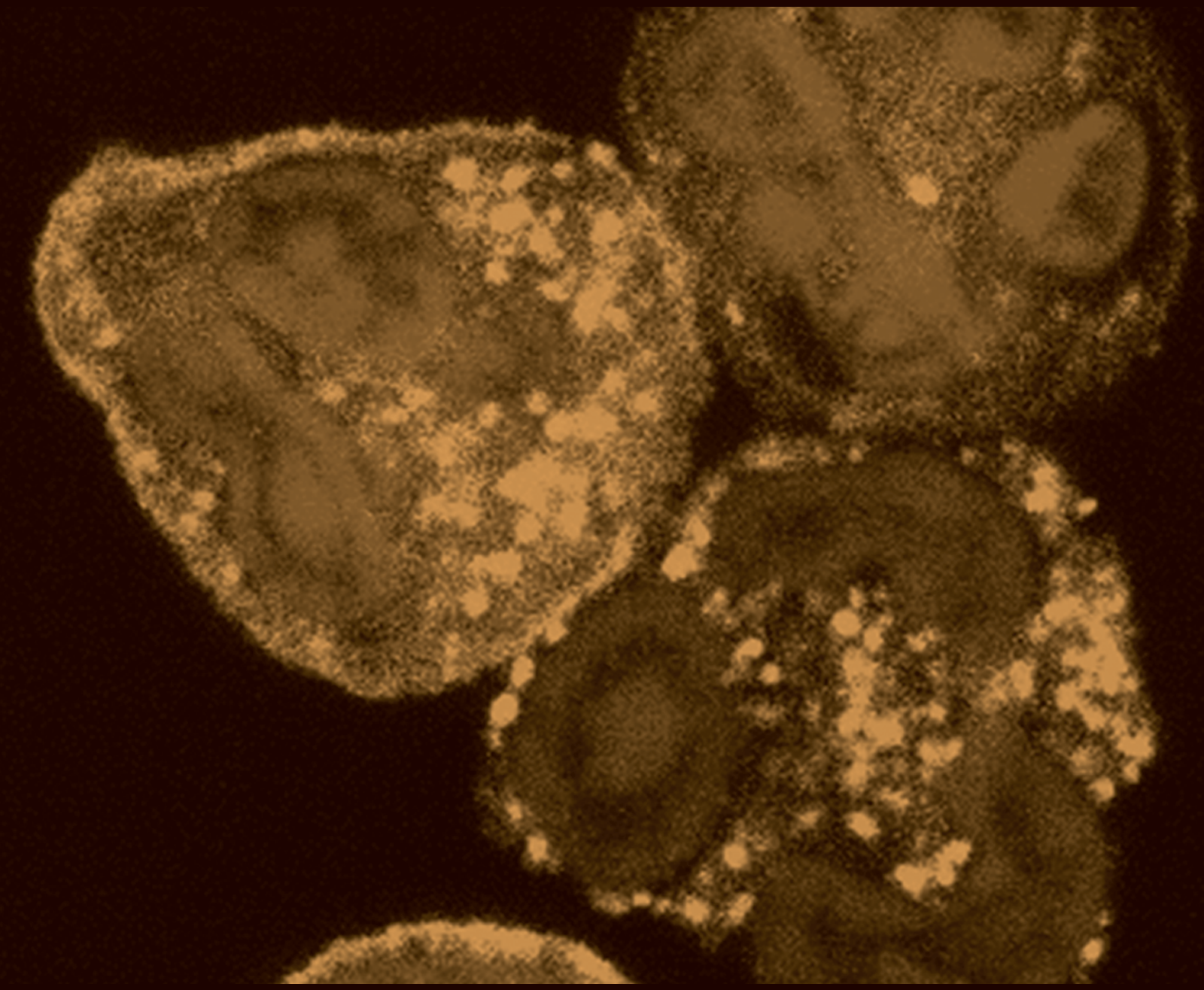
Mediators of Inflammation

Inflammation Is a Key Pathophysiological Feature of Metabolic Syndrome

Guest Editors: Fabrizio Montecucco, François Mach, and Aldo Pende



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Editorial Board

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Editorial

Inflammation Is a Key Pathophysiological Feature of Metabolic Syndrome

Fabrizio Montecucco,[1,2] François Mach,[1] and Aldo Pende[2]

[1] *Division of Cardiology, Department of Internal Medicine, Foundation for Medical Researches, University of Geneva, 64 Avenue de la Roseraie, 1211 Geneva, Switzerland*
[2] *Laboratory of Phagocyte Physiopathology and Inflammation, Department of Internal Medicine, First Medical Clinic, University of Genoa, 6 Viale Benedetto XV, 16100 Genoa, Italy*

Correspondence should be addressed to Fabrizio Montecucco; fabrizio.montecucco@unige.ch

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Metabolic syndrome is a disease clustering different cardiovascular risk disorders, including central obesity, insulin resistance, glucose intolerance, dyslipidemia, and hypertension. In the last decade, different definitions have been produced to better assess the multiplicative cardiovascular risk of metabolic syndrome and potentially improve patient treatment in both primary and secondary prevention. In particular, insulin resistance progressively lost its relevance on metabolic syndrome patients, being considered as a concomitant rather than a diagnostic condition. On the other hand, local organ remodeling and structural alterations (including adipose tissue, heart, liver, and pancreatic β-cells) in metabolic syndrome have been targeted by pathophysiological studies. Given the worldwide pandemic dimensions of metabolic syndrome, a strong scientific effort has been made on the identification of the causal mechanisms and molecules underlying this disease. In addition, both basic research and clinical studies investigating prognostic circulating biomarkers of metabolic syndrome have shown some soluble mediators (such as inflammatory cytokines, adipocytokines, and coagulation factors) as crucial players in the development of metabolic syndrome. More recently, the alterations in free fatty acid metabolism have also indicated these molecules as critical mediators of inflammation in metabolic syndrome. This special issue was focused on these and other soluble mediators as promising candidates to better assess the cardiovascular risk in metabolic syndrome. In particular, the pathophysiological activities of adipocytokines, cytokines, and chemokines as well as the controversies on the role of insulin resistance have been discussed. F. Renna et al. investigated the role of the enzyme cyclooxygenase-2 (COX-2) in an experimental rat model of spontaneous hypertension and metabolic syndrome. Using the COX-2 specific antagonist lumiracoxib, the authors showed that the treatment with this drug was able to reverse vascular remodeling and inflammation, confirming the potential role for COX-2 in the pathophysiology of atherogenesis. H. Qu et al. investigated the potential relationship between a novel systemic biomarker (i.e., plasma progranulin [PGRN]) as well as interleukin- (IL-) 6 with insulin resistance in Chinese patients with normal glucose tolerance ($n = 88$) and type 2 diabetes ($n = 80$). Results showed that systemic PGRN concentrations were significantly increased in the diabetic patients as compared to normal glucose control group. Importantly, PGRN levels were positively related with patient weight, central obesity, inflammation, and insulin resistance (assessed by HOMA-IR). C. L. Reading et al. focused on the potential anti-inflammatory benefits of the insulin sensitizer, HE3286. Importantly, this drug was able to decrease insulin resistance in metformin-treated subjects, indicating a therapeutic potential to restore metabolic homeostasis in type 2 diabetes. O. Bădulescu et al. investigated the potential association between diabetes mellitus and other cardiovascular risk factors (such as dyslipidemia) in three different groups of patients: diabetic without ischemic-cardiopathy-related disorders, diabetic with clinical ischemic cardiopathy, and nondiabetic with ischemic-cardiopathy-related disorders, respectively. The results showed that diabetic patients have an increased thrombotic risk as well as highest

levels of interleukin-1-beta and lipids. Therefore, this study shows that the diagnostic conditions of metabolic syndrome might differently affect the cardiovascular risk in human beings. F. Rodriguez et al. investigated the role of the adipocytokine resistin on different aspects (such as inflammation, food intake, and gonadal function) both *in vitro* in rat adenopituitary cells and *in vivo* in fed and fasting rats. The authors showed that exogenous administration of resistin increased β-oxidation and inhibited metabolic enzymes involved on lipid synthesis. This study indicated that resistin might have a regulatory and homeostatic role on lipid metabolism within the pituitary gland.

F. Wasinski et al. investigated the potential benefits of physical exercise and caloric restriction *in vivo* on inflammatory cells, resident within adipose tissue, in obese mice. Both exercise and caloric restriction improved body mass, number of resident immune cells in the adipose tissue, and serum levels of inflammatory molecules in obese animals. However, selective mediators were modified by these therapeutic approaches, indicating potential different antiinflammatory pathways, suggesting that the combination of both strategies might be very promising to reduce inflammation in obesity. Potential controversies on the role of obesity on the cardiovascular risk have been particularly debated. B. K. Cole et al. investigated the inflammatory role of 12/15-lipoxygenase activity in white adipose tissue from obese mice with fat-specific deletion of this enzyme. Knockout mice showed improvements in fasting glucose levels and metabolism when compared to controls. In addition, a reduced inflammation and macrophage infiltration characterized adipose tissue of knockout mice. These results suggest that specific deletion of 12/15-lipoxygenase in adipose tissue can protect from the deleterious effects of inflammation. Two articles have also been included about a condition accelerating atherosclerosis. A review article by I. Ferraz-Amaro et al. focused on potential common mediators between metabolic syndrome and chronic inflammatory diseases, such as rheumatoid arthritis (RA). The authors provided a complete overview on the potential role of adipocytokines that could influence atherogenesis in both diseases. Biological therapies, including anti-TNF-α drugs, have been indicated to potentially improve atheroprogression and insulin resistance in RA patients. Finally, P. H. Dessein et al. performed a clinical study on 277 black African subjects (of whom 119 had RA) investigating the potential association between adipocytokine serum levels, RA, and lipid profile. Only in RA subjects, adiponectin concentrations were associated with a favorable lipid profile and blood pressure. No associations were observed for leptin. This clinical study partially confirmed a potential pathophysiological relevance for adiponectin in chronic inflammatory diseases. The present special issue on mediators of inflammation represents an update on pathophysiological mediators that can be targeted by novel and more selective treatments in metabolic syndrome and concomitant chronic inflammation. We hope that the reader will find some novel input for future researches.

Fabrizio Montecucco
François Mach
Aldo Pende

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Research Article

Resistin Regulates Pituitary Lipid Metabolism and Inflammation *In Vivo* and *In Vitro*

F. Rodriguez-Pacheco,[1,2,3] M. G. Novelle,[1,2] M. J. Vazquez,[1,2] E. Garcia-Escobar,[3,4] F. Soriguer,[3,4] G. Rojo-Martinez,[3,4] E. García-Fuentes,[3,4] M. M. Malagon,[2,5] and C. Dieguez[1,2]

[1] *Department of Physiology, School of Medicine, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[2] *CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Spain*
[3] *Endocrinology and Nutrition Service, Carlos Hayà Hospital, Fundación IMABIS, 29009 Málaga, Spain*
[4] *CIBER Diabetes and Metabolic Illness (CIBER-DEM), Spain*
[5] *Department of Cellular Biology, Physiology and Inmunology, School of Siences, University of Córdoba, Córdoba, Spain*

Correspondence should be addressed to F. Rodriguez-Pacheco; paqui.endocrino@gmail.com

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The adipokine resistin is an insulin-antagonizing factor that also plays a regulatory role in inflammation, immunity, food intake, and gonadal function and also regulates growth hormone (GH) secretion in rat adenopituitary cells cultures with the adipokine. Although adipose tissue is the primary source of resistin, it is also expressed in other tissues, including the pituitary. The aim of this study is to investigate the possible action of resistin on the lipid metabolism in the pituitary gland *in vivo* (rats in two different nutritional status, fed and fast, treated with resistin on acute and a chronic way) and *in vitro* (adenopituitary cell cultures treated with the adipokine). Here, by a combination of *in vivo* and *in vitro* experimental models, we demonstrated that central acute and chronic administration of resistin enhance mRNA levels of the lipid metabolic enzymes which participated on lipolysis and moreover inhibiting mRNA levels of the lipid metabolic enzymes involved in lipogenesis. Taken together, our results demonstrate for the first time that resistin has a regulatory role on lipid metabolism in the pituitary gland providing a novel insight in relation to the mechanism by which this adipokine can participate in the integrated control of lipid metabolism.

1. Introduction

Resistin, also known as found in inflammatory zone 3 (FIZZ3) is an adipocyte-derived hormone known to promote insulin resistance, impair adipocyte differentiation, and to promote inflammation [1–5] and that was originally identified in rats as a protein secreted by adipocytes that is under the control of different humoral signals and nutritional status; nutrition and metabolism regulate this adipoine. Resistin is decreased in fasting situations [6], whereas circulating resistin is increased in obese insulin resistant rodents [7] and humans [8]. Despite adipose tissue being the more relevant source of this protein, it has been recently reported that resistin is also expressed in the hypothalamus and in the pituitary gland [8, 9]. Central resistin administration appears to have a dual effect on metabolic homeostasis, first by acutely inhibiting feeding [10] and second by controlling glucose homeostasis and inducing hepatic insulin resistance [8, 11]. Recently, it has been demonstrated that central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion and even that this regulation is opposite in peripheral organs in comparison with central effect [12]. The authors demonstrate that the anorectic effect of resistin is associated with the low levels of mRNA expression of orexigenic (agouti-related protein and neuropeptide Y) and the increased mRNA expression of anorexigenic (cocaine and amphetamine-regulated transcript) neuropeptides in the arcuate nucleus of the hypothalamus. Also they show that resistin exerts a nutritional status dependent inhibitory effect on hypothalamic fatty acid metabolism, as indicated by increased phosphorylation levels of both AMP-activated protein kinase and its downstream target

acetyl-coenzyme A carboxylase, associated with decreased expression of fatty acid synthase in the hypothalamus. In addition, it is demonstrated that chronic central resistin infusion results in decreased body weight and major changes in peripheral expression of lipogenic enzymes, in a tissue-specific and nutrition-dependent manner [12].

In previous studies we tested the resistin effect on GH release *in vitro*. It has been demonstrated that the adipoine induces GH release in a dose fashion on adenopituitary cells culture at 4 h of exposition. This effect is even higher at 24 h of resistin treatment [13]. These results implicate the existence of resistin receptors in the pituitary, a receptor which it has not been cloned yet.

The aim of this study was to characterize the effect of acute and chronic resistin administration in the regulation of pituitary lipid metabolism. In addition since this adipokine have been reported to be involved in the inflammation process linked to obesity, we also studied its effects on the expression of the most-known proinflammatory cytokines, namely, TNF-alpha and Il-6 [14].

We have studied the expression of enzymes; fatty acid synthase, FAS; malonyl CoA decarboxylase, MCD; carnitina-palmitoil transferase, CPT-1; lipoprotein lipase, LPL; the proinflammatory cytokines interleukin 6, IL-6; tumor necrosis factor alfa, TNF-α.

2. Materials and Methods

2.1. Reagents. The fetal bovine serum (FBS) was obtained from Fisher Scientific Biobloce. Trizol reagent and MML-V reverse transcriptase (RT) were purchased from Invitrogen (Paisley, UK). Dulbecco's modified Eagle's medium (DMEM), collagenase from Clostridium histolyticum, deoxiribonuclease I crude lyophilized, hyaluronidase type I-S from Bovine test, Dispase II (neutral protease, grade II) from Roche Diagnostics, SL. (Barcelona, Spain). Resistin was obtained from Phoenix Pharmaceuticals Inc. (Karlsruhe, Germany); 10 μg/rat dissolved in 5 μL of saline.

2.2. Animals. Male Sprague Dawley rats (300–350 g) were housed in a temperature-controlled room, with a 12-h light, 12-h dark cycle (lights from 0800 to 2000 h). The experiments were performed in agreement with the International Law on Animal Experimentation, and the experimental protocols have been approved by the Ethics Committee of the University of Santiago de Compostela. Intracerebroventricular (ICV) cannulae chronic ICV cannulae were implanted under ketamine/xylazine anesthesia as previously described [11, 15, 16].

The correct location of the cannula in the lateral ventricle was confirmed by methylene blue staining. Animals were individually caged and allowed to recover for 1 wk before experiment. During the postoperative recovery period, the rats were handled regularly under nonstressful conditions.

2.3. Acute Resistin Treatment. One group of rats was fed ad libitum, and the other group was deprived of food for 12 h (nocturnal fasting). Rats then received either a single ICV injection of resistin (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany; 10 μg/rat dissolved in 5 μL of saline) or vehicle. The rats were killed 1.5 h after injection. Treatments started at 0800 h and were carried out in the light phase.

2.4. Chronic Resistin Treatment. Brain infusion cannulae were stereotaxically placed into the lateral ventricle as described above. A catheter tube was connected from the brain infusion cannula to an osmotic minipump flow moderator (model 2001D or 2ML2; Alza Corp., Palo Alto, CA, USA). An sc pocket on the dorsal surface of the animal was created using blunt dissection, and the osmotic minipump was inserted. The incision was closed with sutures, and the rats were kept warm until fully recovered. The rats were then infused with either vehicle or resistin (10 μg/day) for 6 d. On day 4, one group of rats was fed ad libitum, and the other group was deprived of food for the final two days.

2.5. Pituitary Cell Dispersion and Culture. Isolated cells from rat anterior pituitary were obtained using an enzymatic dispersion protocol. Briefly, for each experiment, three to four anterior pituitaries were pooled, minced, and enzymatically dissociated by incubation in DMEM supplemented with collagenase 0.4%, deoxyribonuclease 0.01%, hyaluronidase 0.1%, and dispase II 0.2% at 37°C in a 5% CO_2 atmosphere, and the tissues were mechanically dispersed every 10 minutes until a homogeneous cellular suspension was obtained. Cellular viability, as estimated by the trypan blue test, was always above 90%.

Dispersed anterior pituitary cells were plated at a density of 300,000 cells onto 24-well culture plates in 1 mL DMEM supplemented with 10% FBS and 0.1% antibiotic-antimycotic solution.

Cells were incubated at 37°C in a 5% CO_2 atmosphere, and medium was replaced by fresh DMEM-FBS after 48 h of culture. After a 3-d culture period, medium was removed, and cells were preincubated in 1 mL serum-free DMEM for 2 h to stabilize basal hormone secretion. Medium was then replaced with fresh DMEM containing increasing doses of resistin or the corresponding control vehicle and incubated for 4 h at 37°C.

Medium samples were collected at the end of the experiments and were stored at −20°C until hormone determinations by RIA.

Cells in the culture plates were processed for RNA extraction as indicated below.

2.6. Measurement of Growth Hormone by RIA. GH levels in culture media were measured in a volume of 25–50 μL using a double antibody method and radioimmunoassay kits kindly supplied by the NIH (Dr. A. F. Parlow, NIDDK National Hormone and Peptide Program; Torrance, CA, USA). Rat GH-I-7 was labeled with ^{125}I using the chloramine-T method and Iodo-Gen Pre-coated iodination tubes (Pierce, Rockford, IL, USA), respectively. Hormone concentration was expressed using the reference preparations GH-RP-2 as standards. Intra- and interassay coefficients of variation were below 6% and 9% for GH. The sensitivity of the assay was

5 pg/tube for GH. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

2.7. Real-Time Quantitative PCR of Enzymes Involved in Lipid Metabolism. The mRNA levels of acetyl-coenzyme A carboxylase (ACC)α, IL-6, TNF α, and lipoprotein lipase (LPL) were studied by using real-time PCR (TaqMan; Applied Biosystems, Foster City, CA, USA) by using specific primers and probes published as supplemental data on The Endocrine society's journals Online web site at http://endo.endojournals.org/) as previously described [17, 18]. All reactions were carried out using the following cycling parameters: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min [17, 18].

For the analysis of the data, the input value of the gene of interest was standardized to the 18S value for the sample group and was expressed compared with the average value for the vehicle-treated group. We used six to eight rats per group.

2.8. Statistical Analysis. Data are expressed as mean ± SEM in relation (%) to vehicle-treated rats. Statistical significance was determined by t-Student's test when two groups were compared or by ANOVA and post-hoc two-tailed Bonferroni test when more than two groups were compared. $P < 0.05$ was considered significant. The program used for the analysis was GraphPad Prism.

3. Results

3.1. Central Acute Administration of Resistin Does Not Regulate Pituitary Fatty Acid Metabolism. Central acute resistin administration induced no changes in the mRNA expression of FAS, MCD, CPT-1, LPL, and the proinflammatory cytokines IL-6 and TNF-α suggesting that pituitary gland fatty acid metabolism is not regulated by central acute resistin treatment (Figure 1).

3.2. Central Chronic Administration of Resistin Regulates Pituitary Fatty Acid Metabolism. Central chronic administration of resistin was associated with a marked decreased in the expression levels of FAS and LPL indicating that resistin does not participate on lipid synthesis. We also observed that CPT-1 and MCD mRNA levels are higher than in fasting conditions and in presence of resistin. With respect to the proinflammatory cytokines, resistin diminished mRNA levels of both IL-6 and TNF-α on fasted rats (Figure 2).

3.3. Effect of Resistin on Rat Pituitary Cell Cultures. For knowing if resistin has a direct participation on pituitary lipid metabolism, we have examined the effect of increasing doses of resistin (10^{-14}–10^{-6} M) on mRNA levels by rat anterior pituitary cell cultures exposed to the adipokine for 4 h. As shown in Figure 3, when resistin is administered for 4 h, significantly decreased FAS shows low levels of mRNA with 10^{-8} M and 10^{-6} M resistin concentrations. The LPL shows decreased levels of the enzyme expression at the highest resistin doses employed. With respect to CPT-1 enzyme, resistin induces an enhanced transferase enzyme expression at 10^{-8} and 10^{-6} M doses of the adipokine. The two proinflammatory cytokines that we have studied showed low levels of the mRNA at 10^{-6} M. TNF-alpha even presents low levels at other resistin concentrations (10^{-10} M to 10^{-6} M).

4. Discussion

Although resistin was initially suggested to promote insulin resistance and adipocyte differentiation, recent data indicate that this hormone also plays a pleiotropic role in rodents, immunity, food intake, gonadal function, and hypothalamic and peripheral lipid metabolism regulation [19]. A recent study of our group added a new role to resistin, regulating pituitary somatotrope cell function. Resistin enhances GH release through the activation of multiple signaling pathways [13].

Specifically, resistin enhanced GH release on ad libitum feeding rats when resistin was administered in an acute (1.5 h) or a chronic (6 d) way increasing the transcripts numbers of the pituitary transcription factor Pit-1. It is important to confirm whether resistin-induced changes in mRNA expression of Pit-1 correlate with hormone protein levels, because it has been recently reported that POMC altered gene expression is not always linked to specific changes in α-MSH hypothalamic protein content [20]. Further work, using different experimental techniques as HPLC combined with RIA or proteomic analysis, will help to clarify these issues [21].

Previous work in our group reported that 4 h of resistin administration to dispersed rat anterior pituitary cells increased GH release [13]. Therefore, our results confirm the regulatory role of resistin on GH secretion with rats fed ad libitum. It is widely accepted that the regulation of the secretion of GH from the anterior pituitary gland is under the reciprocal control of two hypothalamic hormones, the stimulatory GHRH found in the arcuate nucleus and the inhibitory hormone, somatostatin (SRIF), synthesized in the periventricular nucleus [22]. Our results suggest that resistin might regulate GHRH or SRIF secretion to obtain GH release, or as it has been reported previously, resistin could act directly over somatotropes to regulate GH release [13].

That resistin regulates lipid metabolism centrally and in the periphery as has been reported by Kjems and coworkers [12]. Herein, we have expanded the research to the pituitary gland, studying if resistin regulates fatty acid metabolism in the gland *in vivo* and in adenopituitary cells cultures *in vitro*.

We demonstrate that chronic central resistin administration during 6 d but, no acute administration during 90 min, modified the mRNA levels of the fatty acid metabolism enzymes in the pituitary gland. Besides, resistin diminished the IL-6 and TNF-α mRNA levels on the rat with resistin chronic treatment and on the rat adenopituitary cultures exposed to the adipokine. In detail, resistin diminished mRNA levels of FAS in the pituitary gland and enhanced mRNA levels of CPT-1 and MCD when resistin was administered during 6 d to rats, but there was no effect when the adipokine was administered in an acute way (90 min). Our data showed that central chronic resistin treatment causes a reduction in the number of transcripts of fat-promoting

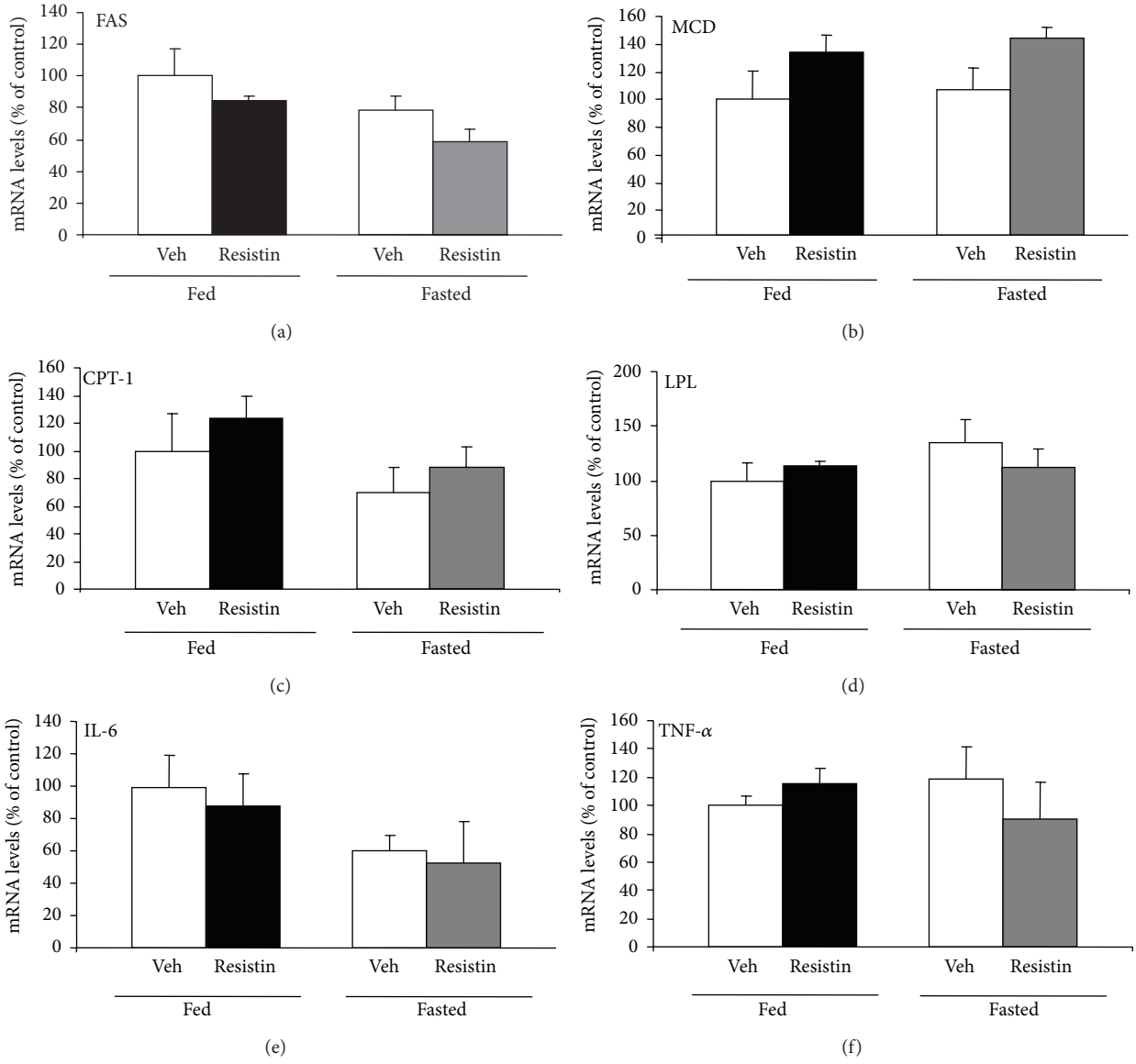

Figure 1: Effect of central acute administration of resistin on pituitary lipid metabolism enzymes and proinflammatory cytokines. Mean + SEM mRNA levels of FAS, MCD, CPT-1, LPL, IL-6, and TNF-alpha in the pituitary gland of fed and fasted rats following administration (I.C.V) of vehicle (Veh) or resistin (10 ug/rat). Samples were obtained 90 min later. $N = 6$–8 rats per group assay.

enzymes in the gland and that the adipokine promotes to β-oxidation in a nutrition-independent fashion. The physiological significance of this effect in the pituitary gland is intriguing. We confirmed that fatty acid metabolism enzymes regulation in the gland is opposite to that described by other authors in other peripheral tissues as white adipose tissue and liver [12]. The reason why resistin increases CPT-1 and MCD in pituitary and diminishes FAS expression is unclear; however, it indicates that pituitary fatty acid metabolism enzymes expression may be regulated by mechanisms distinct from those operating in adipocytes and hepatocytes. Other enzymes such as leptin also have different roles depending on whether they act at the central or peripheral level [23]. On the other hand, our results are similar in part with the results of Kjems and coworkers published on the hypothalamus in which central resistin diminished FAS mRNA levels [12].

We suggest that, as in the hypothalamus, low levels of pituitary ACC mRNA may be a compensatory physiological mechanism that prevents harmful high levels of malonyl-CoA produced in the gland after FAS inhibition [24]. Respect to CPT-1 and MCD, our results showed that CPT-1 mRNA levels are enhanced in resistin-treated fed rats within the same time frame, suggesting that malonyl-CoA levels are changed, as a consequence of ACC inactivation and MCD activation that maybe derived from the low ACC mRNA levels and the high MCD mRNA levels.

Recent data point to the fact that central resistin induces hepatic insulin resistance by increasing the expression of

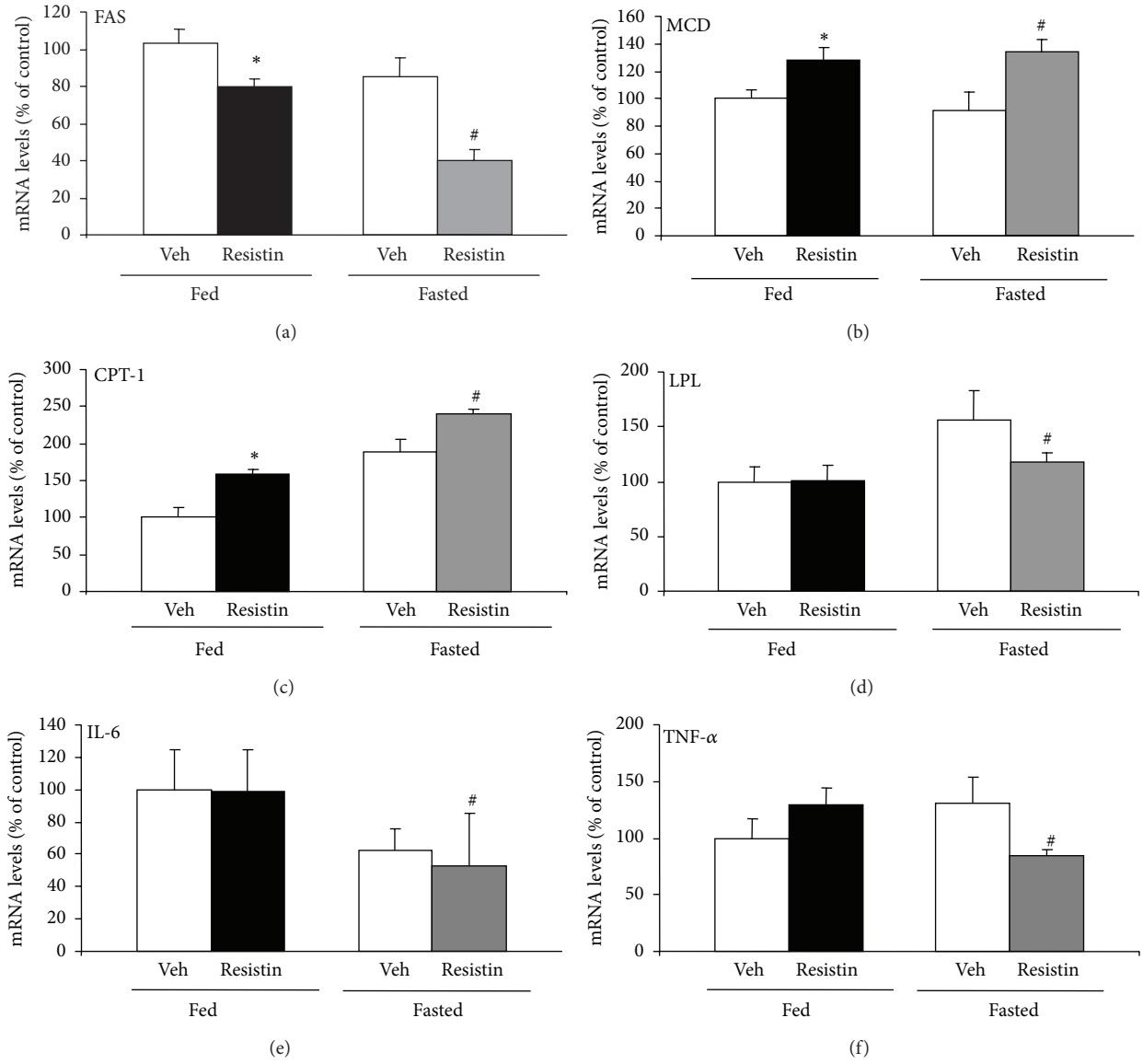

Figure 2: Effect of central chronic administration of resistin on pituitary lipid metabolism enzymes and proinflammatory cytokines. Mean + SEM mRNA levels of FAS, MCD, CPT-1, LPL, IL-6, and TNF-alpha in the pituitary gland of fed and fasted rats following administration (I.C.V.) of vehicle (Veh) or resistin (10 ug/day/over six days. $N = 6$–8 rats per group assay; $^{*}P < 0,05$ versus fed vehicle; $^{\#}P < 0.05$ versus fasted vehicle.

proinflammatory cytokines, such as IL-6 and TNFα, via an unidentified mechanism mediated by the autonomic nervous system [8, 11]. Those alterations in proinflammatory adipocytokines may be due, in part, to the excess accumulation of fatty acids and triglycerides in the liver after central treatment with resistin [25–27]. Therefore, the no increase of fat deposition due to tilt toward the β-oxidation might at least partially explain the low mRNA levels of IL-6 and TNF-α on the gland after resistin treatment centrally and directly on the adenopituitary cells cultures. Further investigation is necessary to clarify this issue.

Finally, we investigated the effect of resistin on lipid metabolism on pituitary cells cultures, and we found the same result observed *in vivo,* resistin diminished the mRNA levels of the enzymes involved in regulating triglyceride uptake and lipid metabolism, such as LPL, ACC, FAS, SCD-1, and key transcriptional factors regulating lipid metabolism, such as SREBP-1c and enhanced the transcripts numbers of CPT-1 and MCD when resistin was administered during 4 h on adenopituitary cells cultures. We found significant activity at resistin 10^{-12} and resistin 10^{-10} M, which is within the concentration range of circulating resistin in rat [28]. Noteworthy, the effect of resistin did not follow a typical dose-response pattern in receptor gene expression, an observation that has been reported for this and other signalling molecules [29–32]. Although the reasons are unclear at present, several

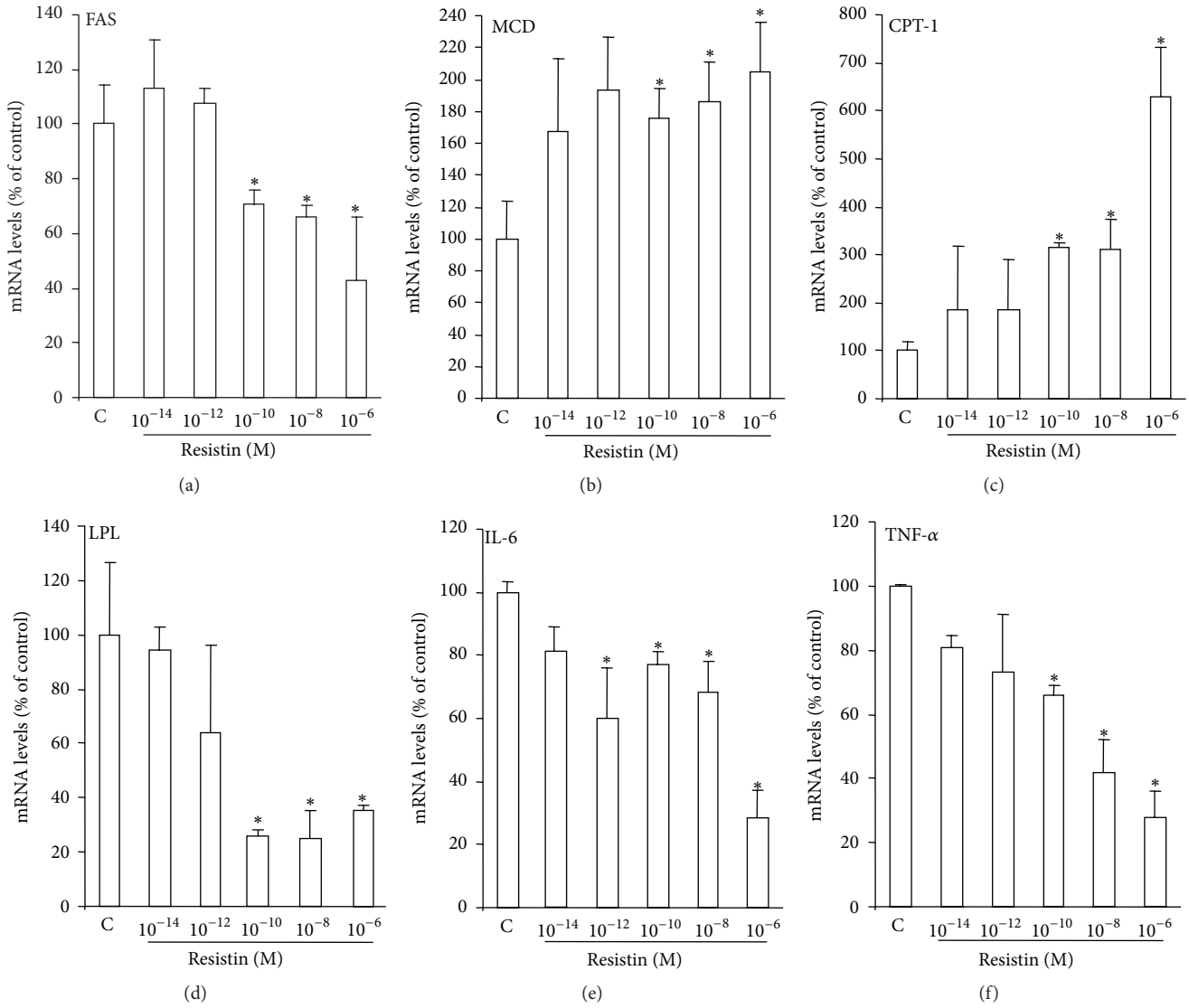

Figure 3: Effect of resistin on mRNA levels of lipid metabolism enzymes and proinflammatory cytokines *in vitro*. Mean + SEM mRNA levels of ACCα, CPT-1, FAS, LPL, MCD, SCD-1, SREBP, IL-6, and TNF-alpha. After 3 days of culture, dispersed rat pituitary cells were incubated in medium alone (C, Control) or in the presence of resistin (10^{-14}–10^{-6} M) for 4 h. After culture, cells were harvested, and receptor mRNA levels were determined by real-time RT-PCR. Enzymes band intensities were determined and adjusted by the signal intensity for HPRT. The averaged results were then calculated and expressed as a percentage of vehicle-treated control levels (100%). Data are the mean (±SEM) of three separate experiments. At least three replicate wells were evaluated per treatment in each experiment. $^{*}P < 0,05$ versus corresponding control.

possibilities can be put forward. It is possible that, depending on its concentration, resistin might induce different structural conformations of its receptor(s) and/or the selective interaction of resistin receptor(s) with other receptors (i.e., homo- or heterodimerization), which in turn might modify resistin activity as it has been reported to occur for other receptors in response to their corresponding ligands [33]. Anyhow, it is yet unknown whether the effects of resistin are mediated by one or several receptors because none has been identified. If there is more than one receptor for resistin, it could be possible that they may have different affinities for resistin with different biological effects that could explain the atypical dose-response curves observed herein. However, until more data, these explanations are presently speculative. Resistin could be inducing to β-oxidation against to promote fat storage. Other peptides as ghrelin or GHRH being GH secretagogues have a similar behaviour on lipid metabolism in the pituitary (preliminary data from our laboratory). Besides, resistin diminished the IL-6 and TNF-α mRNA levels on rat adenopituitary cultures exposed to the adipokine as occured in the *in vivo* experiments. These results show that resistin promotes the β-oxidation in the pituitary gland, decreasing the levels of proinflammatory cytokines and that the adipokine does this action directly on anterior pituitary cells, showing that the pituitary gland could be a target in the control of diseases caused by insulin resistance.

In summary we show that administration of resistin to rats *in vivo* and to adenopituitary cell cultures *in vitro* evoked important effects on pituitary.

Chronic infusion of central administration of resistin increased enzymes mRNA levels implicated on lipid β-oxidation activity and inhibited mRNA expression levels of the enzymes involved on lipid synthesis *in vivo* and *in vitro.*

Chronic administration of the adipokine decreased IL-6 and TNF-α transcripts numbers *in vivo* and on primary cultures experiments.

When viewed together, these results provide evidence that pituitary is a direct and indirect target of resistin action for lipid metabolism regulation of the gland itself. It is feasible that pituitary adipokine expression serves as a link between peripheral metabolic signals. Pituitary control fat storage and metabolism could provide another important step in unraveling the interactions between center, periphery, and adipocytokines, which will improve our understanding of metabolic syndrome and obesity.

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References

[1] W. Zhang, B. Chai, J. Y. Li, H. Wang, and M. W. Mulholland, "Effect of des-acyl ghrelin on adiposity and glucose metabolism," *Endocrinology*, vol. 149, no. 9, pp. 4710–4716, 2008.

[2] M. Kovacs, R. D. Kineman, A. V. Schally, M. Zarandi, K. Groot, and L. A. Frohman, "Effects of antagonists of growth hormone-releasing hormone (GHRH) on GH and insulin-like growth factor I levels in transgenic mice overexpressing the human GHRH gene, an animal model of acromegaly," *Endocrinology*, vol. 138, no. 11, pp. 4536–4542, 1997.

[3] J. Cordoba-Chacon, M. D. Gahete, J. P. Castano, R. D. Kineman, and R. M. Luque, "Homologous and heterologous *in vitro* regulation of pituitary receptors for somatostatin, growth hormone (GH)-releasing hormone, and ghrelin in a nonhuman primate (*Papio anubis*)," *Endocrinology*, vol. 153, no. 1, pp. 264–272, 2012.

[4] W. J. Brown, L. Williams, J. H. Ford, K. Ball, and A. J. Dobson, "Identifying the energy gap: magnitude and determinants of 5-year weight gain in midage women," *Obesity*, vol. 13, no. 8, pp. 1431–1441, 2005.

[5] K. Wynne, S. Stanley, B. McGowan, and S. R. Bloom, "Appetite control," *Journal of Endocrinology*, vol. 184, no. 2, pp. 291–318, 2005.

[6] C. Verdich, A. Flint, J. P. Gutzwiller et al., "A meta-analysis of the effect of glucagon-like peptide-1 (7–36) amide on Ad Libitum energy intake in humans," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 9, pp. 4382–4389, 2001.

[7] S. Mojsov, G. C. Weir, and J. F. Habener, "Insulinotropin: glucagon-like peptide I (7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas," *Journal of Clinical Investigation*, vol. 79, no. 2, pp. 616–619, 1987.

[8] M. A. Nauck, M. M. Heimesaat, C. Orskov, J. J. Holst, R. Ebert, and W. Creutzfeldt, "Preserved incretin activity of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus," *Journal of Clinical Investigation*, vol. 91, no. 1, pp. 301–307, 1993.

[9] V. Matheeussen, L. Baerts, G. de Meyer et al., "Expression and spatial heterogeneity of dipeptidyl peptidases in endothelial cells of conduct vessels and capillaries," *Biological Chemistry*, vol. 392, no. 3, pp. 189–198, 2011.

[10] M. A. Nauck, E. Bartels, C. Orskov, R. Ebert, and W. Creutzfeldt, "Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7–36) amide infused at near-physiological insulinotropic hormone and glucose concentrations," *Journal of Clinical Endocrinology and Metabolism*, vol. 76, no. 4, pp. 912–917, 1993.

[11] D. J. Drucker, J. Philippe, S. Mojsov, W. L. Chick, and J. F. Habener, "Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 10, pp. 3434–3438, 1987.

[12] L. L. Kjems, J. J. Holst, A. Vølund, and S. Madsbad, "The influence of GLP-1 on glucose-stimulated insulin secretion: Effects on β-cell sensitivity in type 2 and nondiabetic subjects," *Diabetes*, vol. 52, no. 2, pp. 380–386, 2003.

[13] N. Mentis, I. Vardarli, L. D. Köthe et al., "GIP does not potentiate the antidiabetic effects of GLP-1 in hyperglycemic patients with type 2 diabetes," *Diabetes*, vol. 60, no. 4, pp. 1270–1276, 2011.

[14] M. Dalamaga, G. Sotiropoulos, K. Karmaniolas, N. Pelekanos, E. Papadavid, and A. Lekka, "Serum resistin: a biomarker of breast cancer in postmenopausal women? Association with clinicopathological characteristics, tumor markers, inflammatory and metabolic parameters," *Clinical Biochemistry*, 2013.

[15] J. J. Holst and C. F. Deacon, "Glucagon-like peptide-1 mediates the therapeutic actions of DPP-IV inhibitors," *Diabetologia*, vol. 48, no. 4, pp. 612–615, 2005.

[16] M. B. Toft-Nielsen, M. B. Damholt, S. Madsbad et al., "Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 8, pp. 3717–3723, 2001.

[17] S. Reichlin, "Growth hormone content of pituitaries from rats with hypothalamic lesions," *Endocrinology*, vol. 69, pp. 225–230, 1961.

[18] R. Guillemin, P. Brazeau, P. Bohlen, F. Esch, N. Ling, and W. B. Wehrenberg, "Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly," *Science*, vol. 218, no. 4572, pp. 585–587, 1982.

[19] L. T. O. Lee, F. K. Y. Siu, J. K. V. Tam et al., "Discovery of growth hormone-releasing hormones and receptors in nonmammalian vertebrates," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 7, pp. 2133–2138, 2007.

[20] S. A. Berry, C. H. Srivastava, L. R. Rubin, W. R. Phipps, and O. H. Pescovitz, "Growth hormone-releasing hormone-like messenger ribonucleic acid and immunoreactive peptide are present in human testis and placenta," *Journal of Clinical Endocrinology and Metabolism*, vol. 75, no. 1, pp. 281–284, 1992.

[21] E. Tomas, T. S. Tsao, A. K. Saha et al., "Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16309–16313, 2002.

[22] E. Tomas, T. S. Tsao, A. K. Saha et al., "Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16309–16313, 2002.

[23] M. Zakikhani, R. Dowling, I. G. Fantus, N. Sonenberg, and M. Pollak, "Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells," *Cancer Research*, vol. 66, no. 21, pp. 10269–10273, 2006.

[24] T. Ciampani, A. Fabbri, A. Isidori, and M. L. Dufau, "Growth hormone-releasing hormone is produced by rat Leydig cell in culture and acts as a positive regulator of Leydig cell function," *Endocrinology*, vol. 131, no. 6, pp. 2785–2792, 1992.

[25] S. Matsubara, M. Sato, M. Mizobuchi, M. Niimi, and J. Takahara, "Differential gene expression of growth hormone (GH)-releasing hormone (GRH) and GRH receptor in various rat tissues," *Endocrinology*, vol. 136, no. 9, pp. 4147–4150, 1995.

[26] H. Kiaris, M. Koutsilieris, A. Kalofoutis, and A. V. Schally, "Growth hormone-releasing hormone and extra-pituitary tumorigenesis: therapeutic and diagnostic applications of growth hormone-releasing hormone antagonists," *Expert Opinion on Investigational Drugs*, vol. 12, no. 8, pp. 1385–1394, 2003.

[27] A. Bagnato, C. Moretti, J. Ohnishi, G. Frajese, and K. J. Catt, "Expression of the growth hormone-releasing hormone gene and its peptide product in the rat ovary," *Endocrinology*, vol. 130, no. 3, pp. 1097–1102, 1992.

[28] C. T. Lim, B. Kola, and M. Korbonits, "AMPK as a mediator of hormonal signalling," *Journal of Molecular Endocrinology*, vol. 44, no. 2, pp. 87–97, 2010.

[29] W. W. Winder and D. G. Hardie, "Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise," *The American Journal of Physiology*, vol. 270, no. 2, pp. E299–E304, 1996.

[30] M. C. Towler and D. G. Hardie, "AMP-activated protein kinase in metabolic control and insulin signaling," *Circulation Research*, vol. 100, no. 3, pp. 328–341, 2007.

[31] G. R. Steinberg and S. B. Jørgensen, "The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity," *Mini Reviews in Medicinal Chemistry*, vol. 7, no. 5, pp. 519–526, 2007.

[32] V. Locatelli and A. Torsello, "Pyruvate and satiety: can we fool the brain?" *Endocrinology*, vol. 146, no. 1, pp. 1–2, 2005.

[33] H. Motoshima, B. J. Goldstein, M. Igata, and E. Araki, "AMPK and cell proliferation—AMPK as a therapeutic target for atherosclerosis and cancer," *Journal of Physiology*, vol. 574, no. 1, pp. 63–71, 2006.

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Research Article

Rheumatoid Arthritis Impacts on the Independent Relationships between Circulating Adiponectin Concentrations and Cardiovascular Metabolic Risk

Patrick H. Dessein,[1] Gavin R. Norton,[1] Margaret Badenhorst,[1] Angela J. Woodiwiss,[1] and Ahmed Solomon[2]

[1] *Cardiovascular Pathophysiology and Genomics Research Unit, School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, P.O. Box 1012, Melville 2109, Johannesburg 2193, South Africa*
[2] *Department of Rheumatology, Charlotte Maxeke Johannesburg Academic Hospital, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg 2193, South Africa*

Correspondence should be addressed to Patrick H. Dessein; dessein@telkomsa.net

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Adiponectin and leptin are likely involved in the pathophysiology of rheumatoid arthritis (RA) and therefore potential new therapeutic targets. Adiponectin inhibition could be expected to enhance cardiovascular metabolic risk. However, it is unknown whether RA changes the influence of adipokines on cardiovascular metabolic risk. We determined whether RA impacts on the independent relationships of circulating leptin and adiponectin concentrations with cardiovascular risk factors and carotid intima-media thickness (cIMT) in 277 black African subjects from a developing population; 119 had RA. RA impacted on the relationships of adiponectin concentrations with lipid concentrations and blood pressure, independent of confounders including adiposity (interaction $P < 0.05$). This translated into an association of adiponectin concentrations with more favorable lipid variables including HDL cholesterol ($P = 0.0005$), non-HDL cholesterol ($P = 0.007$), and triglyceride ($P = 0.005$) concentrations, total cholesterol-HDL cholesterol ($P = 0.0002$) and triglycerides-HDL cholesterol ($P = 0.0003$) ratios, and higher systolic ($P = 0.0006$), diastolic ($P = 0.0004$), and mean blood pressure ($P = 0.0007$) in RA but not non-RA subjects. Leptin was not associated with metabolic risk after adjustment for adiposity. The cIMT did not differ by RA status, and adipokine concentrations were unrelated to atherosclerosis. This study suggests that leptin and adiponectin inhibition may not alter overall cardiovascular risk and disease in RA.

1. Introduction

Since the identification of leptin in 1994, adipose tissue is no longer regarded as a passive reservoir for energy storage [1]. Rather, it comprises a highly active metabolic and endocrine organ that produces a large number of bioactive peptides [1–4]. These molecules are referred to as adipokines and substantially modulate metabolic cardiovascular risk factors including insulin resistance and atherogenesis as well as inflammatory and immune responses [5–9]. Leptin and adiponectin are the most studied adipokines. Leptin is primarily involved in food intake and energy expenditure but is also proinflammatory [1, 2]. Leptin production is increased in obesity and hyperleptinemia that reflects leptin resistance enhances metabolic risk [6]. By contrast, adiponectin is anti-inflammatory and improves metabolic risk, and its production decreases with increasing adiposity [1, 2, 5, 7].

Investigations in patients with RA have mostly shown increased serum leptin and adiponectin concentrations, and both adipokines are also produced in inflamed joints [1, 10–19]. These findings support their involvement in the immune response in RA. Indeed, leptin induces interleukin-8 in RA synovial fibroblasts [20] but also has anabolic effects through stimulation of the expression of cartilage growth factors [1, 10]. In contrast to its anti-inflammatory role in the metabolic syndrome, adiponectin induces gene expression

and protein synthesis in several effector cells involved in the pathophysiology of RA that result in the production of an extensive range of proinflammatory and prodestructive molecules [1, 10, 21–23]. Accordingly, leptin and adiponectin were proposed as potential new therapeutic targets in RA [23, 24]. However, whereas rheumatoid arthritis (RA) is associated with markedly enhanced metabolic cardiovascular risk and disease [25–27], inhibition of adiponectin would be expected to further enhance metabolic risk [5, 7].

Available data on the potential contribution of adipokine metabolism to enhanced CVD in RA is mostly derived in white subjects from developed populations. Overall, serum adipokine concentrations as well as adipokine polymorphisms were found to be unrelated to atherosclerosis and cardiovascular event rates in RA [18, 28–31]. Importantly in the present context, the presence of autoimmunity can alter the impact of adipokines on cardiovascular metabolic risk and disease [32, 33]. Indeed, in a recent investigation by Hahn and colleagues, twice weekly intraperitoneally administered leptin increased proinflammatory high density lipoprotein scores and atherosclerosis in high fat diet fed lupus prone mice but not in nonautoimmune controls [32]. The same group also reported independent relationships between serum leptin and proinflammatory lipid concentrations as well as atherosclerosis in patients with lupus [33]. Of additional importance in the present context, differences between black and white subjects in the gene encoding adiponectin allele's frequencies as well as body types and metabolic risk factors according to ADPC genotypes were reported [34]. Whether RA modifies the influence of adipokines on metabolic risk, and atherosclerosis is currently unknown. In order to elucidate the potential anticipated effects of leptin and adiponectin inhibition on metabolic cardiovascular risk in RA, in the present investigation, we examined the impact of RA on the independent relationships of the respective adipokines with metabolic cardiovascular risk factors and carotid intima-media thickness (cIMT) in black African subjects that form part of a developing population.

2. Methods

2.1. Patients. The present investigation was conducted according to the principles outlined in the Helsinki declaration. The Committee for Research on Human Subjects of the University of Witwatersrand approved the protocols (approval numbers: M02-04-72 and renewed as M07-04-69 in non-RA subjects and M06-07-33 in RA subjects). Participants gave informed, written consent. The present study design has previously been described [35–42]. Briefly, 119 African black patients that met the 1988 American College of Rheumatology criteria for RA [43] were enrolled at the Charlotte Maxeke Johannesburg Academic Hospital and Milpark Hospital [35–38]. All invited patients agreed to participate. All patients used disease modifying agents for rheumatic disease (DMARD) at the time of the study. Age and sex matched non-RA subjects ($n = 158$) were participants in a population study on cardiovascular risk and disease that is also conducted in Johannesburg [39–42]. This investigation comprises randomly recruited nuclear families of black African descent with siblings older than 16 years. Serum leptin concentrations were measured in all non-RA and 112 of non-RA subjects and those of adiponectin in all RA and 77 of the non-RA subjects. Serum C-reactive protein (CRP) concentrations and carotid intima-media thickness (cIMT) were determined in 123 and 91 of the non-RA subjects. The other recorded variables did not differ in non-RA subjects with and without adiponectin, CRP, and carotid ultrasound assessments. Apart from the latter three investigations, data were missing in fewer than 5% for any of the recorded characteristics in all participants.

2.2. Baseline Characteristics. We recorded demographic features, life style factors comprising alcohol use (at least one unit per month), and exercise (at least once per month) that included time spent in walking, for example, to reach public transportation, cardiovascular, and nonsteroidal anti-inflammatory drug (NSAID) use. Height, weight, and waist and hip circumference were measured using standard approaches. Abdominal obesity and fat distribution were estimated by waist circumference and waist-hip ratio, respectively. CRP concentrations were determined using immunoturbidimetric methods. In patients with RA, we additionally recorded disease duration, the Clinical Disease Activity Index (CDAI) [44], rheumatoid factor status, and the use of traditional or synthetic DMARD. None of the patients were treated with biological DMARD therapy at the time of the study.

2.3. Metabolic Cardiovascular Risk Factors. Hypertension was defined as an average systolic blood pressure ≥140 or/and diastolic blood pressure ≥90 mmHg or/and current use of antihypertensive medications. Standard laboratory blood tests of renal and liver function, hematological parameters, lipids, and glucose were performed. Dyslipidemia was diagnosed when the atherogenic index; that is, the cholesterol-HDL cholesterol ratio was >4, and proatherogenic non-HDL cholesterol concentrations were calculated by subtracting HDL cholesterol from total cholesterol concentrations [35–38, 45]. We documented smoking habits. Diabetes was identified as the use of glucose lowering agents or a fasting plasma glucose ≥7 mmol/L.

2.4. Carotid Artery Atherosclerosis. Carotid artery intima-media thickness (cIMT) measurements were made using a linear array 7.5 MHz probe attached to a high resolution B-mode ultrasound machine (SonoCalc IMT, Sonosite Inc, Bothell, Wash, USA) in both RA and non-RA subjects, as recently described [35, 37, 38]. This equipment involves the application of a unique semiautomated border detection program that was previously documented to provide highly reproducible intra- and interrater results in other as well as our settings [35, 37, 38, 46]. Carotid artery plaque is currently identified in our RA patients [35, 37, 38] but not in non-RA subjects, and hence results on plaque are not shown in the present report.

2.5. Leptin and Adiponectin Concentrations. Leptin and adiponectin concentrations were measured using solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) (QuantakineHS, R&D Systems, Inc., Minneapolis, MN, USA). The lower detection limit was 7.8 pg/mL for leptin and 0.246 ng/mL for adiponectin. The inter- and intraassay coefficients of variation were 4.4 and 3.2%, respectively for leptin and 6.5 and 3.5%, respectively, for adiponectin.

2.6. Data Analysis. Dichotomous variables are expressed as proportions or percentages and continuous variables as mean (SD). Nonnormally distributed characteristics were logarithmically transformed prior to statistical analysis and for these variables geometric means (SD) are given.

Disparities in baseline characteristics between RA and non-RA subjects were compared using the Students t-test and univariate logistic regression analysis as appropriate. Associations of RA with metabolic cardiovascular risk factors and cIMT were investigated in multivariable logistic and continuous regression models with consistent adjustment for demographic characteristics since age differed numerically by RA status and for antihypertensive, statin, and glucose lowering therapy as appropriate.

The relationships of RA with serum adipokine concentrations were first assessed by the Students t-test and subsequently in confounder adjusted multivariable linear regression models.

The associations between baseline characteristics and adipokine concentrations and of adipokine concentrations with metabolic cardiovascular risk factors and cIMT were investigated in confounder adjusted linear regression models. The impact of RA on these relationships was determined by the addition of interaction terms (RA × variable of interest) and their individual terms to the models and in stratified analysis, that is, in RA and non-RA subjects separately.

Statistical computations were made using the GB Stat program (Dynamic Microsystems, Inc, Silver Spring, MD, USA) and SAS software, version 9.1 (The SAS Institute, Cary, NC).

3. Results

3.1. Baseline Characteristics in Subjects with and without RA. Table 1 shows the baseline characteristics in the study participants. Non-RA subjects consumed alcohol more frequently, exercised more extensively, and smoked more cigarettes per day than patients with RA. Except for waist-hip ratio, adiposity indices were reduced in RA compared to non-RA participants. Patients with RA employed antihypertensives more often and in larger numbers. None of the non-RA subjects used statins. Serum C-reactive protein concentrations were similar in RA and non-RA subjects. Mean (SD) disease duration and CDAI were 12.8 (9.2) years and 8.1 (1.6), respectively, in patients with RA; 17.6% experienced clinical RA remission (CDAI < 2.8) [44] at the time of the study. Seventy-seven percent tested rheumatoid factor positive. Methotrexate, chloroquine, sulphasalazine, leflunomide, azathioprine, tetracycline, cyclophosphamide, penicillamine, and prednisone were employed by 91.1, 79.8, 24.4, 20.2, 16.8, 10.1, 5.6, 4.2, and 1.7% of patients, respectively; none were using biological agents.

3.2. Metabolic Cardiovascular Risk Factor Profiles and Carotid Atherosclerosis in Subjects with and without RA. Table 2 gives the metabolic cardiovascular risk factors and atherosclerosis results in subjects with and without RA. The prevalence of hypertension, diabetes, blood pressure values, and glucose concentrations did not differ significantly between participants with and without RA. By contrast, lipid characteristics were consistently more favorable in subjects with RA compared to those without RA. The cIMT did not differ by RA status.

3.3. Serum Leptin and Adiponectin Concentrations in Subjects with and without RA. The geometric mean (SD) circulating leptin and adiponectin concentrations were 28 330.88 (2.75), 12 114.63 (2.35) pg/mL, 11.25 (1.83), and 7.28 (2.05) ng/mL in non-RA and RA participants, respectively ($P < 0.0001$ for both).

In all participants, eight of the baseline characteristics (Table 1) were associated with leptin or/and adiponectin concentrations: in age adjusted analysis, female sex related to leptin and adiponectin concentrations (partial $R = 0.405$ ($P < 0.0001$) and 0.141 ($P = 0.049$), resp.); in age and sex adjusted analysis, BMI, waist, angiotensin converting inhibitor, and statin use were associated with leptin concentrations (partial $P = 0.634$ ($P < 0.0001$), partial $R = 0.559$ ($P < 0.0001$), partial $R = -0.126$ ($P = 0.04$), and partial $R = -0.158$ ($P = 0.009$), resp.), and BMI, waist, waist-hip ratio, CRP concentrations and angiotensin converting enzyme inhibitor, diuretic, and statin use were associated with adiponectin concentrations (partial $R = -0.142$, $P = 0.05$), partial $R = -0.284$ ($P < 0.0001$), partial $R = -0.182$ ($P = 0.01$), partial $R = -0.224$ ($P = 0.005$), (partial $R = -0.254$ ($P < 0.0001$), partial $R = -0.185$ ($P = 0.01$), and partial $R = -0.164$ ($P = 0.02$), resp.).

When age as well as the potential confounders of sex, BMI (for leptin), waist (for adiponectin), CRP concentrations and angiotensin converting inhibitor, diuretic, and statin use were adjusted for, leptin and adiponectin concentrations remained higher in non-RA compared to RA subjects ($P < 0.0001$ and 0.0002, resp.).

3.4. The Impact of RA on the Relationships between Serum Adipokine Concentrations, Metabolic Risk Factors and Carotid Atherosclerosis. Table 3 gives the age, sex, antihypertensive agent, statin, and glucose lowering drug use (as appropriate) adjusted associations of adipokine concentrations with metabolic cardiovascular risk factors and cIMT. RA impacted on the relationships between leptin concentrations and blood pressure, lipid and glucose concentrations and cIMT. These results translated into an association of leptin concentrations with high diastolic and mean blood pressure in RA but not non-RA subjects and a relationship of leptin concentrations with a high total cholesterol-HDL cholesterol ratio, triglyceride concentrations and the triglycerides-HDL cholesterol

Table 1: Baseline characteristics in subjects with and without rheumatoid arthritis.

Characteristic	Rheumatoid arthritis		P
	Present (n = 119)	Absent (n = 158)	
Demographics			
Age	55.8 (10.2)	56.5 (10.9)	0.6
Female (%)	89.1	86.1	0.4
Lifestyle factors			
Alcohol use (%)	0.8	17.7	0.006
Units per week, n^*	0.01 (0.07)	0.27 (1.09)	0.001
Exercise (%)	40.3	43.0	0.6
Hours per week, n^*	0.01 (1.00)	1.7 (2.4)	0.0001
Smoking (%)	3.4	8.2	0.1
Cigarettes per day	0.05 (0.37)	0.15 (0.69)	0.06
Anthropometric measures			
BMI, kg/m^2	29.3 (6.6)	33.7 (8.0)	<0.0001
Waist circumference, cm	93.3 (13.4)	97.5 (15.1)	0.02
Waist-hip ratio*	0.86 (0.12)	0.85 (0.10)	0.4
Cardiovascular drugs			
Antihypertensive agents			
Use (%)	54.6	40.0	0.02
Number	1.0 (1.1)	0.5 (0.7)	<0.0001
Diuretic (%)	39.5	38.6	0.9
ACEI (%)	39.5	7.0	<0.0001
CCB (%)	17.7	5.1	0.002
BB (%)	3.4	0	—
ARB (%)	0.8	0	—
Glucose lowering agents			
Oral glucose lowering agent (%)	13.5	10.1	0.4
Insulin (%)	0.8	2.5	0.6
Statin (%)	19.4	0	—
NSAID (%)	6.7	4.4	0.3
Systemic inflammation			
CRP (mg/L)*	7.0 (3.1)	6.7 (3.1)	0.7

Results are expressed as mean (SD) or proportions/percentages. N: number; BMI: body mass index; ACEI: angiotensin converting enzyme inhibitor; CCB: calcium channel blocker, BB: beta blocker, ARB: angiotensin receptor blocker; NSAID: nonsteroidal anti-inflammatory agent; CRP: C-reactive protein.
*Nonnormally distributed variables for which geometric mean (SD) is given.
†Denotes active RA.

ratios in non-RA but not non-RA participants. RA impacted on the relationships between adiponectin concentrations, and blood pressure and lipid concentrations. These results translated into an association of adiponectin concentrations with high systolic and mean blood pressure as well as low LDL and high HDL cholesterol concentrations and low total-HDL cholesterol and triglycerides-HDL cholesterol ratios in RA but not non-RA subjects, and adiponectin concentrations were more strongly associated with non-HDL cholesterol concentrations and the triglycerides-HDL cholesterol ratio in RA compared to non-RA subjects.

We repeated the analyses in Table 3 with further adjustment for adiposity indices as well as other potential confounders (see above). As shown in Table 4, the associations of leptin concentrations with metabolic risk factors were no longer significant in either RA or non-RA subjects. However, adiponectin concentrations remained strongly associated with high systolic, diastolic, and mean blood pressure as well as low LDL and non-HDL cholesterol and triglyceride concentrations, high HDL cholesterol concentrations, and low total-HDL cholesterol and triglycerides-HDL cholesterol ratios in RA, whereas no associations with metabolic risk factors were present any longer in non-RA subjects. Adiponectin concentrations remained unassociated with cIMT in both RA and non-RA participants. The lack of significance for the associations of adipokine concentrations with triglyceride concentrations and the triglycerides-HDL cholesterol ratio despite the presence of relatively large partial correlation coefficients in non-RA subjects was due to the presence of large standard errors of the regression coefficients in the respective models (>0.135 compared to <0.096 in RA and non-RA subjects, resp.).

We reevaluated the associations of adiponectin concentrations with high blood pressure values in RA (Table 4)

Table 2: Metabolic cardiovascular risk factor profiles and carotid atherosclerosis in subjects with and without rheumatoid arthritis.

Characteristic	Rheumatoid arthritis		
	Present (n = 119)	Absent (n = 158)	
Categorical variables			OR (95% CI)*
Hypertension (%)	74.0	65.2	1.47 (0.87 to 2.48)
Total C-HDL C ratio > 4	**21.7**	**33.8**	**0.53 (0.29 to 0.96)**
Diabetes (%)	16.0	12.0	1.46 (0.74 to 2.88)
Continuous variables			P*
Blood pressure values			
SBP, mmHg	140 (25)	137 (22)	0.3
DBP, mmHg	86 (15)	87 (13)	0.5
MBP, mmHg	104 (17)	104 (15)	0.9
Lipid values			
Total C, mmol/L	**4.7 (0.9)**	**5.1 (1.2)**	**0.02**
HDL C†, mmol/L	**1.48 (1.34)**	**1.39 (1.32)**	**0.04**
Total C-HDL C ratio	**3.2 (1.1)**	**3.7 (1.3)**	**0.0002**
LDL C, mmol/L	**2.6 (0.8)**	**3.0 (1.0)**	**0.003**
Non HDL C, mmol/L	**3.1 (0.9)**	**3.6 (1.2)**	**0.0001**
Trig†, mmol/L	**1.1 (1.7)**	**1.2 (1.6)**	**0.03**
Trig-HDL C ratio†	**0.73 (2.05)**	**0.87 (1.85)**	**0.01**
Cigarettes smoked per day†, n	0.05 (0.37)	0.15 (0.68)	0.08
Glucose†, mmol/L	5.3 (1.4)	5.5 (1.4)	0.1
Carotid atherosclerosis			
cIMT, mm	0.694 (0.098)	0.704 (0.121)	0.5

Results are expressed in mean (SD) or proportions/percentages. Significant associations are shown in bold. C: cholesterol; HDL: high density lipoprotein; Trig: triglycerides; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; LDL: low density lipoprotein; n: number; CHD: coronary heart disease; CVD: cardiovascular disease; cIMT: carotid intima-media thickness.

* Adjusted for age and sex with additional adjustment for antihypertensive, statin, and glucose lowering therapy in models that included blood pressure, lipid, and glucose variables, respectively.

† Nonnormally distributed variables for which geometric means (SD) are given.

in additional models. This revealed that when other potential confounders including life style factors (smoking, exercise status, and alcohol use) and leflunomide use [47] were additionally adjusted for, the respective relationships were unaltered (partial R = 0.347, 0.270, and 0.329 and P = 0.0004, 0.007, and 0.0008 for systolic, diastolic, and mean blood pressure, resp.).

4. Discussion

In the present study performed in black African people from a developing population, comprehensive cardiovascular risk factor assessment in both persons with and without RA allowed us to compare the relationships of circulating adipokine concentrations with metabolic cardiovascular risk factors and cIMT between both groups. The most novel finding produced by this investigation is that RA impacts consistently on several potentially important independent adiponectin concentration-metabolic cardiovascular risk factor associations that translate into disparities in the respective relationships in RA compared to non-RA subjects. Leptin concentrations were not independently related to cardiovascular risk. Neither leptin nor adiponectin concentrations were associated with atherosclerosis. Leptin antagonism reduced disease severity in a preclinical animal model of rheumatoid arthritis [24], and several studies have indicated that adiponectin is involved in the progression of RA [1, 10, 21–23, 48, 49]. Therefore, our findings have important potential implications in the management of RA as related to the possible use of leptin and adiponectin inhibition in RA [23, 24].

The absence of independent relationships of leptin concentrations in both non RA and RA subjects and adiponectin concentrations in non-RA subjects with metabolic risk, as found in our study, suggests that leptin and adiponectin are markers of fat mass rather than independent metabolic risk factors in the respective groups. In contrast, adiponectin concentrations associated strongly and favorably with all recorded lipid variables except for total and LDL cholesterol as reported in the population at large [1, 2, 5, 7], but consistently with high systolic and diastolic as well as mean blood pressure in RA subjects. The latter relationships in RA persisted even after additional potential determinants of hypertension including lifestyle factors and leflunomide use were accounted for. Our finding that adiponectin concentration-metabolic risk factor relationships differed by RA status suggests that the influence of adiponectin on metabolic cardiovascular risk factors as identified in non-RA subjects cannot be merely extrapolated to patients with RA.

Table 3: Impact of RA on the age and sex adjusted relationships between serum adipokine concentrations and metabolic risk factors and carotid atherosclerosis.

Potential characteristic	Leptin*					Adiponectin*				
	Interaction P	RA (n = 112)		Non-RA (n = 158)		Interaction P	RA (n = 119)		Non-RA (n = 77)	
		Partial R	P	Partial R	P		Partial R	P	Partial R	P
SBP	0.6	0.110	0.3	0.019	0.8	0.0009	0.257	0.005	0.093	0.4
DBP	0.0008	0.248	0.009	0.044	0.6	<0.0001	0.174	0.06	−0.009	0.9
MBP	0.09	0.201	0.04	0.034	0.7	<0.0001	0.229	0.01	0.037	0.8
Total C	0.0003	−0.022	0.8	0.123	0.1	0.2	−0.113	0.2	0.040	0.7
HDL C*	0.9	−0.117	0.2	−0.089	0.3	<0.0001	0.442	<0.0001	0.224	0.06
Total C-HDL C ratio	<0.0001	0.095	0.3	0.178	0.03	<0.0001	−0.466	<0.0001	−0.202	0.08
LDL C	<0.0001	−0.014	0.9	0.148	0.07	0.06	−0.187	0.049	−0.069	0.6
Non HDL C	0.002	0.048	0.6	0.159	0.05	0.0008	−0.322	0.0005	−0.021	0.9
Trig*	1.0	0.124	0.2	0.161	0.046	0.8	−0.366	<0.0001	−0.295	0.01
Trig-HDL C ratio*	0.9	0.139	0.2	0.163	0.045	0.0009	−0.456	<0.0001	−0.347	0.002
Glucose*	0.0003	0.084	0.4	0.082	0.3	0.1	−0.142	0.1	−0.152	0.2
cIMT	0.006	0.144	0.1	0.112	0.3	0.2	−0.076	0.4	−0.008	1.0

Additional adjustment was made for antihypertensive agent use, statins and oral glucose lowering agent, and insulin use in models that included blood pressure, lipid variables, and glucose concentrations, respectively. SBP: systolic blood pressure; DBP: diastolic blood pressure; C: cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; trig: triglycerides; CRP: C-reactive protein; cIMT: carotid intima-media thickness.
*Logarithmically transformed in view of nonnormal distribution.

Table 4: Impact of RA on the independent relationships of serum leptin and adiponectin concentrations with metabolic risk factors and carotid atherosclerosis.

Potential characteristic	Leptin*				Adiponectin*			
	RA (n = 112)		Non-RA (n = 158)		RA (n = 119)		Non-RA (n = 77)	
	Partial R	P	Partial R	P	Partial R	P	Partial R	P
SBP	−0.048	0.6	−0.018	0.9	0.331	0.0006	0.067	0.7
DBP	0.109	0.3	0.014	0.9	0.280	0.004	−0.050	0.8
MBP	0.042	0.7	−0.000	1.0	0.329	0.0007	0.001	1.0
Total C	−0.035	0.7	0.091	0.3	−0.115	0.3	−0.018	0.9
HDL C*	−0.113	0.3	0.009	0.9	0.335	0.0005	0.103	0.6
Total C-HDL C ratio	−0.076	0.5	0.068	0.5	−0.363	0.0002	−0.120	0.5
LDL C	−0.024	0.8	0.064	0.5	−0.149	0.1	−0.039	0.8
Non HDL C	0.038	0.7	0.087	0.4	−0.263	0.007	−0.036	0.8
Trig*	0.120	0.2	0.093	0.3	−0.271	0.005	−0.281	0.1
Trig-HDL C ratio*	0.136	0.2	0.066	0.5	−0.347	0.0003	−0.291	0.1
Glucose*	0.012	0.9	−0.020	0.8	−0.125	0.2	−0.151	0.4
cIMT	0.123	0.2	0.023	0.9	−0.096	0.3	0.008	1.0

Age, sex, body mass index (for leptin), waist (for adiponectin), C-reactive protein concentrations, angiotensin converting enzyme inhibitors and diuretic use, and stain therapy were adjusted for in each model. Additional adjustment was made for the use of any antihypertensive agents, oral glucose lowering agent and insulin use in models that included blood pressure and glucose concentrations, respectively. SBP: systolic blood pressure; DBP: diastolic blood pressure; C: cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; trig: triglycerides; CRP: C-reactive protein; cIMT: carotid intima-media thickness.
*Logarithmically transformed in view of nonnormal distribution.

The cIMT was similar in RA compared with non-RA subjects. Hence, the relative potential adverse influence of circulating adiponectin on blood pressure may be counterbalanced by its beneficial impact on lipid metabolism, thereby resulting in an overall neutral effect on atherosclerosis in RA. Taken together, our results suggest that interventions that alter the production or inhibit the effects of adiponectin may influence individual metabolic risk factors but not overall adiponectin-mediated cardiovascular risk and disease in RA. Interestingly, glucocorticoid [50] and synthetic and biologic DMARD [51–53] can also alter adiponectin production in RA. Leptin inhibition would not be expected to influence either leptin-mediated metabolic risk or atherosclerosis in RA.

Previous studies reported higher or similar serum leptin and adiponectin concentrations in RA compared to non-RA subjects [1, 10–19]. In the present study, we found that RA is associated with reduced circulating concentrations

of both adipokines. Congruent with this finding and of likely importance in the present context, Ukkola and colleagues recently reported disparities in body composition, the insulin response to glucose and plasma lipid concentrations according to the different alleles of the gene encoding adiponectin in black and white subjects [34]. Reported findings and our results therefore strongly suggest that adipokine production in RA and the influence of circulating adipokines on metabolic cardiovascular risk factors are population specific and, hence, also argue against extrapolation of findings on adipokine metabolism from one population to another in cardiovascular risk management.

Hypertension is associated with reduced adiponectin concentrations, and low adiponectin levels were shown to increase the risk of hypertension in the population at large [5, 54, 55]. The strong and independent association of adiponectin concentrations with blood pressure in our patients with RA is therefore unexpected. We included only black Africans, whereas the impact of adiposity on cardiovascular risk may differ by population grouping [35]. Indeed, in a previous investigation in 33 white patients with RA that were treated with the tumor necrosis factor-alpha antagonist infliximab, we found that adiponectin concentrations were inversely related to atherogenic lipid ratios and plasma glucose concentrations but not to blood pressure [56].

Our cross-sectional study design precludes, however, drawing inferences on the direction of causality. Thus, our findings on adiponectin-blood pressure relationships in RA could conceptually also have resulted from a compensatory increase of adiponectin production in response to refractory hypertension, as observed in our patients, and caused by factors other than adiponectin, and in an attempt to reduce blood pressure values. A compensatory increase in adiponectin production in the presence of high grade inflammation and in an attempt to reduce inflammation was previously also postulated to underlie increased adiponectin serum concentrations in patients with RA from developed populations [10, 11]. Further prospective longitudinal and mechanistic studies are required to elucidate the relationship between circulating adiponectin concentrations and blood pressure in RA. Also, whether the positive adiponectin concentration-blood pressure relationships in RA as found in the present investigation translate in accelerated incident cardiovascular risk and disease merits additional investigation.

The present study has further limitations. Carotid artery plaques are more strongly associated with CAD and lipids than cIMT that relates more closely to stroke and blood pressure [35]. Nevertheless, both cIMT and plaque predict future cardiovascular event rates in RA and non-RA subjects irrespective of population grouping [57–60]. We measured circulating total adiponectin concentrations. Amongst its different isoforms, it is high molecular weight adiponectin that reportedly confers the vascular-protective activities in the general population [61].

In conclusion, considering previously reported findings, this study suggests that altered adipokine production in RA is population specific. RA modifies adiponectin concentration-metabolic risk factor relationships. Individual cardiovascular risk factors and particularly serum lipid concentrations require close monitoring upon employing interventions that alter adiponectin production or inhibit its effects in RA. However, whereas leptin and adiponectin inhibition could improve disease activity, this intervention may also not result in altered overall cardiovascular and disease in RA.

Conflict of Interests

The authors declare that they have no conflict of interest. This includes the fact that they do not have a direct financial relation with the trademarks mentioned in the paper.

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References

[1] E. E. Kershaw and J. S. Flier, "Adipose tissue as an endocrine organ," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 6, pp. 2548–2556, 2004.

[2] R. S. Ahima, "Adipose tissue as an endocrine organ," *Obesity*, vol. 14, supplement, pp. 242S–249S, 2006.

[3] F. Montecucco and F. Mach, "Update on therapeutic strategies to increase adiponectin function and secretion in metabolic syndrome," *Diabetes, Obesity and Metabolism*, vol. 11, no. 5, pp. 445–454, 2009.

[4] R. Gomez, J. Conde, M. Scotece, J. J. Gomez-Reino, F. Lago, and O. Gualillo, "What's new in our understanding of the role of adipokines in rheumatic diseases?" *Nature Reviews Rheumatology*, vol. 7, no. 9, pp. 528–536, 2011.

[5] T. Kadowaki, T. Yamauchi, N. Kubota, K. Hara, K. Ueki, and K. Tobe, "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1784–1792, 2006.

[6] P. Zimmet, E. J. Boyko, G. R. Collier, and M. De Courten, "Etiology of the metabolic syndrome: potential role of insulin resistance, leptin resistance, and other players," *Annals of the New York Academy of Sciences*, vol. 892, no. 1, pp. 25–44, 1999.

[7] H. Ekmekci and O. B. Ekmekci, "The role of adiponectin in atherosclerosis and thrombosis," *Clinical and Applied Thrombosis/Hemostasis*, vol. 12, no. 2, pp. 163–168, 2006.

[8] J. Beltowski, "Leptin and atherosclerosis," *Atherosclerosis*, vol. 189, no. 1, pp. 47–60, 2006.

[9] G. Matarese, C. Mantzoros, and A. La Cava, "Lepti and adipocytokines: bridging the gap between immunity and atherosclerosis," *Current Pharmaceutical Design*, vol. 13, no. 36, pp. 3676–3680, 2007.

[10] R. Krysiak, G. Handzlik-Orlik, and B. Okopien, "The role of adipokines in connective tissue diseases," *European Journal of Nutrition*, vol. 51, no. 50, pp. 513–528, 2012.

[11] M. Otero, R. Logo, R. Gomez et al., "Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 9, pp. 1198–1201, 2006.

[12] L. Šenolt, K. Pavelka, D. Housa, and M. Haluzík, "Increased adiponectin is negatively linked to the local inflammatory

process in patients with rheumatoid arthritis," *Cytokine*, vol. 35, no. 5-6, pp. 247–252, 2006.

[13] W. Tan, F. Wang, M. Zhang, D. Guo, Q. Zhang, and S. He, "High adiponectin and adiponectin Receptor 1 expression in synovial fluids and synovial tissues of patients with rheumatoid arthritis," *Seminars in Arthritis and Rheumatism*, vol. 38, no. 6, pp. 420–427, 2009.

[14] G. Palmer and C. Gabay, "A role for leptin in rheumatic diseases?" *Annals of the Rheumatic Diseases*, vol. 62, no. 10, pp. 913–915, 2003.

[15] A. Seven, S. Guzel, M. Aslan, and V. Humuryudan, "Serum and synovial leptin levels and markers of inflammation in rheumatoid arthritis," *Rheumatology International*, vol. 29, no. 7, pp. 743–747, 2009.

[16] M. Bokarewa, D. Bokarew, O. Hultgren, and A. Tarkowski, "Leptin consumption in the inflamed joints of patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 62, no. 10, pp. 952–956, 2003.

[17] A. Schaffler, A. Ehling, E. Neumann et al., "Adipocytokines in synovial fluid," *Journal of the American Medical Association*, vol. 290, no. 13, p. 1709, 2003.

[18] M. Scotece, J. Conde, R. Gomez et al., "Role of adipokines in atherosclerosis: interferences with cardiovascular complications in rheumatic diseases," *Mediators of Inflammation*, vol. 2012, Article ID 125458, 14 pages, 2012.

[19] H. R. Young, J. Solus, T. Sokka et al., "Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 60, no. 7, pp. 1906–1914, 2009.

[20] K. M. Tong, D. C. Shieh, C. P. Chen et al., "Leptin induces IL-8 expression via leptin receptor, IRS-1, PI3K, Akt cascade and promotion of NF-κB/p300 binding in human synovial fibroblasts," *Cellular Signalling*, vol. 20, no. 8, pp. 1478–1488, 2008.

[21] K. W. Frommer, A. Schaffler, C. Buchler et al., "Adiponectin isoforms: a potential therapeutic target in rheumatoid arthritis?" *Annals of the Rheumatic Diseases*, vol. 71, no. 10, pp. 1724–1732, 2012.

[22] A. Ehling, A. Schaffler, H. Herfarth et al., "The potential of adiponectin in driving arthritis," *Journal of Immunology*, vol. 176, no. 7, pp. 4468–4478, 2006.

[23] K. W. Frommer, B. Zimmermann, F. M. P. Meier et al., "Adiponectin-mediated changes in effector cells involved in the pathophysiology of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 62, no. 10, pp. 2886–2899, 2010.

[24] L. Otvos Jr., W. H. Shao, A. S. Vanniasinghe et al., "Toward understanding the role of leptin and leptin receptor antagonism in preclinical models of rheumatoid arthritis," *Peptides*, vol. 32, no. 8, pp. 1567–1574, 2011.

[25] P. H. Dessein, A. E. Stanwix, and B. I. Joffe, "Cardiovascular risk in rheumatoid arthritis versus osteoarthritis: acute phase response related decreased insulin sensitivity and high-density lipoprotein cholesterol as well as clustering of metabolic syndrome features in rheumatoid arthritis," , *Arthritis Research*, vol. 4, no. 5, 2002.

[26] M. A. Gonzalez-Gay, C. Gonzalez-Juanatey, and J. Martin, "Rheumatoid arthritis: a disease associated with accelerated atherogenesis," *Seminars in Arthritis and Rheumatism*, vol. 35, no. 1, pp. 8–17, 2005.

[27] P. H. Dessein and B. I. Joffe, "When is a patient with rheumatoid arthritis at risk for cardiovascular disease?" *Journal of Rheumatology*, vol. 33, no. 2, pp. 201–203, 2006.

[28] M. Ozgen, S. S. Koca, N. Dagli, M. Balin, B. Ustundag, and A. Isik, "Serum adiponectin and vaspin levels in rheumatoid arthritis," *Archives of Medical Research*, vol. 41, no. 6, pp. 457–463, 2010.

[29] M. A. Gonzalez-Gay, C. Gonzalez-Juanatey, L. Rodriguez-Rodriguez, J. A. Miranda-Filloy, J. Martin, and J. Llorca, "Lack of association between adipokines and ghrelin and carotid intima-media thickness in patients with severe rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 29, no. 2, pp. 358–359, 2011.

[30] Y. H. Rho, C. P. Chung, J. F. Solus et al., "Adipocytokines, insulin resistance, and coronary atherosclerosis in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 62, no. 5, pp. 1259–1264, 2010.

[31] M. García-Bermúdez, C. González-Juanatey, L. Rodríguez-Rodríguez et al., "Lack of association between LEP rs2167270 (19 G>A) polymorphism and disease susceptibility and cardiovascular disease in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 29, no. 2, pp. 293–298, 2011.

[32] B. H. Hahn, E. V. Lourenço, M. McMahon et al., "Pro-inflammatory high-density lipoproteins and atherosclerosis are induced in lupus-prone mice by a high-fat diet and leptin," *Lupus*, vol. 19, no. 8, pp. 913–917, 2010.

[33] M. McMahon, B. J. Skaggs, L. Sahakian et al., "High plasma leptin levels confer increased risk of atherosclerosis in women with systemic lupus erythematosus, and are associated with inflammatory oxidised lipids," *Annals of the Rheumatic Diseases*, vol. 70, no. 9, pp. 1619–1624, 2011.

[34] O. Ukkola, M. Santaniemi, T. Rankinen et al., "Adiponectin polymorphisms, adiposity and insulin metabolism: HERITAGE family study and Oulu diabetic study," *Annals of Medicine*, vol. 37, no. 2, pp. 141–150, 2005.

[35] A. Solomon, G. R. Norton, A. J. Woodiwiss, and P. H. Dessein, "Obesity and carotid atherosclerosis in African black and Caucasian women with established rheumatoid arthritis: a cross-sectional study," *Arthritis Research and Therapy*, vol. 14, no. 2, article R67, 2012.

[36] A. Solomon, B. F. Christian, G. R. Norton, A. J. Woodiwiss, and P. H. Dessein, "Risk factor profiles for atherosclerotic cardiovascular disease in black and other Africans with established rheumatoid arthritis," *Journal of Rheumatology*, vol. 37, no. 5, pp. 953–960, 2010.

[37] P. H. Dessein, G. R. Norton, B. I. Joffe, A. T. Abdool-Carrim, A. J. Woodiwiss, and A. Solomon, "Metabolic cardiovascular risk burden and atherosclerosis in African black and Caucasian women with rheumatoid arthritis: a cross-sectional study," *Clinical and Experimental Rheumatology*, vol. 31, no. 1, pp. 53–61, 2013.

[38] A. Solomon, A. J. Woodiwiss, A. T. Abdool-Carrim, B. A. Stevens, G. R. Norton, and P. H. Dessein, "The carotid artery atherosclerosis burden and its relation to cardiovascular risk factors in black and white Africans with established rheumatoid arthritis: a cross-sectional study," *Journal of Rheumatology*, vol. 39, no. 9, pp. 1798–1806, 2012.

[39] M. Redelinghuys, G. R. Norton, N. M. Janse van Rensburg et al., "Lack of independent association between C-reactive protein and central aortic hemodynamics in black Africans with high risk of cardiovascular disease," *The American Journal of Hypertension*, vol. 24, no. 10, pp. 1094–1101, 2011.

[40] G. R. Norton, M. Maseko, E. Libhaber et al., "Is prehypertension an independent predictor of target organ changes in

young-to-middle-aged persons of African descent?" *Journal of Hypertension*, vol. 26, no. 23, pp. 2279–2287, 2008.

[41] A. J. Woodiwiss, N. Molebatsi, M. J. Maseko et al., "Nurse-recorded auscultatory blood pressure at a single visit predicts target organ changes as well as ambulatory blood pressure," *Journal of Hypertension*, vol. 27, no. 2, pp. 287–297, 2009.

[42] G. R. Norton, O. H. Majane, M. J. Maseko et al., "Brachial blood pressure-independent relations between radial late systolic shoulder-derived aortic pressures and target organ changes," *Hypertension*, vol. 59, no. 4, pp. 885–592, 2012.

[43] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[44] A. Gülfe, D. Aletaha, T. Saxne, and P. Geborek, "Disease activity level, remission and response in established rheumatoid arthritis: performance of various criteria sets in an observational cohort, treated with anti-TNF agents," *BMC Musculoskeletal Disorders*, vol. 10, article 41, 2009.

[45] P. H. Dessein, B. F. Christian, and A. Solomon, "Which are the determinants of dyslipidemia in rheumatoid arthritis and does socioeconomic status matter in this context?" *Journal of Rheumatology*, vol. 36, no. 7, pp. 1357–1361, 2009.

[46] A. D. Gepner, C. E. Korcarz, S. E. Aeschlimann et al., "Validation of a carotid intima-media thickness border detection program for use in an office setting," *Journal of the American Society of Echocardiography*, vol. 19, no. 2, pp. 223–228, 2006.

[47] V. F. Panoulas, G. S. Metsios, A. V. Pace et al., "Hypertension in rheumatoid arthritis," *Rheumatology*, vol. 47, no. 9, pp. 1286–1298, 2008.

[48] J. T. Giles, D. M. van der Heijde, and J. M. Bathon, "Association of circulating adiponectin levels with progression of radiographic joint destruction in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 70, no. 9, pp. 1562–1568, 2011.

[49] I. R. Klein-Wieringa, M. P. van der Linden, R. Knevel et al., "Baseline serum adipokine levels predict radiographic progression in early rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 63, no. 9, pp. 2567–2574, 2011.

[50] P. Härle, P. Sarzi-Puttini, M. Cutolo, and R. H. Straub, "No change of serum levels of leptin and adiponectin during anti-tumour necrosis factor antibody treatment with adalimumab in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 7, pp. 970–971, 2006.

[51] C. Popa, M. G. Netea, J. de Graaf et al., "Circulating leptin and adiponectin concentrations during tumor necrosis factor blockade in patients with active rheumatoid arthritis," *Journal of Rheumatology*, vol. 36, no. 4, pp. 724–730, 2009.

[52] T. B. Laurberg, J. Frystyk, T. Ellingsen et al., "Plasma adiponectin in patients with active, early, and chronic rheumatoid arthritis who are steroid- and disease-modifying antirheumatic drug-naive compared with patients with osteoarthritis and controls," *Journal of Rheumatology*, vol. 36, no. 9, pp. 1885–1891, 2009.

[53] I. L. Engvall, B. Tengstrand, K. Brismar, and I. Hafström, "Infliximab therapy increases body fat mass in early rheumatoid arthritis independently of changes in disease activity and levels of leptin and adiponectin: a randomised study over 21 months," *Arthritis Research and Therapy*, vol. 12, no. 5, article R197, 2010.

[54] Y. Okamoto, S. Kihara, T. Funahashi, Y. Matsuzawa, and P. Libby, "Adiponectin: a key adipocytokine in metabolic syndrome," *Clinical Science*, vol. 110, no. 3, pp. 267–278, 2006.

[55] L. Wang, J. E. Manson, J. M. Gaziano et al., "Plasma adiponectin and the risk of hypertension in white and black postmenopausal women," *Clinical Chemistry*, vol. 58, no. 10, pp. 1438–1445, 2012.

[56] M. A. Gonzalez-Gay, J. Llorca, M. T. Garcia-Unzueta et al., "High-grade inflammation, circulating adiponectin concentrations and cardiovascular risk factors in severe rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 26, no. 4, pp. 596–603, 2008.

[57] C. Gonzalez-Juanatey, J. Llorca, J. Martin, and M. A. Gonzalez-Gay, "Carotid intima-media thickness predicts the development of cardiovascular events in patients with rheumatoid arthritis," *Seminars in Arthritis and Rheumatism*, vol. 38, no. 5, pp. 366–371, 2009.

[58] M. R. Evans, A. Escalante, D. F. Battafarano, G. L. Freeman, D. H. O'Leary, and I. Del Rincõn, "Carotid atherosclerosis predicts incident acute coronary syndromes in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 63, no. 5, pp. 1211–1220, 2011.

[59] P. Greenland, J. S. Alpert, G. A. Beller et al., "2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines," *Journal of the American College of Cardiology*, vol. 56, no. 25, pp. e50–e130, 2010.

[60] S. A. Peters, H. M. den Ruijter, M. L. Bots, and K. G. Moons, "Improvements in risk stratification for the occurrence of cardiovascular disease by imaging subclinical atherosclerosis: a systematic review," *Heart*, vol. 98, no. 3, pp. 177–184, 2012.

[61] H. Kobayashi, N. Ouchi, S. Kihara et al., "Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin," *Circulation Research*, vol. 94, no. 4, pp. e27–e31, 2004.

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Research Article

Exercise and Caloric Restriction Alter the Immune System of Mice Submitted to a High-Fat Diet

Frederick Wasinski,[1] Reury F. P. Bacurau,[2] Milton R. Moraes,[1] Anderson S. Haro,[1] Pedro M. M. Moraes-Vieira,[3] Gabriel R. Estrela,[1] Edgar J. Paredes-Gamero,[1] Carlos C. Barros,[4] Sandro S. Almeida,[1] Niels O. S. Câmara,[3] and Ronaldo C. Araujo[1]

[1] *Department of Biophysics, Federal University of São Paulo, 04023-062 São Paulo, SP, Brazil*
[2] *School of Arts, Sciences and Humanities, University of Sao Paulo, Avenue Arlindo Bettio 1000, 03828-000 São Paulo, SP, Brazil*
[3] *Department of Immunology, Laboratory of Transplantation Immunobiology, Institute of Biomedical Sciences, University of São Paulo, 05508-900 São Paulo, SP, Brazil*
[4] *Department of Nutrition, School of Nutrition, Federal University of Pelotas, 96010-610 Pelotas, RS, Brazil*

Correspondence should be addressed to Ronaldo C. Araujo; araujo.ronaldo@unifesp.br

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As the size of adipocytes increases during obesity, the establishment of resident immune cells in adipose tissue becomes an important source of proinflammatory mediators. Exercise and caloric restriction are two important, nonpharmacological tools against body mass increase. To date, their effects on the immune cells of adipose tissue in obese organisms, specifically when a high-fat diet is consumed, have been poorly investigated. Thus, after consuming a high-fat diet, mice were submitted to chronic swimming training or a 30% caloric restriction in order to investigate the effects of both interventions on resident immune cells in adipose tissue. These strategies were able to reduce body mass and resulted in changes in the number of resident immune cells in the adipose tissue and levels of cytokines/chemokines in serum. While exercise increased the number of NK cells in adipose tissue and serum levels of IL-6 and RANTES, caloric restriction increased the CD4+/CD8+ cell ratio and MCP-1 levels. Together, these data demonstrated that exercise and caloric restriction modulate resident immune cells in adipose tissues differently in spite of an equivalent body weight reduction. Additionally, the results also reinforce the idea that a combination of both strategies is better than either individually for combating obesity.

1. Introduction

Chronic, low-grade inflammation is associated with insulin resistance, type 2 diabetes, and several types of cancer [1, 2]. These detrimental conditions are also associated with obesity [3]. As visceral and subcutaneous adipocytes increase in size, monocytes and CD4+ and CD8+ T cells migrate to adipose tissue (AT) [4] initiating the release of proinflammatory mediators (e.g., IL-1β, IL-6, RANTES, MCP-1, and IL-18) inducing local insulin resistance [5]. Thus, the expanded ATs and their populations of resident immune cells constitute the main microenvironment in which proinflammatory cytokines are produced and released in the organism [6].

Physical activity and caloric restriction (CR) are both nonpharmacological strategies recommended to reduce obesity [7]. Although the beneficial effects of exercise are well described in skeletal muscle and the liver, the same is not true for AT [7–9]. Additionally, there is little information concerning the effects of both weight reduction strategies on immune cell populations that reside in AT. In relation to CR, though its importance in reducing body weight is unquestionable, consumption of a healthy diet requires such marked lifestyle changes that many individuals are unable to comply with one of them.

Thus, it is important to investigate whether physical exercise and CR are able to promote a healthy lifestyle while

maintaining a high-fat (HF) diet. The aim of this study was investigate the effect of physical exercise or CR on AT immune cells in diet-induced obese mice.

2. Materials and Methods

2.1. Animals. Male C57BL/6 ($n = 20$, 5 per group) mice (aged 8–12 weeks; 23–26 g) were obtained from the Animal Care Facility at the Federal University of São Paulo (UNIFESP). All animals were housed in standard, individual cages and had access to water and food. To examine the changes in stromal vascular cell populations in adipose tissue under conditions of diet-induced obesity, we divided the C57BL/6 mice into four groups and fed them either a standard chow diet (6% fat, Nuvilab mod. CR-1) or a high-fat diet (D12451, 45% Kcal fat, Research Diets). At 16 weeks, the mice were further subdivided into the following groups: (1) a control group fed a normal, low-fat (LF) chow; (2) a control group fed a high-fat (HF) diet; (3) a dietary restriction group fed a 30% high-fat diet (HFREST); and (4) an exercise group fed a high-fat diet that participated in 60 minutes of swimming (HFEX). Food consumption was controlled every day. Based on the quantity of high-fat diet chow consumed and using of the macronutrient composition as reference, we calculated the energy intake. The 30% caloric restriction was designed taking HF consumption as a reference.

All procedures were previously reviewed and approved by the internal ethical committee of the institution.

2.2. Exercise Protocol. The HFEX animals were subject to swimming sessions in a swimming system adapted for mice with water heated to 30°C. The 300 liter tank had 10 lanes and was fitted with air pumps that maintained the mice in constant motion. Swimming sessions began with 15 minutes in the first week and gradually increased in length until the mice were able to swim for 60 minutes a day. At this point, the HFEX mice were subjected to swimming sessions 5 times per week for 6 weeks. Both the exercise and the dietary restriction groups were subject to their respective intervention for 6 weeks.

The mice were anesthetized with ketamine/xylazine for blood collection via retroorbital venous plexus and then killed by cervical dislocation. The blood was centrifuged at 1000 g for 10 minutes. The serum was removed and stored at −80°C for future analysis. We collected a 1 g sample of adipose inguinal from each group and subjected the sample to enzymatic degradation. All animals were weighed weekly until the end of the experiments.

2.3. Isolation of the Stromal Vascular Fraction (Sfv) and Flow Cytometry. After sacrificing the mouse, inguinal adipose tissue (IAT) was extracted, weighed, and subjected to enzymatic degradation as previously described [10]. After the isolation of the IAT SFV cells, 200 μL of FCS washing buffer (1x PBS, 2% SFC) was added, and the solution was centrifuged for 5 minutes. After the supernatant was discarded, the pellet was resuspended in FCS and centrifuged for 5 minutes at 600 g. The cells were stained with anti-CD8 (Caltag-FITC-Medsystems, Buckingham, UK), anti-CD4 (blue-Pacific-BioLegend), anti-F4/80 (PerCP-Bioscience), and anti-NK.1 (PE-Bioscience) antibodies. The stromal cells were acquired via FACS in a Canto II flow cytometer (BD, Becton Dickinson, NJ, USA). The data analyses were completed using the program FlowJo 8.7.4. (Tree Star Inc., Ashland, OR, USA).

2.4. Analysis of Cytokines in Serum. Serum samples were stored at −80°C. The panel used for the Milliplex Mouse cytokine/chemokine immunoassay included the following cytokines: MCP-1, RANTES, TNF-alpha (tumor necrosis factor), IL-6, and IL1-β. Testing was conducted in accordance with the procedures previously described by the manufacturer (Milliplex Mouse cytokine/chemokine panel).

2.5. Glucose Tolerance Test. The glucose tolerance test (GTT) was carried out in animals fasted for 12 hours. To avoid stress, there was an interval of 7 days between tests. Glycemia was measured using a glucometer (Accu-Chek Advantage) measuring blood drops obtained from the tail vein. For GTT 1 g glucose per kg of body weight (BW) was injected intraperitoneally. Glucose levels were determined at baseline, 0, 15, 30, 60, and 120 min after the injection of glucose.

2.6. Statistical Analysis. The data were presented as the mean ± standard error in the descriptive text and graphics. All experiments were compared using One Way ANOVA followed by post-hoc Tukey test. Significant differences were determined when the P value was less than 0.05 ($P < 0.05$). The graphics were developed in Prism 5.0.

3. Results

Animals subjected to the HF diet consumed more calories compared with mice from the LF diet group (Figure 1(a)). Higher caloric consumption was accompanied by an increase in mouse total body mass (Figure 1(b)). Swimming combined with the HF diet was able to reduce mouse body weight similar to that observed in animals administered caloric restriction (Figure 1(b)). There was no difference in inguinal adipose tissue (IAT) and brown adipose tissue (BAT) between trained animals and those submitted to caloric restriction (Figures 1(c) and 1(d)); however, trained animals presented more BAT than animals from LF group (Figure 1(d)).

It is known that obesity is associated with systemic, low-grade inflammation. Therefore, we investigated the effects of a HF diet, exercise, and diet restriction in our study groups by evaluating the serum levels of several proinflammatory cytokines. We observed increased levels of IL-1β in the HF diet group and a reduction of this cytokine in both intervention groups (exercise and caloric restriction) (Figure 2(a)). TNF-α serum levels were not affected by the changes in diet and exercise investigated in this study (Figure 2(b)).

In addition to exerting both pro- and anti-inflammatory functions, IL-6 also plays important roles in both obesity and physical exercise. While the HF diet did not change IL-6 levels, exercise training increased its levels (Figure 2(c)).

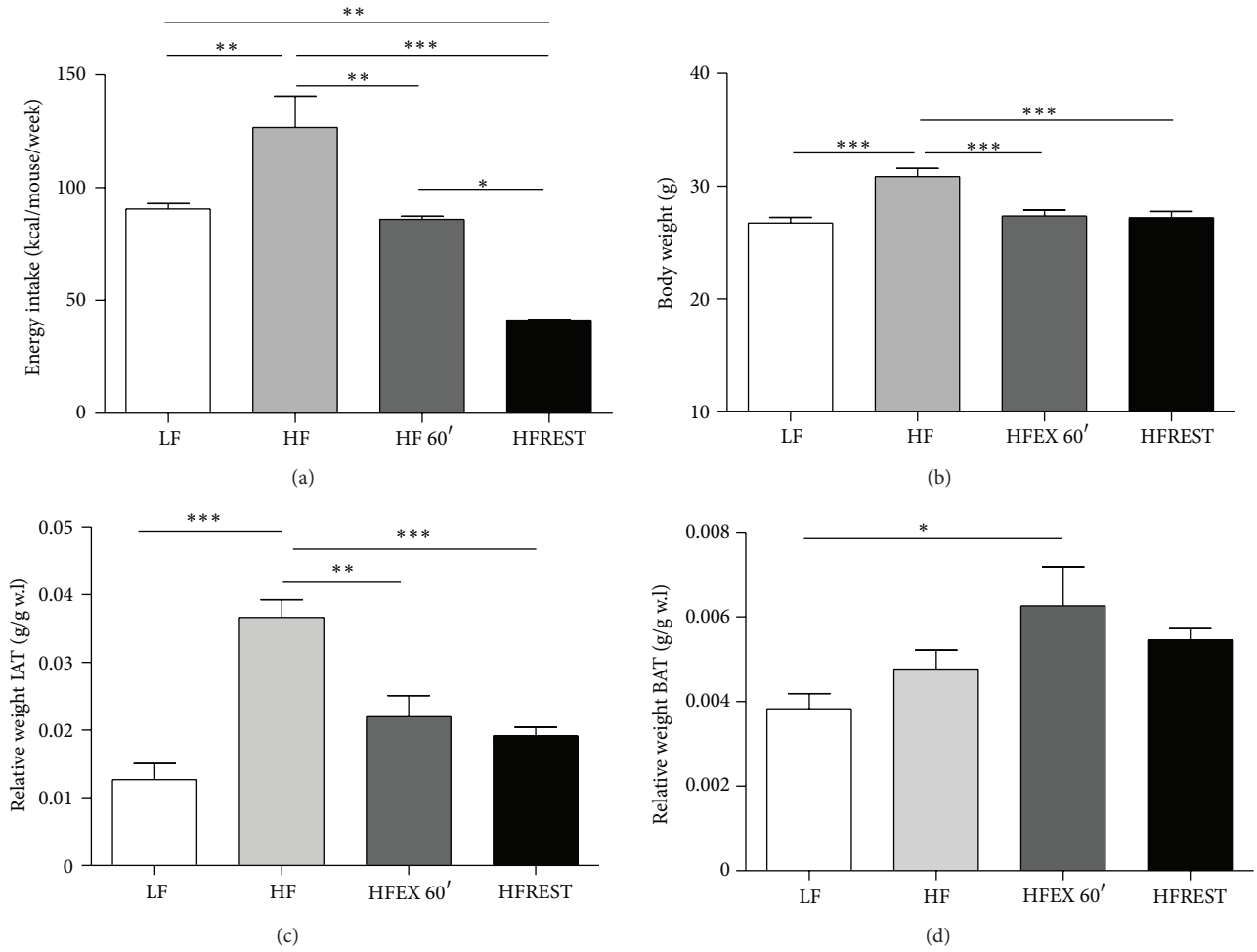

Figure 1: (a) Weekly caloric intake of animals subjected to the control diet (LF), high-fat diet (HF), high-fat diet with exercise (HFEX 60′), and high-fat diet with 30% food restriction (HFREST). (b) Mouse body weights (g). (c) Relative weight of IAT (inguinal adipose tissue). (d) Relative weight of BAT (brown adipose tissue). Mean ± SEM, $n = 5$ mice per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Reduction of body weight was unable to induce changes in IL-6 levels as no differences were observed in animals from the caloric restriction group (Figure 2(c)).

Animals subjected to swimming had significantly increased RANTES serum levels (regulated upon activation, normal T cells expressed and secreted). The increases in body mass and IAT observed in animals submitted to the HF diet did not lead to changes in RANTES levels, albeit AT lymphocyte infiltration during obesity is expected. Nevertheless, physical exercise increased RANTES serum levels, while caloric restriction did not change its levels (Figure 2(d)). Moreover, MCP-1 (monocyte chemoattractant protein-1) was reduced by exercise and was not affected by caloric restriction (Figure 2(e)).

Because immune cells that reside in obese AT actively secrete proinflammatory cytokines and chemokines, we evaluated the effect of the HF diet and both interventions on AT leukocyte populations. We observed a reduction in CD4+ and CD8+ T lymphocytes in AT in animals submitted to both interventions in comparison with animals consuming the HF diet (Figures 3(a) and 3(b)). Because CD8+ cells were more reduced than CD4+ cells, an increased CD4+/CD8+ ratio was observed (Figure 3(e)). The natural killer cell marker (NK1.1) was affected only by caloric restriction (Figure 3(c)). Also, we observed that both exercise and caloric restriction were able to reverse the increased macrophage infiltration observed in the HF diet group (Figure 3(d)).

Concerning glucose tolerance test, it was observed that only caloric restriction was able to improve this parameter in comparison to HF group (Figure 4).

4. Discussion

Immune cells reside in lean and obese adipose tissue but exhibit different characteristics in each condition. As adipocytes increase in size, these immune cells change in terms of number and functionality. Moreover, such changes in immune cells contribute actively to the establishment of local and systemic low-grade inflammation [11]. Regular exercise is an important nonpharmacological strategy for treating obesity as it protects against the increases in body

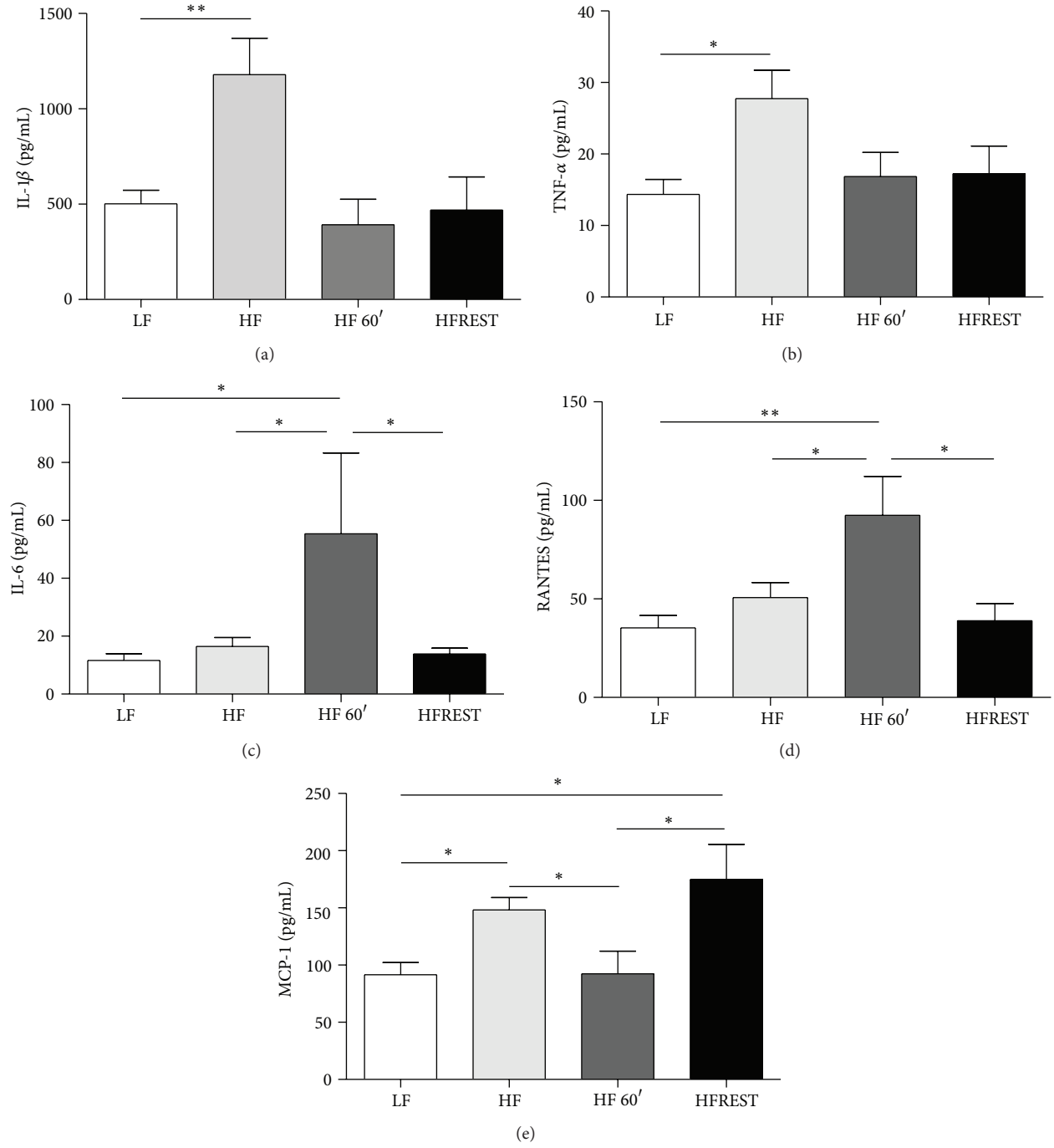

Figure 2: Cytokine concentrations (pg/mL) in mouse serum subjected to control diet (LF, $n = 12$), high-fat diet (HF, $n = 12$), high-fat diet with exercise 60′ (HFEX 60′, $n = 8$), and high-fat diet with 30% food restriction (HFREST, $n = 8$). (a) IL-1β, (b) TNF-α, (c) IL-6, (d) RANTES, and (e) MCP-1. Mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

mass and counterbalances several deleterious consequences due to its anti-inflammatory effects [12]. Normally, exercise intervention with dietary modifications is suggested to combat obesity; the combination of both interventions works better than exercise alone [13–15]. However, little is known about the benefits of exercise when dietary modifications are not prescribed.

Because our results demonstrated that chronic exercise is able to counterbalance several immune changes induced by HF (or promotes different changes), it appears that this intervention is beneficial even when a high-fat diet is maintained. It was previously demonstrated that AT expansion reduces the number of resident NK cells. NK cells produce significant amounts of gamma interferon (IFN-γ), promoting

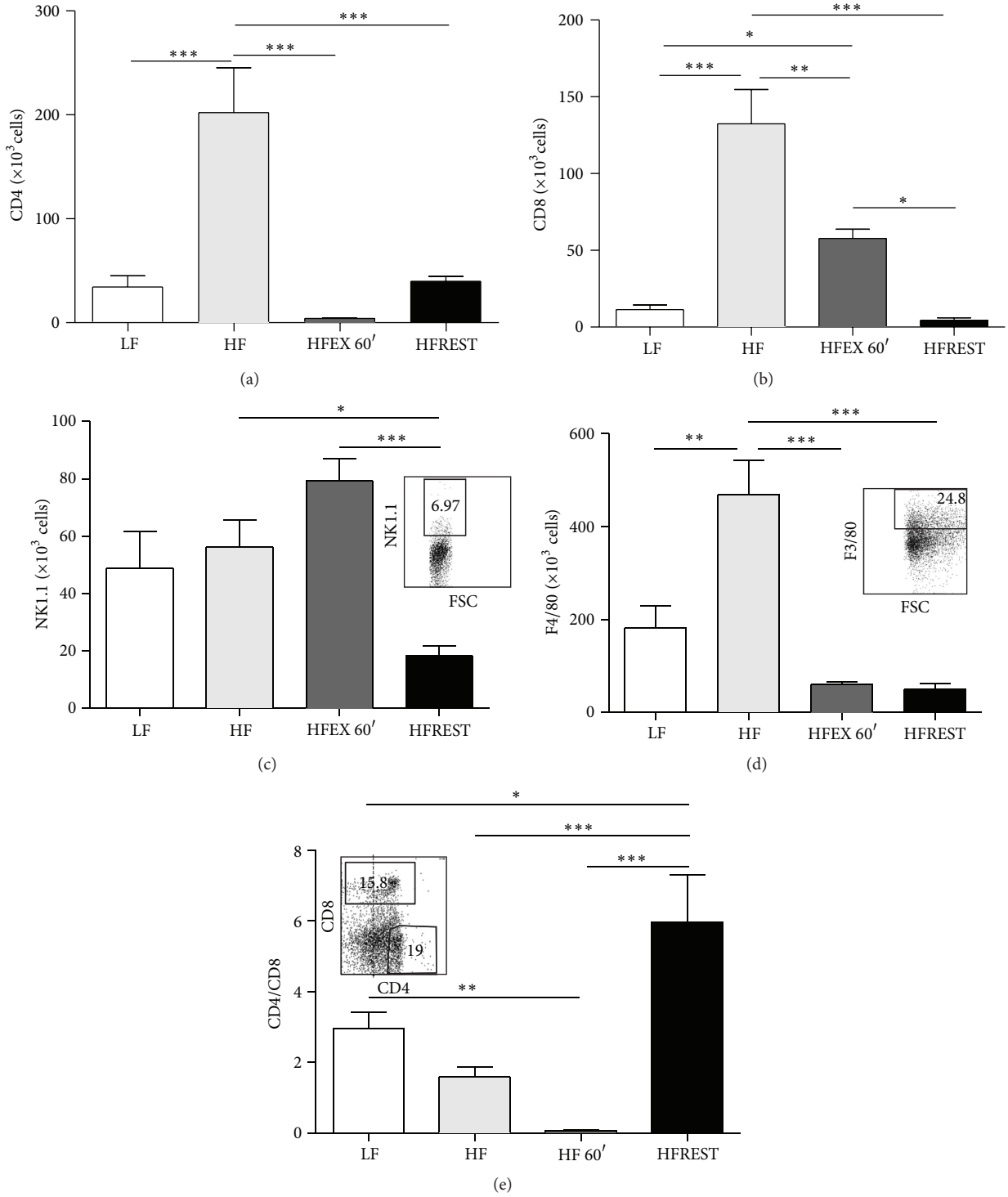

Figure 3: Quantification of cells expressing CD4, CD8, NK1.1, and F4/80 (macrophage) in inguinal adipose tissue of mice subjected to control diet (LF), high-fat diet (HF), high-fat diet with exercise (HFEX 60′), and high-fat diet with 30% food restriction (HFREST). (a) CD4 cells, (b) CD8 cells, (c) NK1.1 cells, (d) F4/80 cells, and (e) ratio of CD4/CD8. Mean ± SEM, $n = 5$ mice per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

an inflammatory state in AT. Studies have also shown that reducing the presence of inflammatory cells in AT improves glucose tolerance in IFN-γ-deficient mice [16]. IFN-γ is also able to inhibit the Hedgehog signaling pathway involved in adipocyte differentiation [17]. The ability of exercise to promote an increase in the number of NK cells in AT may also reflect another type of cell infiltration, such as NKT, that appears to display a protective role. The absence of changes

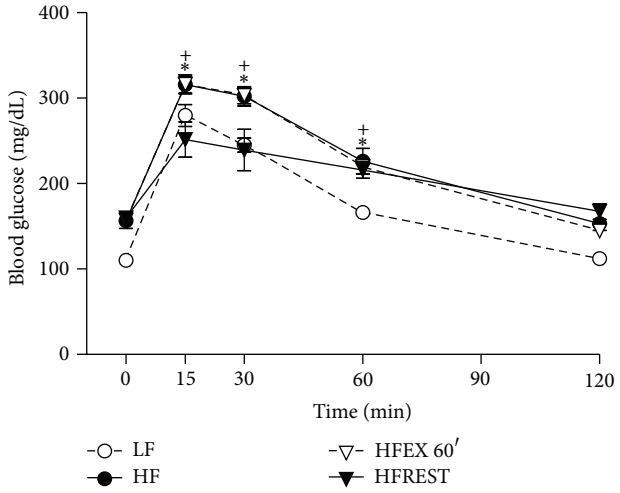

Figure 4: Glucose tolerance test in mouse subjected to control diet (LF), high-fat diet (HF), high-fat diet with exercise 60′ (HFEX 60′), and high-fat diet with 30% food restriction (HFREST). The glucose levels of LF were different from the other groups at time 0, 30, and 60 minutes ($P < .05$). Mean ± SEM, $n = 5$ mice per group ($^{+}P < 0.05$, HFEX 60′ versus HFREST; $^{*}P < 0.05$, HF versus HFREST).

in the number of NK cells corroborates with two previous studies which reported that loss of NK cells has little or no effect on metabolic parameters after a 45% HF diet for 26 weeks or 60% of the same diet for 12 weeks [18, 19].

In concordance with previous results [20, 21] we observed an increased frequency of macrophages in obese AT. Macrophages are responsive to TLR stimuli producing marked amounts of proinflammatory cytokines (e.g., IL-12, TNF, IL-1β, and IL-6) increasing AT inflammatory response [22]. Additionally, these immune mediators are involved in insulin resistance and type 2 diabetes in obese organisms [23]. Thus, our data regarding macrophages and adipose tissue from the HF diet animals were in accordance with previous studies that showed that an increased infiltration of macrophages in AT was observed. Although we did not evaluate the macrophage profile (e.g., M1 and M2) in AT, we verified that the increase in macrophage number and MCP-1, an important molecule in the recruitment of these cells [24], promoted by HF, was reversed by exercise.

In this study, increased macrophage infiltration due to HF was followed by an increased number of CD8+ T cells in the AT. Conversely, macrophage reduction promoted by exercise and caloric restriction was followed by a decreased number of CD8+ T cells. Adaptive T cells are also related to macrophage infiltration in AT [25]. CD8+ T cells increase 3 to 4 times in the AT of humans and animals subjected to high-fat diets and produce large amounts of cytokines and chemokines. Nishimura and et al. [25] reported that CD8+ T cell neutralization reduced macrophage infiltration and insulin resistance in mice fed with a high-fat diet. The adoptive transfer of CD8+ T cells to mice deficient in this cell population aggravates inflammation in the AT. Together, these data suggest that CD8+ T cells are activated in the AT of obese mice and that these lymphocytes induce macrophage activation and migration to AT.

CD4+ T cells also play a pivotal role in the progression of obesity and are associated with inflammation via cytokine secretion. In Rag-1 KO mice, reconstitution of CD4+ T cells reduced the increment in body weight, adipocytes size, glucose tolerance, and insulin signaling [26, 27]. In this sense, it is tempting to speculate that the increase of CD4+ T cells due to a HF diet could compensate for the increased inflammation in AT. The reduction of these cells induced by exercise and caloric restriction suggests that inflammation improvement in AT induces a reduction in CD4+ T cells.

In the blood as well as in most tissues, the CD4+/CD8+ T-cell ratio is generally greater than 2 to 1. HF consumption induced a reduction in this ratio in AT. It is important to note that only caloric restriction was able to restore the CD4+ and CD8+ numbers to the levels observed in LF group, and this could be related to the different responses observed in the glucose tolerance test.

Since immune cells that reside in adipose tissue are an important source of proinflammatory cytokines and chemokines in obesity, we decided to investigate whether an HF diet and interventions affected cytokines. The HF diet reduced MCP-1 and increased RANTES in serum compared with the control. RANTES is a potent chemoattractant for several cell types [28], including NK cells [29]; its increase in the serum of control animals could be due to the higher number of NK cells in these animals.

Interleukin-6 is a widely studied cytokine in exercise. It has been demonstrated to be increased by up to 100 times in exhaustive acute exercise promoted by skeletal muscle glycogen depletion [12]. Investigations on the effects of chronic exercise on IL-6 revealed that physical training reduces its levels. Our data, in opposition, demonstrated that chronic swimming increased IL-6. Higher increases in IL-6 levels, in response to exercise, have been associated with the reduction of glycogen stores [12]. In our study, mice were subjected to an HF diet during initial swimming training, and this diet was maintained throughout the study. This difference in study design could explain the discrepancy among our data and those from other studies that investigated the effect of chronic exercise on IL-6 levels. Our results show that the anti-inflammatory effects of exercise are present regardless of whether the exercise promotes body mass reduction [12]. In our study, all the changes promoted by exercise were accompanied by a reduction in body mass, AT, and caloric intake. Interesting, it seems that these reductions were not the only factor in determining the changes in AT cell populations or serum cytokines. Although exercise and caloric restriction have both induced a reduction in body mass and AT mass, the biological repercussions of both interventions were different. For example, fewer NK and CD4+ cells were observed in response to changes in diet as compared to exercise.

Additionally, the effects of dietary restriction on MCP-1, RANTES, and IL-6 levels were different in comparison to those observed in the trained group. These observations suggest that exercise and caloric restriction, thought to be able to attain the same goal, proceed by different mechanisms [30]. Another example of this statement is reduction of food

intake promoted by exercise. In accordance with previous studies [31, 32], such effect could be related to the effect of exercise on leptin sensitivity on central nervous system. Trained animals also presented an enhancement of BAT in comparison to LF group and it is tempting to speculate that a change in thermogenesis could influence body weight reduction in trained animals. Therefore, our results suggest that the effect of chronic exercise was not restricted to caloric expenditure due exercise practice.

A few limitations must also be discussed. Because the changes we observed in the levels of circulating cytokines were not strictly related to the local number of resident immune cells, it is important to note that the absence of detection experiments of adipose tissue cytokines constitute a limitation of our study. Also, note that exercise intensity is an important factor for adaptations to occur. Thus, other different intensities from that which we evaluated could induce different immune changes from those observed herein, and this must be considered as a limitation of our design.

5. Conclusions

Our data demonstrate that both exercise and caloric restriction were able to counterbalance the deleterious effects induced by an HF diet. The interventions induced a reduction in body mass and body fat. However, these reductions could not explain all the results because the effects of dietary restriction and exercise were not the same. The exercise appears to affect innate immunity (i.e., NK1.1), while dietary restriction influenced adaptive immunity (i.e., CD4+/CD8+ ratio). Both interventions affected cytokine and chemokine levels in different manners.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgment

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References

[1] A. H. Mokdad, B. A. Bowman, E. S. Ford, F. Vinicor, J. S. Marks, and J. P. Koplan, "The continuing epidemics of obesity and diabetes in the United States," *Journal of the American Medical Association*, vol. 286, no. 10, pp. 1195–1200, 2001.

[2] P. M. Moraes-Vieira, E. J. Bassi, R. C. Araujo, and N. O. Camara, "Leptin as a link between the immune system and kidney-related diseases: leading actor or just a coadjuvant?" *Obesity Reviews*, vol. 13, no. 8, pp. 733–743, 2012.

[3] S. Haffner and H. Taegtmeyer, "Epidemic obesity and the metabolic syndrome," *Circulation*, vol. 108, no. 13, pp. 1541–1545, 2003.

[4] H. Yang, Y. H. Youm, B. Vandanmagsar et al., "Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance," *Journal of Immunology*, vol. 185, no. 3, pp. 1836–1845, 2010.

[5] A. S. Greenberg and M. S. Obin, "Obesity and the role of adipose tissue in inflammation and metabolism," *American Journal of Clinical Nutrition*, vol. 83, no. 2, pp. 461S–465S, 2006.

[6] V. Z. Rocha and P. Libby, "The multiple facets of the fat tissue," *Thyroid*, vol. 18, no. 2, pp. 175–183, 2008.

[7] R. H. Coker, R. H. Williams, S. E. Yeo et al., "The impact of exercise training compared to caloric restriction on hepatic and peripheral insulin resistance in obesity," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 11, pp. 4258–4266, 2009.

[8] N. Kawanishi, H. Yano, T. Mizokami et al., "Exercise training attenuates hepatic inflammation, fibrosis and macrophage infiltration during diet induced-obesity in mice," *Brain, Behavior, and Immunity*, vol. 26, no. 6, pp. 931–941, 2012.

[9] A. D. Krisan, D. E. Collins, A. M. Crain et al., "Resistance training enhances components of the insulin signaling cascade in normal and high-fat-fed rodent skeletal muscle," *Journal of Applied Physiology*, vol. 96, no. 5, pp. 1691–1700, 2004.

[10] S. Gesta, M. Blüher, Y. Yamamoto et al., "Evidence for a role of developmental genes in the origin of obesity and body fat distribution," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 17, pp. 6676–6681, 2006.

[11] S. Sun, Y. Ji, S. Kersten, and L. Qi, "Mechanisms of inflammatory responses in obese adipose tissue," *Annual Review of Nutrition*, vol. 32, pp. 261–286, 2012.

[12] A. M. W. Petersen and B. K. Pedersen, "The anti-inflammatory effect of exercise," *Journal of Applied Physiology*, vol. 98, no. 4, pp. 1154–1162, 2005.

[13] L. H. Colbert, M. Visser, E. M. Simonsick et al., "Physical activity, exercise, and inflammatory markers in older adults: findings from the health, aging and body composition study," *Journal of the American Geriatrics Society*, vol. 52, no. 7, pp. 1098–1104, 2004.

[14] M. L. Kohut, D. A. McCann, D. W. Russell et al., "Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of β-blockers, BMI, and psychosocial factors in older adults," *Brain, Behavior, and Immunity*, vol. 20, no. 3, pp. 201–209, 2006.

[15] E. Goldhammer, A. Tanchilevitch, I. Maor, Y. Beniamini, U. Rosenschein, and M. Sagiv, "Exercise training modulates cytokines activity in coronary heart disease patients," *International Journal of Cardiology*, vol. 100, no. 1, pp. 93–99, 2005.

[16] V. Z. Rocha, E. J. Folco, G. Sukhova et al., "Interferon-γ, a Th1 cytokine, regulates fat inflammation: a role for adaptive immunity in obesity," *Circulation Research*, vol. 103, no. 5, pp. 467–476, 2008.

[17] J. Todoric, B. Strobl, A. Jais et al., "Cross-talk between interferon-γ and hedgehog signaling regulates adipogenesis," *Diabetes*, vol. 60, no. 6, pp. 1668–1676, 2011.

[18] M. E. Kotas, H. Y. Lee, M. P. Gillum et al., "Impact of CD1d deficiency on metabolism," *PLoS One*, vol. 6, no. 9, Article ID e25478.

[19] B. S. Mantell, M. Stefanovic-Racic, X. Yang, N. Dedousis, I. J. Sipula, and R. M. O'Doherty, "mice lacking NKT cells but with a complete complement of CD8+ T-Cells are not protected against the metabolic abnormalities of diet-induced obesity," *PLoS One*, vol. 6, no. 6, Article ID e19831, 2011.

[20] H. Kanda, S. Tateya, Y. Tamori et al., "MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity," *Journal of Clinical Investigation*, vol. 116, no. 6, pp. 1494–1505, 2006.

[21] C. N. Lumeng, J. L. Bodzin, and A. R. Saltiel, "Obesity induces a phenotypic switch in adipose tissue macrophage polarization," *Journal of Clinical Investigation*, vol. 117, no. 1, pp. 175–184, 2007.

[22] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2003.

[23] A. C. Konner and J. C. Bruning, "Toll-like receptors: linking inflammation to metabolism," *Trends in Endocrinology & Metabolism*, vol. 22, no. 1, pp. 16–23, 2011.

[24] A. Ito, T. Suganami, A. Yamauchi et al., "Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue," *Journal of Biological Chemistry*, vol. 283, no. 51, pp. 35715–35723, 2008.

[25] S. Nishimura, I. Manabe, M. Nagasaki et al., "CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity," *Nature Medicine*, vol. 15, no. 8, pp. 914–920, 2009.

[26] P. Mombaerts, J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou, "RAG-1-deficient mice have no mature B and T lymphocytes," *Cell*, vol. 68, no. 5, pp. 869–877, 1992.

[27] Y. Shinkai, G. Rathbun, K. P. Lam et al., "RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement," *Cell*, vol. 68, no. 5, pp. 855–867, 1992.

[28] T. J. Schall, K. Bacon, R. D. R. Camp, J. W. Kaspari, and D. V. Goeddel, "Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes," *Journal of Experimental Medicine*, vol. 177, no. 6, pp. 1821–1825, 1993.

[29] P. Loetscher, M. Seitz, I. Clark-Lewis, M. Baggiolini, and B. Moser, "Activation of NK cells by CC chemokines: chemotaxis, Ca2+ mobilization, and enzyme release," *Journal of Immunology*, vol. 156, no. 1, pp. 322–327, 1996.

[30] Q. Wang, X. D. Perrard, J. L. Perrard et al., "Differential effect of weight loss with low-fat diet or high-fat diet restriction on inflammation in the liver and adipose tissue of mice with diet-induced obesity," *Atherosclerosis*, vol. 219, no. 1, pp. 100–108, 2011.

[31] M. A. Cornier, E. L. Melanson, A. K. Salzberg et al., "The effects of exercise on the neuronal response to food cues," *Physiology & Behavior*, vol. 105, no. 4, pp. 1028–1034, 2012.

[32] D. Stensel, "Exercise, appetite and appetite-regulating hormones: implications for food intake and weight control," *Annals of Nutrition and Metabolism*, vol. 57, supplement 2, pp. 36–42, 2011.

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Research Article

Plasma Progranulin Concentrations Are Increased in Patients with Type 2 Diabetes and Obesity and Correlated with Insulin Resistance

Hua Qu, Huacong Deng, and Zhenping Hu

Department of Endocrinology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

Correspondence should be addressed to Huacong Deng; huacong_deng@163.com

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Insulin resistance (IR) is considered to be one of the most important pathogenesis of glycolipid metabolism disorders. However, the molecular mechanism responsible for IR is not fully understood. Recently, the chronic inflammation has been proposed to be involved in the pathogenesis of IR. In this study, we aim to investigate the concentrations of plasma progranulin in Chinese patients with obesity (OB) and type 2 diabetes mellitus (T2DM), and its relationship to IR. Plasma progranulin concentrations were significantly higher in the T2DM patients than in the normal glucose tolerant (NGT) subjects ($P < 0.01$). Within the T2DM and the NGT patients, the concentrations of progranulin were significantly higher in obese subjects than that in the normal weight subjects (225.22 ± 34.39 ng/mL versus 195.59 ± 50.47 ng/mL and 183.79 ± 61.63 ng/mL versus 148.69 ± 55.27 ng/mL, $P < 0.05$). Plasma progranulin concentrations correlated positively with weight, waist circumferences, BMI, HbA1c, TG, IL-6, FINS and HOMA-IR ($P < 0.05$), while correlated negatively with HOMA-β ($P < 0.05$). Multiple linear regression analysis showed that BMI, HbA1c, IL-6 and TG correlated independently with circulating progranulin concentrations ($P < 0.05$). These results suggested that Plasma progranulin concentrations were higher in Chinese patients with type 2 diabetes and obesity and correlated closely with glycolipid metabolism, chronic inflammation and IR.

1. Introduction

Progranulin, also known as granulin/epithelin precursor (GEP), acrogranin, and PC cell-derived growth factor (PCDGF), is a 593 amino acid growth factor [1, 2]. Progranulin gene can be found in adipose tissue, epithelial tissue, gastrointestinal tract, reproductive organs, and so forth [3]. The widespread expression of PGRN gene makes it act in many physiological and pathophysiological processes, such as the inflammatory response, the injury-induced tissue repair, and the tumor formation progression [3, 4]. Previous studies have demonstrated that increased gene expression of PGRN stimulates cancer cell division, invasion, and against anoikis, promoting tumor formation [2]. Loss-of-function mutations of PGRN gene lead to the onset of some neurodegenerative diseases such as frontotemporal dementia [5, 6]. Recent studies showed that PGRN could restrain rheumatoid arthritis by binding directly to tumor necrosis factor receptors (TNFR) and play an anti-inflammatory role in the processes [7]. It has been reported that circulating PGRN levels are elevated in patients with type 2 diabetes [8]. Moreover, increased plasma PGRN levels are associated with impaired glucose tolerance rather than impaired fasting glucose [9]. Although type 2 diabetes is often accompanied by obesity, the respective role of elevation of circulating PGRN levels in obesity and type 2 diabetes remains to be established.

Insulin resistance (IR) is a key feature of type 2 diabetes and obesity [10]; as yet the molecular mechanism responsible for IR is not fully understood. Recently, the chronic inflammation has been proposed to be involved in the pathogenesis of IR [11, 12]. Compared to the healthy subjects, plasma levels of the proinflammatory markers are increased (e.g., TNF-α, IL-6, and resistin), while the anti-inflammatory markers are decreased (e.g., adiponectin, leptin) in the obese subjects with type 2 diabetes [11, 12]. PGRN has been shown to promote IR in obese mice and 3T3-L1 adipocytes through IL-6. However,

the effect of PGRN on IR in obese patients with type 2 diabetes is still not known. Thus, we investigated the changes in plasma PGRN concentrations in Chinese patients with obesity and type 2 diabetes and analyzed the relationship of PGRN with IR.

2. Methods

2.1. Subjects. Eighty patients with newly diagnosed type 2 diabetes and 88 subjects with normal glucose tolerance (NGT) were recruited in this study, and age of the subjects ranged from 40 to 75 years. 75 g oral glucose tolerance test (OGTT) was performed in all 168 subjects. All of the T2DM patients were newly diagnosed and had not received any antidiabetes treatments including diet, exercise, and medications. The diagnoses of T2DM were based on the diagnostic criteria of World Health Organization (WHO) in 1999, and then according to the WHO-Western Pacific Region diagnostic criteria (2000) [13] defined obesity as BMI $\geqslant 25\,kg/m^2$. All subjects were divided into four subgroups: NGT-normal weight (NGT-NW) subgroup, NGT-obesity (NGT-OB) subgroup, T2DM-NW subgroup, and T2DM-OB subgroup.

2.2. Exclusion Criteria

(1) Smoking and drinking history.

(2) Acute and chronic complications of diabetes.

(3) Sustained hypertension, coronary heart disease.

(4) Acute and chronic inflammatory diseases as determined by clinical symptom of infection, blood leukocyte $>7\times10^9$/L, or high-sensitivity C-reactive protein (hs-CRP) >5.0 mg/dL;

(5) Hepatic or renal disease and systemic corticosteroid treatment.

(6) Women who were currently pregnant and breastfeeding were also excluded from this study.

The study was approved by the Ethical Committee of Chongqing Medical University. Signed informed consents were obtained from all participants in this study.

2.3. Study Measurements

2.3.1. Clinical Evaluation of Subjects. Standardized protocols were used to measure height, body weight, waist circumferences, hip circumferences, and blood pressure (BP) in all subjects. Height, waist, and hip circumferences were measured to minimum recorded unit 0.1 cm, body weight was measured to an accuracy of ±0.2 kg, and blood pressure was measured twice with a standard mercury manometer with the subjects seated and was used for the second measurement. Body mass index (BMI) and waist to hip ratio (WHR) were calculated.

Overnight fasting blood samples were collected for the determination of fasting plasma glucose (FPG), HbA1c, fasting insulin (FINS), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and liver and kidney functions. Blood samples also were collected after 2 hours of a 75 g OGTT for determined the 2 h plasma glucose (2hPG). All of the blood samples were separated within 1 h and then frozen at −80°C until used in this study, all within 3 month period. Glucose was assayed by glucose oxidase method. HbA1c was measured by isoelectric focusing. Fasting insulin (FINS) was measured in serum by RIA using human insulin as standard (Linco, St Charles, MO, USA). Lipid profiles, liver and kidney functions were detected by biochemical autoanalyzer (Beckman CX-7 Biochemical Autoanalyser, Brea, CA, USA).

2.3.2. Assessment of Plasma Progranulin and IL-6 Concentrations. Plasma PGRN and IL-6 concentrations were determined by enzyme-linked immunosorbent assays according to the manufacturers' instructions. (Human ELISA kit, CUSABIO Science Co, Ltd, China). All samples were run in duplicate and repeated if there was a >15% difference between duplicates. No significant cross-reactivity or interference was observed.

2.3.3. Related Calculation Formulas. Body mass index (BMI) formula is weight in kilograms divided by height in meters squared.

The Homeostasis Model Assessment for insulin resistance (HOMA-IR) was computed as follows: Fasting insulin (mU/L) × Fasting plasma glucose (mmol/L)/22.5.

The Homeostasis Model Assessment for β-cell function (HOMA-β) was calculated using the following formula: 20 × Fasting insulin (mU/L)/Fasting plasma glucose (mmol/L) − 3.5.

2.4. Statistical Analysis. SPSS software, version 19.0 (IBM, Armonk, NY), was used for all statistical analyses. Data are expressed as mean values ± standard deviation (SD). Before statistical analysis, nonnormally distributed parameters were logarithmically transformed to approximate a normal distribution. Independent-samples t-tests were used to compare continuous variables between the 2 groups. Analysis of variance and Student-Newman-Keuls tests were performed for multiple and pairwise comparisons, respectively. Interrelationships between variables were analyzed by Pearson correlation analysis. Multiple linear regression analyses were used to determine independent predictors of plasma PGRN. P values <0.05 were considered to be statistically significant.

3. Results

3.1. The Clinical Characteristics. The anthropometric and metabolic parameters in different subgroups were shown in Table 1. Between NGT and T2DM groups, there were no significant differences in age, weight, BMI, waist circumferences, and WHR. Compared to NGT group, the T2DM group exhibited higher levels of SBP, FPG, 2hPG, FINS, HbA1c, TG, LDL-c, IL-6, hs-CRP, and HOMA-IR ($P < 0.05$ or $P < 0.01$). The levels of HDL-c, HOMA-β were significantly higher in

Table 1: Anthropometric and metabolic characteristics of the study groups.

	NGT		T2DM	
	NGT-NW	NGT-OB	T2DM-NW	T2DM-OB
N (M/F)	46 (15/31)	42 (20/22)	37 (11/26)	43 (18/25)
Age (year)	58.54 ± 5.96	59.10 ± 7.43	61.65 ± 6.96	60.86 ± 7.22
Weight (kg)	55.83 ± 5.74	68.79 ± 8.04[b]	55.54 ± 6.27	69.30 ± 8.51[f]
BMI (kg/cm^2)	22.36 ± 1.75	27.81 ± 3.10[b]	22.97 ± 1.47	27.78 ± 2.25[f]
WC (cm)	78.17 ± 5.72	91.69 ± 5.42[b]	80.38 ± 6.20	91.81 ± 5.43[f]
WHR	0.86 ± 0.04	0.92 ± 0.05[b]	0.87 ± 0.05	0.91 ± 0.04[f]
SBP (mm Hg)	118.41 ± 9.45	122.79 ± 9.61	133.46 ± 15.65[bd]	127.43 ± 14.45[bd]
DBP (mm Hg)	74.20 ± 6.99	74.67 ± 7.25	74.49 ± 10.09	75.52 ± 8.81[ace]
FPG (mmol/L)	5.25 ± 0.33	5.40 ± 0.43	7.28 ± 1.34[bd]	7.46 ± 1.13[bd]
2hPG (mmol/L)	6.28 ± 1.03	6.00 ± 1.14	13.58 ± 2.99[bd]	13.09 ± 3.30[bd]
FINS (mU/L)	5.61 ± 2.58	8.87 ± 4.76[a]	7.58 ± 3.88	12.27 ± 7.04[bdf]
HbA1c (%)	5.49 ± 0.32	5.57 ± 0.35	6.79 ± 0.78[bd]	6.79 ± 0.80[bd]
TC (mmol/L)	4.43 ± 0.91	4.82 ± 1.00[a]	4.59 ± 0.84	4.85 ± 0.92[a]
TG (mmol/L)	1.07 ± 0.51	1.48 ± 0.84[a]	1.50 ± 0.80[a]	2.04 ± 1.37[bce]
HDL-c (mmol/L)	1.43 ± 0.27	1.28 ± 0.32[a]	1.31 ± 0.35	1.14 ± 0.23[bce]
LDL-c (mmol/L)	2.47 ± 0.69	2.84 ± 0.78[a]	2.55 ± 0.64	2.82 ± 0.70[a]
PGRN (ng/mL)	148.69 ± 55.27	183.79 ± 61.63[a]	195.59 ± 50.47[b]	225.22 ± 34.39[bde]
IL-6 (pg/mL)	2.57 ± 1.78	3.73 ± 2.69	4.46 ± 3.37[a]	7.68 ± 3.14[bde]
hsCRP (mg/dL)	0.24 ± 0.13	0.35 ± 0.26[a]	0.32 ± 0.18[a]	0.43 ± 0.29[bdf]
HOMA-IR	1.32 ± 0.64	2.12 ± 1.14[a]	2.43 ± 1.22[b]	4.07 ± 2.49[bdf]
HOMA-β	93.42 ± 39.60	90.90 ± 44.27	63.51 ± 32.09[bd]	43.96 ± 26.08[bde]

Data are presented as means ± SD. NGT: normal glucose tolerance; T2DM: type 2 diabetes mellitus; NW: normal weight; OB: obesity; BMI: body mass index; WC: waist circumference; WHR: waist hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; 2hPG: 2h postchallenge plasma glucose; FINS: fasting serum insulin; HOMA-IR: Homeostasis Model Assessment for insulin resistance; HOMA-β: Homeostasis Model Assessment for beta-cell function; TC: total cholesterol; TG: triglyceride; HDL-c: high-density lipoprotein-cholesterol; LDL-c: low-density lipoprotein-cholesterol; hs-CRP: high-sensitivity C-reaction protein. [a]$P < 0.05$ compared with NGT-NW, [b]$P < 0.01$ compared with NGT-NW; [c]$P < 0.05$ compared with NGT-OB, [d]$P < 0.01$ compared with NGT-OB, [e]$P < 0.05$ compared with T2DM-NW, and [f]$P < 0.01$ compared with T2DM-NW.

NGT group than in T2DM group ($P < 0.01$). SBP was higher in T2DM-NW and T2DM-OB subgroups than in NGT-NW subgroup, while DBP was higher in T2DM-OB subgroup than in T2DM-NW subgroup ($P < 0.05$). The levels of BMI, waist circumferences, FINS, HOMA-IR, TC, TG, LDL-c, and hs-CRP were significantly higher in NGT-OB subgroup than in NGT-NW subgroup, while HDL-c were higher in NGT-NW subgroup than in NGT-OB subgroup ($P < 0.05$ or $P < 0.01$). Compared to NGT-OB subgroup, the levels of SBP, DBP, FPG, 2hPG, HbA1c, FINS, HOMA-IR, IL-6, hs-CRP, and TG were significantly increased in T2DM-OB subgroup, while the HOMA-β and HDL-c levels were decreased in T2DM-OB subgroup ($P < 0.05$ or $P < 0.01$). Compared to T2DM-NW subgroup, the levels of BMI, waist circumferences, WHR, DBP, FINS, HOMA-IR, IL-6, hs-CRP, and TG were higher in T2DM-OB subgroup. The HDL-c and HOMA-β were higher in T2DM-NW subgroup than that in T2DM-OB subgroup ($P < 0.05$ or $P < 0.01$).

3.2. The Changes in Plasma Progranulin Concentrations in Obesity and Type 2 Diabetes. There were no significant differences in plasma PGRN *concentrations* between men and women ((190.11 ± 56.63) versus (185.70 ± 59.54) ng/mL, $P = 0.636$). Compared to NGT group, T2DM group displayed a significant increase in the PGRN *concentrations* (165.44 ± 60.67 versus 211.52 ± 44.84 ng/mL, $P < 0.01$, Figure 1). Plasma PGRN *concentrations* in four subgroups are the followings: T2DM-OB subgroup (225.22 ± 34.39 ng/mL), T2DM-NW subgroup (195.59 ± 50.47 ng/mL), NGT-OB subgroup (183.79 ± 61.63 ng/mL), and NGT-NW subgroup (148.69 ± 55.27 ng/mL). T2DM-OB subgroup had significant higher levels of PGRN than those in T2DM-NW subgroup ($P < 0.05$) and in NGT-OB subgroup ($P < 0.01$).

Bivariate correlation analysis revealed that the plasma PGRN *concentrations* correlated positively and significantly with weight, waist circumferences, HOMA-IR (Figure 2(a)) and FINS ($r = 0.175, 0.191, 0.228, 0.172$, resp., $P < 0.05$, Table 2). The PGRN *concentrations* also correlated positively and significantly with BMI, SBP, FPG, 2hPG, HbA1C, TG, and IL-6 significantly ($r = 0.286, 0.256, 0.348, 0.292, 0.276, 0.221, 0.383$, resp., $P < 0.01$, Table 2), while correlating negatively and significantly with HOMA-β ($r = -0.225$, $P < 0.05$, Figure 2(b)). Multivariable linear regression models revealed that BMI, HbA1c, IL-6, and TG were independently related with the plasma PGRN levels ($\beta = 0.191, 0.473, 0.181, 0.151$, resp., $P < 0.05$). The multiple regression equation was as follows: $Y_{\mathrm{PGRN}} = -57.828 + 3.237X_{\mathrm{BMI}} + 30.596X_{\mathrm{HbA1c}} + 3.130X_{\mathrm{IL\text{-}6}} + 8.722X_{\mathrm{TG}}$.

Table 2: Univariate associations between plasma PGRN levels and metabolic parameters.

	r	P value
Weight (kg)	0.175	0.023
BMI (kg/m^2)	0.286	<0.001
WC (cm)	0.191	0.013
WHR	0.042	0.589
SBP (mmHg)	0.256	0.001
DBP (mmHg)	0.138	0.074
FPG (mmol/L)	0.348	<0.001
2hPG (mmol/L)	0.292	<0.001
FINS (mU/L)	0.172	0.025
HbA1c (%)	0.276	<0.001
TC (mmol/L)	0.126	0.103
TG (mmol/L)	0.221	0.004
HDL-c (mmol/L)	−0.110	0.156
LDL-c (mmol/L)	0.059	0.450
IL-6 (pg/mL)	0.383	<0.001
HOMA-IR	0.228	0.003
HOMA-β	−0.225	0.003

WHR: waist hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: Homeostasis Model Assessment for insulin resistance; HOMA-β: Homeostasis Model Assessment for beta-cell function; TC: total cholesterol; TG: triglyceride; HDL-c: high-density lipoprotein-cholesterol; LDL-c: low-density lipoprotein-cholesterol.

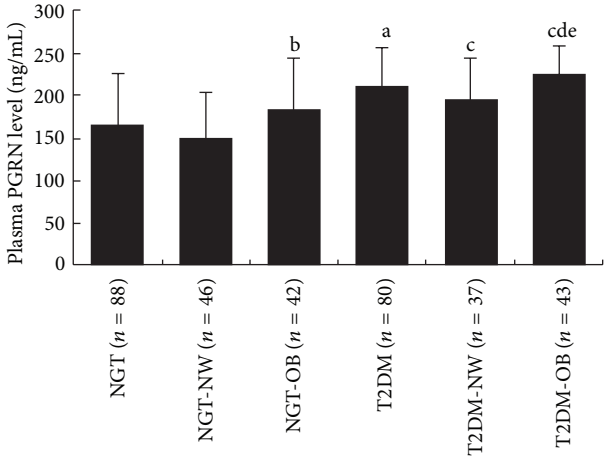

Figure 1: Plasma PGRN levels in different groups. Data are presented as means ± SD. [a]$P < 0.01$ compared with NGT; [b]$P < 0.05$ compared with NGT-NW; [c]$P < 0.01$ compared with NGT-NW; [d]$P < 0.01$ compared with NGT-OB; [e]$P < 0.05$ compared with T2DM-NW.

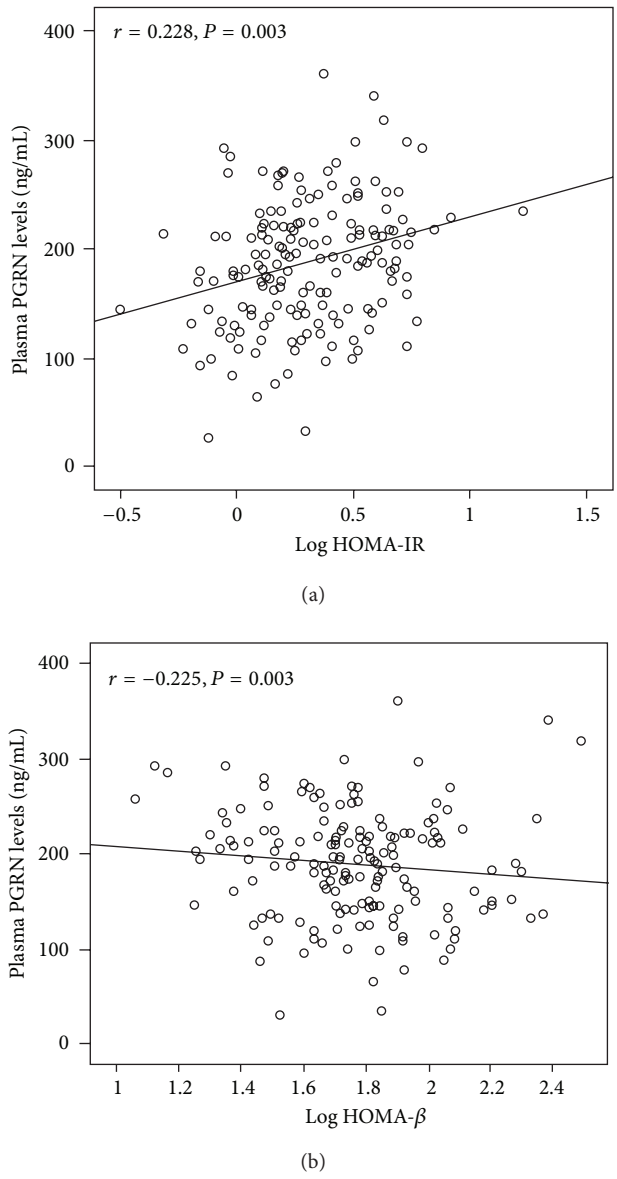

Figure 2: Scatter plots showing the correlation of plasma PGRN levels with HOMA-IR and HOMA-β in all subjects. (a) The plasma PGRN levels positively correlated with HOMA-IR. (b) The plasma PGRN levels negatively correlated with HOMA-β.

4. Discussion

PGRN is a 68–88 kDa growth factor with seven and one-half granulin (GRN) motifs connected by short linker domains. It was originally discovered by Anakwe and Gerton in 1990 [14]. PGRN catches our attention because it is a secreted protein with inflammatory properties and was recently identified as a novel adipokine. In addition, the changes of circulating PGRN brought about by obesity and type 2 diabetes, respectively, in Chinese subjects are still unclear.

In our study, we found that plasma PGRN concentrations were 1.3-fold ($P < 0.01$) higher in normal-weight type 2 diabetes patients, compared to those of healthy subjects. The PGRN concentrations were lower than those in previous study which conducted by Youn et al. in the Korean population, and this may be because our subjects have lower BMI which may contribute to the increase of PGRN concentrations in type 2 diabetes patients. In addition, the most recent study reported that renal elimination is a major route for circulation PGRN [15]. Therefore, we tested serum creatinine for all subjects and only recruited the population

with normal kidney function. Correlation analysis showed that plasma PGRN levels were positively correlated to FPG and 2hPG. Multivariable linear regression models revealed that HbA1c was an independent predictor of plasma PGRN levels. These results indicate that type 2 diabetes could increase plasma PGRN concentrations independently, and the circulating PGRN concentrations could increase with deteriorating glucose metabolism.

Gene expression analysis in previous studies revealed that both isolated adipocytes and cells of the adipose tissue matrix express progranulin gene [8, 16]. Progranulin gene expression is significantly higher in visceral fat compared with subcutaneous fat [8]. Matsubara et al. [16] find that ablation of PGRN gene protects against HFD-induced obesity in mice. Moreover, recombinant mouse PGRN (rmPGRN) treatment shows a higher probability that mice will become obese when fed a HFD [16]. That research demonstrates a significant relationship between PGRN and obesity. In the present study, we found that obese patients with normal glucose tolerance have 1.2-fold ($P < 0.05$) higher plasma PGRN concentrations compared to healthy subjects. Furthermore, obese type 2 diabetes patients have 1.5-fold ($P < 0.01$) higher PGRN concentrations than healthy subjects. There was an indication that obesity could elevate the plasma PGRN concentrations independently, and both obesity and abnormal glucose tolerance contribute to the increase of plasma PGRN concentrations. In our study, we also found a significant positive correlation between PGRN and waist circumferences. Furthermore, we found that plasma PGRN concentrations positively correlated to LDL-c and TG concentrations and negatively correlated to HDL-c. Those findings suggested that PGRN is associated with central obesity and lipid metabolism disorders in Chinese subjects.

Both type 2 diabetes and obesity are associated with a state of chronic low-grade inflammation which is characterized by increased pro-inflammatory factors and decreased anti-inflammatory factors. Those pro-inflammatory factors increase the accumulation of macrophages in adipose tissue, while the increased macrocytes could stimulate the release of pro-inflammatory factors from adipose tissue. Youn and colleagues [8] found that PGRN promote macrophages infiltration into white adipose tissues by ERK pathway in vitro and induce inflammatory response in adipose tissue. A recent study demonstrates that rmPGRN could stimulate the adipocytes to release more IL-6 and the increased secretion of IL-6 by TNF-α was completely blocked by ablation of PGRN gene in 3T3-L1 adipose cells [16]. Those findings suggested that PGRN could promote inflammatory response by increasing the secretion of proinflammatory factors. In our study, we found that IL-6 levels were higher in patients with both diabetes and obesity than in those with diabetes and normal-weight and those with obesity and normal glucose tolerance. Moreover, the IL-6 levels were lowest in normal subjects. We also found that PGRN correlated positively with IL-6, which was an independent predictor of PGRN. Taking these together, the results suggested that concentrations of plasma PGRN and the extent of chronic inflammation are enhanced along with the aggravation of glycolipid metabolic disorders.

Insulin resistance is a key feature of obesity and type 2 diabetes and can directly result in hyperinsulinemia. Recently, a report shows that PGRN could induce insulin resistance through stimulating IL-6 expression in adipocytes [16]. Meanwhile, many studies find that IL-6 could increase the expression of cytokine signaling-3 (SOCS3) via activation of JAK-STAT signaling pathway in adipocytes to inhibit tyrosine phosphorylation of insulin receptor substrate (IRS-1), leading to impaired insulin signaling [17–20]. In our research, we found that plasma PGRN levels correlated significantly and positively with HOMA-IR and FINS, while negatively correlating with HOMA-β. Those results indicated that circulating PGRN levels correlated significantly positively with insulin resistance and negatively correlated with pancreatic β-cell function. Combined with the aforementioned relationship between PGRN and IL-6 in our study, we speculated that one of the mechanisms responsible for PGRN-induced insulin resistance may be associated with increased IL-6 levels. However, other possible factors may play a role in this process as well and need to be investigated further.

As yet, there are some limitations in our study that require emphasis. First, the sample size is small, and the nonsignificant associations between PGRN and some factors become statistically significant if larger samples were studied. Second, our study is a cross-sectional research, and therefore causality of PGRN and IL-6 cannot be established.

In conclusion, we demonstrated for the first time that plasma PGRN concentrations increased in Chinese patients with type 2 diabetes and obesity. The concentrations of plasma PGRN correlated closely with the glycolipid metabolic disorder, chronic inflammation, and insulin resistance, suggesting PGRN may contribute to the pathogenesis of insulin resistance in humans. Thus PGRN could be a potential therapeutic target for management of type 2 diabetes and obesity.

Conflict of Interests

The authors have no conflicts of interests to disclose.

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References

[1] R. Daniel, E. Daniels, Z. He, and A. Bateman, "Progranulin (acrogranin/PC cell-derived growth factor/granulin-epithelin precursor) is expressed in the placenta, epidermis, microvasculature, and brain during murine development," *Developmental Dynamics*, vol. 227, no. 4, pp. 593–599, 2003.

[2] Z. He and A. Bateman, "Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis," *Journal of Molecular Medicine*, vol. 81, no. 10, pp. 600–612, 2003.

[3] D. Tolkatchev, S. Malik, A. Vinogradova et al., "Structure dissection of human progranulin identifies well-folded granulin/epithelin modules with unique functional activities," *Protein Science*, vol. 17, no. 4, pp. 711–724, 2008.

[4] K. Sleegers, N. Brouwers, and C. Van Broeckhoven, "Role of progranulin as a biomarker for Alzheimers disease," *Biomarkers in Medicine*, vol. 4, no. 1, pp. 37–50, 2010.

[5] P. Bossù, F. Salani, A. Alberici et al., "Loss of function mutations in the progranulin gene are related to pro-inflammatory cytokine dysregulation in frontotemporal lobar degeneration patients," *Journal of Neuroinflammation*, vol. 8, article 65, 2011.

[6] N. Finch, M. Baker, R. Crook et al., "Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members," *Brain*, vol. 132, no. 3, pp. 583–591, 2009.

[7] W. Tang, Y. Lu, Q. Y. Tian et al., "The growth factor progranulin binds to tnf receptors and is therapeutic against inflammatory arthritis in mice," *Science*, vol. 332, no. 6028, pp. 478–484, 2011.

[8] B. S. Youn, S. I. Bang, N. Klöting et al., "Serum progranulin concentrations may be associated with macrophage infiltration into omental adipose tissue," *Diabetes*, vol. 58, no. 3, pp. 627–636, 2009.

[9] A. Tönjes, M. Fasshauer, J. Kratzsch, M. Stumvoll, and M. Bluher, "Adipokine pattern in subjects with impaired fasting glucose and impaired glucose tolerance in comparison to normal glucose tolerance and diabetes," *PLoS One*, vol. 5, no. 11, Article ID e13911, 2010.

[10] G. S. Hotamisligil, "Inflammation and metabolic disorders," *Nature*, vol. 444, no. 7121, pp. 860–867, 2006.

[11] J. P. Sutherland, B. McKinley, and R. H. Eckel, "The metabolic syndrome and inflammation," *Metabolic Syndrome and Related Disorders*, vol. 2, no. 2, pp. 82–104, 2004.

[12] R. H. Eckel, S. M. Grundy, and P. Z. Zimmet, "The metabolic syndrome," *Lancet*, vol. 365, no. 9468, pp. 1415–1428, 2005.

[13] E. Anuurad, K. Shiwaku, A. Nogi et al., "The new BMI criteria for asians by the regional office for the western pacific region of WHO are suitable for screening of overweight to prevent metabolic syndrome in elder Japanese workers," *Journal of Occupational Health*, vol. 45, no. 6, pp. 335–343, 2003.

[14] O. O. Anakwe and G. L. Gerton, "Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal gylcoprotein, acrogranin, during guinea pig spermatogenesis," *Biology of Reproduction*, vol. 42, no. 2, pp. 317–328, 1990.

[15] J. Richter, D. Focke, T. Ebert et al., "Serum levels of the adipokine progranulin depend on renal function," *Diabetes Care*, vol. 36, no. 2, pp. 410–414, 2013.

[16] T. Matsubara, A. Mita, K. Minami et al., "PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue," *Cell Metabolism*, vol. 15, no. 1, pp. 38–50, 2012.

[17] K. Ueki, T. Kondo, and C. R. Kahn, "Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms," *Molecular and Cellular Biology*, vol. 24, no. 12, pp. 5434–5446, 2004.

[18] H. Shi, I. Tzameli, C. Bjørbæk, and J. S. Flier, "Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling," *Journal of Biological Chemistry*, vol. 279, no. 33, pp. 34733–34740, 2004.

[19] B. Emanuelli, P. Peraldi, C. Filloux et al., "SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-α in the adipose tissue of obese mice," *Journal of Biological Chemistry*, vol. 276, no. 51, pp. 47944–47949, 2001.

[20] P. Peraldi, C. Filloux, B. Emanuelli, D. J. Hilton, and E. Van Obberghen, "Insulin induces suppressor of cytokine signaling-3 tyrosine phosphorylation through janus-activated kinase," *Journal of Biological Chemistry*, vol. 276, no. 27, pp. 24614–24620, 2001.

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Research Article

Role of Cox-2 in Vascular Inflammation: An Experimental Model of Metabolic Syndrome

Nicolás F. Renna,[1,2] Emiliano R. Diez,[2,3] Carina Lembo,[2] and Roberto M. Miatello[1,2]

[1] *Department of Pathology, School of Medicine, National University of Cuyo, Avenida Libertador No. 80, Centro Universitario, CP 5500 Mendoza, Argentina*
[2] *Institute of Experimental Medicine and Biology of Cuyo (IMBECU)-CONICET, Mendoza, Argentina*
[3] *Department of Morphophysiology, School of Medicine, National University of Cuyo, Avenida Libertador No. 80, Centro Universitario, CP 5500 Mendoza, Argentina*

Correspondence should be addressed to Nicolás F. Renna; nicolasrenna@fcm.uncu.edu.ar

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The objective of this work was to demonstrate the role of COX-2 enzyme at the vascular in experimental model of metabolic syndrome. SHR male WKY rats were employed; they were distributed in 8 groups ($n = 8$ each): control (W); W + L: WKY rats receiving 20 mg/kg of lumiracoxib by intraesophageal administration; SHR; SHR + L: SHR + 20 mg/kg of lumiracoxib by intraesophageal administration; Fructose-Fed Rats (FFR): WKY rats receiving 10% (w/v) fructose solution in drinking water during all 12 weeks; FFR + L: FFR + 20 mg/kg of lumiracoxib by intraesophageal administration; Fructose-Fed Hypertensive Rats (FFHR): SHR receiving 10% (w/v) fructose solution in drinking water during all 12 weeks; and FFHR + L: FFHR + 20 mg/kg of lumiracoxib by intraesophageal administration. Metabolic variables, blood pressure, morphometric variables, and oxidative stress variables were evaluated; also MMP-2 and MMP-9 (collagenases), VCAM-1, and NF-κB by Westernblot or IFI were evaluated. FFHR presented all variables of metabolic syndrome; there was also an increase in oxidative stress variables; vascular remodeling and left ventricular hypertrophy were evidenced along with a significant increase in the expression of the mentioned proinflammatory molecules and increased activity and expression of collagenase. Lumiracoxib was able to reverse vascular remodeling changes and inflammation, demonstrating the involvement of COX-2 in the pathophysiology of vascular remodeling in this experimental model.

1. Introduction

The traditional view of atherosclerosis as a lipid storage disease falls apart against the large and growing evidence that inflammation is at the center of all stages of the disease, from the initial injury until the final stage of thrombotic complications that compromise blood flow. Advances in the understanding of vascular inflammation have resulted in a radical change in the way vascular diseases are approached. With increased awareness of the active role of the vessel and its complex interactions with cytokines and immune cells, this concept unites disorders previously thought be different. Understanding atherosclerosis as a vascular inflammation disease is the basis of a new approach for risk stratification and treatment [1].

Matrix metalloproteinases (MMPs) play an important role in maintaining homeostasis of extracellular structures. MMPs are induced by cytokines and by cell-cell and cell-matrix interactions. Examples of the increased presence of MMPs in clinical pathology are the SCA, specifically in the vulnerable region of the plaque [2]. Exposure to oxidized low density lipoproteins (ox-LDL) or TNF-α induces the expression of MT3-MMP, an MMP expressed in the atherosclerotic plaque of macrophages [3].

C-reactive protein (CRP) presents a rapid and dramatic response to an inflammatory stimulus. Ultrasensitive C-reactive protein (hsCRP) has a very important role in the detection of vascular inflammation and cardiovascular risk prediction. There is evidence that CRP is involved in atherosclerosis, especially at its beginning. Proinflammatory

cytokines production in monocytes and macrophages is stimulated by PCR [4]. CAMs expression is mediated by CRP, allowing the increase of leukocyte adhesion and migration [5–7].

Spontaneously hypertensive rats (SHR) provide a model of genetic hypertension that allows studying essential hypertension. By administrating carbohydrate diets to rats it is possible to induce insulin resistance, hyperinsulinemia, dyslipidemia, and hypertension. Fructose-Fed-Rats (FFR) provides a useful experimental model for the study of the interaction factors shaping metabolic syndrome. This combined model (FFHR) is representative of hypertensive individuals who eat a modern Western diet rich in refined sugars. Some authors postulate this dual model as the most appropriate for extrapolating results to human models [8, 9].

This experimental model has proved in previous works its utility for the study of the interaction factors shaping insulin resistance syndrome [10], including endothelial dysfunction, the decrease, at the cardiovascular level, of the activity of the endothelial isoform of nitric oxide synthase (eNOS), and the increase in the proliferation of vascular smooth muscle cells [11], and has also provided evidence involving RAS in its pathophysiology [12].

The objective of this work was to demonstrate that vascular inflammation and oxidative stress are involved in the pathophysiologic mechanisms of structural and functional vascular changes (remodeling) associated to the experimental model of metabolic syndrome through administration of lumiracoxib (L) as COX-2 specific anti-inflammatory.

2. Methods

2.1. Animals and Experimental Design. All procedures were performed according to institutional guidelines for animal experimentation; protocol was submitted and approved by the Institutional Committee for Laboratory Animal Use and Care (CICUAL) of the School of Medicine, UNCuyo. Thirty-day-old male Wistar Kyoto (WKY) rats and SHR were fed a standard commercial chow diet ad libitum and housed in a room under conditions of controlled temperature (20°C) and humidity, with a 12-hour light/dark cycle during a 12-week experimental period. Lumiracoxib (L) was administrated to respective groups during the last six weeks. Study groups were divided as follows.

(i) Control (W): WKY receiving food and drinking water (DW) ad libitum;

(ii) SHR: receiving food and DW ad libitum;

(iii) Fructose-Fed Rats (FFR): WKY receiving 10% (w/v) fructose (Parafarm, Buenos Aires, Argentina) solution in DW during all 12 weeks;

(iv) Fructose-Fed Hypertensive Rats (FFHR): SHR receiving 10% (w/v) fructose solution in DW during all 12 weeks;

(v) FFR + L:FFR receiving 20 mg/kg L by intraesophageal administration;

(vi) FFHR + L:FFHR receiving 20 mg/kg L by intraesophageal administration.

At the end of the experimental period, rats were anesthetized with sodium pentobarbital (50 mg/Kg ip), blood samples were taken, and arteries and organs were aseptically excised for measurements.

2.2. Systolic Blood Pressure Measurement. Systolic blood pressure (SBP) was monitored indirectly in conscious prewarmed slightly restrained rats by the tail-cuff method and recorded on a Grass Model 7 polygraph (Grass Instruments Co., Quincy, MA, USA). The rats were trained in the apparatus several times before measurement.

2.3. Biochemical Determinations

2.3.1. HOMA Index and Intraperitoneal Glucose Tolerance Test. Fasting plasma insulin was assayed by ACS:180SE automated chemiluminescence system (Bayer, Germany). Plasma glucose levels were assayed using a commercial colorimetric method (Wiener Lab., Argentina). Homeostasis model assessment (HOMA) was used as an index to measure the degree of insulin resistance; it was calculated using the following formula: (insulin (μU/mL) × glucose (mmol/L)/22.5) [13].

Three days before the end of the experimental period, a glucose tolerance test (GTT) was performed. Rats fasted overnight were slightly anesthetized with pentobarbital, and glucose was administered (2 g/Kg ip). Blood samples were taken by tail bleeding at 0, 30, 60, and 90 minutes after injection to determine plasma glucose concentration. The total area under the curve was calculated as mmol/L/90 min.

Assessment of the Lipid Profile. At the end of the experimental period blood samples were drawn from the animals, after fasting for 12 hours. Total plasma cholesterol, HDL-cholesterol, and triglycerides were assessed using photocolorimetric enzymatic methods (Wiener Lab., Rosario, Argentina). Data are expressed in mmol/L.

2.4. Oxidative Stress Determinations

2.4.1. Measurement of Plasma Thiobarbituric Acid-Reactive Substances (TBARS). In order to demonstrate the effect of increased oxidative stress at the vascular level, plasma lipid peroxidation was assessed by TBARS concentration. This method was based on the reaction between plasma malondialdehyde, a product of lipid peroxidation, and thiobarbituric acid, as has been previously described [13]. No correction for sample protein content was necessary because of the nature of sample [14].

2.5. Measurement of Vascular NAD(P)H-Oxidase Activity. The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H-oxidase activity in a segment of thoracic aorta, as previously described [14]. To assess NAD(P)H-oxidase activity, NADPH (500 μmol/L) was added, and chemiluminescence was immediately measured in a liquid scintillation counter (LKB Wallac Model 1219 Rack-Beta Scintillation Counter, Finland) set in the out-of-coincidence

mode. Time-adjusted and normalized-to-tissue-weight scintillation counters were used for calculations. Measurements were repeated in the absence and presence of diphenyleneiodnium (DPI) (10-6 mol/L), which inhibits flavin-containing enzymes, including NAD(P)H oxidase [15, 16].

2.6. eNOS Activity in Homogenates of Cardiac and Arterial Tissue. The activity of Ca^{2+}/calmodulin-dependent endothelial nitric oxide synthase, (eNOS) was measured in mesenteric arteries homogenates and in left ventricle cardiac tissue, by conversion of L-[3H]arginine into L-[3H]citrulline. Values were corrected according to protein contents in the homogenates (Bradford method) and to incubation time and are expressed as dpm/mg protein/min. The material obtained from each animal was processed independently [17].

2.7. Relative Heart Weight. In order to evaluate cardiac hypertrophy, we measured relative heart weight (RHW). Briefly, heart was separated from the great vessels, dropped into a buffered saline solution (PBS), blotted with tissue paper to remove blood, and weighed. Total heart weight was corrected according to the ratio between heart weight (milligrams) and 100 grams of the total body weight before killing.

2.8. Measurement of High-Sensitive C-Reactive Protein (hs-CRP) Concentration. Plasma hs-CRP concentrations were measured using a turbidimetric assay (Bayer Advia 1650, AG Leverkusen). Data are expressed in mg/L.

2.9. Tissue Preservation. Tissue samples for histopathology were processed as has been previously reported [15]. Samples from all rats were used for these observations. Anesthetized animals were briefly perfused with PBS (298 mOsmol/Kg H_2O, pH 7.40, 4°C) to clear out the blood. Mesenteric arteries were perfused in vivo with the same solution through the mesenteric artery during 5 min. For histological studies, arteries were also perfused with 4% paraformaldehyde solution for 10 min and fixed by paraffin. Five μm-thick tissue slices were transversely cut across the mesenteric tissue on a microstate (Microm HM, Germany) and processed for histological studies. Similar procedure was applied for heart tissue preservation, by aortic retrograde perfusion.

2.10. Quantitative Histomorphometry to Determine Cardiac Hypertrophy. Histomorphological analyses were conducted on slices from the outer (free) wall of the left ventricle (LV) of the heart. Estimations of cardiomyocyte area were made from sections stained with Masson trichrome solution. Areas with transverse sections of myofibers were selected. The contour of the fibers was then drawn manually. Total myocardiocyte area was expressed as square micrometer (μm^2).

2.11. Arterial Structure. Changes in the structure of arterial walls were assessed by measuring the media layer in mesenteric arteries. Dissected mesenteric vascular beds were fixed in 10% formaldehyde, dehydrated, embedded in paraffin, and later cut in microtome. The slices were dyed and examined as has been previously described [15]. Nontransverse sectioned arteries were excluded from investigation. The lumen to media ratio (i.e., internal diameter to medial thickness) (L/M) was then calculated. Fifty slices from each animal were processed and analyzed to obtain an average value for each rat. Average values were then used for final analysis.

2.12. SDS-PAGE and Immunoblot Analysis. Mesenteric tissue was washed in PBS and proteins extracted in cold 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and a protease inhibitor mixture (P2714, Sigma). After sonication for 15 s (3 times with 10 s intervals) and extraction for 30 min at 4°C, sample extracts were clarified by centrifugation at 14,000 ×g for 20 min and used immediately or stored at −20°C. Proteins were separated on 10% polyacrylamide slab gels and transferred to 0.22 μm nitrocellulose membranes (GE, Germany). Nonspecific reactivity was blocked by incubation for 1 h at room temperature in 5% nonfat dry milk dissolved in washing buffer (PBS, pH 7.6, 0.2% Tween 20). Blots were incubated with anti-p65 and anti-VCAM-1 antibodies (0.2 μg/mL in blocking solution) for 60 min at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit-IgG and swine anti-goat-IgG dissolved in blocking buffer were used as secondary antibodies (0.25 μg/mL, 45 min at room temperature). Excess first and second antibodies were removed by washing 5 times for 5 min in blocking solution. Detection was accomplished with enhanced chemiluminescence system (ABC, Dako System) and subsequent exposure to Kodak X-AR film (Eastman Kodak) for 5–30 s.

2.13. Immunohistochemistry and Digital Confocal Microscopy (IHC)

2.13.1. Determination of Transcription Factors (WB). Rabbit anti-rat NF-κB p65 subunit [Rel A], C-terminus antibody, was obtained from Millipore International Inc. (Amsterdam, The Netherlands) (AB1604b), and goat anti-rat VCAM-1 (C-19) antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) (sc-1504). Tissue sections were cut at 3 μm thickness from paraffin-embedded blocks. Deparaffinized sections were used to determine inflammatory response. Tissue was permeabilized in 1% Triton X-100 for 15 min, rinsed well with PBS, and blocked with sterile filtered 10% normal rabbit serum for 20 min. All antibody solutions were microfuged for 20 min before use. The antibodies were 1 : 1000 diluted. Primary incubations lasted 1 hour at 21-22°C, followed by extensive washes in PBS with Triton X-100, six times for 5 min each. Secondary antibodies, anti-rabbit IgG TR and anti-goat IgG FITC (Sigma-Aldrich), were diluted in PBS alone in compliance with the manufacturer's instructions.

Images were collected with Nikon EZ-C1 3.00 software on a Nikon Diaphot TMD microscope equipped for fluorescence with a xenon lamp and filter wheels (Sutter Instruments, Novato, CA, USA), fluorescent filters (Chroma, Brattleboro, VT, USA), cooled charge-coupled device camera (Cooke,

Table 1: The above values correspond to metabolic and cardiovascular variables.

Variables	W	FFR	SHR	FFHR	FFHR + L
SBP (mmHg)	118 ± 0.8	140 ± 1.8*	172 ± 2.0$^{*\wedge}$	182 ± 1.1$^{*\wedge\#}$	165 ± 0.9$^{*\wedge\dagger}$
HOMA (μU/mL insulin × mmol/L glucose)/22.5	4.22 ± 1.1	11.9 ± 1.3*	8.1 ± 2.2*	15.1 ± 2.5*	13.2 ± 2.1*
Fast glucemia (mmol/L)	4.0 ± 1.1	6.8 ± 1.3*	5.2 ± 1.3*	6.92 ± 2.1*	6.5 ± 2.1*
Tryglycerides (mg/dL)	72.5 ± 1.9	109 ± 1.8*	115 ± 2.4*	149 ± 2.2$^{*\wedge\#}$	140.9 ± 2.4*
Relative heart weight (mg/100 g corporal weight)	229 ± 2.5	302 ± 2.1*	425 ± 4.4*	475 ± 2.6$^{*\wedge\#}$	389 ± 2$^{*\wedge\dagger}$
Vascular NAD(P)H-oxidase activity (cpm/mg)	14.5 ± 3.3	68 ± 1.4*	149 ± 2.6*	366 ± 12$^{*\wedge\#}$	197 ± 2.3$^{*\wedge\#\dagger}$
TBARS (mmol/L)	39 ± 3.2	118 ± 5.4*	110 ± 3.9*	171 ± 2.6$^{*\wedge\#}$	101 ± 2.7$^{*\wedge\#\dagger}$
Arterial eNOS activity (dpm·mg P/min)	82.0 ± 2	62 ± 1.5*	81.9 ± 2.6	50.6 ± 1.9$^{*\wedge\#}$	71.4 ± 1.1$^{*\wedge\#\dagger}$
L/M relationship	13.1 ± 1.4	9.5 ± 1.2*	8.9 ± 2.1*	7.4 ± 1.2$^{*\#}$	11.5 ± 1.1$^{*\wedge\#\dagger}$
PCRus	1.18 ± 0.1	3.2 ± 0.3*	4.5 ± 0.1*	6.7 ± 0.2$^{*\wedge\#}$	1.1 ± 0.2$^{*\wedge\#}$

$^{*}P < 0.001$ versus WKY; $^{\wedge}P < 0.001$ versus SHR; $^{\#}P < 0.01$ versus FFR. †versus FFHR.

Tonawanda, NY, USA), and stepper motor (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Multifluor images were merged, deconvolved, and renormalized using EZ-C1 3.00 Thumbnail software.

2.13.2. Determination of Matrix Metalloproteinases. Anti-MMP-2 was obtained from Chemicon International Inc. (MAB3308), and anti-MMP-9 antibody was obtained from Chemicon (MAB3309). Tissue sections were cut at 5 μm thickness from paraffin-embedded blocks. Deparaffinized sections were used to determine inflammatory response. Tissue was permeabilized in 1% Triton X-100 for 15 min, rinsed well with PBS, and blocked with sterile filtered 10% normal rabbit serum for 20 min. All antibody solutions were microfuged for 20 min before use. The antibodies were diluted 1 : 500. Primary incubations were done for 1 hour at 21-22°C, followed by extensive washes in PBS with Triton X-100, generally six times for 5 min each. Secondary antibodies, Cy5 and FITC IgG (Sigma-Aldrich), were diluted in PBS alone in compliance with the manufacturer's instructions.

Images were collected with Nikon EZ-C1 3.00 software on a Nikon Diaphot TMD microscope equipped for fluorescence with a xenon lamp and filter wheels (Sutter Instruments, Novato, CA, USA), fluorescent filters (Chroma, Brattleboro, VT, USA), cooled charge-coupled device camera (Cooke, Tonawanda, NY, USA) and stepper motor (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Multi-fluor images were merged, deconvolved, and renormalized using EZ-C1 3.00 Thumbnail software.

Activity of Matrix Metalloproteinases 9 and 2 (Collagenases). A sample of mesenteric tissue homogenates is obtained using 50 ug of total protein; subsequently a polyacrylamide gel co-polymerized with gelatin was used. Composition is as follows: 2880 uL bidistilled water (BD, milliQ), 800 uL gelatin 10 mg/mL (Sigma, USP, etc.) (end conc: 1 mg gelatin/mL of gel), 2160 uL acrylamide/bis 30%, 2160 uL Tris-Cl 1.5 M pH 8.8, 80 uL SDS 10% (Sigma), 80 uL of ammonium persulfate 10%, and 6 uL TEMED. A third of the sample buffer was used based on the total sample volume with proteins. The run was stopped when the phenol red reached the lower edge of the gel and was starting to overflow it. Then, the gels were washed to remove the SDS. The gels were washed in 30–50 mL of Triton X-100 2.5% for 20–30 min with continuous agitation. Incubation in 50 mL (per gel) of this solution at 37°C for 12 h approximately Afterwards the gels were stained with Coomassie blue R-250 for 12 h. For higher contrast it was used at a concentration of 0.5% (w/v) instead of 0.1%. Gels were decolorated with solution of methanol, acetic acid, and water. Metalloprotease activity was evaluated based on mean optical density of light bands on a dark blue background.

2.14. Reagents. The drug Lumiracoxib in pure state was provided by Novartis Basel.

Unless otherwise noted, reagents were purchased from Sigma Chemical Co, MO, USA.

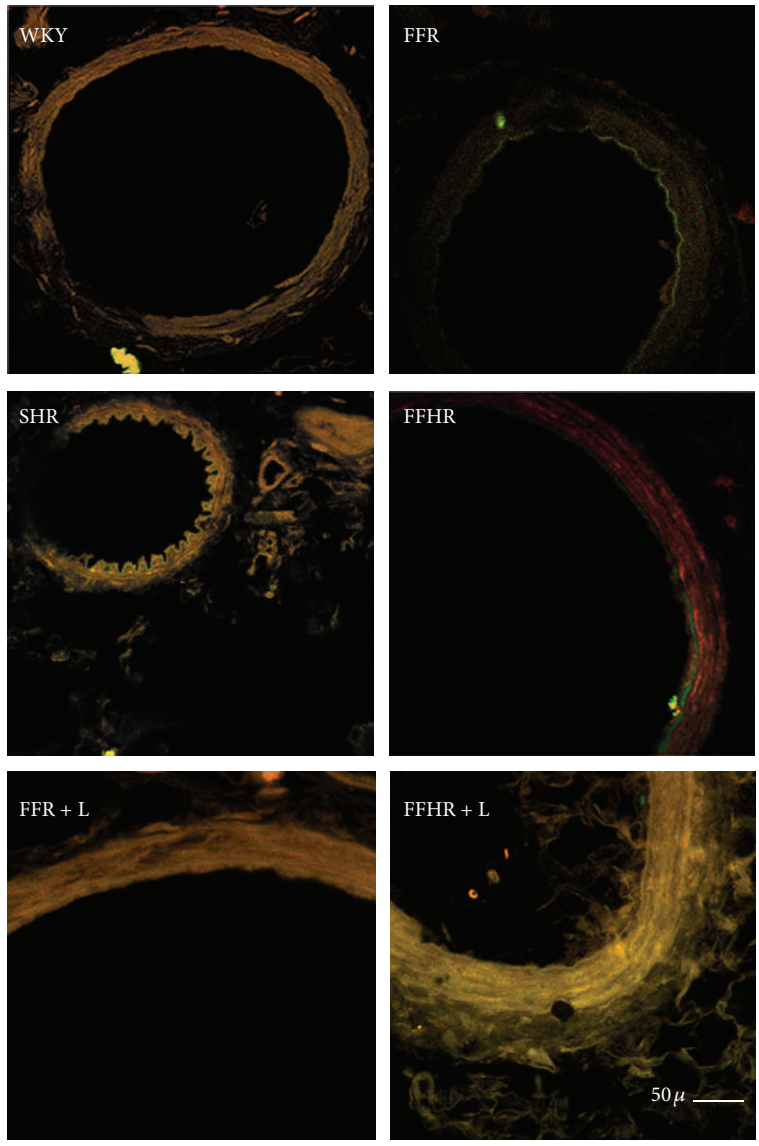
2.15. Statistical and Data Analysis. Data are expressed as mean ± SEM. The statistical significance of data comparison between all groups was assessed by one-way ANOVA followed by Bonferroni posttest. A two-sided P value of less than 0.05 was considered significant.

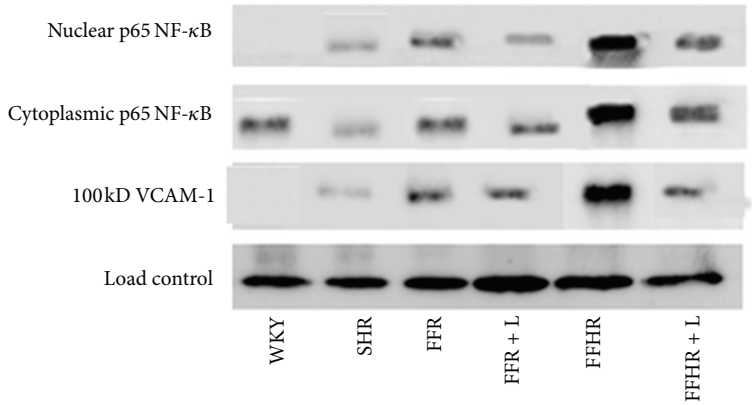
3. Results

Levels of systolic blood pressure increased gradually throughout the entire experimental period in animals of groups FFR, SHR, and FFHR, reaching significant differences with respect to the control group at the end of the protocol (Table 1). Chronic treatment with L significantly decreased TA values in groups FFR, FFHR, and SHR but not to the values of the control group (Table 1).

Furthermore, FFR and FFHR groups are characterized as a model of metabolic syndrome according to the increase of the HOMA index, fasting blood glucose, triglycerides, decreased HDL-cholesterol, and arterial hypertension. Animals in the experimental groups FFR and FFHR which received L did not have significantly modified values of fasting blood glucose, triglycerides, and HDL-cholesterol (Table 1).

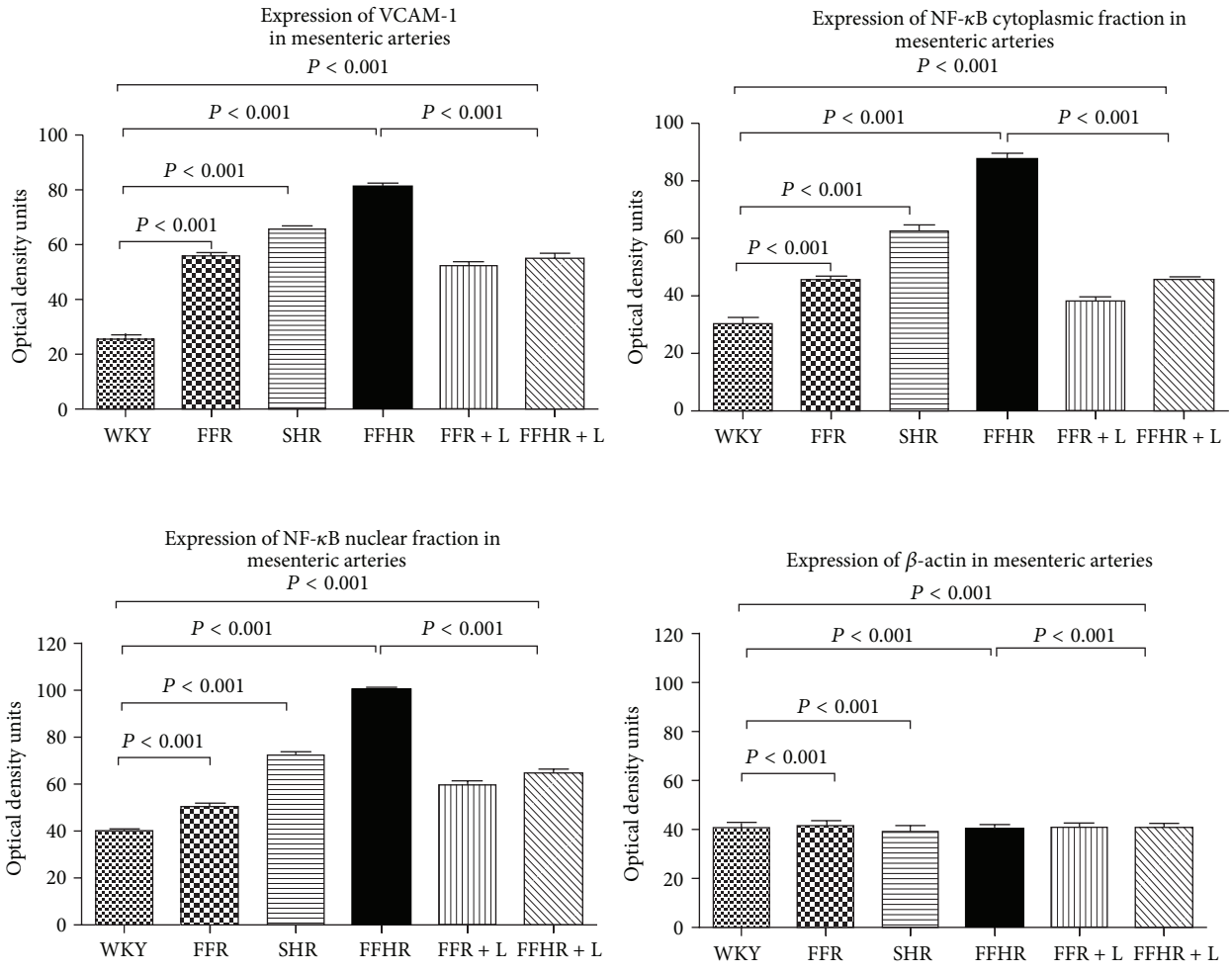
Oxidative stress variables were evaluated in the four experimental models. NADPH oxidase activity increased significantly in all experimental models but did so exponentially in the FFHR model. On the other hand eNOS





(a)

Figure 1: Continued.



(b)

Figure 1: Cytoplasmatic and nuclear p-65 fraction of NF-κB and VCAM-1 expression in mesenteric arteries by WB and IHC. up panel shows the WB representative membrane and which analyzed anti-VCAM-1-FITC and anti-p65-TRITC, the results were obtained by optic density of the bands revealed for each group. top panel shows microphotographs obtained by laser ICM 600x of mesenteric tissue.

activity decreased significantly in the experimental models with fructose feeding (Table 1). Lipid peroxidation was evaluated from TBARS, which showed significant increases in all three experimental models, the FFHR model being the most affected. After chronic treatment with L, aortic NAD(P)H oxidase activity significantly decreased in groups FFR, SHR, and FFHR. Endothelial eNOS activity normalized in the experimental models FFHR and FFR after treatment with L, and these results were statistically significant.

In addition, cardiac remodeling was evaluated based on relative cardiac weight (RHW) (Table 1). Experimental models FFR, SHR, and FFHR showed cardiac remodeling, which decreased, after chronic treatment with L, in the experimental groups studied but only the FFHR group showed statistically significance.

Vascular remodeling, as discussed previously, was evaluated based on the M/L ratio. In experimental models FFR, and FFHR a significant reduction of the media/lumen ratio was observed, demonstrating the presence of eutrophic remodeling of the mesenteric arteries studied. After chronic treatment with L, we demonstrated an increase of this variable in the three experimental models, which caused the remodeling to decrease. It should be noted that this increase in the remodeling index, although statistically significant, did not reach normal values, which is an important fact when analyzing the involvement of COX-2 in the pathophysiological mechanisms.

After demonstrating the presence of cardiovascular remodeling in these experimental models, we studied the presence of inflammatory markers.

At the systemic level, C-reactive protein (hsCRP) was evaluated; it showed significant differences among FFR and FFHR models, demonstrating the presence of inflammation in these groups. After treatment with a COX-2 specific

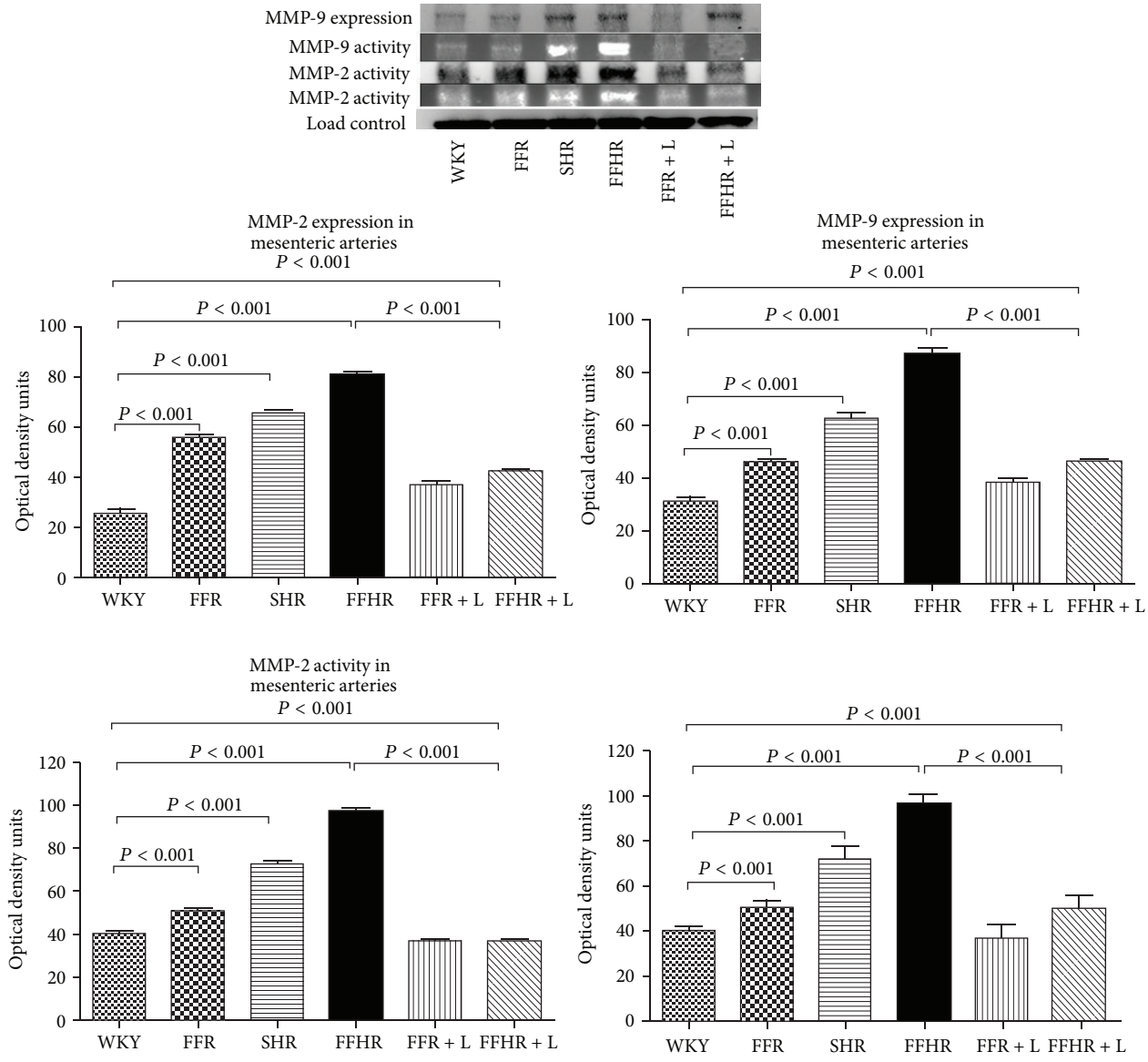


Figure 2: A representative polyacrylamide gel for Western blot and zymography for collagenases (MMP-2 and MMP-9). This image showed activity and expression of collagenases contrasted for each experimental group. The picture below shows the bar graph with statistical analysis.

antagonist, these variables significantly decreased in the three experimental models, reaching normal values and demonstrating the participation of this pathophysiological pathway in the systemic inflammatory process.

At the vascular level, the expression of these proteins was assessed by Western blot as shown in Figure 1. In experimental models FFR, FFHR, and SHR the expression of these markers increased. After chronic treatment with L, the translocation of NF-κB to the nucleus and the expression of VCAM-1 at the cellular membrane level were both reduced in the experimental models FFR and FFHR (Figure 1).

The expression of the aforementioned markers was studied by IFI; they were exposed in the vascular wall by laser colocalization microscopy. The experimental models studied showed different expression patterns of NF-κB and VCAM-1. This result was previously reported by our laboratory [18]. Model FFHR presents an important expression of NF-κB in the entire vascular wall (intima, media, and adventitia) as well as endothelial expression of VCAM-1, while in other models both markers were present only in the endothelium. Treatment with L significantly reduced the expression of these markers, as demonstrated by WB, although inflammation at the adventitia level was higher in the FFHR model (Figure 1).

We also analyzed two markers of vascular remodeling, MMP-2 and MMP-9. The expression was analyzed using WB and the activity by zymography. In models FFR and SHR the activity and the expression of these MMPs increased moderately, while the FFHR model presents maximum activity and

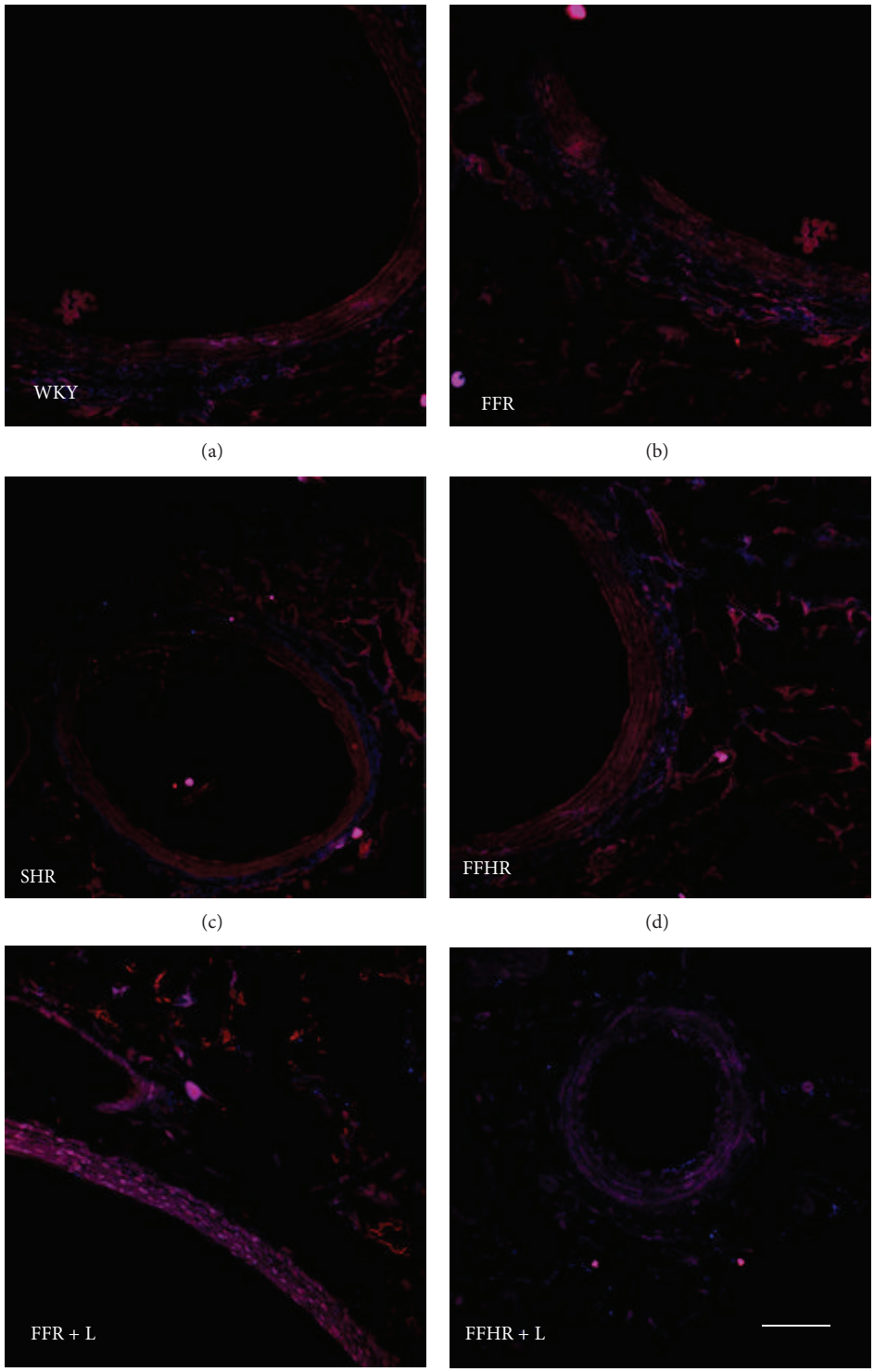


Figure 3: Representative figure showing the expression of MMP-2 (blue: Cy5) and MMP-9 (red: TRICT) merge mode in mesenteric arteries. You can see an increased level of tagging in adventitia for MMP-2 and MMP-9 on experimental models with vascular remodeling: SHR, FFR, and FFHR. These changes are reversed after administration of L. Microphotographs obtained by laser ICM 600x of mesenteric tissue.

expression of both collagenases (Figure 2). Chronic treatment with L significantly reduced the activity and expression of the MMPs, although in the FFHR model this decrease did not reach the values of the control group and persisted primarily at the adventitial level. Figure 2 shows a representative image of the gels and the statistical analysis. Figure 3 shows expression by IFI of representative photomicrographs of these antibodies, with the aim of obtaining a vascular level location of this expression. MMP-2 and MMP-9 are observed predominately at the adventitia level.

This finding provides further evidence of the importance of this vascular layer present in vascular remodeling of metabolic syndrome.

4. Conclusions

The most important finding of this study was the demonstration that ciclioxigenasa-2 participates in the cardiovascular remodeling associated with metabolic syndrome and the reversal of this syndrome after chronic treatment with a specific antagonist, lumiracoxib (L).

We demonstrate that chronic treatment with L did not modify the metabolic variables associated with metabolic syndrome of models FFHR and FFR. It partially modified the oxidative stress variables but reduced total lipid peroxidation as demonstrated by TBARS, reducing vascular damage and probably reducing the expression of redox-sensitive genes such as NF-κB, an important initiator of inflammation.

Both vascular and cardiac remodeling showed significant differences after treatment with L, mainly in the FFHR model. This was also evidenced in microscopic sections of mesenteric arteries showing less inflammation and decreased expression of extracellular matrix metalloproteinases.

The initiation of the atherosclerotic process is associated, according to some authors, with the presence of endothelial dysfunction (as demonstrated in this model) and local inflammation in a first stage, after activating macrophages that synthesize IL-6, which interacts with hepatocytes and initiates the release of acute phase reactants such as CRP. NF-κB is a redox-sensitive transcription factor of great potency for initiating and perpetuating the inflammatory response; its most important transcriptional product at the vascular level is VCAM-1, a marker both of inflammation and of endothelial dysfunction according to several authors. The number of these two molecules increased in the entire vascular wall in the FFHR model and only in the intima layer in FFR and SHR models, as if they were intermediate products of WKY rats and FFHR. Epigenetics may explain the differential expression in this model, as fructose is a hidden genes demethylation in SHR that culminates, at the vascular level, showing a completely swollen vessel. Chronic treatment with L altered the expression of inflammatory molecules in the models that received fructose, although at a microscopic level there remains a residue of inflammation in the adventitia.

The remodeling of the vessels can demonstrate that collagenases, which perform the work "softening" the matrix, are present in increased numbers [19]. Although the fact that they have increased does not imply the presence of remodeling, for that it should be demonstrated that their activity has also increased and that is why zymography used. Both MMP-2 and MMP-9 increased in the models studied. Chronic treatment with L modified the expression and activity of MMP-2 and MMP-9, increasing at a microscopic level the media/lumen ratio and thereby improving the vascular microenvironment.

Other authors have also shown the involvement of COX-2 in vascular pathophysiology; Dinarello et al. suggest that a detrimental action of COX-2 has also been described in hypertensive patients and represents one mechanism whereby COX-2 may promote atherosclerosis; also they find that vascular COX-2 downregulation represents a prominent mechanism whereby statins may attenuate the development of the atherosclerotic process in normocholesterolemic hypertensive patients [20].

As a final conclusion, cyclooxygenase actively participates in inflammation process and in the remodeling of the vessels, being an important factor that in the future will allow us to use these results to pharmacologically improve these antagonists so that they can be used in clinical practice as therapeutic targets or as adjuvants in more complete drug therapies the way monoclonal antibodies are used now [21].

Acknowledgments

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References

[1] P. Libby, "Inflammation and cardiovascular disease mechanisms1-3," *American Journal of Clinical Nutrition*, vol. 83, supplement 2, pp. 456S–460S, 2006.

[2] Z. S. Galis, G. K. Sukhova, M. W. Lark, and P. Libby, "Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques," *The Journal of Clinical Investigation*, vol. 94, no. 6, pp. 2493–2503, 1994.

[3] H. Uzui, A. Harpf, M. Liu et al., "Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines," *Circulation*, vol. 106, no. 24, pp. 3024–3030, 2002.

[4] V. Pasceri, J. T. Willerson, and E. T. H. Yeh, "Direct proinflammatory effect of C-reactive protein on human endothelial cells," *Circulation*, vol. 102, no. 18, pp. 2165–2168, 2000.

[5] C. Y. Ho, C. K. Wong, E. K. Li, L. S. Tam, and C. W. K. Lam, "Elevated plasma concentrations of nitric oxide, soluble thrombomodulin and soluble vascular cell adhesion molecule-1 in patients with systemic lupus erythematosus," *Rheumatology*, vol. 42, no. 1, pp. 117–122, 2003.

[6] R. Elhage, J. Jawien, M. Rudling et al., "Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice," *Cardiovascular Research*, vol. 59, no. 1, pp. 234–240, 2003.

[7] M. Peichev, A. J. Naiyer, D. Pereira et al., "Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors," *Blood*, vol. 95, no. 3, pp. 952–958, 2000.

[8] G. M. Reaven, "Role of insulin resistance in human disease," *Diabetes*, vol. 37, no. 12, pp. 1595–1607, 1988.

[9] O. A. Carretero and S. Oparil, "Essential hypertension. Part I: definition and etiology," *Circulation*, vol. 101, no. 3, pp. 329–335, 2000.

[10] R. Miatello, N. Risler, C. Castro, S. González, M. Rüttler, and M. Cruzado, "Aortic smooth muscle cell proliferation and endothelial nitric oxide synthase activity in fructose-fed rats," *American Journal of Hypertension*, vol. 14, no. 11 I, pp. 1135–1141, 2001.

[11] N. Risler, C. Castro, M. Cruzado, S. González, and R. Miatello, "Early changes in proteoglycans production by resistance arteries smooth muscle cells of hypertensive rats," *American Journal of Hypertension*, vol. 15, no. 5, pp. 416–421, 2002.

[12] N. Renna, M. Vazquez, M. Lama, E. González, and R. Miatello, "Effect of chronic aspirin administration on an experimental model of metabolic syndrome," *Clinical and Experimental Pharmacology and Physiology*, vol. 36, no. 2, pp. 162–168, 2009.

[13] J. F. Keaney Jr., J. M. Gaziano, A. Xu et al., "Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 24, pp. 11880–11884, 1993.

[14] R. F. Furchgott and J. V. Zawadzki, "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine," *Nature*, vol. 288, no. 5789, pp. 373–376, 1980.

[15] L. J. Ignarro, R. E. Byrns, G. M. Buga, and K. S. Wood, "Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical," *Circulation Research*, vol. 61, no. 6, pp. 866–879, 1987.

[16] T. Michel and O. Feron, "Nitric oxide synthases: which, where, how, and why?" *The Journal of Clinical Investigation*, vol. 100, no. 9, pp. 2146–2152, 1997.

[17] I. Fleming and R. Busse, "Signal transduction of eNOS activation," *Cardiovascular Research*, vol. 43, no. 3, pp. 532–541, 1999.

[18] N. Renna, C. Lembo, E. Diez, and R. Miatello, "Vascular repair by endothelial progenitor cells ina an experimental model of metabolic syndrome," in *Handbook on Metabolic Syndrome: Classification, Risk Factors and Health Impact*, C. M. L. Garcia and P. A. P. Gonzalez, Eds., pp. 287–303, 2012.

[19] P. Zamani, P. Ganz, P. Libby et al., "Relationship of antihypertensive treatment to plasma markers of vascular inflammation and remodeling in the Comparison of Amlodipine versus Enalapril to Limit Occurrences of Thrombosis study," *American Heart Journal*, vol. 163, no. 4, pp. 735–740, 2012.

[20] C. Dinarello, A. Simon, and J. van der Meer, "Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases," *Nature Reviews Drug Discovery*, vol. 11, no. 8, pp. 633–652, 2012.

[21] A. Virdis, R. Colucci, D. Versari et al., "Atorvastatin prevents endothelial dysfunction in mesenteric arteries from spontaneously hypertensive rats role of cyclooxygenase 2-derived contracting prostanoids," *Hypertension*, vol. 53, no. 6, pp. 1008–1016, 2009.

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Review Article

Metabolic Syndrome in Rheumatoid Arthritis

Iván Ferraz-Amaro,[1] Carlos González-Juanatey,[2] Raquel López-Mejias,[3] Leyre Riancho-Zarrabeitia,[3] and Miguel A. González-Gay[3]

[1] *Division of Rheumatology, Hospital Universitario de Canarias, 38320 Santa Cruz de Tenerife, Spain*
[2] *Division of Cardiology, Lucus Augusti University Hospital, 27004 Lugo, Spain*
[3] *Division of Rheumatology, Hospital Universitario Marqués de Valdecilla, IFIMAV, Avenida de Valdecilla s/n, 39008 Santander, Spain*

Correspondence should be addressed to Miguel A. González-Gay; miguelaggay@hotmail.com

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Insulin resistance is an essential feature of the metabolic syndrome that has been linked to rheumatoid arthritis (RA). Understanding how inflammation arising in one tissue affects the physiology and pathology of other organs remains an unanswered question with therapeutic implications for chronic conditions including obesity, diabetes mellitus, atherosclerosis, and RA. Adipokines may play a role in the development of atherogenesis in patients with RA. Biologic therapies, such as TNF-α antagonists, that block proinflammatory cytokines have beneficial effects on the insulin resistance that is often observed in patients with RA.

1. What Is Metabolic Syndrome?

The term "metabolic syndrome" (MS) refers to a clustering of specific cardiovascular (CV) disease risk factors including central obesity, hypertension, high triglycerides, and low HDL levels whose underlying pathophysiology is thought to be related to insulin resistance [1]. Some clinical studies [2] debate whether MS is a distinct pathophysiologic entity or simply reflects an association of CV risk factors, while others [3] argue that each individual component of the MS confers an increased risk of CV-related death but this risk is more pronounced when the MS itself is present. Nevertheless, it is thought that the more components of the MS that are evident, the higher is the CV mortality rate, and many studies have shown that patients diagnosed with the MS have more prevalent CV disease or are at greater risk of developing CV complications.

It is estimated that around a quarter of the world's adult population have MS [4] and they are twice as likely to die from and three times as likely to have a heart attack or stroke compared with people without the syndrome [3]. People with MS have a fivefold greater risk of developing type 2 diabetes [5]. In addition, MS has also been associated with several obesity-related disorders including fatty liver disease with steatosis, fibrosis and cirrhosis [6], hepatocellular and intrahepatic cholangiocarcinoma, chronic kidney disease [7], polycystic ovary syndrome [8], sleep-disordered breathing, including obstructive sleep apnea [9], and hyperuricemia and gout [10].

There are five definitions for the MS [11–14] (Table 1). However, the relative value of the different metabolic syndrome definitions in terms of prognosis and management has been established to be similar. For example, when data from the Framingham population were examined using ATPIII, IDF, and EGIR definitions of the MS, associations for incident type 2 diabetes and for CV disease were found to be equivalent [15]. Nevertheless, the National Cholesterol Education Program (NCEP/ATPIII) and International Diabetes Federation (IDF) definitions are the most widely used. The WHO, ATPIII, and IDF definitions include type 2 diabetes as syndrome traits. Experts do not all agree that type 2 diabetes should be part of the definition, as the importance of the syndrome is that it identifies patients at increased risk for the development of diabetes.

In summary, an MS indicates the presence of a series of risk factors that are associated with the risk of CV complications.

Table 1: Five current definitions of metabolic syndrome.

Required	NCEP ATP3 2005	IDF 2006 Waist = 94 cm (men) or = 80 cm (women)	EGIR 1999 Insulin resistance or fasting hyperinsulinemia in top 25 percent	WHO 1999 Insulin resistance in top 25 percent; glucose = 6.1 mmol/L (110 mg/dL); 2-hour glucose = 7.8 mmol/L (140 mg/dL)	AACE 2003 High risk of insulin resistance; or BMI = 25 kg/m^2; or waist = 102 cm (men) or = 88 cm (women)
Number of abnormalities	3	2	2	2	2
Glucose	mmol/L (100 mg/dL) or drug treatment for elevated blood glucose	5.6 mmol/L (100 mg/dL) or diagnosed diabetes	6.1–6.9 mmol/L (110–125 mg/dL)		6.1 mmol/L (110 mg/dL); 2-hour glucose 7.8 mmol/L (140 mg/dL)
HDL cholesterol	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women); or drug treatment for low HDL-C	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women); or drug treatment for low HDL-C	<1.0 mmol/L (40 mg/dL)	<0.9 mmol/L (35 mg/dL) (men); <1.0 mmol/L (40 mg/dL) (women)	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women)
Triglycerides	1.7 mmol/L (150 mg/dL) or drug treatment for elevated triglycerides	1.7 mmol/L (150 mg/dL) or drug treatment for high triglycerides	2.0 mmol/L (180 mg/dL) or drug treatment for dyslipidemia	1.7 mmol/L (150 mg/dL)	1.7 mmol/L (150 mg/dL)
Obesity	Waist = 102 cm (men) or = 88 cm (women)		Waist = 94 cm (men) or = 80 cm (women)	Waist/hip ratio > 0.9 (men) or >0.85 (women) or BMI = 30 kg/m^2	
Hypertension	130/85 mmHg or drug treatment for hypertension	130/85 mmHg or drug treatment for hypertension	140/90 mmHg or drug treatment for hypertension	140/90 mmHg	130/85 mmHg

NCEP: National Cholesterol Education Program; IDF: International Diabetes Federation; EGIR: Group for the Study of Insulin Resistance; WHO: World Health Organization; AACE: American Association of Clinical Endocrinologists.
HDL-C: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, BMI: body mass index.

2. Metabolic Syndrome and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disorder of unknown etiology that if uncontrolled may lead to destruction and deformity of joints due to erosion of cartilage and bone. Epidemiologic data suggest that RA is an independent risk factor for CV disease [26, 27]. The development of accelerated atherosclerosis and increased risk of CV disease in patients with RA may be influenced by the occurrence of MS [28]. An association between inflammatory activity of RA and MS has also been suggested (Table 2). Also high incidence of MS has been reported in patients with RA. With respect to this, in a series of 283 patients and 226 controls, Da Cunha et al. [16] reported that 39% of RA patients met criteria for MS while these criteria were only fulfilled in 19% of controls ($P = 0.001$). These authors also found increased prevalence of waist circumference, elevated blood pressure, and increased fasting glucose in this series of RA patients when compared with controls. In this study the risk of having MS was significantly higher in RA patients than controls (odds ratio (OR) = 1.87 (95% confidence interval (CI) = 1.17–3.00), $P = 0.01$); and disease activity score-28 (DAS28) was significantly higher in RA patients with MS than in those without MS (3.59 ± 1.27 versus 3.14 ± 1.53; $P = 0.01$). Disease duration, the presence of rheumatoid factor, and extra-articular manifestations were similar in RA patients with and without MS in this study. Nevertheless, the frequency of MS in RA varies according to the criteria used for the assessment. In this regard, using the WHO criteria in 154 patients with RA and 85 controls, Chung et al. [17] observed the presence of MS in 42% of RA patients with long-standing disease, in 31% of RA patients with early arthritis, and in 11% of the controls. In the same study, when NCEP criteria were used, the prevalence of MS was 30% in RA patients with long-standing disease, 22% in patients with RA patients with early arthritis and 10% in controls, respectively. In this paper, coronary-artery atherosclerosis was studied by electron beam computed tomography, and RA patients with MS were found to have a higher coronary-artery calcification score (OR = 2.02 (95% CI 1.03–3.97), $P = 0.04$).

The association of RA and MS was confirmed in patients with short disease duration. With respect to this, Dao et al. [18] assessed the presence of MS in 105 women with RA and disease duration less than 3 years and 105 age-matched healthy women. Different definitions for MS were tested in this study (Joint Consensus, International Diabetes Federation, National Cholesterol Education Program 2004 and 2001, European Group for Study of Insulin Resistance, and World Health Organization). The authors observed that the frequency of MS in women with RA varied from 16.2% to 40.9% according to the different definitions. However, it was higher than in matched controls (10.5% to 22.9%). Therefore, MS frequency was significantly higher in this series of patients with RA than in healthy controls. When individual components of MS were assessed, hypertension, ($P < 0.001$), high-density lipoprotein cholesterol levels ($P < 0.001$), and abdominal obesity ($P = 0.019$) were found more commonly observed in RA patients than in matched controls. After adjusting for age and physical activity, higher erythrocyte sedimentation rate (OR = 1.52 (95% CI 1.07 to 3.20), $P = 0.04$), disease activity score (OR = 1.74 (95% CI 1.29–2.79), $P = 0.01$), health assessment questionnaire score (OR = 1.58 (95% CI 1.20–2.37), $P = 0.03$), and less methotrexate use (OR = 0.74 (95% CI 0.55–0.96), $P = 0.02$) remained significant independent predictors of the presence of MS in women with RA. Another interesting study by Crowson et al. [19] in 232 patients with RA with no overt CV disease and 1241 non-RA subjects without CV disease showed that RA patients were significantly more likely to have increased waist circumference and elevated blood pressure than non-RA subjects without CV disease. The authors concluded that RA patients were more commonly classified as having MS, and that MS was associated with Health Assessment Questionnaire Disability Index, large-joint swelling, and uric acid levels, but not with C-reactive protein or RA therapies.

Regarding therapy used in the management of RA, Toms et al. [20] showed that methotrexate therapy, unlike other disease modifying antirheumatic drugs (DMARDs) or glucocorticoids, was independently associated with a reduced risk to suffer MS, suggesting a drug-specific mechanism, and making methotrexate a good first-line DMARD in RA patients at high risk of developing MS. In another studies the same authors reported the presence of MS in 40.1% of 398 patients with RA. However, its prevalence did not differ significantly between the different glucocorticoid-exposure groups [29].

Mok et al. [21] assessed the prevalence of the MS in patients with RA, ankylosing spondylitis (AS), and psoriatic arthritis. For this purpose, 930 patients were studied (699 with RA, 122 with AS, and 109 with psoriatic arthritis; 70% women, mean ± standard deviation age 51.1 ± 12.7 years). In this study, the prevalence of MS was significantly higher in psoriatic arthritis (38%) than RA (20%) or AS (11%; $P < 0.001$). The ORs for the MS compared to age- and sex-matched controls were 0.98 (95% CI 0.78–1.23), $P = 0.88$; 0.59 (95% CI 0.30–1.15), $P = 0.12$; and 2.68 (95% CI 1.60–4.50), $P < 0.001$, respectively, for RA, AS, and psoriatic arthritis. Patients with psoriatic arthritis had a significantly higher prevalence of impaired fasting glucose (30%; $P < 0.001$), low HDL cholesterol (33%; $P < 0.001$), high triglycerides level (21%; $P = 0.008$), central obesity (65%; $P < 0.001$), and high blood pressure (56%; $P = 0.045$). They concluded that patients with psoriatic arthritis, but not RA or AS patients, have a significantly higher prevalence of MS compared to the general population.

In another study by Zonana-Nacach et al. [22], 107 RA patients and 85 systemic lupus erythematosus where compared regarding APTIII definitions for MS. They reported that the frequency of obesity and abnormal waist circumference was similar in patients with RA and systemic lupus erythematosus. It was also the case for the frequency of MS in both diseases was also similar (17%). In their series, MS was significantly associated with older age, lower education levels, lower income, and smoking. In RA patients, MS was significantly associated with a shorter period of methotrexate therapy, with pain, and with health assessment questionnaire scores.

Table 2: Metabolic syndrome and rheumatoid arthritis.

Reference	RA/controls	Association	MS definition used	Comments
Da Cunha et al. [16]	283/226	Yes	NCEP	MS associated with disease activity. Increased prevalence of waist circumference, blood pressure, and fasting glucose in this RA population when compared to controls.
Chung et al. [17]	154/85	Yes	NCEP, WHO	88 with early RA and 66 with long-standing RA. RA patients with MS had an increased risk of having higher coronary-artery calcification score; this association of RA and MS has been also observed when early RA was considered.
Dao et al. [18]	105/105	Yes	NCEP, WHO IDF, EGIR	Early RA already had higher prevalence of MS compared with healthy controls. Higher systemic inflammatory markers, disease activity and disability scores, and less methotrexate use were independent predictors associated with the presence of MS in women with early RA.
Crowson et al. [19]	232/1241	Yes	NCEP	Only RA patients with no overt cardiovascular disease were considered. RA patients were significantly more likely to have increased waist circumference and elevated blood pressure than non-RA subjects Significantly more RA patients were classified as having MS.
Toms et al. [20]	400/—	Yes	NCEP, WHO IDF, EGIR	MS prevalence rates varied from 12.1% to 45.3% in RA according to the definition used. Methotrexate use, but not other DMARDs or glucocorticoids, was associated with significantly reduced chance of having MS in RA.
Mok et al. [21]	699 RA 122 AS 109 PsA	No	Asian criteria for central obesity	The prevalence of MS was significantly higher in PsA (38%) than RA (20%) or AS (11%; $P < 0.001$). Patients with PsA, but not those with RA or AS, have a significantly higher prevalence of MS compared to the general population. In RA patients, MS was related to pain and functional status, indicating disease activity.
Zonana-Nacach et al. [22]	107 RA/85 LES	Yes	NCEP	The frequency of MS in RA and SLE patients was similar and associated with smoking.
Karimi et al. [23]	92/96	No	NCEP, WHO	The duration of RA was associated with MS.
Sahebari et al. [24]	120/431	No	NCEP, IDF	The prevalence of MS was significantly higher in the control group. There was no association between the DAS28 and the presence of MS components by either definition.
Karvounaris et al. [25]	200/400	No	NCEP	Risk of having moderate-to-high disease activity (DAS28 > 3.2) was significantly higher in patients with MS compared with those with no MS components.

NCEP: National Cholesterol Education Program; IDF: International Diabetes Federation; EGIR: Group for the Study of Insulin Resistance; WHO: World Health Organization.
MS: metabolic syndrome, DAS28: disease activity score, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, DMARDs: disease modifying antirheumatic drugs.
PsA: psoriatic arthritis, AS: ankylosing spondylitis.
The term Association means if an association between RA and MS was observed.
Toms, Mok and Zonana did not study healthy controls.

With regard to the relationship between MS and subclinical atherosclerosis in patients with RA, using carotid ultrasonography, Dessein et al. [30] investigated the associations of MS features and MS definitions with common carotid artery intima-media thickness and carotid plaques in 74 RA patients. They concluded that MS was associated with carotid artery intima-media thickness ($P = 0.04$) but not with the presence of carotid plaques ($P > 0.1$).

There are three exceptions in the literature with regard to the association between MS and RA. With respect to this, using NCEP and WHO criteria, in a case-control study that encompassed 92 RA patients and 96 healthy controls, Karimi et al. [23] did not find differences between groups regarding MS. The only differences observed were a higher frequency of hypertension patients with RA than in the controls and significantly longer duration of the disease in RA patients with MS compared to those without MS. Also, in a series of 200 outpatients with RA and 400 age and sex-matched controls, Karvounaris et al. [25], found a high, albeit comparable to the control population, prevalence of MS in middle-to-older aged patients with RA. However, in this study, in a multivariate logistic regression analysis adjusting for demographics and RA treatment modalities, the risk of having moderate-to-high disease activity (DAS28 > 3.2) was significantly higher in patients with MS than in those without MS components (OR 9.24 (95% CI 1.49–57.2), $P = 0.02$).

Finally, in a series of 120 patients with RA and 431 age- and sex-matched controls study, Sahebari et al. disclosed that the prevalence of IDF or ATPIII MS was significantly higher in controls [24]. In this series the presence of RA was not associated with an increased risk of MS.

A recent study on a random sample of 499 patients with RA disclosed that Vitamin D deficiency was associated independently with an increased risk of hyperlipidemia (OR 1.72 (95% CI 1.10–2.45)) and MS (OR 3.45 (95% CI 1.75–6.80)) in adjusted models [31]. In this regard, another recent study suggested that 25-hydroxyvitamin D may play a protective role against MS in patients with RA [32].

Taking together all these considerations, we can conclude that MS is not uncommon in patients with RA.

3. Metabolic Syndrome and Obesity

The underlying cause of the MS is still a challenging question. However, insulin resistance and central obesity are considered to play a key role in the development of MS. Central (abdominal) obesity is independently associated with each of the other MS components and is generally a prerequisite risk factor for the diagnosis of this condition. Although genetics, physical inactivity, ageing, or hormonal changes may also have a causal effect, the chronic and subacute inflammatory state that accompanies obesity has recently been suggested as a potentially unifying pathogenic link [33]. More than a passive storage depot, adipose tissue seems to be a dynamic and metabolically active organ with the ability to elaborate mediators with widespread effects on metabolism, immune function, and vascular homeostasis.

Areas of active investigation focus on the molecular bases of metabolic inflammation and potential pathogenic roles in insulin resistance, diabetes, and CV disease. An increased accumulation of macrophages occurring in obese adipose tissue has emerged as a key process in metabolic inflammation. Recent studies have also begun to unravel the heterogeneity of adipose tissue macrophages, and their physical and functional interactions with adipocytes, endothelial cells, and other immune cells within the adipose tissue microenvironment [33].

In conclusion, as shown for insulin resistance, obesity has also a relevant role in the development of MS.

4. Obesity and Rheumatoid Arthritis

Obesity is highly prevalent in patients with RA [34]. Changes in body composition have been found in patients with RA, with reduced fat-free mass and increased fat mass and, thus, with little or no weight loss or with a maintained body mass index. This condition has been named "rheumatoid cachexia" and is believed to accelerate morbidity and mortality in RA and has also been linked to MS [35, 36].

Abdominal adiposity has been shown to be increased in RA. In a comparative study that included 131 patients with RA and 121 controls, Giles et al. [37] disclosed similar body mass index and waist circumference between both groups. However, the adjusted abdominal visceral fat area was 45 cm^2 higher (representing a 51% difference) in men with RA patients than in men from the control group ($P = 0.005$) but not significantly different according to RA status in women. In the same study, the adjusted mean abdominal subcutaneous fat area was 119 cm^2 higher in women with RA (representing a 68% difference) than in women from the control group ($P < 0.001$) but not significantly different according to RA status in men. These authors found that the presence of increased visceral fat area was associated with a significantly higher adjusted probability of having an elevated fasting glucose, hypertension, or the composite definition of the MS for the RA group compared with controls. Within the RA group, rheumatoid factor seropositivity and higher cumulative prednisone exposure were significantly associated with a higher mean adjusted visceral fat area. Higher C-reactive protein levels and lower sharp radiographic scores were significantly associated with both visceral and subcutaneous fat areas. Similar results were described in a report on 80 outpatients with RA [35]. In that study, patients with fat mass index above the 50th percentile and patients with rheumatoid cachexia had the highest frequencies of hypertension and MS. Treatment with glucocorticoids and mean dose given did not differ between those who were cachectic and those not [35].

Thus, these studies confirmed the presence of an abnormal body composition in RA and that these abnormalities are related to factors associated with increased CV risk. Most people who are overweight are also *overfat*, but the two are not the same. Overfat is often found in patients with RA. However, Giles et al. [37] showed that the development of MS in patients with RA is due to the presence of a specific altered pattern of fat content. In this regard, for a similar body mass

index in patients and controls, the presence of increased visceral fat was associated with a significantly higher adjusted probability of fulfilling the composite definition of the MS in the individuals with RA than in controls. This means the development of MS is not only due to the presence of fat content but the result of a specific pattern of fat deposition.

Obesity but not specifically MS was classically considered to be an important risk factor for the developing RA [38]. However, further studies indicated that obesity is not a strong predisposing factor for RA. Methodological differences and a strict standardization for possible confounders may explain that the association of obesity with RA development had not been confirmed in other studies [39–41].

However, obesity has emerged as a protective risk factor for radiographic joint damage [42].

In summary, adiposity and obesity are often present in patients with RA and are associated with increased risk of MS in patients with RA.

5. Insulin Resistance and Rheumatoid Arthritis

Insulin resistance is an essential feature of MS that has been linked to RA [43]. In a study that included 94 patients with RA, Dessein and Joffe [44] observed that insulin resistance was associated with markers for inflammation such as C-reactive protein and erythrocyte sedimentation rate and disease activity scores. On the other hand, beta-cell function showed an inverse correlation with DAS28 and swollen and painful joint. Chung et al. [45] studied insulin resistance in 104 patients with RA and compared the results with those of 124 cases of systemic lupus erythematosus. They found that patients with RA have a higher insulin resistance index than systemic lupus erythematosus patients, and that insulin resistance was directly correlated with levels of interleukin 6, tumor necrosis factor-(TNF-) α, C-reactive protein, and erythrocyte sedimentation rate or coronary calcification [41]. Others studies have also confirmed the association between insulin resistance and RA [46–49].

In conclusion, insulin resistance is frequently observed in patients with RA.

6. Adipokines, Inflammation, and Cardiovascular Risk in Rheumatoid Arthritis

The adipose tissue is a multifunctional organ. Besides the central role of lipid storage, it has a major endocrine function secreting several hormones [50]. These various protein signals have been given the collective name "adipocytokines" or "adipokines." These molecules are mediators of immune response and inflammation [51]. Adipokines exert potent modulatory actions on target tissues and cells involved in rheumatic disease, including cartilage, synovium, bone, and various immune cells [52]. White adipose tissue-derived cytokines mediate between obesity-related exogenous factors (nutrition and lifestyle) and the molecular events that lead to the development of MS, inflammation, and CV disease [53]. In this regard, a complex adipokine-mediated interaction among white adipose tissue, CV disease, and RA has been described [54].

In RA adipocytes and their surrounding macrophages produce a range of adipokines that regulate systemic inflammation [55]. In this regard, the adipokine resistin was initially considered to be only implicated in insulin resistance and type II diabetes mellitus. However, more recent studies have shown that resistin plays an important function in inflammation. Although resistin can be detected at very low levels in human adipose tissue, it is found in peripheral blood mononuclear cells (PBMCs) [56], and resistin gene expression in PBMC is upregulated by proinflammatory cytokines such as TNF-α [57]. In some studies, high levels of resistin were found in synovial fluid from patients with RA [58]. However, in other studies plasma resistin levels in RA patients were similar to those found in healthy controls. As pointed out by Gomez et al., this discrepancy may be due to the increased permeability of inflamed synovial membrane in patients with RA [59].

In assessing a series of patients with RA in treatment with the anti-TNF-α monoclonal antibody infliximab for severe disease refractory to conventional DMARD therapy including methotrexate, we found positive correlations between markers of inflammation, in particular with C-reactive protein, and resistin levels [60]. Also, TNF-α blockade yielded a rapid reduction in the levels of resistin in these patients [60]. These observations support a potential role of resistin in the inflammatory cascade in RA.

Adiponectin, another important adipokine, is an especially promising candidate in explaining the link between obesity, metabolism, and systemic inflammation [54, 61]. Low circulating adiponectin concentrations constitute an MS feature and circulating adiponectin has antiinflammatory, antiatherogenic, and antidiabetic properties [62, 63]. In patients with RA undergoing anti-TNF-α therapy due to severe disease high-grade inflammation was independently and negatively correlated with circulating adiponectin concentrations [64]. Low adiponectin concentrations further clustered and correlated with MS features such as dyslipidemia and high plasma glucose that have been reportedly to contribute to the accelerated atherogenesis of patients with RA [64]. These findings may suggest that low circulating adiponectin levels may be implicated in the development of CV disease associated to RA. However, the interaction of high-grade inflammation with low circulating adiponectin concentrations does not likely to be TNF-α mediated in RA [64]. In this regard, no association between adiponectin and carotid intima-media wall thickness, a surrogate marker of CV events in RA [65], was observed in patients with RA [66]. In keeping with these negative results, no association between functional adiponectin—ADIPOQ rs266729 and ADIPOQ rs1501299 polymorphisms—and CV disease was found in patients with RA [67].

Leptin is another important adipokine. This peptide plays an important role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. Leptin is also a proinflammatory adipocyte-derived factor that

operates in the cytokine network by linking immune and inflammatory processes to the neuroendocrine system [68, 69]. Leptin acts as a modulator of T-cell activity and plays a key role in some autoimmune inflammatory diseases such as type 1 diabetes [70]. This adipokine is produced by stimulation of inflammatory cytokines such as TNF-α and interleukin-1. Importantly, leptin exerts many potential atherogenic effects and high leptin concentrations predict incident CV disease in non-RA subjects [71]. Recent studies have shown that high leptin levels may play an important role in the development of CV disease associated to obesity including atherosclerosis. Leptin exerts many atherogenic effects such as induction of endothelial dysfunction, stimulation of inflammatory reaction, oxidative stress, reduction of paraoxonase activity, platelet aggregation, migration, hypertrophy, and proliferation of vascular smooth muscle cells [71].

In patients with RA, circulating leptin levels have been described as either higher or unmodified in comparison to healthy controls [54, 69]. In patients with RA undergoing anti-TNF-α therapy, a correlation between serum leptin levels and VCAM-1 was observed [72]. This is of potential interest as biomarkers of endothelial dysfunction-endothelial cell activation have been found elevated in patients with RA and anti-TNF-α therapy improved endothelial dysfunction [73, 74] and led to a reduction of the levels of some of these endothelial cell activation biomarkers [75]. However, no immediate change in serum leptin levels upon anti-TNF-α-infliximab infusion was observed [72]. These results were in keeping with those obtained by other investigators who found an absence of change plasma leptin concentrations after several weeks' treatment with TNF-α blockers [76, 77].

It is known that MS features are independently associated with atherosclerosis in RA [17, 30]. However, in contrast to what was reported in non-RA subjects, only body mass index but not insulin resistance, blood pressure, or the lipid profile was related to leptin concentrations in patients with severe RA [72].

A potential association between the adipokine visfatin, also called pre-B-cell colony-enhancing factor, and inflammation has been proposed in the last years. Circulating levels of visfatin are correlated with the amount of visceral fat [78], even though visfatin is produced also by endotoxin-stimulated neutrophils. Also, visfatin synthesis is regulated by numerous factors, among other corticosteroids, TNF-α, interleukin-6, and growth hormone [79]. Otero et al. showed higher circulating visfatin levels in patients with RA in comparison to healthy subjects [54]. However, up to now it cannot be excluded the direct effect of different proinflammatory factors in the production of visfatin in patients with RA. Haider et al. [80] demonstrated in young healthy nonobese subjects, that visfatin concentrations are increased by hyperglycemia, and this effect was prevented by coinfusion of insulin or somatostatin. These findings may have high relevance in patients with RA who often present a MS that included insulin resistance, which, in turn, is improved following TNF-α blockade [81]. However, in a study that included RA patients with severe disease undergoing anti-TNF-α-infliximab therapy visfatin levels were not associated with inflammation or MS and infliximab infusion did not yield significant changes in visfatin levels [82].

In summary, an abnormal adipokine profile is often found in patient with RA.

7. Effect of Anti-TNF-α Therapy on Insulin Resistance in Patients with Rheumatoid Arthritis

A disturbed glucose metabolism is included within the constellation of CV risk factors of the MS. In patients with RA insulin resistance is closely related to the presence of a chronic proinflammatory state [41]. In the general population TNF-α production is increased under chronic hyperglycemia and TNF-α has negative effect on insulin sensitivity [83]. TNF-α is also an important mediator of insulin resistance in obesity and diabetes through its ability to decrease the tyrosine kinase activity of the insulin receptor. This pivotal proinflammatory cytokine also impedes insulin-glucose-mediated uptake in the skeletal muscle [84].

In a cross-sectional study using acute phase responses as surrogate markers of systemic inflammation, Dessein et al. disclosed a consistent association with insulin resistance in patients with RA [85]. In their series of patients with RA the impaired insulin sensitivity was significantly associated with both low HDL cholesterol and high triglycerides, which are abnormalities that reflect the lipid component of atherogenic dyslipidemia observed in this chronic inflammatory disease [85]. In a further longitudinal study, the same investigators found that the initiation of DMARDs in combination with pulsed corticosteroids, which were employed only at the onset and as bridge therapy to accelerate the DMARD response, yielded an improvement of insulin sensitivity and a reduction in atherogenic dyslipidemia [86]. Therefore, these results reinforce the claim that high-grade systemic inflammation in RA clearly clustered with insulin resistance and its suppression was associated with an improvement of the MS in which insulin resistance has an important role [86].

In line with the above, several studies have also assessed the effect of the TNF-α antagonists on the mechanisms associated with accelerated atherogenesis in RA, including specifically the effect of these biologic agents on insulin resistance. These studies have been conducted using the most common commercially available TNF-α antagonists that neutralize TNF-α in patients with severe RA refractory to conventional DMARD therapy. The first studies were performed using infliximab, a chimeric IgG1 α monoclonal antibody binding TNF-α. Some others were performed using etanercept, a protein composed of two p75 TNF-α receptors fused to the Fc portion of IgG1. Less frequently, the TNF-α antagonist used to undertake the study was adalimumab, a fully humanized IgG1α monoclonal antibody [87].

We observed an immediate reduction in the serum insulin levels following infliximab infusion in most patients undergoing this biologic therapy because severe RA refractory to DMARD therapy [81]. Moreover, statistically significant reduction in the insulin/glucose index was observed.

Most patients experienced improvement of insulin resistance manifested by a decrease in the Homeostasis Model Assessment of Insulin Resistance (HOMA) index. Moreover, a significant improvement of insulin sensitivity was also observed in most patients [81].

Several studies were also conducted to determine long-term effect of TNF-α antagonists on insulin resistance [87]. These studies assessed the effect of these biologic agents on insulin resistance in patients undergoing TNF-α blocker therapy after several weeks or months of periodical treatment with these drugs. In most cases, insulin resistance was assessed by the HOMA and the Quantitative Insulin Sensitivity Check Index (QUICKI), as previously described [81]. In one of them, Kiortsis et al. showed reduction in HOMA and increase of QUICKI in the subgroup of RA patients with initially had the highest tertile of insulin resistance [88]. In a prospective study of RA patients with active disease, Seriolo et al. observed a significant decrease of the HOMA index and increase of QUICKI in infliximab-treated RA patients for 24 weeks [89]. Similar improvement of HOMA has been shown by other authors [90]. Also, additional evidence using hyperinsulinemic euglycemic clamps in nondiabetic RA patients was observed [91]. A recent study has confirmed that anti-TNF-α therapy improves insulin resistance, beta-cell function, and reverted defects in the insulin signaling cascade in active RA patients with high insulin resistance [92]. In this study that included patients treated with different TNF-α blockers, mainly with infliximab, an improvement of insulin sensitivity was also observed [92].

Interestingly, as previously described [81], the improvement of insulin sensitivity correlated negatively with the bcaseline BMI [91]. With respect to this, a recent report has shown that the improvement of insulin resistance in patients with RA undergoing anti-TNF-α therapy is impaired by the presence of obesity [93]. In this regard, obesity at the onset of treatment with TNF-α blockers leads to a reduction of the beneficial effect of these drugs on MS associated to RA. Therefore, weight-loss and exercise should be considered in the management of RA patients undergoing anti-TNF-α blockers to improve the effect of these drugs on insulin sensitivity [94].

Taking together all these considerations, anti-TNF-α therapy exerts beneficial metabolic effects by the reduction of insulin resistance and improvement of insulin sensitivity.

8. Conclusion

In light of our paper, there seems to be ample evidence supporting a relationship between MS and RA. Understanding how inflammation arising in one tissue affects the physiology and pathology of other organs remains an unanswered question with therapeutic implications for chronic conditions including obesity, diabetes mellitus, atherosclerosis, and RA.

Adipokines may influence the development of atherogenesis in these patients. Biologic therapies that block proinflammatory cytokines seem to have beneficial effects on the insulin resistance observed in patients with RA.

Conflict of Interests

The authors have no conflict of interests to declare.

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References

[1] R. Kahn, J. Buse, E. Ferrannini, and M. Stern, "The metabolic syndrome: time for a critical appraisal—joint statement from the American Diabetes Association and the European Association for the Study of Diabetes," *Diabetes Care*, vol. 28, no. 9, pp. 2289–2304, 2005.

[2] O. Bayturan, E. M. Tuzcu, A. Lavoie et al., "The metabolic syndrome, its component risk factors, and progression of coronary atherosclerosis," *Archives of Internal Medicine*, vol. 170, no. 5, pp. 478–484, 2010.

[3] B. Isomaa, P. Almgren, T. Tuomi et al., "Cardiovascular morbidity and mortality associated with the metabolic syndrome," *Diabetes Care*, vol. 24, no. 4, pp. 683–689, 2001.

[4] D. W. Dunstan, P. Z. Zimmet, T. A. Welborn et al., "The rising prevalence of diabetes and impaired glucose tolerance: the Australian diabetes, obesity and lifestyle study," *Diabetes Care*, vol. 25, no. 5, pp. 829–834, 2002.

[5] M. P. Stern, K. Williams, C. González-Villalpando, K. J. Hunt, and S. M. Haffner, "Does the metabolic-syndrome improve identification of individuals at risk of type 2 diabetes and/or cardiovascular disease?" *Diabetes Care*, vol. 27, no. 11, pp. 2676–2681, 2004.

[6] M. Hamaguchi, T. Kojima, N. Takeda et al., "The metabolic syndrome as a predictor of nonalcoholic fatty liver disease," *Annals of Internal Medicine*, vol. 143, no. 10, pp. 722–I70, 2005.

[7] J. Chen, P. Muntner, L. L. Hamm et al., "The metabolic syndrome and chronic kidney disease in U.S. adults," *Annals of Internal Medicine*, vol. 140, no. 3, pp. 167–I39, 2004.

[8] R. Pasquali, A. Gambineri, B. Anconetani et al., "The natural history of the metabolic syndrome in young women with the polycystic ovary syndrome and the effect of long-term oestrogen-progestagen treatment," *Clinical Endocrinology*, vol. 50, no. 4, pp. 517–527, 1999.

[9] M. S. M. Ip, B. Lam, M. M. T. Ng, W. K. Lam, K. W. T. Tsang, and K. S. L. Lam, "Obstructive sleep apnea is independently associated with insulin resistance," *American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 5, pp. 670–676, 2002.

[10] H. K. Choi, E. S. Ford, C. Li, and G. Curhan, "Prevalence of the metabolic syndrome in patients with gout: the Third National Health and Nutrition Examination Survey," *Arthritis Care and Research*, vol. 57, no. 1, pp. 109–115, 2007.

[11] K. G. M. M. Alberti, P. Zimmet, and J. Shaw, "The metabolic syndrome—a new worldwide definition," *The Lancet*, vol. 366, no. 9491, pp. 1059–1062, 2005.

[12] S. M. Grundy, J. I. Cleeman, S. R. Daniels et al., "Diagnosis and management of the metabolic syndrome: an American Heart

Association/National Heart, Lung, and Blood Institute scientific statement," *Circulation*, vol. 112, no. 17, pp. 2735–2752, 2005.

[13] S. Genuth, K. G. Alberti, and P. Bennett, "Follow-up report on the diagnosis of diabetes mellitus," *Diabetes Care*, vol. 26, no. 11, pp. 3160–3167, 2003.

[14] J. I. Cleeman, "Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III)," *Journal of the American Medical Association*, vol. 285, no. 19, pp. 2486–2497, 2001.

[15] J. B. Meigs, M. K. Rutter, L. M. Sullivan, C. S. Fox, R. B. D'Agostino, and P. W. F. Wilson, "Impact of insulin resistance on risk of type 2 diabetes and cardiovascular disease in people with metabolic syndrome," *Diabetes Care*, vol. 30, no. 5, pp. 1219–1225, 2007.

[16] V. R. Da Cunha, C. V. Brenol, J. C. T. Brenol et al., "Metabolic syndrome prevalence is increased in rheumatoid arthritis patients and is associated with disease activity," *Scandinavian Journal of Rheumatology*, vol. 41, no. 3, pp. 186–191, 2012.

[17] C. P. Chung, A. Oeser, J. F. Solus et al., "Prevalence of the metabolic syndrome is increased in rheumatoid arthritis and is associated with coronary atherosclerosis," *Atherosclerosis*, vol. 196, no. 2, pp. 756–763, 2008.

[18] H. H. Dao, Q. T. Do, and J. Sakamoto, "Increased frequency of metabolic syndrome among Vietnamese women with early rheumatoid arthritis: a cross-sectional study," *Arthritis Research and Therapy*, vol. 12, no. 6, article R218, 2010.

[19] C. S. Crowson, E. Myasoedova, J. M. Davis et al., "Increased prevalence of metabolic syndrome associated with rheumatoid arthritis in patients without clinical cardiovascular disease," *Journal of Rheumatology*, vol. 38, no. 1, pp. 29–35, 2011.

[20] T. E. Toms, V. F. Panoulas, H. John, K. M. Douglas, and G. D. Kitas, "Methotrexate therapy associates with reduced prevalence of the metabolic syndrome in rheumatoid arthritis patients over the age of 60- more than just an anti-inflammatory effect? A cross sectional study," *Arthritis Research & Therapy*, vol. 11, no. 4, p. R110, 2009.

[21] C. C. Mok, G. T. C. Ko, L. Y. Ho, K. L. Yu, P. T. Chan, and C. H. To, "Prevalence of atherosclerotic risk factors and the metabolic syndrome in patients with chronic inflammatory arthritis," *Arthritis Care and Research*, vol. 63, no. 2, pp. 195–202, 2011.

[22] A. Zonana-Nacach, E. Santana-Sahagún, F. J. Jiménez-Balderas, and A. Camargo-Coronel, "Prevalence and factors associated with metabolic syndrome in patients with rheumatoid arthritis and systemic lupus erythematosus," *Journal of Clinical Rheumatology*, vol. 14, no. 2, pp. 74–77, 2008.

[23] M. Karimi, S. Mazloomzadeh, S. Kafan, and H. Amirmoghadami, "The frequency of metabolic syndrome in women with rheumatoid arthritis and in controls," *International Journal of Rheumatic Diseases*, vol. 14, no. 3, pp. 248–254, 2011.

[24] M. Sahebari, L. Goshayeshi, Z. Mirfeizi et al., "Investigation of the association between metabolic syndrome and disease activity in rheumatoid arthritis," *TheScientificWorldJournal*, vol. 11, pp. 1195–1205, 2011.

[25] S. A. Karvounaris, P. I. Sidiropoulos, J. A. Papadakis et al., "Metabolic syndrome is common among middle-to-older aged Mediterranean patients with rheumatoid arthritis and correlates with disease activity: a retrospective, cross-sectional, controlled, study," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 28–33, 2007.

[26] C. Meune, E. Touzé, L. Trinquart, and Y. Allanore, "Trends in cardiovascular mortality in patients with rheumatoid arthritis over 50 years: a systematic review and meta-analysis of cohort studies," *Rheumatology*, vol. 48, no. 10, pp. 1309–1313, 2009.

[27] J. A. Aviña-Zubieta, H. K. Choi, M. Sadatsafavi, M. Etminan, J. M. Esdaile, and D. Lacaille, "Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies," *Arthritis Care and Research*, vol. 59, no. 12, pp. 1690–1697, 2008.

[28] L. Cavagna, N. Boffini, G. Cagnotto, F. Inverardi, V. Grosso, and R. Caporali, "Atherosclerosis and rheumatoid arthritis: more than a simple association," *Mediators of Inflammation*, vol. 2012, Article ID 147354, 8 pages, 2012.

[29] T. E. Toms, V. F. Panoulas, K. M. J. Douglas, H. R. Griffiths, and G. D. Kitas, "Lack of association between glucocorticoid use and presence of the metabolic syndrome in patients with rheumatoid arthritis: a cross-sectional study," *Arthritis Research and Therapy*, vol. 10, no. 6, article R145, 2008.

[30] P. H. Dessein, M. Tobias, and M. G. Veller, "Metabolic syndrome and subclinical atherosclerosis in rheumatoid arthritis," *Journal of Rheumatology*, vol. 33, no. 12, pp. 2425–2432, 2006.

[31] J. F. Baker, N. N. Mehta, D. G. Baker et al., "Vitamin D, metabolic dyslipidemia, and metabolic syndrome in rheumatoid arthritis," *American Journal of Medicine*, vol. 125, no. 10, pp. 1035.e09–1036.e15, 2012.

[32] L. Goshayeshi, H. Saber, M. Sahebari et al., "Association between metabolic syndrome, BMI, and serum vitamin D concentrations in rheumatoid arthritis," *Clinical Rheumatology*, vol. 31, no. 8, pp. 1197–1203, 2012.

[33] G. R. Romeo, J. Lee, and S. E. Shoelson, "Metabolic syndrome, insulin resistance, and roles of inflammation- mechanisms and therapeutic targets," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 8, pp. 1771–1776, 2012.

[34] A. Stavropoulos-Kalinoglou, G. S. Metsios, Y. Koutedakis, and G. D. Kitas, "Obesity in rheumatoid arthritis," *Rheumatology*, vol. 50, no. 3, Article ID keq266, pp. 450–462, 2011.

[35] A. C. Elkan, N. Håkansson, J. Frostegård, T. Cederholm, and I. Hafström, "Rheumatoid cachexia is associated with dyslipidemia and low levels of atheroprotective natural antibodies against phosphorylcholine but not with dietary fat in patients with rheumatoid arthritis: a cross-sectional study," *Arthritis Research and Therapy*, vol. 11, no. 2, article R37, 2009.

[36] R. Roubenoff, "Rheumatoid cachexia: a complication of rheumatoid arthritis moves into the 21st century," *Arthritis Research and Therapy*, vol. 11, no. 2, article 108, 2009.

[37] J. T. Giles, M. Allison, R. S. Blumenthal et al., "Abdominal adiposity in rheumatoid arthritis: association with cardiometabolic risk factors and disease characteristics," *Arthritis and Rheumatism*, vol. 62, no. 11, pp. 3173–3182, 2010.

[38] C. S. Derdemezis, P. V. Voulgari, and A. A. Drosos, "Obesity, adipose tissue and rheumatoid arthritis: coincidence or more complex relationship?" *Clinical and Experimental Rheumatology*, vol. 29, no. 4, pp. 712–727, 2011.

[39] J. R. Cerhan, K. G. Saag, L. A. Criswell, L. A. Merlino, and T. R. Mikuls, "Blood transfusion, alcohol use, and anthropometric risk factors for rheumatoid arthritis in older women," *Journal of Rheumatology*, vol. 29, no. 2, pp. 246–254, 2002.

[40] T. Bartfai, J. Waalen, and J. N. Buxbaum, "Adipose tissue as a modulator of clinical inflammation: does obesity reduce the prevalence of rheumatoid arthritis?" *Journal of Rheumatology*, vol. 34, no. 3, pp. 488–492, 2007.

[41] L. A. Rodriguez, L. B. Tolosa, A. Ruigmez, S. Johansson, and M. A. Wallander, "Rheumatoid arthritis in UK primary care incidence and prior morbidity," *Scandinavian Journal of Rheumatology*, vol. 38, no. 3, pp. 173–177, 2009.

[42] A. H. M. Van Der Helm-van Mil, S. M. Van Der Kooij, C. F. Allaart, R. E. M. Toes, and T. W. J. Huizinga, "A high body mass index has a protective effect on the amount of joint destruction in small joints in early rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 67, no. 6, pp. 769–774, 2008.

[43] I. Ferraz Amaro, F. Díaz González, C. González Juanatey, and M. A. González Gay, "Insulin resistance and rheumatoid arthritis," *Reumatologia Clinica*, vol. 7, no. 2, pp. 124–129, 2011.

[44] P. H. Dessein and B. I. Joffe, "Insulin resistance and impaired beta cell function in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 9, pp. 2765–2775, 2006.

[45] C. P. Chung, A. Oeser, J. F. Solus et al., "Inflammation-associated insulin resistance: differential effects in rheumatoid arthritis and systemic lupus erythematosus define potential mechanisms," *Arthritis and Rheumatism*, vol. 58, no. 7, pp. 2105–2112, 2008.

[46] G. La Montagna, F. Cacciapuoti, R. Buono et al., "Insulin resistance is an independent risk factor for atherosclerosis in rheumatoid arthritis," *Diabetes and Vascular Disease Research*, vol. 4, no. 2, pp. 130–135, 2007.

[47] P. H. Dessein, G. R. Norton, A. J. Woodiwiss, B. I. Joffe, and A. Solomon, "Independent role of conventional cardiovascular risk factors as predictors of c-reactive protein concentrations in rheumatoid arthritis," *Journal of Rheumatology*, vol. 34, no. 4, pp. 681–688, 2007.

[48] O. N. Pamuk, E. Unlu, and N. Cakir, "Role of insulin resistance in increased frequency of atherosclerosis detected by carotid ultrasonography in rheumatoid arthritis," *Journal of Rheumatology*, vol. 33, no. 12, pp. 2447–2452, 2006.

[49] P. H. Dessein, B. I. Joffe, A. Stanwix, A. S. Botha, and Z. Moomal, "The acute phase response does not fully predict the presence of insulin resistance and dyslipidemia in inflammatory arthritis," *Journal of Rheumatology*, vol. 29, no. 3, pp. 462–466, 2002.

[50] P. Trayhurn and I. S. Wood, "Adipokines: inflammation and the pleiotropic role of white adipose tissue," *British Journal of Nutrition*, vol. 92, no. 3, pp. 347–355, 2004.

[51] F. Lago, C. Dieguez, J. Gómez-Reino, and O. Gualillo, "Adipokines as emerging mediators of immune response and inflammation," *Nature Clinical Practice Rheumatology*, vol. 3, no. 12, pp. 716–724, 2007.

[52] R. Gómez, J. Conde, M. Scotece, J. J. Gómez-Reino, F. Lago, and O. Gualillo, "What's new in our understanding of the role of adipokines in rheumatic diseases?" *Nature Reviews Rheumatology*, vol. 7, no. 9, pp. 528–536, 2011.

[53] O. Gualillo, J. R. González-Juanatey, and F. Lago, "The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives," *Trends in Cardiovascular Medicine*, vol. 17, no. 8, pp. 275–283, 2007.

[54] M. Otero, R. Logo, R. Gomez et al., "Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 9, pp. 1198–1201, 2006.

[55] M. Scotece, J. Conde, R. Gómez et al., "Role of adipokines in atherosclerosis: interferences with cardiovascular complications in rheumatic diseases," *Mediators of Inflammation*, vol. 2012, Article ID 125458, 14 pages, 2012.

[56] L. Patel, A. C. Buckels, I. J. Kinghorn et al., "Resistin is expressed in human macrophages and directly regulated by PPARγ activators," *Biochemical and Biophysical Research Communications*, vol. 300, no. 2, pp. 472–476, 2003.

[57] S. Kaser, A. Kaser, A. Sandhofer, C. F. Ebenbichler, H. Tilg, and J. R. Patsch, "Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro," *Biochemical and Biophysical Research Communications*, vol. 309, no. 2, pp. 286–290, 2003.

[58] A. Schäffler, A. Ehling, E. Neumann et al., "Adipocytokines in synovial fluid," *Journal of the American Medical Association*, vol. 290, no. 13, pp. 1709–1710, 2003.

[59] R. Gomez, F. Lago, J. Gomez-Reino, C. Dieguez, and O. Gualillo, "Adipokines in the skeleton: influence on cartilage function and joint degenerative diseases," *Journal of Molecular Endocrinology*, vol. 43, no. 1, pp. 11–18, 2009.

[60] M. A. Gonzalez-Gay, M. T. Garcia-Unzueta, C. Gonzalez-Juanatey et al., "Anti-TNF-α therapy modulates resistin in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 26, no. 2, pp. 311–316, 2008.

[61] J. Conde, M. Scotece, R. Gómez et al., "Adipokines: biofactors from white adipose tissue. A complex hub among inflammation, metabolism, and immunity," *BioFactors*, vol. 37, no. 6, pp. 413–420, 2011.

[62] N. Ouchi, S. Kihara, Y. Arita et al., "Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin," *Circulation*, vol. 100, no. 25, pp. 2473–2476, 1999.

[63] M. Ryo, T. Nakamura, S. Kihara et al., "Adiponectin as a biomarker of the metabolic syndrome," *Circulation Journal*, vol. 68, no. 11, pp. 975–981, 2004.

[64] M. A. Gonzalez-Gay, J. Llorca, M. T. Garcia-Unzueta et al., "High-grade inflammation, circulating adiponectin concentrations and cardiovascular risk factors in severe rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 26, no. 4, pp. 596–603, 2008.

[65] C. Gonzalez-Juanatey, J. Llorca, J. Martin, and M. A. Gonzalez-Gay, "Carotid intima-media thickness predicts the development of cardiovascular events in patients with rheumatoid arthritis," *Seminars in Arthritis and Rheumatism*, vol. 38, no. 5, pp. 366–371, 2009.

[66] M. A. Gonzalez-Gay, C. Gonzalez-Juanatey, L. Rodriguez-Rodriguez, J. A. Miranda-Filloy, J. Martin, and J. Llorca, "Lack of association between adipokines and ghrelin and carotid intima-media thickness in patients with severe rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 29, no. 2, pp. 358–359, 2011.

[67] L. Rodríguez-Rodríguez, M. García-Bermúdez, C. González-Juanatey et al., "Lack of association between ADIPOQ rs266729 and ADIPOQ rs1501299 polymorphisms and cardiovascular disease in rheumatoid arthritis patients," *Tissue Antigens*, vol. 77, no. 1, pp. 74–78, 2011.

[68] H. Tilg and A. R. Moschen, "Adipocytokines: mediators linking adipose tissue, inflammation and immunity," *Nature Reviews Immunology*, vol. 6, no. 10, pp. 772–783, 2006.

[69] M. Scotece, J. Conde, R. Gómez et al., "Beyond fat mass: exploring the role of adipokines in rheumatic diseases," *TheScientificWorldJournal*, vol. 11, pp. 1932–1947, 2011.

[70] M. Otero, R. Lago, R. Gómez, F. Lago, J. J. Gómez-Reino, and O. Gualillo, "Leptin: a metabolic hormone that functions like a proinflammatory adipokine," *Drug News and Perspectives*, vol. 19, no. 1, pp. 21–26, 2006.

[71] J. Beltowski, "Leptin and atherosclerosis," *Atherosclerosis*, vol. 189, no. 1, pp. 47–60, 2006.

[72] M. A. Gonzalez-Gay, M. T. Garcia-Unzueta, A. Berja et al., "Anti-TNF-α therapy does not modulate leptin in patients with severe rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 27, no. 2, pp. 222–228, 2009.

[73] C. Gonzalez-Juanatey, J. Llorca, A. Sanchez Andrade, C. Garcia-Porrua, J. Martin, and M. A. Gonzalez-Gay, "Short-term adalimumab therapy improves endothelial function in patients with rheumatoid arthritis refractory to infliximab," *Clinical and Experimental Rheumatology*, vol. 24, no. 3, pp. 309–312, 2006.

[74] C. Gonzalez-Juanatey, T. R. Vazquez-Rodriguez, I. Gomez-Acebo et al., "Anti-TNF-alpha-adalimumab therapy is associated with persistent improvement of endothelial function without progression of carotid intima-media wall thickness in patients with rheumatoid arthritis refractory to conventional therapy," *Mediators of Inflammation*, vol. 2012, Article ID 674265, 8 pages, 2012.

[75] M. A. Gonzalez-Gay, M. T. Garcia-Unzueta, J. M. De Matias et al., "Influence of anti-TNF-α infliximab therapy on adhesion molecules associated with atherogenesis in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 24, no. 4, pp. 373–379, 2006.

[76] C. Popa, M. G. Netea, T. R. D. S. Radstake, P. L. Van Riel, P. Barrera, and J. W. M. Van Der Meer, "Markers of inflammation are negatively correlated with serum leptin in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 64, no. 8, pp. 1195–1198, 2005.

[77] P. Härle, P. Sarzi-Puttini, M. Cutolo, and R. H. Straub, "No change of serum levels of leptin and adiponectin during anti-tumour necrosis factor antibody treatment with adalimumab in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 7, pp. 970–971, 2006.

[78] A. Fukuhara, M. Matsuda, M. Nishizawa et al., "Visfatin: a protein secreted by visceral fat that Mimics the effects of insulin," *Science*, vol. 307, no. 5708, pp. 426–430, 2005.

[79] J. Berndt, N. Klöting, S. Kralisch et al., "Plasma visfatin concentrations and fat depot-specific mRNA expression in humans," *Diabetes*, vol. 54, no. 10, pp. 2911–2916, 2005.

[80] D. G. Haider, G. Schaller, S. Kapiotis, C. Maier, A. Luger, and M. Wolzt, "The release of the adipocytokine visfatin is regulated by glucose and insulin," *Diabetologia*, vol. 49, no. 8, pp. 1909–1914, 2006.

[81] M. A. Gonzalez-Gay, J. M. De Matias, C. Gonzalez-Juanatey et al., "Anti-tumor necrosis factor-α blockade improves insulin resistance in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 24, no. 1, pp. 83–86, 2006.

[82] M. A. Gonzalez-Gay, T. R. Vazquez-Rodriguez, M. T. Garcia-Unzueta et al., "Visfatin is not associated with inflammation or metabolic syndrome in patients with severe rheumatoid arthritis undergoing anti-TNF-α therapy," *Clinical and Experimental Rheumatology*, vol. 28, no. 1, pp. 56–62, 2010.

[83] M. Fukuzawa, J. Satoh, X. Qiang et al., "Inhibition of tumor necrosis factor-α with anti-diabetic agents," *Diabetes Research and Clinical Practice*, vol. 43, no. 3, pp. 147–154, 1999.

[84] G. S. Hotamisligil, P. Peraldi, A. Budavari, R. Ellis, M. F. White, and B. M. Spiegelman, "IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α- and obesity-induced insulin resistance," *Science*, vol. 271, no. 5249, pp. 665–668, 1996.

[85] P. H. Dessein, A. E. Stanwix, and B. I. Joffe, "Cardiovascular risk in rheumatoid arthritis versus osteoarthritis: acute phase response related decreased insulin sensitivity and high-density lipoprotein cholesterol as well as clustering of metabolic syndrome features in rheumatoid arthritis," *Arthritis Research*, vol. 4, no. 5, p. R5, 2002.

[86] P. H. Dessein, B. I. Joffe, and A. E. Stanwix, "Effects of disease modifying agents and dietary intervention on insulin resistance and dyslipidemia in inflammatory arthritis: a pilot study," *Arthritis Research*, vol. 4, no. 6, p. R12, 2002.

[87] M. A. Gonzalez-Gay, C. Gonzalez-Juanatey, T. R. Vazquez-Rodriguez, J. A. Miranda-Filloy, and J. Llorca, "Insulin resistance in rheumatoid arthritis: the impact of the anti-TNF-α therapy: annals of the New York Academy of Sciences," *Annals of the New York Academy of Sciences*, vol. 1193, pp. 153–159, 2010.

[88] D. N. Kiortsis, A. K. Mavridis, S. Vasakos, S. N. Nikas, and A. A. Drosos, "Effects of infliximab treatment on insulin resistance in patients with rheumatoid arthritis and ankylosing spondylitis," *Annals of the Rheumatic Diseases*, vol. 64, no. 5, pp. 765–766, 2005.

[89] B. Seriolo, S. Paolino, C. Ferrone, and M. Cutolo, "Impact of long-term anti-TNF-α treatment on insulin resistance in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 26, no. 1, p. 159, 2008.

[90] L. S. Tam, B. Tomlinson, T. T. Chu, T. K. Li, and E. K. Li, "Impact of TNF inhibition on insulin resistance and lipids levels in patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 26, no. 9, pp. 1495–1498, 2007.

[91] F. C. Huvers, C. Popa, M. G. Netea, F. H. J. Van Den Hoogen, and C. J. Tack, "Improved insulin sensitivity by anti-TNFα antibody treatment in patients with rheumatic diseases," *Annals of the Rheumatic Diseases*, vol. 66, no. 4, pp. 558–559, 2007.

[92] I. Stagakis, G. Bertsias, and S. Karvounaris, "Anti-tumor necrosis factor therapy improves insulin resistance, beta cell function and insulin signaling in active rheumatoid arthritis patients with high insulin resistance," *Arthritis Research and Therapy*, vol. 14, no. 3, p. R141, 2012.

[93] A. Stavropoulos-Kalinoglou, G. S. Metsios, and V. F. Panoulas, "Anti-tumour necrosis factor alpha therapy improves insulin sensitivity in normal-weight but not in obese patients with rheumatoid arthritis," *Arthritis Research & Therapy*, vol. 14, no. 4, p. R160, 2012.

[94] M. A. González-Gay and C. González-Juanatey, "Rheumatoid arthritis: obesity impairs efficacy of anti-TNF therapy in patients with RA," *Nature Reviews Rheumatology*, vol. 8, no. 11, pp. 641–642, 2012.

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Research Article

An Anti-Inflammatory Sterol Decreases Obesity-Related Inflammation-Induced Insulin Resistance and Metabolic Dysregulation

Chris L. Reading, Jaime Flores-Riveros, Dwight R. Stickney, and James M. Frincke

Harbor Therapeutics, Inc., San Diego, CA 92122, USA

Correspondence should be addressed to Chris L. Reading; creading@harbortx.com

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Obesity-related inflammation-induced insulin resistance and metabolic dysregulation were investigated in retrospective analysis of placebo hematologic and metabolic laboratory data from trials associated with increasing chronic low-grade inflammation and body mass index. Studies included healthy subjects and those with progressive stages of metabolic dysregulation, including type 2 diabetes mellitus with uncontrolled hemoglobin $A1_c$. Intrasubject variances in erythroid and metabolic values increased with metabolic dysregulation. Random effects were demonstrated in treatment-naïve diabetes for erythroid, glucose, and HbA1c fluctuations. The anti-inflammatory insulin sensitizer, HE3286, was tested for its ability to decrease obesity-related inflammation-induced insulin resistance and metabolic dysregulation in diabetes. HE3286 significantly decreased erythroid and metabolic variances and improved 1,5-anhydroglucitol (a surrogate of postprandial glucose) compared to the placebo group. HE3286 HbA1c decrease correlated with weight loss and inversely with baseline monocyte chemoattractant protein-1 (MCP-1) in metformin-treated diabetics. Normalization of HbA1c to the 84-day average hemoglobin revealed that HE3286 HbA1c decrease correlated with high baseline MCP-1 and MCP-1 decrease in treatment-naïve diabetics. HE3286 decreased insulin resistance, increased the frequency of decreased day 84 HbA1c in metformin-treated subjects, and decreased day 112 HbA1c in treatment-naïve diabetics. HE3286 may be useful to restore metabolic homeostasis in type 2 diabetes.

1. Introduction and Purpose

HE3286 (17α-ethynylandrost-5-ene-3β,7β,17β-triol) is a chemical derivative of the natural mammalian sterol androst-5-ene-3β,7β,17β-triol (βAET). βAET exhibits anti-inflammatory activity in rodent models, is elevated in plasma of obese subjects with normal glucose disposal, and may play a compensatory role in preventing development of metabolic syndrome (reviewed in [1]). βAET is pharmaceutically unsuitable, due to poor oral bioavailability and its propensity for oxidative inactivation by 17β-hydroxysteroid dehydrogenase [1]. HE3286 is stabilized against oxidation at position 17 and consequently orally bioavailable, does not bind to any known nuclear steroid hormone receptors, and is pharmacologically unrelated to androgens, estrogens, corticosteroids, or peroxisome proliferators [1]. HE3286 has shown broad anti-inflammatory activity in animal models of rheumatoid arthritis, ulcerative colitis, multiple sclerosis, lung inflammation, autoimmune type 1 diabetes, and neuroinflammation (reviewed in [1]). In these models, nuclear factor kappa B (NFκB) activation and proinflammatory cytokine production were consistently suppressed. Furthermore, HE3286 was not markedly immunosuppressive in rodent models of ovalbumin immunization, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* infection, Coxsackievirus B3 myocarditis, delayed-type hypersensitivity, and mitogen-induced proliferation, or in the human mixed lymphocyte reaction assay (reviewed in [1]).

Obesity induces an insulin-resistant state in adipose tissue [2], liver, and muscle and is a strong risk factor for the development of type 2 diabetes mellitus [3]. In adipose tissue, MCP-1 and tumor necrosis factor alpha (TNFα) play

dominant proinflammatory roles [2]. Adiposity-induced inflammation-stimulated kinases phosphorylate insulin receptor substrate-1 on serine residues and inhibit insulin signaling [4]. Two recent publications report the activity of HE3286 against *in vitro* inflammatory responses and *in vivo* rodent models of obesity-induced inflammation and insulin resistance [5, 6]. HE3286 suppressed endotoxin-induced NFκB activation, reporter gene expression, nuclear localization, and p65 phosphorylation in mouse macrophages and decreased phosphorylation of the proinflammatory extracellular signal-regulated (Erk1/2), IkappaB (Ikk), Jun N-terminal (Jnk), and p38 mitogen-activated protein (p38 Mapk) kinases. HE3286 also attenuated TNFα-stimulated inflammation and TNFα-induced adipocyte-stimulated macrophage chemotaxis [5, 6]. HE3286 treatment of diabetic *db*/*db* mice, insulin-resistant diet-induced obese mice, and genetically obese *ob*/*ob* mice suppressed progression to hyperglycemia and markedly improved glucose clearance. This effect appeared to be consequent to reduced insulin resistance, since HE3286 lowered blood insulin levels in both *db*/*db* and *ob*/*ob* mice. In these studies HE3286 suppressed levels of the chemokine monocyte chemoattractant protein-1 (MCP-1), along with its cognate receptor, C-C motif chemokine receptor-2, in white adipose tissue [6]. In Zucker diabetic fatty rats, HE3286 downregulated inflammatory cytokine and chemokine expression in both liver and adipose tissues and suppressed macrophage migration into adipose tissue. HE3286 normalized fasting and fed glucose levels, improved glucose tolerance, and enhanced skeletal muscle and liver insulin sensitivity, as assessed by hyperinsulinemic, euglycemic clamp studies. In addition, HE3286 reduced liver cholesterol and triglyceride content, leading to a feedback elevation of low-density lipoprotein (LDL) receptor and decreased total serum cholesterol [5]. Recently, we have reported that HE3286 binds to Erk1/2, Lrp1, and Sirt2 [7] and proposed that the HE3286-mediated decrease in hyperactivation of Erk1/2 may be causal for its metabolic [8] and anti-inflammatory activities.

In a clinical study in obese, impaired glucose tolerance (IGT) subjects, HE3286 significantly increased the frequency of insulin-resistant subjects with improved day 29 insulin-stimulated glucose disposal, increased HDL cholesterol, and decreased day 28 CRP compared to placebo-treated subjects [9]. Based on baseline glucose clamp studies, insulin-resistant subjects had elevated inflammatory biomarkers, with lower adiponectin and higher cytokine secretion in LPS-stimulated PBMC. After 28 days of HE3286 treatment, adiponectin levels increased significantly in insulin-resistant subjects, compared to placebo. These results support our hypothesis that obesity-induced inflammation is a significant contributor to metabolic dysregulation and that the anti-inflammatory activity of HE3286 can preferentially benefit the insulin-resistant inflamed subpopulation of obese IGT subjects.

Based on preclinical studies and these foregoing results in IGT subjects, it was conjected that HE3286 might benefit obese inflamed insulin-resistant individuals with type 2 diabetes mellitus (T2DM). A widely accepted clinical endpoint for T2DM is the change in HbA1c, a surrogate marker for the extent of hyperglycemia an individual experiences over time. Traditionally, erythroid hematology values are considered stable in healthy individuals, and hemoglobin and HbA1c turnover is reported to reflect the normal red cell half-life of 38–60 days [10]. In T2DM, the life span of red cells can be altered significantly by inflammation, particularly TNFα-induced oxidative stress [11], obese low-grade systemic inflammatory response syndrome [12], the presence of elevated levels of advanced glycation endproducts on the surface of red cells [13, 14], hypoxia [15], and excessive erythrocytosis [16]. There are reports of large fluctuations in HbA1c in type 1 diabetes [17], especially in subjects with poor glycemic control [18, 19]. This information prompted us to also assess the association of obesity-related chronic low-grade inflammation with hemoglobin concentration and HbA1c variability in uncontrolled T2DM. We retrospectively analyzed the hematologic and metabolic clinical laboratory data for placebo groups from 10 clinical studies that were conducted between 2001 and 2010. These studies included both healthy subjects and individuals in progressive stages of metabolic disease that presented with increased chronic low-grade inflammation coincident with elevated BMI that included dyslipidemic, IGT, and T2DM participants with uncontrolled HbA1c.

With an understanding of the variability associated with progressive adiposity, inflammation, and metabolic disease, we assessed the activity of HE3286 to decrease obesity-induced inflammation and insulin resistance in T2DM.

2. Subjects and Methods

2.1. Studies. This paper reports the activity of HE3286 in T2DM patients with uncontrolled HbA1c. High metabolic and hematologic laboratory value variances were observed in these patients. For comparison, similar parameters were retrospectively analyzed from placebo subjects enrolled in 10 clinical studies conducted by Harbor Therapeutics, Inc. (formerly Hollis-Eden Pharmaceuticals, Inc.) since 2001. These studies included healthy volunteers, dyslipidemic, IGT, and T2DM participants. Only placebo subjects from these studies were used for intercomparison. All studies excluded patients with known liver disease and alcoholism. The protocols and all amendments were reviewed and approved by the relevant institutional review boards, and all studies were conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization/WHO Good Clinical Practice Standards. Experimental studies were conducted with the understanding and informed consent of human subjects.

Details of studies 2100-200, -201, -202, and -203 have been published [20]. These four double-blind, randomized, placebo-controlled, healthy human safety studies were conducted in The Netherlands (Kendle International, Utrecht) and the United States (Parexel International, Baltimore, MD). Two single-dose, dose-escalation studies assured safety and evaluated the pharmacokinetics of androst-5-ene-3β,17β-diol (HE2100) (studies 200 and 202). A multidose, dose escalation study was performed to assess safety and pharmacokinetics and potential early activity of HE2100 (study

201). Early activity, defined by effects on peripheral blood elements, was confirmed by a follow-up study that included elderly subjects and an initial study of bone marrow hematology (study 203).

Details of studies 2200-100, -101, -120, and -130 have also been published [21]. Healthy adult and elderly subjects were randomized to receive three consecutive daily subcutaneous injections of placebo, 50, or 100 mg androst-5-ene-3β,7β,17β-triol (HE2200), followed by 2 months of periodic observation (trial 2200-100), or to receive placebo, 25, or 100 mg HE2200 transmucosally (buccal administration) once daily for five days followed by 2 months of periodic observation (trial 2200-101). Study 2200-120 was a phase II study in healthy hepatitis B-naïve, and elderly (65–85 years old) volunteers, who received hepatitis B vaccine, were randomized to concomitantly receive either 100 mg of HE2200 or placebo equivalent. Subjects received three subcutaneous injections of study drug or placebo prior to the first and second doses of hepatitis B vaccine given 28 days apart. The third dose of vaccine was given at 6 months without HE2200 or placebo treatment, and the study terminated 28 days later.

Study 2200-130 was a phase II study in dyslipidemic subjects, ages 18–70 years, with plasma triglyceride concentrations 1.7–2 mmol/L, total cholesterol levels of 5.7–8.3 mmol/L, and HDL levels of ≤1.2 mmol/L for males and ≤1.4 mmol/L for females. After informed consent was obtained, subjects initiated a Step II AHA diet and discontinued all lipid lowering agents for a six-week run-in period. Each subject's lipid profile at week four of the diet was used to determine eligibility for the study. At six weeks, qualified subjects were randomized to receive 25 or 100 mg of HE2200 or placebo equivalent by buccal administration for 28 days.

HE3286-0102 was a multicenter, double-blind, dose-ranging phase I study designed with 5 cohorts of obese, impaired glucose tolerance (IGT), but otherwise healthy participants [9]. Subjects were screened for fasting blood glucose level of <7.0 mmol/L and a 7.8–11.1 mmol/L 2-hour postprandial glucose following a 75-gram oral glucose load. Oral placebo or HE3286 doses of 4 (2 BID), 5 (QD), 10 (5 BID), and 20 (10 BID) mg were administered daily for 28 days. One-step hyperinsulinemic, euglycemic clamps were performed on the day before the first dose and day 29 in the BID dose groups.

For comparisons, the number of placebo subjects was 70 healthy, 24 dyslipidemic, 13 IGT, 28 treatment-naïve, and 38 metformin-treated T2DM.

2.2. Placebo Data Analysis. Placebo data from 10 clinical studies was analyzed to obtain coefficients of variation (CV) for red blood cells (RBC), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (Hb), hemoglobin A1c (HbA1c), platelets, lymphocytes, monocytes, white blood cells, fasting glucose, insulin, triglycerides, cholesterol, high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL). CVs from each medical condition were compared to those of healthy volunteers for significant differences in magnitude using Welch ANOVA, allowing for unequal differences, and for significant differences in dispersion, using the 2-sided F test. Only placebo values were used to avoid any treatment effects from drug administrations. Pearson correlations between individual subject CVs for each parameter and CVs for HbA1c and for insulin were determined to assess commonality in increased variances between parameters. In addition, intravisit changes in HbA1c were determined for each subject with HbA1c data.

2.3. HE3286-0401 Data Analysis. Data for all placebo- and HE3286-treated T2DM subjects were analyzed for the same parameters by cohort (metformin treated and treatment naïve) and in subgroups (stratified by baseline monocyte chemoattractant protein-1 (MCP-1) in metformin-treated subjects, and by body mass index (BMI) in the MCP-1 selected treatment-naïve subjects). Baseline characteristics were assessed for balance between groups.

Based on the findings of excessive variability in T2DM, including random effects in intravisit Hb, HbA1c values were normalized (nHbA1c) by the 84 day average Hb value for each subject. This was done based on the fact that random effects have a mean of zero, but an excessive dispersion. Thus the average change in placebo subjects over 84 days would be expected to be zero, and true changes in HbA1c due to treatment could be compared.

Correlations were determined for independent variables of baseline MCP-1, baseline tumor necrosis factor alpha (TNFα), BMI, Hb CV, and day 84 changes in homeostatic model assessment of beta cell function (HOMA2 %B), glucose, weight, Hb, TNFα, and MCP-1 with dependent variables Hb CV, HbA1c CV, and changes in HbA1c and 84 day average normalized HbA1c (ΔnHbA1c), in order to understand clinical parameters affecting variability and treatment effects. The magnitude of HE3286 treatment effects compared to placebo was tested in the subgroups of MCP-1 > 40 pg/mL in metformin-treated subjects, and of BMI > 31 in treatment-naïve subjects.

Heteroscedasticity (differences in variances between subgroups) was tested for changes in insulin, C-peptide, fasting glucose, HOMA2 %B and HOMA2 insulin resistance (HOMA2 IR), leptin, HbA1c, insulin, MCP-1, and triglycerides. Subgroup distributions were tested for normality (Shapiro-Wilks W test) for HE3286 and placebo treatment. Differences in dispersions between HE3286 and placebo treatment were analyzed using the 2-sided F test.

2.4. Test Article HE3286. HE3286, 17α-ethynylandrost-5-ene-3β,7β,17β-triol active pharmaceutical ingredient was manufactured by Norac, Azuza, CA, and formulated and filled in gelatin capsules by Eminent Services Corporation, Frederick, MD. All manufacturing procedures were performed according to current good manufacturing practices.

2.5. Study HE32866-0401. The phase II trial design was a double-blind, randomized, placebo-controlled parallel group study of the safety, tolerance, and activity of HE3286 when

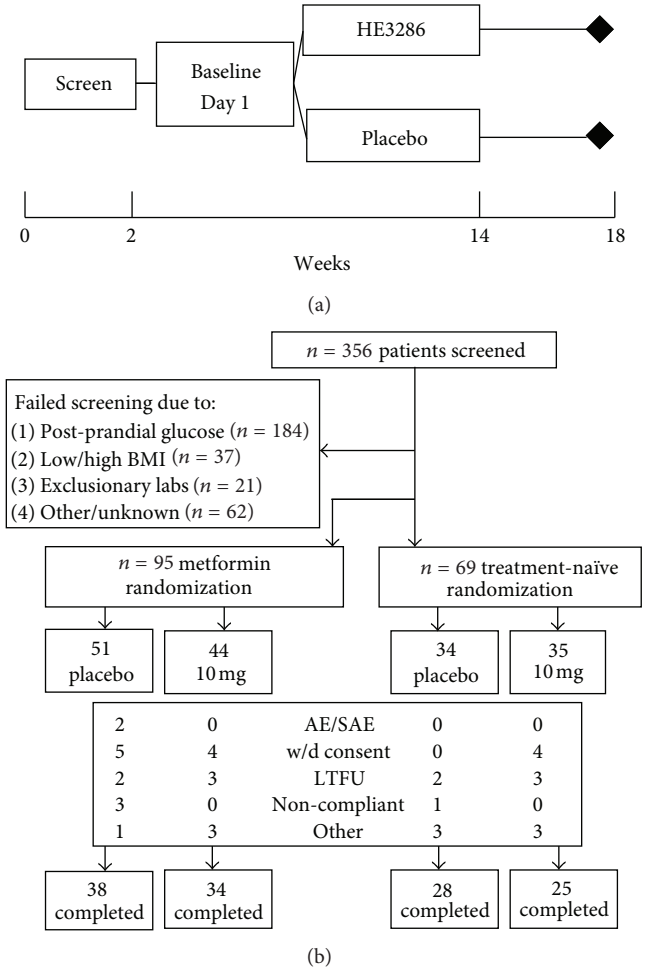

Figure 1: Study HE3286-0401 in type 2 diabetic subjects. HE3286-0401 study design (a) and study flow and numbers (b) for each cohort. BMI: body mass index, w/d: withdrew, and LTFU: lost to followup.

administered orally for 12 weeks to adult T2DM patients (Figure 1(a)). This was an adaptive design to investigate the characteristics of T2DM subjects that respond to HE3286. In cohort 1 of the study, 95 eligible patients, who consented to participate, were randomized 1 : 1 to receive study treatment (HE3286 10 mg/day or placebo) in addition to a stable dose of metformin. Inclusion criteria for cohort 1 included HbA1c $\geq$ 7.5% and fasting glucose $\leq$ 12.5 mmol/L. In cohort 2, 69 subjects who consented to participate and who met a revised eligibility criteria as determined by cohort 1 were randomized 1 : 1 to receive study treatment (HE3286 10 mg/day or placebo) as monotherapy. After the analysis of data from the first stage of the study, the population for cohort 2 was phenotypically enriched by screening for the following: HbA1c 7.0–10.5%, fasting glucose $\leq$ 12.5 mmol/L, BMI $\geq$ 28 kg/m^2, insulin $\geq$ 27.8 pmol/L, C-peptide $\geq$ 0.67 nmol/L, and serum MCP-1 $\geq$ 36 pmol/L. Subjects were screened and enrolled through outpatient clinics. The sponsor selected sites after a site visit to determine site qualifications and the investigator's ability to conduct clinical investigations according to the protocol and current Good Clinical Practice regulations: clinical trial registration: HE3286-0401 NCT00694057 http://www.clinicaltrials.gov.

2.6. Analysis of Variance in Erythroid and Metabolic Parameters in Placebo Comparison Studies. Variance in erythroid parameters was examined by three ways. First, the variances for selected hematologic and metabolic laboratory values, such as the mean coefficient of variation (CV) and the CV range for each individual subject, were determined and compared with those of healthy subjects. Second, the intravisit changes in HbA1c were compared for individual subjects for each condition with those of healthy subjects. Third, intravisit and day 84 changes in HbA1c and other hematology and laboratory parameters were tested for random effects.

2.7. Statistical Analyses. Random effects were tested using Residual Maximum Likelihood (REML) using StatXact, and outliers were examined using Mahalanobis distance (Cytel Software Corporation, Cambridge, MA) in conjunction with SAS software (SAS Institute, Cary, NC). Correlations were tested using Spearman or Pearson correlations, and the hypothesis that placebo participants with clinical conditions have higher frequencies of abnormal hematology and laboratory values than healthy subjects was tested using one-tailed Fisher's exact test. Heteroscedasticity (tests of different variabilities between subpopulations) was tested for normal distributions (Shapiro-Wilks W test), and dispersion was tested using the 2-sided F test (Prism Graph Pad, San Diego, CA). If there were significant differences in variances between groups, they were further examined using a t-test assuming unequal variances, nonparametric Mann-Whitney test, or Fisher's exact test. Due to the exploratory nature of this hypothesis-testing study, P values were not adjusted for multiple comparisons.

2.8. Normalization of HbA1c to Remove Random Effects in HE3286-0401. Through the course of this analysis it was discovered that the inflammatory status of the selected patient population created large and rapid changes in the patient's red cell mass that affected the whole body hemoglobin mass and consequently the fidelity of the HbA1c metric. In order to investigate HE3286 treatment effects on HbA1c in T2DM patients, HbA1c changes were normalized to the day 84 average Hb for each subject, by averaging Hb values acquired at each clinic visit. This is statistically justified based on the fact that random effects have a mean of zero but are characterized with high variances. Normalized HbA1c (nHbA1c) was applied to correct for the inflammation-induced variances found in this T2DM study population with uncontrolled inflammation.

2.9. Details of Normalization of HbA1c Using 84-Day Average Hemoglobin in HE3286-0401. **HbA1c** is reported in units of % hemoglobin (%Hb). **Hb** is the concentration of hemoglobin (reported in units of g/dL). **blood volume** (male) = 0.6041 + 0.3669 $*$ (height in meters)^3 + 0.03219 $*$ (weight in kg). **blood volume** (female) = 0.1833 + 0.3561 $*$ (height in

meters)^3 + 0.03308 ∗ (weight in kg). (Blood volume unit = L). **total Hb mass** = **Hb**∗10∗ **blood volume**(Hb mass units = g). **total HbA1c = HbA1c**∗**total Hb mass** (total HbA1c units = g). **84 day average total Hb mass** = mean of baseline to day 84 **total Hb mass** measurements.

Normalized HbA1c (**nHbA1c**) = 100 ∗ (**total HbA1c/84 day average total Hb mass**)(nHbA1c units = %Hb).

Δ**nHbA1c** represents change in **nHbA1c**.

day 84 Δ**nHbA1c** = **day 84 nHbA1c**−baseline **nHbA1c**.

3. Results

3.1. Retrospective Exploration of Increased Variance with Metabolic Disease Progression. The hypothesis that chronic low-grade inflammation leads to increased variance in laboratory values was explored by a retrospective review of hematology and metabolic clinical parameters from placebo subjects enrolled in 10 clinical studies conducted by Harbor Therapeutics, Inc., since 2001. Only placebo subject data from these studies were used for intercomparisons to exclude study drug effects.

3.2. Variances of Hematology and Laboratory Values between Medical Conditions in Placebo Subjects. Changes in variance (CV means and ranges) for hematologic and metabolic parameters sorted by medical condition are displayed in Figure 2. Dyslipidemic patients showed increased variances in hematocrit, HbA1c, and fasting glucose compared to healthy subjects. Although their lipid parameters were abnormal, their lipid variances were not significantly higher than those of healthy subjects. IGT subjects had significantly higher variances for RBC, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, hemoglobin, and HbA1c. Although they had higher postprandial glucose, their fasting glucose variances were not significantly greater than those of healthy subjects. Metformin-treated T2DM patients had significantly higher variances for RBC, hematocrit, hemoglobin, HbA1c, platelets, fasting glucose, cholesterol, and LDL. Treatment-naïve T2DM patients had significantly elevated variances for RBC, hematocrit, mean cell volume, mean corpuscular hemoglobin, hemoglobin, HbA1c, platelets, fasting glucose, cholesterol, HDL and LDL.

3.3. Intravisit HbA1c Changes in Placebo Subjects. Individual subject intravisit HbA1c changes are presented by medical condition in Figure 3. Healthy subjects HbA1c values were only collected in study 2100-202. Over 28 days, the 8 subjects showed an intravisit median change of 0 and a range from −0.2 to 0.2% Hb, consistent with literature reports. HbA1c was measured in dyslipidemic patients on days 1 and 28, yielding a single intravisit value for 21 patients with a median of 0 and an increased range of −0.5 to 0.5% Hb. Subjects with dysregulated glucose showed a median change of 0 with increased ranges: IGT over 56 days (−0.3 to 0.4% Hb), metformin T2DM over 112 days (−2.2 to 2.0% Hb), and treatment-naïve T2DM over 112 days (−3.4 to 2.8% Hb). Figure 3(f) shows the intravisit changes in HbA1c for each medical condition on the same scale. Intravisit HbA1c changes for individual T2DM patients showed 10-fold increases over healthy subjects. These results indicate that the intravisit variances for individual T2DM patients are increased and distinct from the normal variances in healthy subjects.

Table 1: Correlations with individual treatment-naïve T2DM HbA1c and insulin coefficients of variation.

HbA1c CV[a]	*n*	Pearson correlation	*P* value
Hemoglobin CV	28	0.44	0.018
MCP-1 CV	20	0.44	0.0495
Glucose CV	28	0.58	0.0009
C-reactive protein CV	27	0.39	0.045
HDL CV	27	0.68	<0.0001
LDL CV	22	0.52	0.013
Triglyceride CV	28	0.62	0.0004
Lymphocyte CV	26	0.44	0.0261
Monocyte CV	27	0.56	0.0026
Platelet CV	28	0.42	0.025
Red blood cell CV	28	0.53	0.0036
Hematocrit CV	28	0.48	0.0094
MCV CV	28	0.38	0.048
Insulin CV	*n*	Pearson correlation	*P* value
Glucose CV	28	0.46	0.013
White blood cell CV	28	0.46	0.013
Neutrophil CV	28	0.54	0.0028

[a]HbA1c: hemoglobin A1c; CV: coefficient of variation; MCP-1: monocyte chemoattractant protein 1; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MCV: mean corpuscular volume.

3.4. Correlations with HbA1c and Insulin Variance in Treatment-Naïve Placebo Subjects. Treatment-naïve T2DM patients had the greatest variance in HbA1c. In this group, Pearson correlations were used to investigate the HbA1c and insulin variance relationships with other clinical parameter variances (Table 1). Individual patient HbA1c CV, were correlated with CVs for hemoglobin, MCP-1, glucose, CRP, HDL, LDL, triglycerides, lymphocytes, monocytes, platelets, RBC, hematocrit, and MCV, and insulin CVs were similarly correlated with CVs for glucose, WBC, and neutrophils, indicating dysregulation of multiple hematopoietic and metabolic functions within the same individual. Furthermore, REML analyses demonstrated significant random changes in day 84 glucose ($P < 0.0001$), hemoglobin ($P < 0.0001$), HbA1c ($P = 0.02$), and HOMA2 %B ($P = 0.004$), indicating laboratory results unreflective of the clinical situation.

3.5. Conclusions from the Retrospective Analysis of Placebo Subjects with Medical Conditions. There appears to be a progressive increase in metabolic and hematologic laboratory parameter variances with increased BMI and metabolic disease progression that results in random HbA1c changes. Based on this, intervention with the HE3286 anti-inflammatory agent might confer benefit to this pathology. However, with random HbA1c effects in the placebo

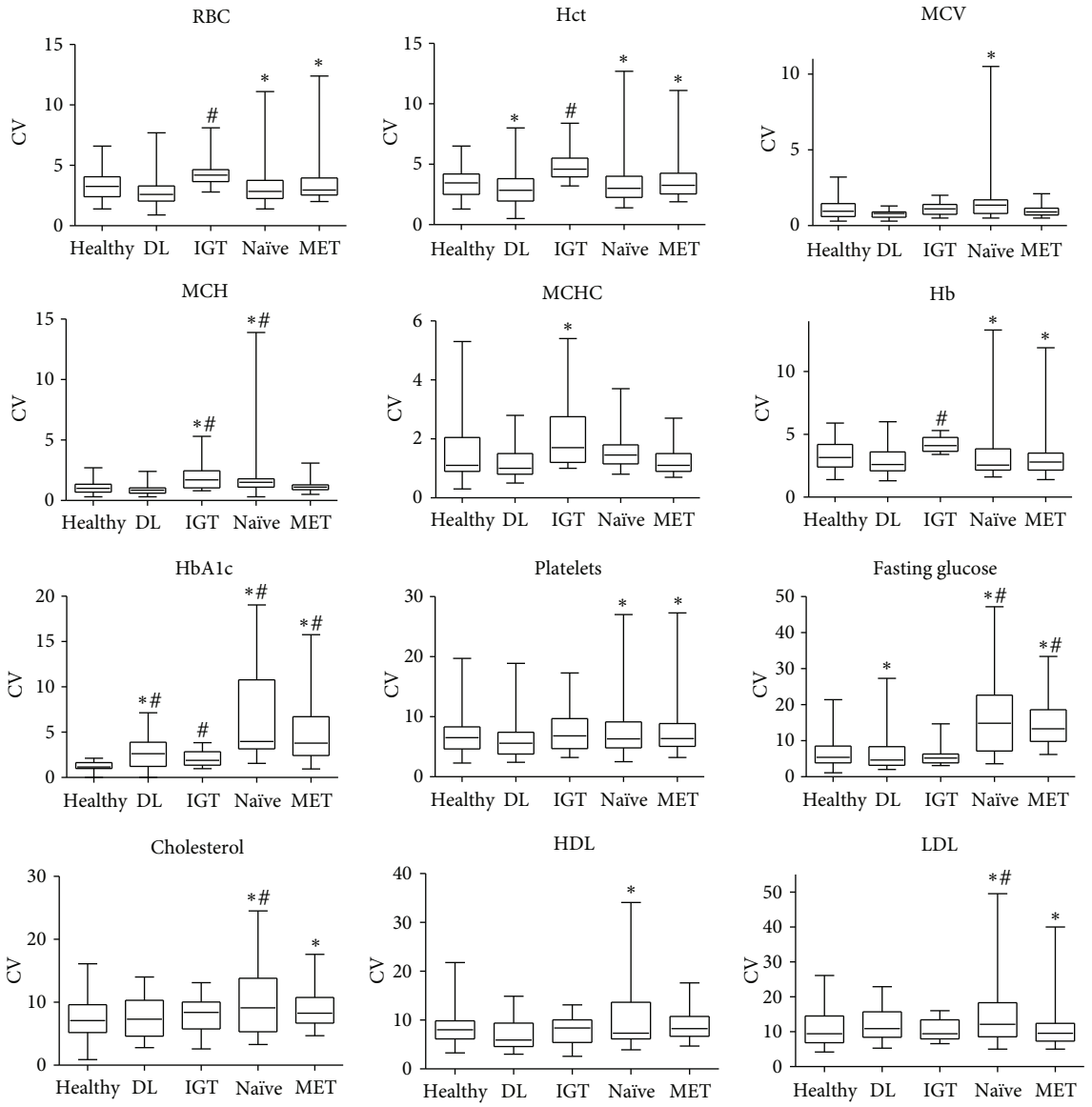

Figure 2: Coefficients of variation for individual subject hematology and chemistry values in placebo comparisons. DL: dyslipidemic placebo group, IGT: impaired glucose tolerant placebo group, Naïve: treatment-naïve uncontrolled T2DM placebo group, MET: uncontrolled T2DM placebo group on a stable dose of metformin, Hb: hemoglobin, Hct: hematocrit, RBC: red blood cell count, MCV: mean cell volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, HbA1c: hemoglobin A1c, HDL: high density lipoprotein cholesterol, LDL: low density lipoprotein cholesterol. $^{\#}$Statistically significant (Welch ANOVA, allowing unequal variance) increase in mean coefficient of variation compared to healthy participants. *Statistically significant dispersion (2-sided F test) in the coefficient of variation compared to healthy participants.

participants, it is difficult to demonstrate significant changes with comparisons to active agents unless a correction is applied to the data. Accordingly, treatment effects were investigated by normalizing HbA1c to the day 84 average total body hemoglobin mass for each patient.

3.6. HE3286-0401. In this study, HE3286 was well tolerated. Seventy-six percent of cohort 1 (metformin treated) and 77% of cohort 2 (treatment naïve) completed the study (Figure 1(b)). Only 1 serious adverse event occurred, a transient asymptomatic elevation from baseline of blood amylase, which resolved on study. This event was considered by the investigator to be possibly related to study medication. There were no clinically significant abnormalities related to any body system, including hypoglycemia and electrocardiograms, attributable to HE3286 administration. There were no detectable differences or trends in adverse events between placebo- and HE3286-treated subjects. No patient died while on study. Baseline demographics and characteristics of each group are presented in Table 2.

Table 2: HE3286-0401 baseline demographics and characteristics of each group.

Cohort 1	All Subjects		MCP-1[a] > 40 pmol/L		MCP-1 ≤ 40 pmol/L	
	HE3286	Placebo	HE3286	Placebo	HE3286	Placebo
n	34	38	22	25	12	13
Age	48 (41–57)[b]	50 (43–59)	49 (40–57)	12(44–61)	48 (41–57)	48 (43–56)
Gender F (%)	19 (56%)	18 (47%)	12 (56%)	12 (48%)	7 (58%)	6 (46%)
Ethnicity						
White/White Hispanic	25 (74%)	27 (71%)	18 (82%)	18 (72%)	7 (58%)	9 (69%)
Black	5 (15%)	7 (18%)	2 (9%)	3 (12%)	3 (25%)	4 (31%)
Asian	3 (9%)	3 (8%)	1 (5%)	3 (12%)	1 (8%)	0 (0%)
Other	1 (3%)	1 (3%)	1 (5%)	1 (4%)	1 (8%)	0 (0%)
MCP-1 (pmol/L)	50 (35–87)	52 (34–70)	61 (50–75)	61 (52–81)	32 (27–36)	18 (20–36)
TNFα (pmol/L)	0 (0–0.1)	0 (0–0.3)	0 (0–0.1)	0 (0–0.4)	0 (0–0)	0 (0–0.4)
BMI (kg/m^2)	29 (25–32)	30 (26–36)	29 (26–32)	29 (25–37)	27 (25–31)	33 (29–36)
HbA1c (%Hb)	8.5 (7.8–9.3)	8.3 (8.0–9.2)	9.0 (7.9–9.6)	8.3 (8.0–9.3)	8.0 (7.8–8.6)	8.5 (8.0–9.2)
Hb (mmol/L)	8.5 (8.1–9.3)	8.5 (8.1–9.3)	8.6 (8.1–9.3)	8.3 (8.1–9.3)	8.0 (7.5–8.7)	9.1 (8.1–9.3)
Hct (proportion)	0.41 (0.37–0.43)	0.41 (0.39–0.44)	0.41 (0.39–0.44)	0.40 (0.39–0.43)	0.39 (0.36–0.42)	0.44 (0.39–0.46)
RBC (10^{12}/L)	4.6 (4.3–4.9)	4.7 (4.4–4.9)	4.6 (4.4–5.0)	4.7 (4.4–5.0)	4.6 (4.3–4.9)	4.7 (4.4–5.1)
Insulin (pmol/L)	56 (28–90)	63 (35–118)	69 (29–97)	56 (28–104)	56 (42–76)	90 (35–188)
C-peptide (nmol/L)	0.77 (0.60–1.0)	0.90 (0.53–1.2)	0.80 (0.50–1.1)	0.70 (0.53–1.1)	0.73 (0.67–0.87)	1.0 (0.53–1.4)
Fasting glucose (mmol/L)	8.9 (7.7–11)	9.2 (7.5–10.1)	9.5 (8.3–11)	8.4 (7.5–10)	8.1 (7.5–8.7)	9.2 (7.2–10)
1,5-Anhydroglucitol (μmol/L)[c]	35 (12–60)	52 (20–85)				
CRP (pmol/L)	30 (14–61)	25 (12–73)	29 (12–49)	13 (11–73)	35 (18–94)	26 (15–57)
HOMA2 %B	47 (36–62)	59 (33–82)	41 (33–57)	58 (30–80)	58 (47–67)	70 (37–88)
HOMA2 IR	2.0 (1.5–2.7)	2.2 (1.4–3.2)	2.3 (1.3–2.9)	2.0 (1.4–2.9)	1.9 (1.6–2.3)	2.8 (1.3–3.6)
Triglycerides (mmol/L)	1.8 (1.3–2.9)	2.0 (1.3–2.5)	1.7 (1.2–3.1)	2.1 (1.3–2.7)	2.0 (1.4–2.6)	2.0 (1.3–2.1)
Cohort 2	All subjects		BMI > 31 kg/m^2		BMI ≤ 31 kg/m^2	
	HE3286	Placebo	HE3286	Placebo	HE3286	Placebo
n	25	28	12	15	13	13
Age	54 (48–60)	53 (46–58)	53 (42–56)	53 (46–55)	55 (49–63)	58 (46–62)
Gender F (%)	10 (40%)	14 (50%)	4 (33%)	9 (60%)	6 (46%)	5 (38%)
Ethnicity						
White/White Hispanic	20 (80%)	27 (96%)	10 (83%)	15 (100%)	10 (77%)	12 (92%)
Black	3 (12%)	0 (0%)	2 (17%)	0 (0%)	1 (8%)	0 (0%)
Asian	2 (8%)	1 (4%)	0 (0%)	0 (0%)	2 (15%)	1 (8%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
BMI (kg/m^2)	31 (29–33)	31 (29–37)	33 (32–36)	36 (32–37)	29 (28–30)	29 (28–30)
MCP-1 (pmol/L)[d]	108 (51–189)	94 (64–117)	97 (49–182)	94 (62–129)	110 (68–195)	95 (67–123)
HbA1c (%Hb)	8.1 (7.5–8.7)	8.5 (7.7–10.2)	7.6 (8.0–8.4)	8.4 (7.5–9.8)	8.4 (7.5–8.8)	9.4 (8.1–10.6)
Hb (mmol/L)	9.4 (8.7–9.9)	8.8 (8.1–9.9)	9.5 (8.7–9.9)	8.9 (8.1–9.9)	8.9 (8.7–9.9)	8.8 (8.1–9.9)
Hct (proportion)	0.45 (0.41–0.46)	0.42 (0.39–0.46)	0.45 (0.43–0.46)	0.44 (0.39–0.46)	0.42 (0.40–0.47)	0.42 (0.38–0.46)
RBC (10^{12}/L)	4.7 (4.5–5.1)	4.7 (4.5–5.1)	4.9 (4.6–5.1)	4.8 (4.5–5.0)	4.7 (4.3–5.0)	4.5 (4.3–5.1)
Insulin (pmol/L)	104 (69–146)	111 (76–153)	118 (76–139)	118 (76–146)	97 (69–153)	104 (69–174)
C-peptide (nmol/L)	1.1 (0.8–1.3)	1.1 (0.9–1.4)	1.2 (0.8–1.3)	1.2 (1.0–1.3)	1.0 (0.8–1.2)	1.0 (0.9–1.5)
Fasting glucose (mmol/L)	8.3 (7.2–9.9)	9.2 (7.3–11)	8.0 (6.7–8.8)	8.2 (6.8–10)	8.5 (7.2–11)	9.3 (8.1–13)
1,5-Anhydroglucitol (μmol/L)[e]	54 (31–83)	102 (35–154)				
CRP (pmol/L)	27 (19–49)	32 (16–48)	27 (22–67)	33 (22–53)	28 (13–48)	30 (12–45)
HOMA2 %B	74 (43–108)	61 (45–89)	87 (47–123)	80 (43–120)	71 (35–85)	56 (46–73)
HOMA2 IR	2.7 (2.2–3.4)	2.8 (2.5–3.8)	2.9 (2.1–3.4)	3.2 (2.5–3.9)	2.6 (2.2–3.3)	2.7 (2.5–4.3)
Triglycerides (mmol/L)	1.6 (1.1–2.7)	2.1 (1.5–3.5)	1.4 (1.1–1.8)	2.0 (1.6–2.8)	1.4 (2.3–3.2)	3.5 (1.4–6.7)

[a]MCP-1: monocyte chemoattractant protein 1; TNFα: tumor necrosis factor alpha; BMI: body mass index; HbA1c: hemoglobin A1c; Hb: hemoglobin; Hct: hematocrit; RBC: red blood cells; CRP: C-reactive protein; HOMA2 %B: homeostatic model assessment of % pancreatic beta cell function, HOMA2 IR: homeostatic model assessment of insulin resistance. [b]Numbers are medians (IQR) or numbers (%). [c]1,5-Anhydroglucitol only 19 retention samples (9 HE3286, 10 placebo, predominantly with MCP > 40 pmol/mL). [d]MCP-1 only 38 samples (18 HE3286, 20 placebo); [e]1,5-Anhydroglucitol only 23 retention samples (9 HE3286, 14 placebo, predominantly from BMI > 31 kg/m^2 subjects).

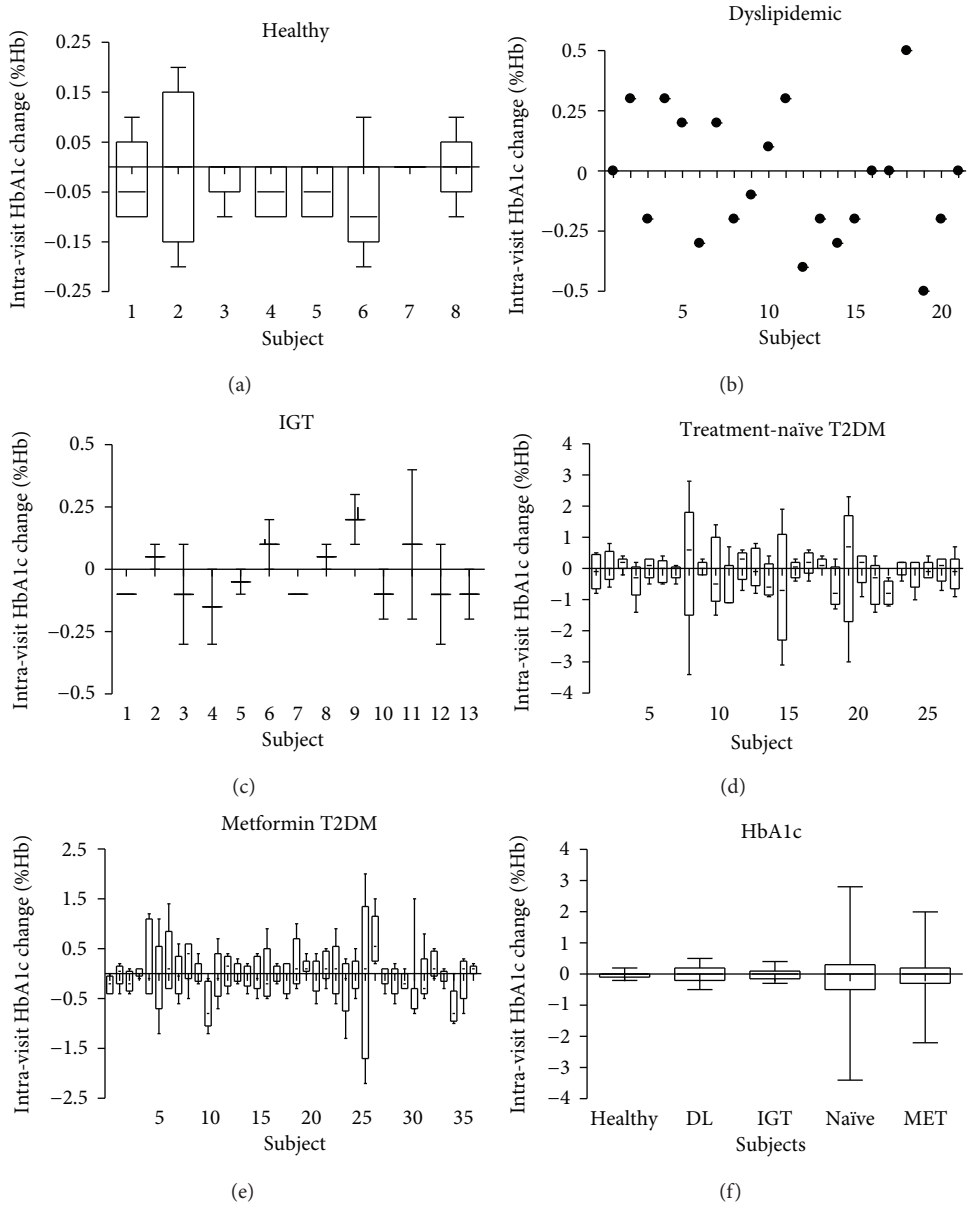

Figure 3: Intrasubject HbA1c changes in placebo comparisons. Bar and whisker plots of the change in HbA1c between visits for individual subjects: (a) healthy subjects from study 2100-202, (b) dyslipidemic subjects from study 2200-130, (c) impaired glucose tolerance subjects from study 3286-0102, (d) treatment-naïve type 2 diabetes patients from HE3286-0401 cohort 2, (e) type 2 diabetic patients on a stable dose of metformin from HE3286-0401 cohort 1, (f) intravisit changes for all subjects from each condition plotted on the same scale. IGT: impaired glucose tolerant, Metformin T2DM: uncontrolled type 2 diabetes mellitus participants on a stable dose of metformin.

3.7. Correlates of HbA1c and Hemoglobin Changes and Variances in HE3286 and Placebo Subjects

3.7.1. Cohort 1. Correlations of ΔHbA1c, HbA1c CV, and Hb CV with other parameters are shown in Table 3. The HE3286 cohort 1 group HbA1c change was negatively correlated with baseline MCP-1 ($P = 0.01$) and change in HOMA2 %B ($P = 0.01$) and positively correlated with glucose change ($P = 0.009$), weight change ($P = 0.007$), and change in Hb ($P = 0.03$). HbA1c change was positively correlated with fasting glucose change in both HE3286 and placebo. The placebo HbA1c change was negatively correlated with baseline TNFα ($P = 0.004$) and positively with change in TNFα ($P = 0.02$). In addition, in the placebo group, intrapatient HbA1c coefficients of variation (CV) were significantly correlated with baseline TNFα ($P = 0.002$). These relationships led to the hypothesis that HE3286 decreased HbA1c in the more inflamed (higher MCP-1) patients, in conjunction with increased pancreatic beta cell function and weight loss, but in placebos, inflammation (TNFα) was primarily contributing to HbA1c changes. The finding that Hb change was positively correlated with HbA1c change suggested the possibility that

Table 3: HE3286-0401 significant correlates of HbA1c and Hb changes.

Group	Dependent	Independent	Test	HE3286				Placebo			
				n	*r*	95% CI	*P*	*n*	*r*	95% CI	*P*
Cohort 1	ΔHbA1c[a]	MCP-1	Spearman	34	−0.42	−0.67 to −0.08	**0.01**				>0.10
		ΔHOMA2 %B	Spearman	34	−0.43	−0.68 to 0.10	**0.01**				>0.10
		ΔGlucose	Spearman	34	0.45	0.11 to 0.69	**0.009**				>0.10
		ΔWeight	Spearman	34	0.45	0.13 to 0.69	**0.007**				>0.10
		ΔHb	Pearson	34	0.36	0.03 to 0.62	**0.03**				>0.10
	ΔHbA1c	TNF*α*	Spearman				>0.10	34	−0.48	−0.71 to −0.16	**0.004**
		ΔGlucose	Spearman				>0.10	38	0.65	0.41 to 0.81	**<0.0001**
		ΔTNF*α*	Spearman				>0.10	32	0.40	0.05 to 0.66	**0.02**
	HbA1c CV	TNF*α*	Pearson				>0.10	34	0.50	0.19 to 0.72	**0.002**
Cohort 2	ΔHbA1c	BMI	Spearman	25	−0.41	−0.70 to −0.008	**0.04**				>0.10
		ΔHOMA2 %B	Pearson	25	−0.50	−0.75 to −0.13	**0.02**				>0.10
		ΔGlucose	Pearson	25	0.61	0.29 to 0.81	**0.001**				>0.10
	HbA1c CV	Hb CV	Pearson				>0.10	28	0.44	0.08 to 0.70	**0.02**
		ΔMCP-1	Pearson				>0.10	20[b]	0.44	0.002 to 0.74	**0.0495**
		ΔWeight	Pearson				>0.10	28	−0.43	−0.69 to −0.07	**0.02**
	Hb CV	MCP-1	Spearman				>0.10	20	0.38	0.03 to 0.70	**0.04**
Cohort 2 BMI > 31	ΔnHbA1c	MCP-1	Spearman Exact	10[b]	−0.68	—[c]	**0.03**				>0.10
		ΔMCP-1	Spearman Exact	9[b]	0.77	—	**0.002**				>0.10

[a]Δ: change in; CV: coefficient of variation; HbA1c: hemoglobin A1c; Hb: hemoglobin; MCP-1: monocyte chemoattractant protein-1; HOMA2 %B: homeostatic model assessment of pancreatic beta cell function; TNF*α*: tumor necrosis factor alpha; BMI: body mass index; nHbA1c: HbA1c normalized to day 84 hemoglobin mass (see Section 2 for details). [b]MCP-1 data unavailable on a portion of participants. [c]Spearman Exact test has no confidence interval.

inflammation-induced effects produce random Hb levels that contribute to increased variance in HbA1c changes.

High intrapatient CVs were observed for both HbA1c (up to 16%) and Hb (up to 12%) in both HE3286 and placebo groups (data not shown). Residual maximum likelihood (REML) analyses indicated a significant random CV component in the intrapatient Hb values, for both the HE3286 and placebo groups ($P < 0.0001$ for each, data not shown).

3.7.2. Cohort 2. Cohort 2 participants were selected using more stringent criteria for MCP-1, BMI, insulin, and C-peptide. TNF*α* was not measured in this group. Consequently, significant correlations observed in cohort 1 for HbA1c change with baseline MCP-1 and changes in weight and in TNF*α* were not observed in the overall cohort 2 group. Table 3 also shows correlations between ΔHbA1c, HbA1c CV, and Hb CV and other parameters in cohort 2. In this overweight to obese population, ΔHbA1c was correlated negatively with baseline BMI ($P = 0.04$) and with HOMA2 %B change ($P = 0.02$) and positively with day 84 change in fasting plasma glucose ($P = 0.001$) for HE3286, but not placebo. Further, we found that the cohort 2 participants (selected for higher inflammation) had higher variances in erythroid hematology values than cohort 1 (see Figure 2), and cohort 2 placebo day 84 changes in HbA1c had a significant random component (REML $P = 0.006$, data not shown). In placebo patients, HbA1c CV was positively correlated with baseline Hb CV ($P = 0.02$) and the ΔMCP-1 inflammation marker ($P = 0.0495$) and negatively correlated with weight change ($P = 0.02$). Hb CV, in turn, was positively correlated with baseline MCP-1 ($P = 0.04$). These relationships led us to the hypothesis that HE3286 decreased HbA1c in patients with higher obesity (BMI), in conjunction with improved pancreatic beta cell function and decreased fasting glucose, that, for placebo patients, HbA1c change and Hb CV were related to inflammation status (MCP-1), and that weight loss in placebos might be related to inflammation effects on malnutrition.

Together, the results from studies in HE3286-0401 T2DM patients suggested that low-grade chronic inflammation develops during metabolic disease progression in the obese diabetic and contributes to dysregulation of metabolic and hematologic homeostasis. If this is correct, then intervention with an anti-inflammatory compound such as HE3286 might lead to restoration of homeostasis, normalization of glucose levels, and a decline in weight. Further, the inflammatory effects on erythropoiesis may be quelled and the utility of ΔHbA1c as a biomarker of glucose control restored.

3.8. HE3286-0401 Treatment Effects

3.8.1. Cohort 1. According to our observations in phase I inflamed obese prediabetics, HE3286 should show benefit in inflamed T2DM individuals. The correlation of baseline MCP-1 in the HE3286 HbA1c response was explored, and significant treatment effects were observed in the more inflamed subjects (baseline serum MCP-1 upper 2 tertiles (>40 pmol/L)). Table 4 displays the HE3286 day 84 treatment

effects on clinical parameters in this subgroup. Significant decreases were observed for HOMA2 IR ($P = 0.02$), C-peptide ($P = 0.04$), Hb ($P = 0.02$), Hct ($P = 0.02$), and RBC ($P = 0.02$) changes in the HE3286 treatment group when compared to the placebo (metformin alone) group.

The effect of HE3286 on ΔnHbA1c in the overall population was not significant. Therefore, the day 84 treatment effect on ΔnHbA1c was investigated in the more inflamed MCP-1 subgroup (Table 4). The median magnitude of the ΔnHbA1c was −0.44% Hb (HE3286, −0.34; placebo, +0.1). The HE3286 data was normally distributed, but placebo was significantly abnormal ($P = 0.0006$, W test, data not shown). This situation necessitated the use of nonparametric methods of data analysis. The HE3286 treatment effect was found to significantly decrease nHbA1c from zero ($P = 0.03$). The frequency of HE3286 patients with decreased nHbA1c was significantly greater than placebo (17/22 versus 9/25, $P = 0.0008$). There were no significant differences between HE3286 and placebo groups at follow-up day 112.

The HE3286 and placebo patients distributions with MCP-1 > 40 pmol/L are shown for ΔnHbA1c in Figure 4(a) and for ΔHOMA2 IR in Figure 4(b). The majority of HE3286 patients showed decreased nHbA1c and HOMA2 IR, whereas the majority of placebos showed increases. These results are consistent with inhibition of NFκB hyperactivation and consequent restoration of normal insulin signaling, consistent with the preclinical HE3286 observations.

3.8.2. Cohort 2. The correlation between baseline BMI and change in HbA1c in the HE3286 group was explored by stratifying participants on the median BMI (31 kg/m^2). The ΔnHbA1c in the HE3286, but not placebo participants (with BMI > 31 kg/m^2), correlated significantly with their baseline MCP-1 ($P = 0.03$) (Table 3). This strengthens the hypothesis that HE3286 benefited the obese inflamed subset of T2DM patients. The ΔnHbA1c also correlated significantly with ΔMCP-1 ($P = 0.002$) in HE3286 (Table 3), but not in placebo participants. Thus the decrease in inflammation (MCP-1) was associated with the decrease in HbA1c with HE3286 treatment.

The effect of HE3286 on nHbA1c in the overall population was not significant. The obese patients with a BMI (>31 kg/m^2), demonstrated a significant treatment effect (t-test) to decrease nHbA1c by 0.6% Hb compared to placebo, but only after exclusion of 2 outliers (Mahalanobis distance). The day 84 distribution of the ΔnHbA1c for BMI > 31 is shown in Figure 4(c) (outliers circled). The variances were much higher in the treatment-naïve patients' parameters compared to uncontrolled metformin-treated patients (Figure 3(f)). We speculated that these two outliers were still subject to inflammation-induced random effects, after only 84 days of treatment and that additional treatment may be necessary to observe effects in these individuals. Because of the lag in ΔHbA1c following glucose excursions, we tested the treatment effects on follow-up day 112. A significant day 112 treatment effect (with no outliers) was found in the high BMI stratum, both by nonparametric and parametric tests (Table 4). HE3286 participants had a significant mean change from baseline (−1.0% Hb, $P = 0.0007$), whereas placebo did not. The mean change compared to placebo was also significant (−0.7% Hb, $P = 0.03$). The HE3286 participants also had a significant median change from baseline (−1.2% Hb, $P = 0.002$), whereas placebo did not. The magnitude of the response in the HE3286 treatment groups was significant (−1.0% Hb, $P = 0.02$) compared to placebo, as was the frequency of subjects with a 0.5% HbA1c decrease (9/12 versus 4/13, $P < 0.05$). The day 112 ΔnHbA1c distributions for BMI > 31 kg/m^2 are shown in Figure 4(d).

3.9. Postprandial Treatment Effect. A significant treatment effect that lowered fasting glucose was not found and was attributed to high metabolic parameter variations. Consequently, the possibility that HE3286 decreased HbA1c through action on postprandial glucose was investigated. Serum 1,5-anhydroglucitol (1,5-AH) is a dietary human metabolite that is reabsorbed by a kidney glucose transporter [22]. The 1,5-AH level declines when blood glucose levels are elevated above 10 mmol/L and likewise increases when the blood glucose level declines.

1,5-AH was measured in a subset of 42 participants (19 from stages 1 and 23 from stage 2) that had available day 84 retention samples. Analysis of 18 patients treated with HE3286 demonstrated that their 1,5-AH concentration increased significantly (+10.4 μmol/L, $P = 0.02$); 24 treated with placebo demonstrated no significant concentration increase (+0.6 μmol/L, $P > 0.1$). The distribution of 1,5-AH responses is shown in Figure 4(e). The majority of HE3286 patients significantly increased 1,5-AH, compared to placebos (15/18 versus 11/24, $P = 0.02$, Fisher's exact test). This outcome indicates that HE3286 had a treatment effect to decrease postprandial glucose excursions compared to placebo, which further supports that it's pharmacologic property is to decrease insulin resistance (see [9] and Figure 4(b)) and lower HbA1c.

3.10. HE3286-0401 Heteroscedasticity in HE3286 and Placebo Groups. Heteroscedasticity (differences in variances between groups) was investigated by analyzing data distributions for normality (Shapiro-Wilks W test) and analyzing dispersion (2-sided F test). In cohort 1 placebo, but not HE3286, day 84 distributions (W test) were significantly abnormal for changes in insulin, C-peptide, fasting glucose, HOMA2 %B, HOMA2 IR, and leptin in all subjects, and for changes in HbA1c, fasting glucose, and HOMA2 %B for MCP-1 > 40 pmol/L participants. Variances for cohort 1 placebo subjects (F test) were also significantly higher than those of HE3286 subjects for insulin, C-peptide, and HOMA2 IR for all subjects.

Cohort 2 placebo, but not HE3286 distributions were abnormal (W test) for the group as a whole for changes in all the following parameters: day 84 nHbA1c, fasting glucose, MCP-1, and triglycerides and day 112 nHbA1c, fructosamine, and HOMA2 %B. In the BMI > 31 subgroup, abnormal distributions were found for changes in all the following parameters: day 84 HOMA2 %B and day 112 insulin, C-peptide, HOMA2 %B, and HOMA2 IR. Variances

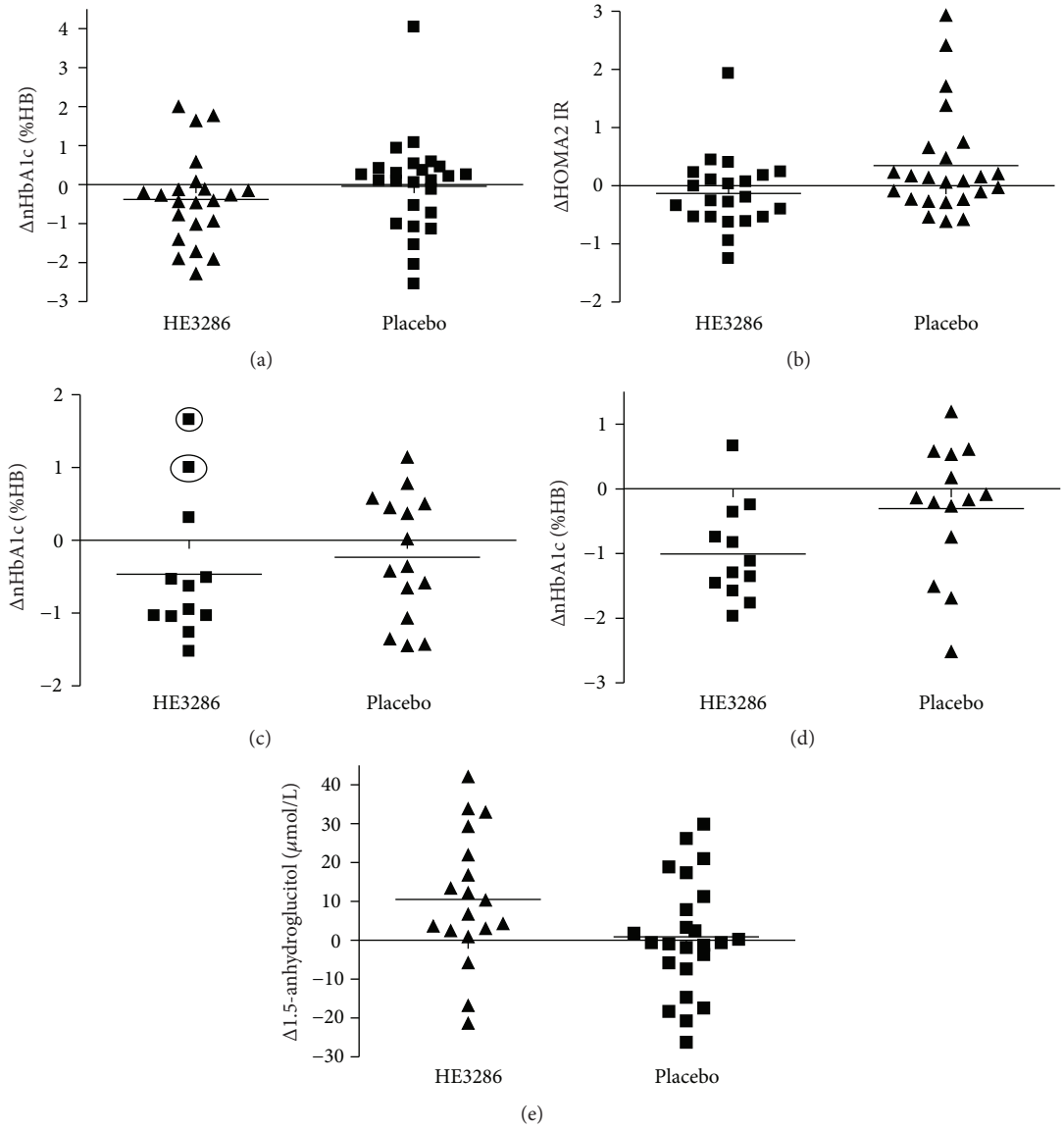

Figure 4: HE3286-0401 distributions of changes by participant. (a) Day 84 cohort 1 baseline MCP-1 > 40 pmol/L individual subject changes in normalized HbA1c by treatment. (b) Day 84 cohort 1 baseline MCP-1 > 40 pmol/L individual subject changes in homeostatic model assessment of insulin resistance by treatment. (c) Day 84 cohort 2 baseline BMI > 31 kg/m^2 individual subject changes in normalized HbA1c by treatment. The circles indicate two outliers detected using Mahalanobis distance. (d) Day 112 cohort 2 baseline BMI > 31 kg/m^2 individual subject changes in normalized HbA1c by treatment. (e) Pooled 1,5 anhydroglucitol changes from day 84 cohort 1 and 2 analyses by treatment. Analyses were performed on a subset of patients with available retention samples, predominantly those in cohort 1 with baseline MCP-1 > 40 pmol/L and in cohort 2 with baseline BMI > 31 kg/m^2.

for cohort 2 placebo subjects as a whole were significantly higher (F test) for changes in all of the following parameters: day 84 insulin, HOMA2 %B, and triglycerides, and day 112 insulin and HOMA2 %B. Variances in placebo were also higher for the BMI > 31 kg/m^2 subgroup for changes in day 84 MCP-1 and triglycerides and day 112 insulin (Table 5).

These differences in distribution and dispersion between groups were not readily evident until day 84 of treatment (data not shown). Together, these findings further support an HE3286 treatment effect that decreases random metabolic effects and restores homeostasis to uncontrolled T2DM patients.

4. Discussion

4.1. Study HE3286-0401. This initial clinical trial of HE3286 in diabetes was designed to take all eligible patients with uncomplicated T2DM even though HE3286 was only qualified in animal models of obese diabetes and subsequently only demonstrated activity in obese individuals that present with inflammation-induced insulin resistance. The strategic intent

Table 4: HE3286-0401 treatment effects in obese inflamed subgroups.

Group	Effect	Value	Change		P	Test[g]
			HE3286	Placebo		
Cohort 1 MCP > 40[a]	ΔHOMA2 IR[c]	Day 84 mean	−0.1	+0.4	**0.02**	
	ΔC-peptide		−0.03	+0.1	**0.04**	
	ΔHb		−0.25	+0.06	**0.02**	t-test
	ΔHct		−0.06	+0.09	**0.02**	
	ΔRBC		−0.05	+0.09	**0.02**	
	ΔnHbA1c	Day 84 median	−0.34		**0.03**	Wilcoxon
		Day 84 median		0.1	—	
		Day 84 numbers	17⇓[e] 5⇑[f]	9⇓ 16⇑	**0.0008**	Fisher's Exact
		Day 84 mean	−0.46	−0.21	—	
		Day 84 mean−2 outliers[d]	−0.82	−0.21	**0.04**	t-test
Cohort 2 BMI > 31[b]	ΔnHbA1c	Day 112 mean	−1.0		**0.0007**	
				−0.3	—	t-test
			−1.0	−0.3	**0.03**	
		Day 112 median	−1.2		**0.002**	Wilcoxon
				−0.16	—	
			−1.2	−0.16	**0.02**	Mann Whitney

[a] Participants with baseline monocyte chemoattractant protein greater than the lowest tertile (40 pmol/L). [b] Participants with baseline body mass index greater than the median (31 kg/m^2). [c] Δ: change in; HOMA2 IR: homeostatic assessment model insulin resistance; Hb: hemoglobin; Hct: hematocrit, RBC: red blood cells; nHbA1c: normalized HbA1c (see Section 2 for details); [d] Two outliers removed (outliers circled in Figure 4(c), Mahalanobis distance); [e] Decrease from zero change; [f] Increase from zero change; [g] Parametric means and t-test used for data with normally distributed data, Nonparametric medians, Wilcoxon, Mann Whitney, and Fisher's Exact test used for abnormally distributed data.

of the study was to identify the responding T2DM population by surveying a broad swath of the constellation of syndromes that are defined by the T2DM condition.

Based on findings in cohort 1, which indicated low BMI individuals were HE3286 nonresponders, and the inclusion criteria in the second cohort of the trial were modified, concentrating the population to elevated weight (BMI) and inflammatory status (MCP-1). Additional criteria included a requirement for detectable insulin and C-peptide levels. This eliminated the patient population that had progressed to lose significant β-cell function and who were no longer able to produce insulin, a population clearly not indicated for treatment with an insulin sensitizer. Notably, these criteria were also imposed on clinical trials with the thiazolidinediones (J. Olefsky, personal communication). In addition treatment-naïve patients were recruited in cohort 2 to remove the potential for metformin to blunt the HE3286 treatment effect and consequently amplify the single agent treatment outcome.

We designed this study to test the hypothesis, based on preclinical data and on molecular studies of HE3286 binding partners, that HE3286 would decrease the hyperactivation of NFkB with consequent restoration of insulin signaling [5, 6], dependent on its interaction with extracellular signal regulated kinase (ERK) 1 and 2 [7] in addition to other binding partners. ERK1 is an important mediator of inflammation-induced insulin resistance [23–25], insulin receptor substrate (IRS)-1 serine (inhibitory) phosphorylation, and the inhibitory effect of TNFα on insulin signaling [26]. HE3286 does not inhibit insulin-mediated ERK activation, but inhibits LPS- and TNFα-stimulated ERK hyperactivation, and IRS-1 serine phosphorylation mediated by IKK and JNK [5, 6]. Coincident HE3286-mediated changes in ERK, IKK, JNK, and p38 MAPK signal transduction may explain the preferential responses observed in high adiposity inflamed T2DM patients. Signal transduction pathways in omental fat are altered in obese, compared to lean individuals. In humans, activation of JNK and p38 MAPK was increased in omental fat (compared to paired subcutaneous fat) from obese, but not lean individuals, and this hyperphosphorylation correlated with clinical parameters of hyperglycemia and insulin resistance [27]. It will be important to further clarify the role of ERK in the activity of HE3286.

4.2. HE3286 Correlates. Data analysis presented here demonstrated that the cohort 1 day 84 changes in the primary end point HbA1c had a significant relationship with expected changes in beta-cell function, fasting glucose, and weight, and also with baseline inflammation status (MCP-1). Surprisingly a relationship with hemoglobin was also detected, a biomarker that is presumed stable over several weeks. Of these covariates associated with HbA1c change, only fasting glucose was significant in placebo patients.

In the enriched cohort 2 population, the HbA1c HE3286 treatment response was no longer dependent on MCP-1 but rather BMI with a statistically significant negative

Table 5: HE3286-0401 Heteroscedasticity[a] Between HE3286 and Placebo Changes from Baseline Values in Laboratory Parameters.

Group	Day	Parameter	HE3286	HE3286 > Placebo	Placebo	Placebo > HE3286
			W test *P*	*F* test *P*	*W* test *P*	*F* test *P*
Cohort 1	84	ΔInsulin[d]	>0.1	>0.1	<**0.0001**	**0.007**
		ΔC-peptide	>0.1	>0.1	<**0.0001**	**0.0495**
		ΔFasting glucose	>0.1	>0.1	**0.02**	>0.1
		ΔHOMA2 %B	>0.1	>0.1	<**0.0001**	>0.1
		ΔHOMA2 IR	>0.1	>0.1	**0.002**	**0.049**
		Δleptin	>0.1	>0.1	**0.005**	>0.1
Cohort 1 MCP-1 > 40[b]	84	ΔHbA1c	>0.1	>0.1	**0.006**	>0.1
		ΔFasting glucose	>0.1	>0.1	**0.02**	>0.1
		ΔHOMA2 %B	>0.1	>0.1	<**0.0001**	>0.1
Cohort 2	84	ΔnHbA1c	>0.1	>0.1	**0.04**	>0.1
		ΔInsulin	>0.1	>0.1	>0.1	**0.004**
		ΔFasting glucose	>0.1	>0.1	**0.03**	>0.1
		ΔHOMA2 %B	>0.1	>0.1	>0.1	**0.006**
		ΔMCP-1	>0.1	>0.1	**0.005**	>0.1
		ΔTriglycerides	>0.1	>0.1	<**0.0001**	**0.007**
	112	ΔnHbA1c	>0.1	>0.1	**0.0007**	>0.1
		ΔInsulin	>0.1	>0.1	>0.1	**0.008**
		ΔFructosamine	>0.1	>0.1	**0.002**	>0.1
		ΔHOMA2 %B	>0.1	>0.1	<**0.0001**	**0.01**
Cohort 2 BMI > 31[c]	84	ΔHOMA2 %B	>0.1	>0.1	**0.007**	>0.1
		ΔMCP-1	>0.1	>0.1	>0.1	**0.009**
		ΔTriglycerides	>0.1	>0.1	>0.1	**0.001**
	112	ΔInsulin	>0.1	>0.1	<**0.0001**	**0.001**
		ΔC-peptide	>0.1	>0.1	<**0.0001**	>0.1
		ΔHOMA2 %B	>0.1	>0.1	<**0.0001**	>0.1
		ΔHOMA2 IR	>0.1	>0.1	<**0.0001**	>0.1
			>0.1	>0.1	>0.1	>0.1

[a] Heteroscedasticity describes differences in variances between groups. [b] Participants with baseline monocyte chemoattractant protein greater than the lowest tertile (40 pmol/L, see results). [c] Participants with baseline body mass index greater than the median (31 kg/m^2, see results). [d] Abbreviations: Δ: change in; HOMA2 %B: homeostatic model assessment of pancreatic beta cell function; HOMA2 IR: homeostatic model assessment of insulin resistance; HbA1c: hemoglobin A1c; nHbA1c: HbA1c normalized to 84 day average hemoglobin mass; MCP-1: monocyte chemoattractant protein-1.

correlation; the higher the BMI the larger the effect on HbA1c decline. Higher BMI subjects presented with higher MCP-1. The cohort 2 outcome remained correlated with expected changes in β-cell function and with fasting glucose.

Thus the general population enrolled in cohort 1 was a very different ensemble of participants than those enrolled in cohort 2. While the relationships of change in HbA1c with changes in β-cell function and fasting glucose remained, the relationship to weight loss was not seen in the cohort 2 participants selected with higher BMI inclusion criteria.

4.3. Placebo Correlates. Cohort 1 placebo group HbA1c change was dependent only on baseline inflammation status (TNFα and day 84 TNFα change). In cohort 2, there were no placebo correlates to HbA1c change. Importantly, fasting glucose change was not correlated with HbA1c change in this group, indicating that glucose levels were uncoupled from the HbA1c surrogate marker. Rather, placebo HbA1c variance (CV) was correlated strictly with inflammation in both cohorts. This was evidenced by correlation to baseline TNFα in cohort 1 and dependent on both changes in MCP-1 and surprisingly hemoglobin CV in cohort 2. Hb CV was in turn dependent on baseline MCP-1 (TNFα was not measured).

In cohort 1 this later dependency on Hb CV was not detected perhaps due to the heterogeneity of the more general patient population (including nonobese and noninflamed diabetics). In cohort 2, placebo HbA1c CV was negatively correlated with weight change, indicating that higher weight led to increased variance. Cohort 2 placebo weight loss was unexpectedly unrelated to HbA1c and glucose control. Since placebo HbA1c CV was correlated with TNFα change in cohort 1, the weight loss associated with higher HbA1c CV in the cohort 2 placebo group is presumed to be related

to inflammation effects on satiety or metabolism leading to changes in caloric intake and energy balance.

4.4. HE3286 Treatment Effects. The significant correlation of changes in HbA1c and hemoglobin was an unexpected observation as hemoglobin is considered an invariant biomass from which HbA1c is formed as a reflection of total hyperglycemia and therefore its status as an FDA approved biomarker. Inspection of individual patient HbA1c changes revealed a high degree of intravisit variance, contrary to its presumed highly controlled and stable total body mass. Further exploration of the hematopoietic elements gathered with the safety data demonstrated these variant effects were not only on the hemoglobin mass but also on other components such as RBC, hematocrit, mean red cell volume, mean corpuscular hemoglobin and platelets, as well as a variety of metabolic parameters including glucose and cholesterol.

Unexpected variance in metabolic and hematologic parameters related to the effects of chronic low-grade inflammation in uncontrolled obese diabetes produced a significant barrier to these analyses and data interpretation. The variances that caused differing distributions and dispersions between treatment and placebo groups' coupled with the HE3286 treatment effect presented significant statistical challenges. Statistically random effects in the treatment-naïve placebo group were demonstrated for day 84 changes in glucose and in the key surrogate parameters hemoglobin, HbA1c and HOMA2 %B. The increasing variances in individual HbA1c change with metabolic disease progression demonstrated median changes of zero for dyslipidemic, IGT, and T2DM patients. Statistically, random effects are assumed to be the realization of a normal distribution with a mean of zero and a variance that can be estimated. In order to investigate HE3286 treatment effects, we were prompted to remove this random component by normalizing HbA1c to the day 84 average total hemoglobin mass (mean 84-day change of zero) for each patient.

In the broadly defined population of metformin-treated T2DM patients (cohort 1), the HE3286 responsive patient population was found in the upper two tertiles of the inflammation marker MCP-1 (≥40 pmol/L). In the inflamed treatment-naïve patients' population studied in cohort 2, the responding population was found above the median BMI (obese subjects, >31 kg/m^2). The magnitude of the treatment response was indeed greater in the treatment-naïve (cohort 2) than metformin-treated patients.

In both cohort 1 and 2, HE3286 treatment was associated with a total Hb mass normalization evidenced by day 84 data distributions and decreased variances in numerous metabolic and erythroid values. For several of these dysregulated parameters, HE3286 did not show a significant correction until day 84. We interpret these results to indicate that HE3286, via its anti-inflammatory activity, decreased inflammation-driven metabolic dysregulation.

HE3286 showed a significant effect to improve insulin resistance in IGT subjects [9] and to decrease HOMA2 IR in cohort 1 T2DM patients, but not in cohort 2. It is possible that since cohort 2 was naïve, previously untreated T2DM patients and showed higher variances, additional improvements would be observed with longer treatment time frames or drug combination therapy.

4.5. Metabolic Disease, Variance, and Random Effects. The relationship between inflammation and increased variances in erythroid and metabolic laboratory parameters was investigated in clinical settings of increasing chronic low-grade inflammation, adiposity, and metabolic dysregulation. Compared to a healthy group, significantly increased variances were observed for hematocrit, and HbA1c for dyslipidemic, IGT, and T2DM patients. RBC and hemoglobin values were also significantly variable, and the fasting glucose was variable in both dyslipidemic and T2DM patients. In treatment-naive T2DM, high variances and random effects were observed in a large number of metabolic and hematologic parameters that the medical community relies on for medical diagnoses. These changes were correlated with increased inflammatory mediators. This data supports our hypothesis that, in obese subjects, adipose tissue inflammation contributes to both metabolic and hematologic dysregulation within the same individuals.

This is the first clinical report of extreme fluctuations in the marker HbA1c in patients with uncontrolled type 2 diabetes mellitus, but there are published data for type 1 diabetes mellitus (T1DM). Fluctuations in %HbA1c of more than 1% occurred in 50% of the patients year to year, and over 9 years the minimum-maximum range was >3% and >5% HbA1c in 55% and 11% of patients, respectively, [17]. In T1DM subjects followed for 4 years, there was high CV for intraindividual HbA1c measurements (15.5 ± 8.1%), which was lower for patients with good glycemic control. Intrasubject variations of fasting glucose and HbA1c (HbA1c 6–8%, with <10% variation in HbA1c over the last two months) were determined in healthy subjects and T1DM patients with good glycemic control [18]. Glucose intrasubject CV, were 5.4% (range 4.6–6.0) for healthy and 30.5% (26.7–35.5) for T1DM. HbA1c CV, were 1.2% (1.1–1.4) for healthy and 1.7% (1.5–1.9) for T1DM with good glycemic control. Longitudinal changes in T1DM glycemic control gave a significant positive association between baseline HbA1c and CV for intraindividual HbA1c ($P < 0.01$) [19].

HbA1c is a useful marker for detection of patients with elevated fasting and postprandial glucose. The ADA recommends that anyone with HbA1c > 7 be treated with additional agents to return them to a glucose-controlled state. In this specific patient population of type 2 diabetes with HbA1c that is uncontrolled according to the ADA recommendations, the authors have found that the basic hypothesis of stable hemoglobin and red cell lifespan allowing extrapolation from HbA1c change and glucose control is flawed, that intravisit fluctuations can be large, and that a change in HbA1c values between two visits is unlikely to reflect a meaningful therapeutic effect on glucose control in this uncontrolled population. Thus, in clinical efficacy studies in patients with poorly controlled HbA1c, the authors recommend that additional tests of glucose control be used for determination of efficacy of new antidiabetic therapies. Numerous publications argue for the improved management by using continuous glucose monitoring, and for the Ptime-averaged effects of

using 1,5-anhydroglucitol [28] to better understand variation in glucose control.

4.6. HE3286-0401 Conclusions. The hypotheses tested in this study appear to be borne out in the high adiposity T2DM patient. HE3286 preferentially improved clinical parameters in obese inflamed insulin-resistant T2DM patients. Since inflammatory changes were driving HbA1c changes in the placebo group for both cohorts 1 and 2, the changes observed with HE3286 treatment appear to be due to its anti-inflammatory activity (i.e., to break the cycle of inflammatory kinase-mediated inhibition of insulin receptor signaling). Furthermore, T2DM subjects that lack chronic, low-grade inflammation lack the specific lesion in the insulin receptor signaling pathway that HE3286 was developed to interdict. Their glucose intolerance arises for other reasons, and therefore they are unaffected by HE3286.

Obese type 2 diabetic incidence is increasing at an alarming rate. Regaining glucose control and metabolic regulation and preventing or delaying macrovascular and microvascular complications could help to contain rising health care costs for end-stage diabetes complications. Understanding which patients are to benefit from a new therapy is now a regulatory consideration. The FDA has published the Critical Path Initiative, with personalized medicine, or the patient-specific information to individualize therapy and disease management as a major theme, and published on the importance of clinical validation of personalized medicine selection criteria in diabetes [29]. Based on estimates of obese, inflamed diabetics in the future at approximately 50% (J. Olefsky, personal communication), HE3286 offers a potentially important personalized medicine for these subjects.

HE3286 is active at low (hormonal level) doses and is an anti-inflammatory insulin sensitizer with a toxicology profile conducive for chronic daily use [1]. In the responsive subpopulations HE3286 significantly decreased HbA1c compared to placebo, by day 84 in metformin-treated subjects with high MCP-1, and by day 112 in treatment-naïve subjects with high BMI. The data presented here in uncontrolled T2DM patients make a compelling argument for further testing of HE3286 in the high adiposity, inflamed T2DM patient subset, using oral glucose tolerance testing, 1,5-AH, or continuous glucose monitoring to assess treatment effects. The correlation or lack thereof with the surrogate marker HbA1c should be confirmed in these uncontrolled patients.

Conflict of Interests

C. Reading, J. F. Riveros, D. Stickney and J. Frincke are or were employees of Harbor Therapeutics, Inc. J. Frincke is a member of the board of directors. C. Reading and J. Frincke are shareholders. The authors have no other conflicts of interest.

References

[1] C. N. Ahlem, M. R. Kennedy, T. M. Page et al., "Studies of the pharmacology of 17α-ethynyl-androst-5-ene-3β,7β,17β-triol, a synthetic anti-inflammatory androstene," *International Journal of Clinical and Experimental Medicine*, vol. 4, no. 2, pp. 119–135, 2011.

[2] K. E. Wellen and G. S. Hotamisligil, "Obesity-induced inflammatory changes in adipose tissue," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1785–1788, 2003.

[3] J. M. Olefsky and C. K. Glass, "Macrophages, inflammation, and insulin resistance," *Annual Review of Physiology*, vol. 72, pp. 219–246, 2009.

[4] S. E. Shoelson, J. Lee, and A. B. Goldfine, "Inflammation and insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1793–1801, 2006.

[5] M. Lu, D. Patsouris, P. Li et al., "A new antidiabetic compound attenuates inflammation and insulin resistance in Zucker diabetic fatty rats," *American Journal of Physiology*, vol. 298, no. 5, pp. E1036–E1048, 2010.

[6] T. Wang, S. Villegas, Y. Huang et al., "Amelioration of glucose intolerance by the synthetic androstene HE3286: link to inflammatory pathways," *Journal of Pharmacology and Experimental Therapeutics*, vol. 333, no. 1, pp. 70–80, 2010.

[7] C. L. Reading, J. M. Frincke, and S. K. White, "Molecular targets for 17alpha-ethynyl-5-androstene-3beta, 7beta, 17beta-triol, an anti-inflammatory agent derived from the human metabolome," *PLoS One*, vol. 7, Article ID e32147, 2012.

[8] N. Barbarroja, R. López-Pedrera, M. D. Mayas et al., "The obese healthy paradox: is inflammation the answer?" *Biochemical Journal*, vol. 430, no. 1, pp. 141–149, 2010.

[9] C. Reading, D. Stickney, J. Flores-Riveros et al., "A synthetic anti-inflammatory sterol improves insulin sensitivity in insulin-resistant obese impaired glucose tolerance subjects," *Obesity*. In press.

[10] R. M. Cohen, R. S. Franco, P. K. Khera et al., "Red cell life span heterogeneity in hematologically normal people is sufficient to alter HbA1c," *Blood*, vol. 112, no. 10, pp. 4284–4291, 2008.

[11] M. Ghashghaeinia, J. C. Cluitmans, A. Akel et al., "The impact of erythrocyte age on eryptosis," *British Journal of Haematology*, vol. 157, pp. 606–614, 2010.

[12] B. Bistrian, "Systemic response to inflammation," *Nutrition Reviews*, vol. 65, no. 12, pp. S170–S172, 2007.

[13] N. Grossin, M. P. Wautier, and J. L. Wautier, "Red blood cell adhesion in diabetes mellitus is mediated by advanced glycation end product receptor and is modulated by nitric oxide," *Biorheology*, vol. 46, no. 1, pp. 63–72, 2009.

[14] H. Vlassara, J. Valinsky, and M. Brownlee, "Advanced glycosylation endproducts on erythrocyte cell surface induce receptor-mediated phagocytosis by macrophages. A model for turnover of aging cells," *Journal of Experimental Medicine*, vol. 166, no. 2, pp. 539–549, 1987.

[15] K. P. Conroy, I. M. Davidson, and M. Warnock, "Pathogenic obesity and nutraceuticals," *Proceedings of the Nutrition Society*, vol. 70, pp. 426–438, 2011.

[16] F. León-Velarde, F. C. Villafuerte, and J. P. Richalet, "Chronic mountain sickness and the heart," *Progress in Cardiovascular Diseases*, vol. 52, no. 6, pp. 540–549, 2010.

[17] B. M. Singh, C. Mcnamara, and P. H. Wise, "High variability of glycated hemoglobin concentrations in patients with IDDM followed over 9 years: what is the best index of long-term glycemic control?" *Diabetes Care*, vol. 20, no. 3, pp. 306–308, 1997.

[18] S. Carlsen, P. H. Petersen, S. Skeie, O. Skadberg, and S. Sandberg, "Within-subject biological variation of glucose and

HbA(1c) in healthy persons and in type 1 diabetes patients," *Clinical Chemistry and Laboratory Medicine*, vol. 49, pp. 1501–1507, 2011.

[19] R. Jorde and J. Sundsfjord, "Intra-individual variability and longitudinal changes in glycaemic control in patients with type 1 diabetes mellitus," *Diabetic Medicine*, vol. 17, no. 6, pp. 451–456, 2000.

[20] D. R. Stickney, J. R. Groothuis, C. Ahlem et al., "Preliminary clinical findings on NEUMUNE as a potential treatment for acute radiation syndrome," *Journal of Radiological Protection*, vol. 30, no. 4, pp. 687–698, 2010.

[21] D. R. Stickney, C. N. Ahlem, E. Morgan, C. L. Reading, N. Onizuka, and J. M. Frincke, "Phase I and Phase II clinical trials of androst-5-ene-3β,7β,17β-triol," *American Journal of Translational Research*, vol. 3, no. 3, pp. 275–283, 2011.

[22] J. Sun, J. T. Dou, X. L. Wang et al., "Correlation between 1, 5-anhydroglucitol and glycemic excursions in type 2 diabetic patients," *Chinese Medical Journal*, vol. 124, pp. 3641–3645, 2011.

[23] J. Jager, V. Corcelle, T. Grémeaux et al., "Deficiency in the extracellular signal-regulated kinase 1 (ERK1) protects leptin-deficient mice from insulin resistance without affecting obesity," *Diabetologia*, vol. 54, no. 1, pp. 180–189, 2011.

[24] S. J. Lee, P. T. Pfluger, J. Y. Kim et al., "A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis," *EMBO Reports*, vol. 11, no. 3, pp. 226–232, 2010.

[25] A. Rodriguez, A. Durán, M. Selloum et al., "Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62," *Cell Metabolism*, vol. 3, no. 3, pp. 211–222, 2006.

[26] B. Emanuelli, D. Eberlé, R. Suzuki, and C. R. Kahn, "Over-expression of the dual-specificity phosphatase MKP-4/DUSP-9 protects against stress-induced insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 9, pp. 3545–3550, 2008.

[27] N. Bashan, K. Dorfman, T. Tarnovscki et al., "Mitogen-activated protein kinases, inhibitory-κB kinase, and insulin signaling in human omental versus subcutaneous adipose tissue in obesity," *Endocrinology*, vol. 148, no. 6, pp. 2955–2962, 2007.

[28] C. M. Hightower, B. Y. Salazar Vazquez, S. Woo Park et al., "Integration of cardiovascular regulation by the blood/endothelium cell-free layer," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, vol. 3, pp. 458–470, 2011.

[29] C. C. Harper, "Personalized medicine in diabetes: regulatory considerations," *Journal of Diabetes Science and Technology*, vol. 3, no. 4, pp. 739–742, 2009.

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Research Article

Interleukin-1-Beta and Dyslipidemic Syndrome as Major Risk Factors for Thrombotic Complications in Type 2 Diabetes Mellitus

Oana Bădulescu,[1] Codruța Bădescu,[2] Manuela Ciocoiu,[1] and Magda Bădescu[1]

[1] *Department of Pathophysiology, University of Medicine and Pharmacy "Grigore T. Popa," Universității Street, No. 16, 700115 Iași, Romania*
[2] *Department of Internal Medicine, University of Medicine and Pharmacy "Grigore T. Popa," Universității Street, No. 16, 700115 Iași, Romania*

Correspondence should be addressed to Magda Bădescu; magda.badescu@gmail.com

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Diabetes mellitus (DM) is a complex disease characterized by chronic hyperglycemia, a known risk factor for accelerated atherosclerosis and vascular disease. The aim of this study was to show that the connection between DM and other risk factors, such as dyslipidemia, inflammatory phenomena, or the development of certain vascular injuries, leads to a high frequency of thrombotic events in diabetic patients compared to the nondiabetic population. The study included one hundred eighty patients divided in the following groups: diabetic without ischemic cardiopathy-related disorders (DM), diabetic with clinical or off-clinical (electrocardiogram, cardiac ultrasound) ischemic cardiopathy-related disorders (DM + IC), and nondiabetic with ischemic cardiopathy-related disorders (IC). We investigated the following parameters: von Willebrand Factor, HDL-cholesterol, LDL-cholesterol, interleukin-1-beta, protein C, and plasminogen activator inhibitor type 1. The results achieved in our study have revealed the highest thrombotic risk among the groups of diabetic patients, which is in direct correlation with the high values of interleukin-1-beta and the modifications of lipid parameters, acknowledging the data in the literature, according to which hyperglycemia alters endothelial functions directly and indirectly by synthesis of growth factors and cytokines and generates metabolic disorders which would explain the high risk for thrombotic events.

1. Introduction

Diabetes mellitus, a disorder with a thrombophilic potential, became an endemic pathology worldwide, alarming not only because of its high incidence, but also because of its serious complications that lead to very costly medical assistance and even death. Numerous clinical observations [1] emphasized the frequent association of high blood pressure, DM, hyperlipidemia, and thrombotic complications, association that is likely to have a common pathogenesis-inflammatory endotheliopathy.

Hyperglycemia may cause vessel damage through the following pathways: advanced glycation end product (AGE) formation, activation of protein kinase C (PKC), and sorbitol accumulation by way of the polyol pathway [2]. These pathways could be linked by an increased production of superoxide anions [3]. The hyperglycemia induced by increased oxidative stress and receptor for advanced glycation end products (RAGE) activation increases the activation of transcription factor-jB (NF-jB) in endothelial and vascular smooth muscle cells. This transcription factor regulates the expression of the genes encoding a number of mediators of atherogenesis such as leukocyte-cell adhesion molecules and chemoattractant proteins that recruit lymphocytes and monocytes into the vascular wall. Activation of the NF-jB pathway may also cause a switch of the endothelial functions toward a prothrombotic condition that, together with an altered platelet metabolism and intraplatelet signaling

Table 1: The repartition of the patients by age and gender.

Age	Number of patients between 61–70 years old	Number of patients between 71–82 years old
Female	49	49
Male	36	46

pathways and with the inflammatory sindrome, contributes to the pathogenesis of atherothrombotic complications in diabetes mellitus.

The physiopathology of the changes in the lipid metabolism of DM is multifactorial and incompletely deciphered. Quantitative anomalies of lipoproteins (LP) consist of increasing levels of triglycerides as the VLDL and IDL and decreased levels of HDLc due to the decrease of the subfraction HDL_2. The decrease of HDLc is due to the rise in its catabolization. Qualitative anomalies of LP include changes in the size of LP (big VLDL particles, small and dense LDLc particles), increase of the contents of triglycerides in LDLc and HDLc, the glycation of apolipoproteins and lipids, and the increase of LDLc susceptibility to oxidation. These anomalies of LP alter their normal metabolism, increase their atherogenic capacity, and contribute to the promotion of accelerated atherogenesis in diabetic patients [4]. Small and dense LDLc fractions are the most atherogenous, increasing the risk of coronary disease up to three times.

The aim of this study was to show that the connection between DM and other risk factors, such as dyslipidemia, inflammatory phenomena, or the development of certain vascular injuries, leads to a high frequency of thrombotic events in diabetic patients compared to the nondiabetic population.

2. Materials and Methods

2.1. Patients. The study comprises 180 patients, 98 females and 82 males (Table 1), with ages between 61 and 82. The patients were followed between June 1, 2009 and December 1, 2009.

The patients were distributed in 6 groups (Table 2).

Group 1: diabetic women with clinical or off-clinical disorders (electrocardiogram, cardiac ultrasound) related to ischemic cardiopathy (41 patients) (DM + IC).

Group 2: diabetic women without ischemic cardiopathy-related disorders (37 patients) (DM).

Group 3: nondiabetic women with ischemic cardiopathy-related disorders (20 patients) (IC).

Group 4: diabetic men with clinical or off-clinical ischemic cardiopathy-related disorders (29 patients) (DM + IC).

Group 5: diabetic men without ischemic cardiopathy-related disorders (33 patients) (DM).

Group 6: nondiabetic men with ischemic cardiopathy-related disorders (20 patients) (IC).

The selection criteria of the diabetic patients were the presence of type 2 diabetes mellitus treated by diet and/or oral antidiabetic drugs and the duration of the disease between 2 and 10 years.

The patients with cardiovascular disorders have been included in the study based on the following criteria: the presence of signs and symptoms of any cardiovascular disease such as effort stable pectoral angina, aggravated pectoral angina, and chronic myocardial infarction; the values of systolic blood pressure >145 mmHg and of diastolic blood pressure >95 mmHg; the presence of arrhythmia of any kind: atrial and ventricular extra-systolic arrhythmias, supraventricular arrhythmias-atrial fibrillation, or atrial flutter of ischemic cause. In the study we also included the patients with the following cardiovascular disorders in personal pathological antecedents: coronary disease under any manifestation form, rheumatismal or non-rheumatismal valvulopathy, ischemic or hypertensive dilated cardiomyopathy, ischemic stroke.

2.2. Analyzed Parameters. von Willebrand Factor (vWF), protein C (PC), plasminogen activator inhibitor type 1 (PAI-1), and interleukin-1-beta (IL-1β) were determined using plasma; venous blood, collected in 0,105 M sodium citrate vacutainer (sodium citrate/blood ratio = 1/9), was centrifuged 15 minutes at 2500 rpm followed immediately by plasma separation and its freezing. Plasma obtained by centrifugation was frozen (no longer than 4 weeks) at −20 degree Celsius until the tests were performed.

(i) *von Willebrand Factor* measurement was performed by an immunoturbidimetry assay(vWF : Ag) on an automatic analyzer, using reagents from the Biomnis Laboratory, France. Reference values = 50–160%.

(ii) *Protein C* was determined by an enzymatic immunoassay test (EIA) using reagents from the Biomnis Laboratory, France. Reference values: 65–140%.

(iii) *PAI-1* was determined using a chromogenic substrate method with reagents from the Biomnis Laboratory, France. Reference values: <10 kU/L.

(iv) *IL-1β*: the test was performed using ELISA method. Reference values: <3.9 ng/mL.

(v) *HDL Cholesterol (HDLc)*: the venous blood collected in a vacutainer without anticoagulant, was centrifuged to obtain the serum, and the test was performed using an enzymatic colorimetric method on a TECAN microplate reader by commercially available kits (Audit Diagnostics, Ireland). Reference values: 65–100 mg/dL.

(vi) *LDL Cholesterol (LDLc)*: was determined according to the Friedewald formula. Reference values: 0–130 mg/dL.

2.3. Statistical Data Interpretation. Data were expressed as mean ($\overline{x}$) ± standard deviation (SD).

The Pearson correlation (r) indicates the degree of linear dependence between the variables (and it ranges from −1

Table 2: The groups of patients taken in the study.

	Diabetic patients with clinical or off-clinical disorders related to ischemic cardiopathy (number of patients) F/M	Diabetic patients without clinical or off-clinical disorders related to ischemic cardiopathy (number of patients) F/M	Nondiabetic patients with ischemic cardiopathy-related disorders (number of patients) F/M
Total number of patients (F/M)	**70** 41/29	**70** 37/33	**40** 20/20
Age between 61 and 70 years (F/M)	**42** 24/18	**28** 15/13	**15** 10/5
Age between 71 and 82 years (F/M)	**28** 17/11	**42** 22/20	**25** 10/15

F: female, M: male.

Table 3: The mean values of monitored parameters in the studied groups.

Group	Gender	vWF (%) $\overline{x} \pm$ SD	IL-1β (ng/mL) $\overline{x} \pm$ SD	HDLc (mg/dL) $\overline{x} \pm$ SD	LDLc (mg/dL) $\overline{x} \pm$ SD	C Prot (%) $\overline{x} \pm$ SD	PAI-1 (kU/L) $\overline{x} \pm$ SD
DM + IC	F versus M	232.1 ± 30.87	16.11 ± 7.51	55.11 ± 7.99	137.2 ± 24.32	56.3 ± 17.09	15.06 ± 3.81
	$P > 0.05$	224.8 ± 64.00	14.37 ± 6.38	56.44 ± 8.94	144.7 ± 37.06	46.8 ± 7.90	15.2 ± 2.74
DM − IC	F versus M	202.0 ± 52.95	14.47 ± 6.74	57.41 ± 12.41	142.1 ± 36.81	65.4 ± 15.02	13.3 ± 3.59
	$P > 0.05$	184.4 ± 59.56	13.84 ± 7.06	58.78 ± 7.87	140.6 ± 37.14	60.1 ± 3.07	12.3 ± 3.13
Non DM + IC	F versus M	185.7 ± 67.79	5.68 ± 2.78	53.45 ± 13.36	169.9 ± 11.94	74.0 ± 25.27	10.8 ± 4.26
	$P > 0.05$	165.6 ± 75.09	6.74 ± 3.91	52.46 ± 7.22	170.4 ± 24.49	73.1 ± 17.02	10.0 ± 2.62

DM + IC: patients with diabetes mellitus and ischemic cardiopathy.
DM − IC: patients with diabetes mellitus without ischemic cardiopathy.
Non DM + IC: patients without diabetes mellitus and with ischemic cardiopathy.
F: female, M: male.

Table 4: The correlation matrix between the monitored parameters in patients from DM + IC group (according to the coefficient of Pearson correlation-r).

Parameter	vWF (%) r	IL-1β (ng/mL) r	HDLc (mg/dL) r	LDLc (mg/dL) r	C Prot (%) r	PAI-1 (kU/L) r	
vWF (%)	1	0.38	−0.33	0.11	−0.11	−0.38	
IL-1β (ng/mL)	**−0.54**	1	0.17	**−0.42**	0.37	−0.12	
HDLc (ng/mL)	0.23	**−0.7**	1	−0.11	−0.03	0.27	Males
LDLc (ng/mL)	0.32	−0.35	0.33	1	**−0.88**	−0.19	
C Protein (%)	**−0.7**	−0.48	**−0.56**	−0.42	1	0.08	
PAI-1 (kU/L)	0.14	**0.66**	**−0.53**	−0.4	−0.12	1	
			Females				

to +1). As it approaches zero there is less of a relationship (closer to uncorrelated). The closer the coefficient is to either −1 or 1, the stronger the correlation between the variables.

Unpaired Student's t-test was performed to determine whether there were significant ($P < 0.05$) differences between groups. The threshold of statistical significance which is unanimously accepted is 95%, namely, $P = 0.05$. The smaller the P value is than this value, the stronger the statistical significance ($P > 0.05$) statistically insignificant.

3. Results and Discussions

The mean values of monitored parameters (Tables 3 , 4, 5, and 6) have not shown significant differences between genders ($P > 0.05$) in any of the investigated groups.

3.1. Groups DM + IC. Within these groups of patients, the following parameters showed the most modified values: vWF, PAI-1, PC, and IL-1β. Regarding the distribution by gender, in females the values of the analyzed parameters have been slightly modified compared to those registered in males, without revealing a significant statistical difference between men and women.

The following significant correlations have been identified among the analyzed parameters.

(i) Females:

(a) indirect correlations between:

(1) vWF and PC ($r = -0.70$),
(2) HDLc and IL-1β ($r = -0.70$),
(3) HDLc and PAI-1 ($r = -0.53$).

Table 5: The correlation matrix between the monitored parameters in patients from DM − IC group (according to the coefficient of Pearson correlation-r).

Parameter	vWF (%)	IL-1β (ng/mL)	HDLc (mg/dL)	LDLc (mg/dL)	C Prot (%)	PAI-1 (kU/L)	
	r	r	r	r	r	r	
vWF (%)	1	−0.37	−0.12	0.12	0.2	0.16	Males
IL-1β (ng/mL)	−0.33	1	**0.55**	0.24	−0.28	0.38	
HDLc (ng/mL)	0.19	0.11	1	−0.38	−0.25	−0.13	
LDLc (ng/mL)	−0.2	−0.39	0.28	1	0.3	0.21	
C Protein (%)	**−0.7**	0.2	0.29	−0.05	1	−0.33	
PAI-1 (kU/L)	−0.45	0.13	−0.23	0.22	0.14	1	
			Females				

Table 6: The correlation matrix between the monitored parameters in patients from non DM + IC group (according to the coefficient of Pearson correlation-r).

Parameter	vWF (%)	IL-1β (ng/mL)	HDLc (mg/dL)	LDLc (mg/dL)	C Prot (%)	PAI-1 (kU/L)	
	r	r	r	r	r	r	
vWF (%)	1	0.22	**0.62**	−0.05	0.13	**0.71**	Males
IL-1β (ng/mL)	**0.6**	1	0.38	**0.49**	0.53	0.02	
HDLc (ng/mL)	−0.48	−0.47	1	0.02	0.53	**0.5**	
LDLc (ng/mL)	0.3	0.29	−0.21	1	0.1	−0.06	
C Protein (%)	−0.4	−0.4	−0.15	0.42	1	0.2	
PAI-1 (kU/L)	**0.88**	**0.7**	**−0.52**	0.47	−0.24	1	
			Females				

(b) direct correlations between:

(1) IL-1β and PAI-1 ($r = 0.66$).

(ii) Males:

(a) indirect correlations between:

(1) LDLc and PC ($r = -0.88$).

(b) direct correlations between:

(1) IL-1β and vWF ($r = 0.38$).

According to the values we recorded, the plasma levels of vWF are considerably higher especially in the group of diabetic patients (DM + IC, DM − IC). The higher plasma levels are probably an expression of diabetes mellitus-related endotheliopathy.

Regarding the von Willebrand Factor, currently no data exists supporting the assumption that the high plasma levels of the von Willebrand Factor represent a high risk factor for cardiovascular disease. However, it has been proved that in patients with angina pectoris this factor is predictive of later cardiovascular events, and it is also present in high levels in acute coronary syndromes [5]. Other researchers found high plasmatic levels of the vWF in different pathological states involving endothelial damage such as endothelial denudation and subendothelial structure exposure (acute and chronic renal failure, hypertension, diabetic nephropathy, and vasculitis) [6]. Persistent high values of this factor were also found in patients with uncomplicated diabetes mellitus or with diabetic nephropathy [7, 8].

In our study, the presence of direct correlation between IL-1β and PAI-1 ($r = 0.66$) supports the idea according to which the prothrombotic characteristics of the endothelium are also expressed under the action of some inflammation mediators (IL-l, tumor factor necrosis-TNF, etc.); recent evidence show that there is a close connection between the inflammatory processes and thrombosis, even when the endothelium suffers no actual morphological injuries [9]. Insulin stimulates PAI-1 synthesis in the hepatic cells and in smaller proportion in the endothelial cells, whereas endotoxins and IL-1, which have a proinflammatory effect, stimulate PAI-1 production in the endothelial cells but has no effect on it in the hepatic cells. The expression of proinflammatory cytokines and other mediators, including adhesion molecules, suggests that the inflammatory process may contribute to the vascular disease in diabetes mellitus. According to recent evidence, TNF-α plasma concentration is connected to the insulin resistance phenomenon, and it may be decreased by diet and weight loss, whereas IL-6 and C reactive protein (CRP) are known as having constantly high levels in type 2 DM [10]. All these compounds may induce a phenotype alteration of endothelial cells and/or monocytes able to trigger tissue factor production increase, which is the main procoagulant agent identified in the atheromatous plaque, to the further alteration of the coagulation and fibrinolysis mechanisms.

The indirect correlation between HDLc and PAI-1 ($r = -0.53$) supports the idea that in obese dyslipidemic patients with hyperinsulinemia the increase of the PAI-1 level leads to higher thrombogenic risk. It is well known that endothelial cells regulate fibrinolysis, as they synthesize and release both

plasminogen activators (tisular plasminogen activator and substances such as urokinase) and PAI-1.

Any quantitative or qualitative deficiency of highly anticoagulant molecules may be suspected in case of subjects with high prothrombotic risk [11]. This was probably the reason why protein C and protein S have been studied in DM. Yet, most of the results that made public so far are contradictory [12–14]. Some studies conducted on these factors revealed lower levels, whereas others detected normal values.

The results of our study shows that the diabetic patients were deficient in these factors. The lowest values were recorded in the group of diabetic patients with associated ischemic cardiopathy (DM + IC) followed by the group of diabetic patients with no cardiovascular diseases (DM − IC). This difference may be accounted for by the glycosylation phenomenon undergone by these proteins. The values of protein C have been indirectly correlated with vWF ($r = -0.70$), highlighting the increased thrombotic risk in this category of patients. In addition to its anticoagulant role, the active PC also increases fibrinolytic activity, both by stimulating the tisular plasminogen activator release from the endothelial cells and by neutralizing the PAI-1 activity. The sum of these effects of PC turns the PC system into one of the most important thrombosis protection mechanisms.

3.2. Groups DM − IC. In these groups of patients, we have obtained results that are similar to those underlined in the lot of the diabetic patients with cardiovascular pathology: increased values for the vWF, IL-1β, and PAI-1 and low values for PC.

The following most important correlations are noticed between the analyzed parameters.

(i) Females:

(a) indirect correlation between:

(1) PC and vWF ($r = -0.70$).

(ii) Males:

(a) indirect correlation between:

(1) PC and PAI-1 ($r = -0.30$).

In accordance with our study results, as far as the diabetic population is concerned, the cardiovascular disorder risk is higher for diabetic women than for diabetic men; unlike the general population, where males are more frequently affected by the cardiovascular disorders, the male gender being even construed as a risk factor. The dyslipidemic syndrome occurs as a risk factor for the macrovascular disease in the case of diabetic women, especially those belonging to the second age category, an aspect that may be connected to the disappearance of hormonal protection, the occurrence of obesity, and to the duration of the disease.

The mechanism linking elevated levels of von Willebrand Factor FVIII to insulin resistance and type 2 diabetes may be related to the presence of endothelial dysfunction and/or inflammation, both involved in the development of insulin resistance [15, 16]. Endothelial cell incubation with high concentrations of glucose led to the increase of the vWF contents. Therefore, the high level of vWF in diabetics reflects the effects of hyperglycemia on the endothelium and the fact that there may be a platelet-vWF interaction, which would explain the atherosclerosis acceleration in these patients.

Taking into account the fact that von Willebrand Factor represents a marker of the endothelial injury and that the functioning of the system of the protein C depends also on the morphological and functional integrity of the vascular endothelium, the indirect correlation that we obtained between these two parameters ($r = -0.70$) is by no means surprising.

Even unexposed to injury factors, the morphofunctionally intact endothelium synthesizes and releases the von Willebrand factor and PAI-1, but, nevertheless, the antithrombotic mechanisms prevail at the endothelial level. However, when endothelium is injured, as, for instance, under the action of certain endotoxins, by means superoxide radicals released from leucocytes, or when they are stimulated by proinflammatory cytokines (IL-1, TNF), there is an accelerated release of thromboplastin and of von Willebrand Factor. Moreover, the endothelium reduces the fibrinolytic activity by the marked increase in the synthesis and release of PAI-1.

The indirect correlation between PC and PAI-1 ($r = -0.30$) evidenced by our study shows an increased risk for thrombotic events to this category of patients.

As for the group of diabetic patients without ischemic cardiopathy (DM − IC), one may assume the existence of a vascular involvement with subclinical symptoms, which is also supported by the fact that endotheliopathy undoubtedly precedes not only the occurrence of cardiovascular conditions but also the very onset of diabetes mellitus. This is due to the fact that the synthesis and release of glycoproteins originating in the endothelium are stimulated by metabolic anomalies [13].

3.3. Groups Non DM + IC. In these groups of patients, we have recorded the most significant alterations of the HDLc and LDLc values. They are an important cardiovascular risk factor in both sexes.

The following most important correlations are noticed between the analyzed parameters.

(i) Females:

(a) indirect correlations between:

(1) PAI-1 and HDLc ($r = -0.52$).

(b) direct correlations between:

(1) vWF and PAI-1 ($r = 0.88$).

(ii) Males:

(a) no indirect correlations have been registered;

(b) direct correlations between:

(1) vWF and PAI-1 ($r = 0.71$).

The results obtained in this group of patients highlight a direct correlation between vWF and PAI-1, a similar aspect met in the groups of diabetic patients.

It has been proven that dyslipidemia is an independent cardiovascular risk factor, and only one in four patients requiring hypolipidemic therapy reaches the recommended target values, in today's worldwide medical practice [17]. Consequently, it is by screening, early diagnosis and intensive dyslipidemia therapy in DM patients that the risk of coronary events may be reduced, that atherosclerotic injury progress may be diminished or prevented, and that already existing atherosclerotic injuries may possibly be recovered.

In many types of vascular-endothelial injuries, a drop in the fibrinolytic capacity of the endothelium was recorded, either by plasminogen activator synthesis decrease or by an increase in the amount of PAI-1 released after the injury, an idea confirmed in our study by the direct correlation between vWF and PAI-1, both in females and males.

Small and dense LDLc particles seem to be the marker of a series of anomalies including HDLc concentration decrease, apoB concentration increase, insulin sensitivity decrease, and procoagulant changes (PAI-1 increase).

The observations on the atherogenic lipoprotein phenotype and its association with different other proatherogenic alterations have raised the hypothesis of specific genetic interdependences between them. The oxidized LDLc has the following effects: it inhibits the NO and the prostacyclin and disturbs the basic vascular tonus; it stimulates the endothelial cells to release a number of active biological factors (the chemotactic protein for monocytes (MCP-1), the molecules of endothelium-leucocytes adhesion (ELAM), and growth factors; it modifies the aggregability of thrombocytes; it may activate the T leukocytes in the atherosclerosis lesion; it may stimulate the proliferation of smooth muscular cells by inducing the expression and the genetic codification of the growth factor derived from the thrombocyte (PDGF) [18]. Arguments for considering the LDLc oxidized as an essential factor in producing the early atheromatous lesions are the presence in high quantity of the oxidized LDLc at the atherosclerotic lesion site and the capacity of the vascular wall cells (monocytes, macrophagous, endothelial cells, and smooth vascular muscular cells) to produce reactive oxygen species ($O_2^{\bullet -}$, H_2O_2) and to induce LDLc oxidation.

Researchers proved, in 1990, that the genetic locus responsible for the occurrence of small and dense LDLc is associated with high levels of TG, apoB, with VLDL and IDL masses, and with HDLc and apoA1 decrease. Moreover, genetic linkage studies showed that the LDLc particle sizes as well as the HDLc and LDLc values present positive correlations with an allele of the hepatic lipase gene. The genetic mutations occurring in the locus of the CTEP (cholesteryl ester transfer protein) on chromosome 16 may influence the LDLc sizes and TG values, due to the role it plays in the reverse cholesterol transport. Peroxisome proliferator-activated receptors (PPARα) play an important role in lipid metabolism. In the liver, PPARα activation regulates the gene expression of apoAI, AII, and CIII and of specific endoenzymes involved in β-oxidation of fatty acids, whereas PPARα activation in the macrophages induces, through complex mechanisms, the expression of the receptors that HDLc interact with during the reverse cholesterol transport process. Recent data supports PPARα involvement in inflammatory response modulation in the arterial endothelium [19].

However, in nondiabetic patients with ischemic cardiopathy (IC), as the endothelial dysfunction is less severe or even absent, the evolution of these parameters was positive after the delivery of the anticoagulant therapy.

To conclude, dyslipidemia [20] is a frequent condition of diabetes and may contribute to the increase of the cardiovascular risk in these patients.

4. Conclusions

The results of our study concerning the researches on lipid metabolism, the proinflammatory activity of plasma, and coagulation parameters have shown that type 2 diabetes mellitus registers a significant frequency in cumulating the main factors of vascular and atherosclerotic risk, the umoral dyslipidemic syndrome (which is characterized by the LDLc increase and HDLc decrease), and the increase in inflammatory markers (IL-1 beta), age, and duration of diabetes, encouraging the increased risk for thrombotic events with lethal potential (increase of vWF, PAI-1, decrease of PC).

Three mechanisms may link the inflammation to the emergence of the diabetes mellitus:

(1) the discovery in diabetic patients of the expression of a new Tanis gene, the protein product of which links seric amiloid A;

(2) the correlation between the inflammatory process and the level of insulinemia according to a trial on rodents, where the administration of the salicylate led to obesity prevention and insulin resistance [21];

(3) the most interesting mechanism, based on the observation that the adiponectin (a protein specific to the adipose tissue which has strong anti-inflammatory properties) [16] is found in small concentrations in obese persons, its concentration increasing as body weight decreases.

Studies in the literature have shown that increased level of inflammatory markers is an independent predictive factor of cardiovascular disease and also that their high plasma levels are correlated with the risk of developing of these clinically manifest diseases. The significantly increased values of interleukin-1-beta evidenced by our study in the groups of diabetic patients require the inclusion of its determination, as well as of other inflammatory markers, in the battery of tests aimed at the prophylaxis of thrombotic events. Beside the inappropriate glycemic control, the insulinresistance, the consecutive hyperinsulinism, an increased flow of fat acids, and proinflammatory cytokines released from the visceral adipose tissue, with direct access to the liver, could induce the procoagulant modifications identified in this study. Hemostasis abnormalities revealed by our study include the increased values of von Willebrand Factor and PAI-1 and decreased levels of protein C. Taking into account the fact that

a significant modification of the plasmatic levels of protein C, von Willebrand Factor and PAI-1 could be identified at diabetic patients without clinically manifest cardiovascular diseases, these modifications with prothrombotic potential have been considered to precede the thrombotic complications, having a predictive role for such complications. Considering the significant reduction in the plasmatic levels of the natural coagulation inhibitors at diabetic patients, the synthesis of these protease inhibitors is construed to be affected by the metabolic abnormalities that characterize diabetes, while the enzymatic glycosylation could reduce their anticoagulant effect. In practice, although the assessment of the haemostatic factors does not stand for a routine conduct yet, it would be important for the clinician to know what factors should be explored and to what extent the correction or treatment of the other associated diseases can improve the haemostasis disorders. The procoagulant modifications signaled in this study sum with the deficit of the fibrinolytic activity and with the hyperactivity of platelets, while the risk of diabetic patients' thrombotic complications must also be evaluated in this context too. Concerning the atherothrombotic process, there is a constant connection between the atherogenic lipoproteins (especially oxidized LDLc and LP(a)) and the vascular endothelium. This relation focuses especially on the depressed fibrinolysis phenomenon, hence, the balance deviation between coagulation and fibrinolysis in favor of the former, and the occurrence of thrombotic accidents. The large number of patients with type 2 diabetes mellitus and macrovascular disease requires the investigation of carbohydrate metabolism in nondiabetic patients with macrovascular disease in different locations, even if they had initially normal blood sugar levels, as macrovascular disease may occur even before the alterations of glucose values. All these data support the hypothesis that nondiabetic glucose levels are a continuous risk factor for cardiovascular disease.

Based on the above mentioned arguments, it may be suggested that the abnormalities of the glycemic balance "per se" may generate prothrombotic modifications of the haemostatic balance. Type 2 diabetes mellitus and its chronic complications, especially the macrovascular disease, take an important place in the medical care system, reducing the life quality of patients suffering from this medical disorder. Only the reduced glycemia values are not enough in order to reduce the rate of complications. The targets of the preventive therapeutic interventions must refer to all the components of the risk factors, including the measures needed to increase the quality of a healthy life. Precocious prevention of the diabetic macrovascular disease must be individualized for each patient, and it must be achieved in parallel to the precocious diagnosis of diabetes mellitus.

Abbreviations

DM: Diabetes mellitus
DM + IC: Diabetic mellitus with cardiovascular disease
DM − IC: Diabetic mellitus without cardiovascular disease
ELAM: The molecules of endothelium-leucocytes adhesion
HDLc: High-density lipoprotein cholesterol
IC: Ischemic cardiopathy
IL-1β: Interleukin-1-beta
LDLc: Low-density lipoprotein cholesterol
LP: Lipoproteins
MCP-1: The chemotactic protein for monocytes-1
NF-jB: Transcription factor-jB
NO: Nitric oxide
PAI-1: Plasminogen activator inhibitor type 1
PDGF: The growth factor derived from the thrombocyte
PKC: Protein kinase C
PPARα: Peroxisome proliferator-activated receptors
RAGE: The receptor for advanced glycation end products
SD: Standard deviation
TG: Triglyceride
VLDL: Very low-density lipoprotein
vWF: von Willebrand Factor.

Ethical Approval

The study protocol was conducted in accordance with the Declaration of Helsinki (1964) and with the understanding and the consent of the human subject. The responsible Ethical Committee has approved the research.

Conflict of Interests

The authors declare that they have no confict of interests.

Authors' Contribution

O. Bădulescu, C. Bădescu, M. Bădescu, and M. Ciocoiu contributed equally to this work.

References

[1] D. Dejica, *Stresul Oxidativ în Bolile Interne*, Casa Cărții de Știință, Cluj-Napoca, Romania, 2000.

[2] M. E. Cooper, F. Bonnet, M. Oldfield, and K. Jandeleit-Dahm, "Mechanisms of diabetic vasculopathy: an overview," *American Journal of Hypertension*, vol. 14, no. 5 I, pp. 475–486, 2001.

[3] T. Nishikawa, D. X. L. Du Edelstein, S. Yamagishi et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, pp. 787–790, 2000.

[4] J. Plutzky, G. Viberti, and S. Haffner, "Atherosclerosis in type 2 diabetes mellitus and insulin resistance: mechanistic links and therapeutic targets," *Journal of Diabetes and its Complications*, vol. 16, no. 6, pp. 401–415, 2002.

[5] M. P. Cucuianu, A. Cristea, and S. Roman, "Comparative behaviour of the components of the factor VIII complex in acute myocardial infarction," *Thrombosis Research*, vol. 30, no. 5, pp. 487–497, 1983.

[6] M. Cucuianu and I. Crâșnic, *Actualități în Patologia Hemostazei și Trombozei*, University Press, Arad, Romania, 1999.

[7] M. H. Borkenstein and W. E. Muntean, "Elevated factor VIII activity and factor VIII-related antigen in diabetic children without vascular disease," *Diabetes*, vol. 31, no. 11, pp. 1006–1009, 1982.

[8] J. Yudkin, "Abnormalities of coagulation and fibrinolzsis in insulin resistance," *Diabetes Care*, vol. 22, supplement 3, pp. C25–C30, 1999.

[9] M. Bădescu, M. Roşca, and C. Bohotin, *Fiziopatologie Generală*, vol. 2, chapter 10, Iasi, Romania, 2000.

[10] A. D. Pradhan, J. E. Manson, N. Rifai, J. E. Buring, and P. M. Ridker, "C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus," *Journal of the American Medical Association*, vol. 286, no. 3, pp. 327–334, 2001.

[11] M. Cucuianu and I. Cârsnic, *Biochimie Clinică—Fundamentare Fiziopatologică*, Cluj-Napoca, Romaina, 1998.

[12] A. Ceriello, D. Giuliano, A. Quatrano et al., "Evidence for a hyperglycemic-dependent decrease of antithrombin III—thrombin complex formation in humans," *Diabetologia*, vol. 33, no. 3, pp. 163–1670, 1990.

[13] P. M. Mannucci, "von Willebrand factor: a marker of endothelial damage?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 9, pp. 1359–1362, 1998.

[14] A. I. Vinik, T. Erbas, T. S. Park, R. Nolan, and G. L. Pittenger, "Platelet dysfunction in type 2 diabetes," *Diabetes Care*, vol. 24, no. 8, pp. 1476–1485, 2001.

[15] S. Haffner, "Insulin resistance, inflammation, and the prediabetic state," *American Journal of Cardiology*, vol. 92, no. 4A, pp. 18j–26j, 2003.

[16] C. M. Streppan, S. T. Bailey, S. Bhat et al., "The hormone resistin links obesity to diabetes," *Nature*, vol. 409, pp. 307–312, 2001.

[17] R. Malmström, C. J. Packard, M. Caslake et al., "Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM," *Diabetologia*, vol. 40, no. 4, pp. 454–462, 1997.

[18] J. A. Colwell and R. W. Nesto, "The platelet in diabetes: focus on prevention of ischemic events," *Diabetes Care*, vol. 26, no. 7, pp. 2181–2188, 2003.

[19] M. R. Taskinen, "Diabetic dyslipidaemia: from basic research to clinical practice," *Diabetologia*, vol. 46, no. 6, pp. 733–749, 2003.

[20] F. Karpe, G. Steiner, K. Uffelman, T. Olivecrona, and A. Hamsten, "Postprandial lipoproteins and progression of coronary atherosclerosis," *Atherosclerosis*, vol. 106, no. 1, pp. 83–97, 1994.

[21] H. N. Gingsberg, "Insulin resistance and cardiovascular disease," *The Journal of Clinical Investigation*, vol. 106, pp. 453–458, 2000.

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Research Article

Adipose Tissue-Specific Deletion of 12/15-Lipoxygenase Protects Mice from the Consequences of a High-Fat Diet

Banumathi K. Cole, Margaret A. Morris, Wojciech J. Grzesik, Kendall A. Leone, and Jerry L. Nadler

Department of Internal Medicine, Strelitz Diabetes Center, Eastern Virginia Medical School, Norfolk, VA 23507, USA

Correspondence should be addressed to Banumathi K. Cole, colebk@evms.edu and Jerry L. Nadler, nadlerjl@evms.edu

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Type 2 diabetes is associated with obesity, insulin resistance, and inflammation in adipose tissue. 12/15-Lipoxygenase (12/15-LO) generates proinflammatory lipid mediators, which induce inflammation in adipose tissue. Therefore we investigated the role of 12/15-LO activity in mouse white adipose tissue in promoting obesity-induced local and systemic inflammatory consequences. We generated a mouse model for fat-specific deletion of 12/15-LO, *aP2-Cre*; *12/15-LO*$^{loxP/loxP}$, which we call ad-12/15-LO mice, and placed wild-type controls and ad-12/15-LO mice on a high-fat diet for 16 weeks and examined obesity-induced inflammation and insulin resistance. High-fat diet-fed ad-12/15-LO exhibited improved fasting glucose levels and glucose metabolism, and epididymal adipose tissue from these mice exhibited reduced inflammation and macrophage infiltration compared to wild-type mice. Furthermore, fat-specific deletion of 12/15-LO led to decreased peripheral pancreatic islet inflammation with enlarged pancreatic islets when mice were fed the high-fat diet compared to wild-type mice. These results suggest an interesting crosstalk between 12/15-LO expression in adipose tissue and inflammation in pancreatic islets. Therefore, deletion of 12/15-LO in adipose tissue can offer local and systemic protection from obesity-induced consequences, and blocking 12/15-LO activity in adipose tissue may be a novel therapeutic target in the treatment of type 2 diabetes.

1. Introduction

Obesity is a rising worldwide epidemic affecting approximately one-third of adults [1]. Obesity is characterized by a persistent exposure to excess nutrients that leads to visceral adiposity and hyperlipidemia, promoting chronic inflammation, insulin resistance, and endoplasmic reticulum stress [2, 3]. This promotes the development of adipocyte dysfunction and systemic decline, including damage to the pancreas, liver, and vascular tissue. Therefore obesity predisposes individuals to the development of type 2 diabetes and cardiovascular disease.

Increasing evidence from our lab suggests that the 12/15-lipoxygenase (12/15-LO) enzyme plays a critical role in promoting adipocyte dysfunction. 12/15-LO oxygenates polyunsaturated fatty acids to generate many proinflammatory lipid mediators (reviewed in [4]). 12/15-LO is largely involved in metabolizing arachidonic acid to form 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and linoleic acid to form 13-hydroperoxyoctadecadienoic acid (13-HPODE), which are further oxidized to 12-hydroxyeicosatetraenoic acid (12(S)-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE), respectively. Our laboratory has demonstrated that 12/15-LO is activated in white adipocytes from mice fed a high-fat diet and from insulin-resistant Zucker obese rats *in vivo* and is also highly expressed in visceral fat of obese human patients [5–7]. Furthermore, addition of 12/15-LO products to 3T3-L1 adipocytes *in vitro* induces inflammation, insulin resistance, and endoplasmic reticulum stress [5, 6, 8]. Finally, global deletion of 12/15-LO in C57BL/6 mice reduces inflammation and endoplasmic reticulum stress in adipose tissue and preserves insulin sensitivity and pancreatic β-cell function when fed a high-fat diet [8–10].

Given that inflammation of adipose tissue is thought to be a preceding step to peripheral metabolic dysfunction [11] and 12/15-LO activity increases inflammation in adipose tissue, we sought to determine whether specific deletion of 12/15-LO in mouse white adipose tissue could protect mice from the local and systemic consequences of a high-fat diet. To this end, we crossed *12/15-LO*$^{loxP/loxP}$ mice to mice harboring the *Cre* transgene driven by the adipocyte-specific *aP2* promoter (*aP2-Cre*) to generate adipose tissue-specific deletion of 12/15-LO. The adipocyte lipid-binding protein (aP2) is thought to function by facilitating intracellular trafficking of lipids [12]. In addition to adipocytes, aP2 is expressed in macrophages and dendritic cells; however, it is expressed in adipocytes approximately 10,000-fold greater [12]. Furthermore, the use of aP2-Cre mice reveals no recombination of loxP targets in macrophages [13]. Wild type and ad-12/15-LO mice were fed a 60% high-fat diet for 16 weeks, and adipose tissue and systemic organ health were assessed. Our results suggest that a high-fat diet activates 12/15-LO in adipose tissue to promote local and systemic inflammation and that deletion of 12/15-LO in adipose tissue can offer systemic protection from the inflammatory consequences of a high-fat diet.

2. Materials and Methods

2.1. Animals and Treatments. *12/15-LO*$^{loxP/+}$ and *aP2-Cre; 12/15-LO*$^{loxP/+}$ mice were generated by Ozgene (Bentley DC, WA, Australia) on a C57BL/6J background. Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a pathogen-free facility at Eastern Virginia Medical School (EVMS), and all experiments were performed in accordance with an animal study protocol approved by the EVMS Institutional Animal Care and Use Committee. Mice were bred to generate homozygous *12/15-LO*$^{loxP/loxP}$ and *aP2-Cre; 12/15-LO*$^{loxP/loxP}$ mice. Male mice were placed on a normal chow or high-fat diet (D12331- consisting of 58% of calories from fat, 16.4% of calories from protein, and 25.5% of calories from carbohydrate, primarily sucrose; Research Diets, New Brunswick, NJ, USA) beginning at 8 weeks of age to 16 weeks ($n = 3$ mice per chow diet group and 5-6 mice per high-fat diet group). Blood glucose was measured using OneTouch Ultra2 Glucose Monitors (LifeScan, Milpitas, CA, USA) from tail vein blood samples. Body weight was measured by placing the mice on a scale using the same scale for each set of measurements. At the end of the 16-week diet regimen, mice were euthanized by CO_2 asphyxiation.

2.2. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT). GTT and ITT were performed as described in [9]. For GTT, mice were fasted overnight and then injected intraperitoneally (IP) with glucose (2 g/kg) in the morning. Blood glucose measurements were performed on tail vein blood samples taken at baseline and at 10, 30, 60, 90, and 120 minutes after injection. The ITT was performed in random-fed mice by IP injection of insulin (0.75 U/kg) in 0.9% NaCl in the afternoon. Blood glucose measurements were performed on tail vein blood samples taken at baseline and at 15, 30, 45, and 60 minutes after injection.

2.3. Serum Measurements. Random-fed mice after the 16-week feeding regimen were euthanized, and blood samples were obtained by cardiac puncture and centrifuged to isolate serum. Insulin and high-molecular-weight adiponectin were measured in serum by ELISA (Mercodia, Uppsala, Sweden and ALPCO Diagnostics, Salem, NH, USA, resp.).

2.4. Adipocyte and Islet Morphometric and Immunohistochemical Analysis. Epididymal adipose and pancreata tissues were isolated and fixed with 10% formalin or Z-fix (Anatech LTD., Battle Creek, MI, USA), respectively, for 4–6 hours at room temperature and embedded in paraffin. 7 μm thick paraffin-embedded tissue sections were then deparaffinized and rehydrated in graduated alcohol in distilled water for processing.

For morphometric analysis of epididymal adipose tissue, sections were counterstained with hematoxylin and eosin and six representative images per section were collected. Adipocyte area was quantified using ImageJ (NIH website: http://rsb.info.nih.gov/ij/download.html). For immunohistochemical analysis of epididymal fat tissue, sections were subjected to antigen retrieval and stained for Mac2 (Cedarlane, Burlington, NC, USA). Detection was performed with the avidin-biotin-peroxidase method and developed with a diaminobenzidine substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA).

For morphometric and immunohistochemical analysis of pancreata tissue, sections were stained for insulin (Abcam, Cambridge, MA, USA) and then with Cy-3-conjugated goat anti-guinea pig (Jackson Immunoresearch Laboratories, West Grove, PA, USA) antibodies. Images of all insulin-stained islets from each section (four sections per mouse) were acquired and measured for islet area, percentage of islet area to pancreas area, and percentage of insulin-secreting β-cell area per islet area. Islet area and insulin staining were quantified using AxioVision Rel. 4.7 (Carl Zeiss, Oberkochen, Germany).

Two-dimensional images were acquired using a Zeiss Plan-Apochromat 10X, 0.45 numerical aperture dry objective lens or a 20X, 0.8 numerical aperture dry objective lens on a Zeiss Axio Observer.Z1 microscope with AxioVision Rel. 4.7 software, and a Zeiss AxioCamMRm (fluorescence) or AxioCamHRc (color) camera (Carl Zeiss). All final images were prepared with Adobe Photoshop CS3 Extended, version 10.0.1.

2.5. Isolation of White Epididymal Adipocytes and Stromal Vascular Cells (SVCs). Isolation of white epididymal adipocytes was based on a protocol previously described [14]. Epididymal fat pads were removed from euthanized mice and minced into fine pieces in Krebs Ringer Hepes-BSA (KRHB) buffer (3 mmol/L glucose, 20 nmol/L adenosine, and 10 mg/mL bovine serum) with 1 mg/mL collagenase. Collagenase digestion was performed at 37°C for 1 hour in a shaking water bath. Once digestion of adipose tissue

Table 1: Primer sequences used in SYBR-based real-time RT-PCR amplification of mouse cDNA.

Mouse genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
12/15-LO	ctctcaaggcctgttcagga	gtccattgtccccagaacct
Actin	aggtcatcactattggcaacga	cacttcatgatggaattgaatgtagtt
Adiponectin	gttgcaagctctcctgttcc	atccaacctgcacaagttcc
CD11c	ctgagagcccagacgaagaca	tgagctgcccacgataagag
Collagen 6 alpha 1	gatgagggtgaagtgggaga	cagcacgaagaggatgtcaa
F4/80	ctttggctatgggcttccagtc	gcaaggaggacagagtttatcgtg
IL-6	gaggataccactcccaacagacc	aagtgcatcatcgttgttcataca
MCP-1	cttctgggcctgctgttca	ccagcctactcattgggatca
Twist-1	cgcacgcagtcgctgaacg	gacgcggacatggaccagg

was complete, the cell suspension was filtered through a 0.4 mm Nitex nylon mesh (Sefar America Inc., Kansas City, MO, USA) and washed several times in KRHB buffer, and the adipocytes were allowed to float and processed for RNA or protein extraction. SVCs were then pelleted from the infranatant and processed for RNA extraction.

2.6. Islet Isolation. Mouse pancreatic islets were isolated following euthanization as described previously [9, 15]. Briefly, the pancreas was perfused through the common bile duct with 5 mL of 1.4 mg/mL collagenase P and then removed and incubated at 37°C for 8–11 minutes in 1 mL Hank's buffered salt solution. Following incubation, pancreatic tissue was centrifuged, resuspended in Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged again to separate islets from acinar tissue. Islets were then processed for RNA extraction.

2.7. RNA Extraction and Real-Time RT-PCR. RNA from adipose tissue or isolated adipocytes was prepared using the RiboPure kit (Ambion, Austin, TX, USA), and RNA from SVCs and islets was prepared using the RNeasy kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. cDNA was made from 5 μg of total RNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) in 20 μL reaction volume using random hexamers (Invitrogen). Primer oligonucleotides (see Table 1) with SYBR Green I (Molecular Probes, Carlsbad, CA, USA) or Taqman probes (actin, IL-12p40, TNFα, IFNγ, IL-1β, CX3CL1, IL-10, PPARγ, Adfp, Fasn, DGAT1, and DGAT2; Applied Biosystems, Carlsbad, CA, USA) were used for PCR. All thermal cycling was performed using the CFX96 Thermal Cycler (Bio-Rad, Hercules, CA, USA). All reactions were performed in triplicate, and the data was normalized to the actin housekeeping gene and evaluated using the $2^{-\Delta\Delta CT}$ method. Expression levels are presented as fold induction/downregulation of transcripts of respective genes relative to control.

2.8. Protein Extraction and Western Blot Analysis. Adipocytes were harvested in RIPA buffer containing protease and phosphatase inhibitors, and equal quantities of protein were separated by SDS-PAGE, transferred to PVDF membrane, and probed at 4°C overnight in either collagen 6 (Abcam) or cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) primary antibody. Detection was performed with secondary HRP-conjugated antibodies and ECL plus (GE Healthcare, Piscataway, NJ, USA) according to manufacturer's instructions. Western blot quantitation was performed by measuring protein band intensities using ImageJ. Protein expression levels were normalized to actin and presented as fold induction/downregulation of band intensities relative to control. All samples represented in the figures and quantified were run on the same gel.

2.9. Statistical Analysis. Data are presented as the means ± SEM. Student's *t*-test was used to establish statistically significant differences between samples. A P value of <0.05 was considered to indicate statistically significant differences.

3. Results

3.1. Generation of Adipose Tissue-Specific Deletion of 12/15-Lipoxygenase. A conditional knockout allele for the mouse 12/15-lipoxygenase gene, *ALOX15*, was generated by Ozgene and contains loxP sites flanking exons 2–5 (Figure 1(a)). In addition, this allele also contains a neo cassette selection marker that is flanked by FRT sites within intron 5. Heterozygous *12/15-LO*$^{(neo)loxP/+}$ mice on a C57BL/6J background were generated and subsequently crossed to a Flp-deleter mouse line on a C57BL/6J background to facilitate Flp recombinase-mediated removal of the neo cassette. In addition, Ozgene crossed *12/15-LO*$^{loxpP/+}$ mice to an aP2-Cre mouse line to generate *aP2-Cre; 12/15-LO*$^{loxP/+}$. We have since bred *12/15-LO*$^{loxpP/+}$ and *aP2-Cre; 12/15-LO*$^{loxP/+}$ mice to homozygosity for the *12/15-LO*^{loxP} allele. *aP2-Cre; 12/15-LO*$^{loxP/loxP}$ mice will be called ad-12/15-LO mice throughout the paper.

To ensure specific deletion of 12/15-lipoxygenase (12/15-LO) from white adipocytes, we isolated epididymal adipocytes from 24-week-old male wild type *12/15-LO*$^{loxP/loxP}$ and ad-12/15-LO mice and measured 12/15-LO mRNA expression. 12/15-LO mRNA expression was significantly reduced by approximately 70% in isolated epididymal adipocytes from ad-12/15-LO adipocytes compared to wild type adipocytes (Figure 1(b)). This reduction in 12/15-LO mRNA expression in adipocytes remained significantly low

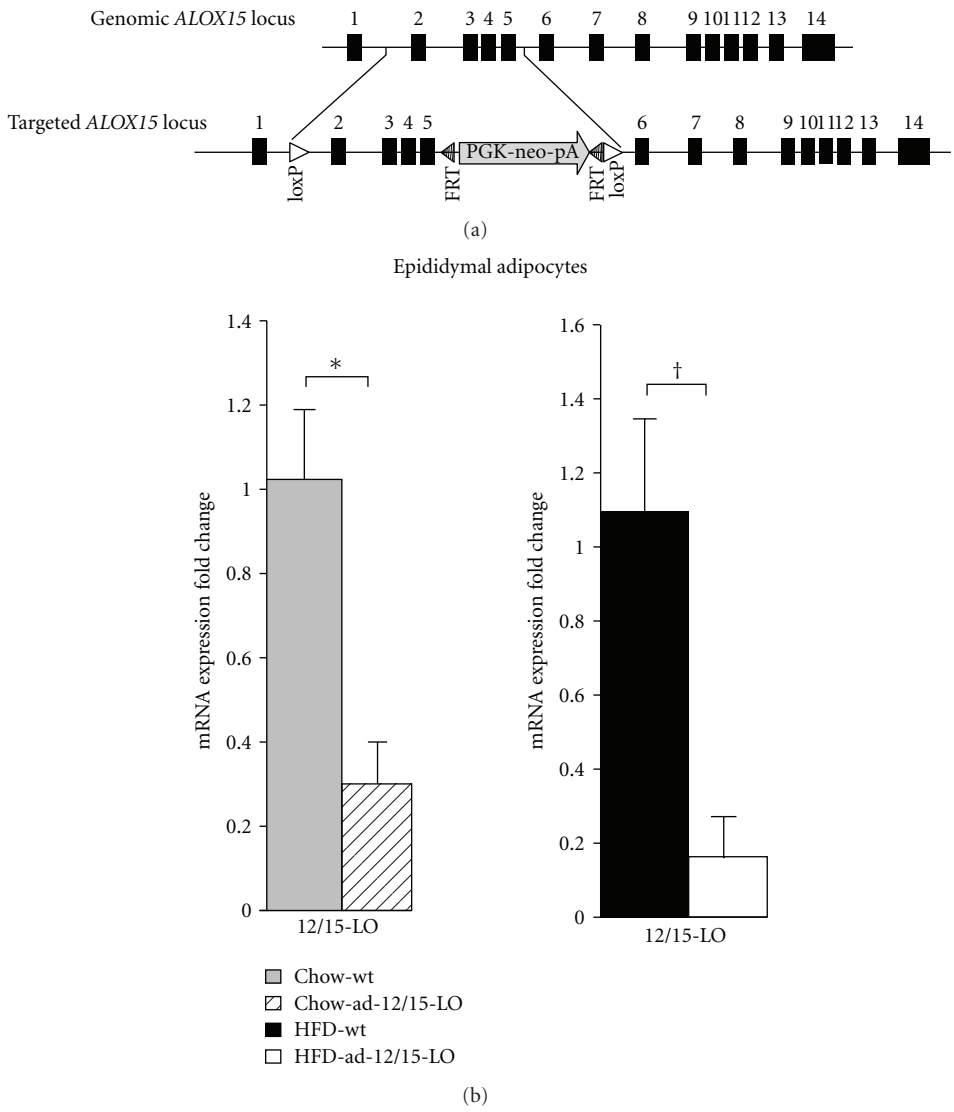

Figure 1: The generation of a targeted conditional knockout allele for the mouse 12/15-lipoxygenase gene, *ALOX15*. (a) The targeted *ALOX15* locus contains loxP sites that flank exons 2 and 5 and a neomycin (neo) cassette selection marker flanked by FRT sites inserted in intron 5. The neo cassette is driven by the mouse phosphoglucokinase gene (PGK) promoter and contains a polyadenylation (pA) signal to terminate the neomycin expression. Exons are depicted as blackened boxes, loxP sites as white triangles, and FRT sites as striped triangles. (b) 12/15-LO mRNA expression was measured in isolated epididymal adipocytes by RT-PCR. All data was normalized to total actin, and the fold changes in expression were calculated relative to control. All data represent the means ± SEM; $n = 3$–6. $^*P < 0.05$ and $^\dagger P < 0.02$ versus control. Chow: chow diet; HFD: high-fat diet; wt: wild type *12/15-LO*$^{loxP/loxP}$. All measurements are performed on 24-week-old mice.

(by approximately 80%) even when age-matched mice were on the high-fat diet for 16 weeks (Figure 1(b)).

3.2. ad-12/15-LO Mice Exhibit Improved Glucose Metabolism When on a High-Fat Diet. 8-week-old male wild type *12/15-LO*$^{loxP/loxP}$ control mice ($n = 5$) and ad-12/15-LO mice ($n = 6$) were placed on a high-fat diet for 16 weeks, along with 8-week-old male chow-fed wild type *12/15-LO*$^{loxP/loxP}$ control mice ($n = 3$), to determine the role of 12/15-LO in adipose tissue in promoting whole-body metabolic dysfunction. At the end of the 16-week feeding regimen, while the high-fat diet-fed groups exhibited significant weight gain compared to chow-fed wild type, no differences in body weight were seen in the high-fat diet-fed wild type and ad-12/15-LO mice (Figure 2(a)). Additionally, fasting blood glucose levels after 15-weeks of feeding were significantly reduced in high-fat diet-fed ad-12/15-LO mice compared to high-fat diet-fed wild type mice and remain unchanged from chow-fed wild type; nonfasting insulin levels after 16-weeks of feeding were also reduced in high-fat diet-fed ad-12/15-LO mice compared to high-fat diet-fed wild type mice (Figure 2(a)). Furthermore, measurement of nonfasting insulin : glucose ratio provides a reading of insulin sensitivity; indeed, this ratio is increased in high-fat diet-fed wild type mice compared to chow-fed wild type

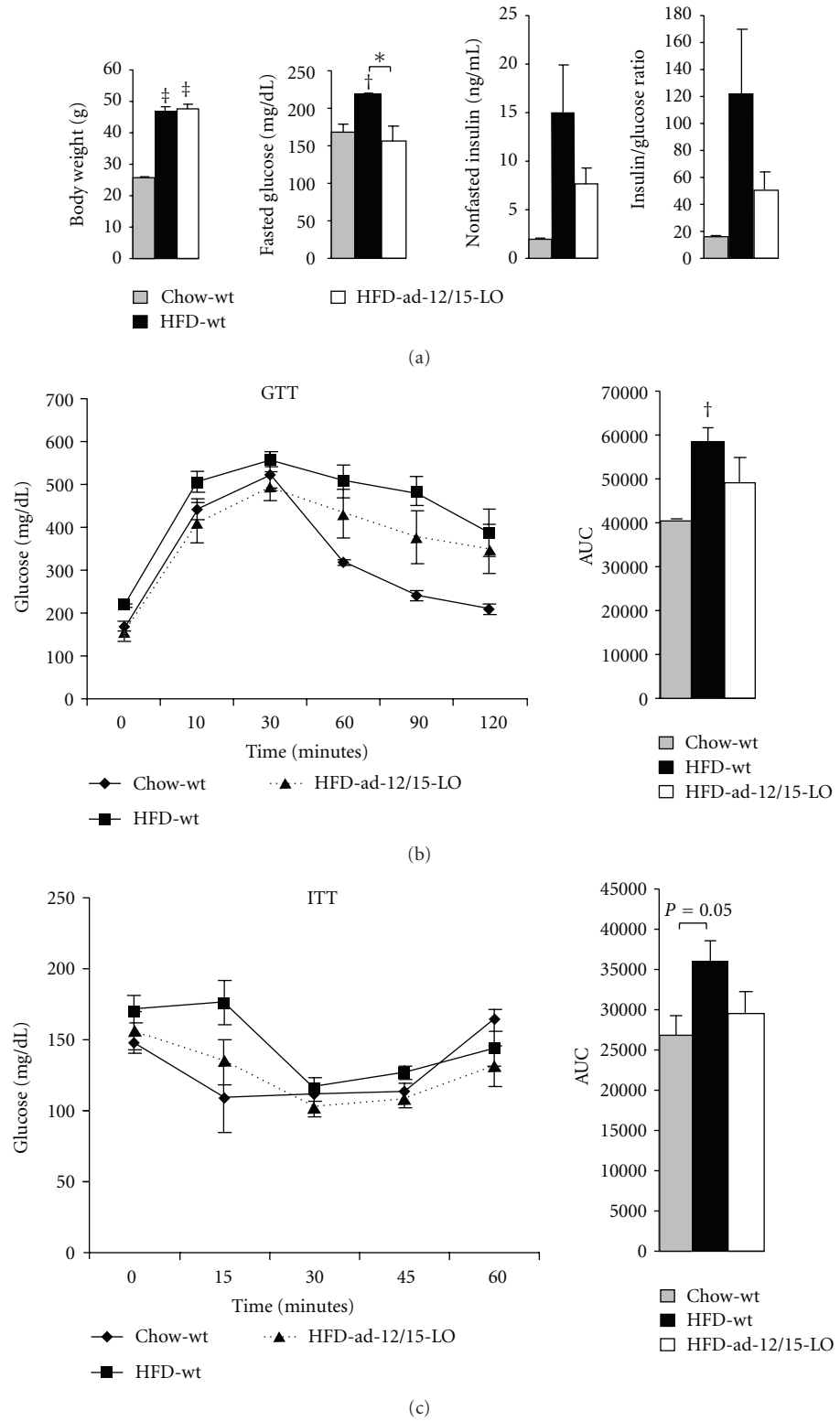

Figure 2: Fat-specific 12/15-LO-deficient mice exhibit improved glucose metabolism when fed a high-fat diet. (a) Body weight, nonfasted serum insulin, and nonfasted serum insulin/nonfasted blood glucose ratio were measured at the end of the 16-week feeding regimen. Fasted blood glucose was measured in overnight fasted mice at 15 weeks of the feeding regimen. Serum insulin levels were measured by ELISA and tail vein blood glucose by glucometers. (b)-(c) Intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed in mice after 15 weeks of feeding, and blood glucose measurements were measured at the indicated time points. Areas under the curves (AUC) are presented for GTT and ITT. All data represent the means ± SEM; $n = 3$–6. $^{*}P < 0.05$, $^{\dagger}P < 0.02$, $^{\ddagger}P < 0.001$ versus control, unless otherwise indicated. Chow: chow diet; HFD: high-fat diet; wt: wild type *12/15-LO*$^{loxP/loxP}$.

mice, and this increase is ameliorated in the high-fat diet-fed ad-12/15-LO mice (Figure 2(a)). Finally, high-fat diet-fed ad-12/15-LO mice exhibited improvements in glucose metabolism compared to high-fat diet-fed wild type mice, as measured by intraperitoneal glucose and insulin tolerance tests (Figures 2(b) and 2(c)).

3.3. Adipose Tissue of ad-12/15-LO Mice on a High-Fat Diet Is Less Inflamed. To examine local effects of 12/15-LO deficiency in adipose tissue when mice are on a high-fat diet, we performed morphometric analysis of epididymal adipose tissue from these mice. Mice on a high-fat diet exhibit enlarged fat pads and adipocytes due to increased triglyceride storage of excess fatty acids [2]. While no significant differences were observed between epididymal adipose tissue weight and adipocyte size in wild type and ad-12/15-LO mice on a high-fat diet, there may be a trend for enlargement of adipocyte size in the ad-12/15-LO mice fed a high-fat diet (Figure 3(a)).

Furthermore, we measured mRNA expression of several key proinflammatory genes in epididymal adipose tissue from the chow- and high-fat diet-fed wild type and ad-12/15-LO mice. Chronic inflammation of adipose tissue is accompanied with increased production of cytokines, such as tumor necrosis factor-α (TNFα) [2]. Indeed, TNFα mRNA expression was significantly upregulated in adipose tissue by the high-fat diet in wild type mice; however, fat-specific 12/15-LO deficiency significantly prevented this upregulation (Figure 3(b)). CX3CL1 (or fractalkine), a cytokine derived from the endothelium that promotes atherogenesis, has recently been shown to also be increased in adipose tissue of obese patients and is associated with inflammation, insulin resistance, and type 2 diabetes in these patients [16, 17]. Interestingly, fat-specific 12/15-LO deficiency was also able to significantly prevent the high-fat diet-induced increase in CX3CL1 mRNA expression (Figure 3(b)). 12/15-LO exerts proinflammatory responses in part by upregulating the expression of interleukin-12 (IL-12) in activated inflammatory cells [5, 6, 18–20]. Indeed, a trend for decreased IL-12p40 mRNA expression was observed in the ad-12/15-LO mice fed a high-fat diet compared to wild type mice. The anti-inflammatory cytokine interleukin-10 (IL-10) [21] and Twist-1, a basic helix-loop-helix transcription factor whose expression in human white adipose tissue is inversely correlated with inflammation and insulin resistance [22], are also increased in high-fat diet-fed ad-12/15-LO mice compared to wild type mice (Figure 3(b)). Other proinflammatory cytokines measured revealed no differences between the wild type and ad-12/15-LO mice on a high-fat diet (interferon-γ (IFNγ), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and interleukin-6 (IL-6); Figure 3(b)).

Adiponectin is an adipose tissue-specific secreted adipokine that is a critical insulin-sensitizer and is downregulated by high-fat diets [23]. Indeed, fat-specific 12/15-LO deficiency ameliorates the significant decrease in adiponectin mRNA expression as seen in isolated epididymal adipocytes from wild type mice on a high-fat diet (Figure 3(c)); a similar effect is observed in serum levels of the active high-molecular-weight form of adiponectin protein (Figure 3(c)) [23].

Furthermore, given the trend for increased adipocyte size in the ad-12/15-LO mice fed a high-fat diet, we examined collagen 6 (Col6). Col 6 is associated with increased fibrosis of inflamed adipose tissue [24]. While Col6 mRNA expression significantly increased in adipose tissue from wild type mice when fed a high-fat diet, fat-specific 12/15-LO deficiency is able to ameliorate this increase (Figure 3(b)); a similar trend was observed for Col6 protein in isolated adipocytes (Figure 3(d)).

3.4. Adipose Tissue of ad-12/15-LO Mice on a High-Fat Diet Exhibits Decreased Macrophage Infiltration. To further probe the inflammatory consequences in adipose tissue of fat-specific 12/15-LO deletion when mice are on a high-fat diet, we examined macrophage infiltration into the epididymal fat pad as this is associated with visceral adiposity [25]. Analysis of markers for activated macrophages, F4/80 and CD11c, in isolated macrophage-containing stromal vascular cells of the epididymal adipose fat pad revealed that fat-specific 12/15-LO deletion was able to prevent the increase in F4/80 and CD11c expression seen in wild type mice fed a high-fat diet (Figure 4(a)). Furthermore, immunohistochemical staining of epididymal adipose tissue for the activated macrophage marker, Mac2, revealed that a high-fat diet significantly increased Mac2 staining in wild type mice but not in ad-12/15-LO mice (Figure 4(b)). Finally, a decrease in cleaved caspase-3 protein in isolated adipocytes from high-fat diet-fed ad-12/15-LO mice compared to wild type suggests a decrease in apoptotic adipocytes (Figure 4(c)). Thus it appears that fat-specific 12/15-LO deficiency is able to reduce the local inflammatory consequences in fat normally rendered by a high-fat diet, resulting in decreased macrophage infiltration.

3.5. Adipose Tissue of ad-12/15-LO Mice on a High-Fat Diet May Be More Adipogenic and Lipogenic. We further examined expression of genes important in adipocyte differentiation and health. Peroxisome proliferator-activated receptor-γ (PPARγ) is a strict requirement for adipocyte differentiation, and formation of new adipocytes generally is thought to indicate the prevalence of healthy adipocytes [26]. Indeed, PPARγ mRNA expression is decreased by a high-fat diet in wild type mice; however, fat-specific 12/15-LO deficiency is able to ameliorate PPARγ decrease when mice are fed a high-fat diet (Figure 4(d)). Additionally, we examined adipose differentiation related protein (Adfp), fatty acid synthase (Fasn), and acyl CoA: diacylglycerol acyltransferases 1 and 2 (DGAT1 and 2) expression. Adfp is a perilipin that plays a role in lipid droplet formation, and stabilization and its expression are associated with increased lipid load, while Fasn is important for the synthesis of long-chain fatty acids [27, 28]. Furthermore, DGAT1 and 2 are important for triglyceride synthesis [29]. Consistent with the observed trend that fat-specific 12/15-LO deficiency may promote larger adipocytes in mice fed a high-fat diet, we observe that

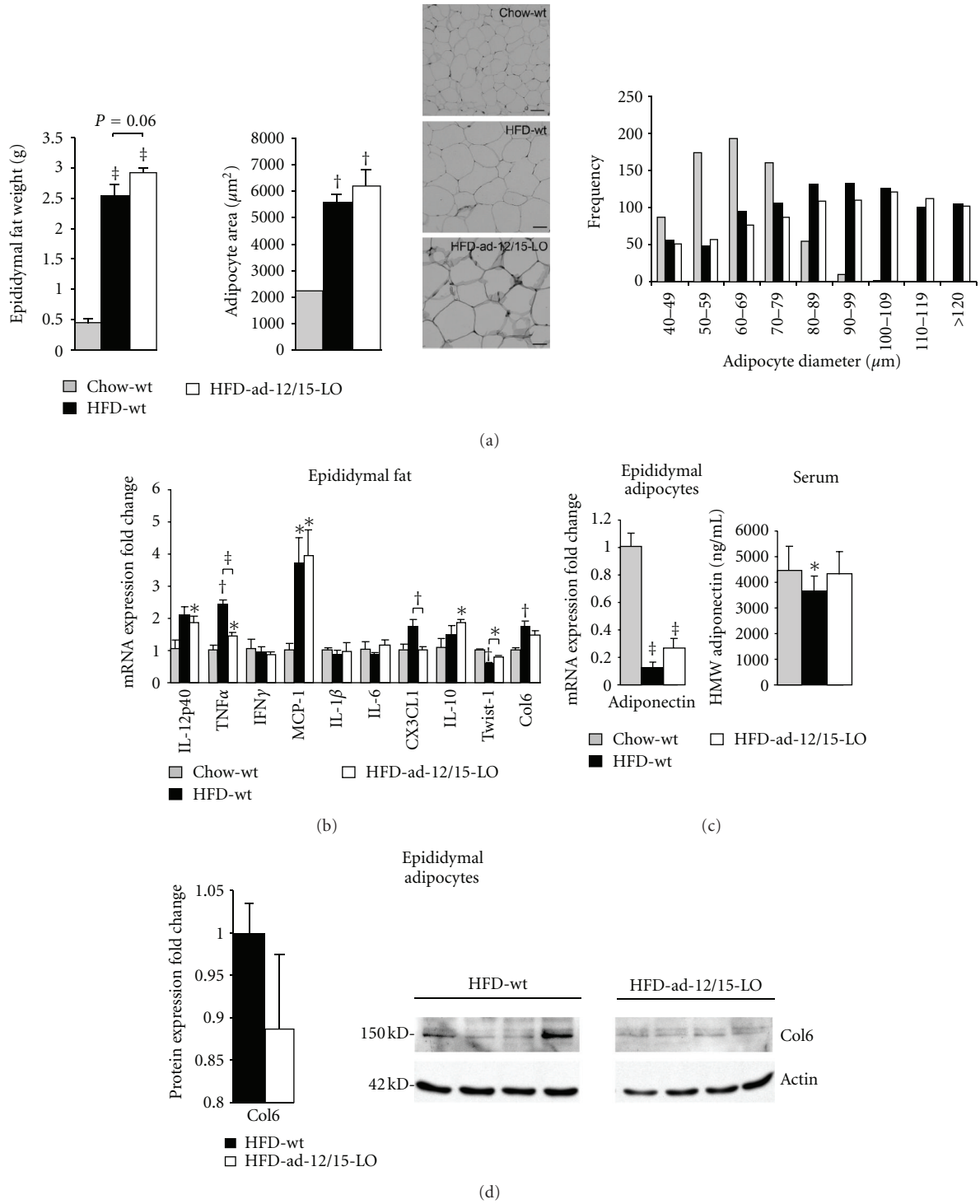

Figure 3: Fat-specific 12/15-LO-deficient mice exhibit decreased inflammation in adipose tissue following a high-fat diet regimen. (a) Epididymal adipose tissue weight and adipocyte size were measured in mice after the 16-week feeding regimen. Hematoxylin and eosin stained sections of epididymal adipose tissue are shown. Scale bar = 50 μM. (b) mRNA measurements of key proinflammatory and anti-inflammatory genes were examined by RT-PCR in epididymal adipose tissue from mice. (c) Adiponectin mRNA and high-molecular-weight (HMW) adiponectin protein were measured in isolated epididymal adipocytes and serum by RT-PCR and ELISA, respectively. (d) Protein measurement of collagen 6 (Col6) was performed from isolated epididymal adipocytes and quantified. Representative western blots are shown, and separate panels for each antibody represent the same exposure from the same gel. All mRNA and protein data were normalized to total actin, and the fold changes in expression were calculated relative to control. All data represent the means ± SEM; $n = 3$–6. $^{*}P < 0.05$, $^{\dagger}P < 0.02$, $^{\ddagger}P < 0.001$ versus control, unless otherwise indicated. Chow: chow diet; HFD: high-fat diet; wt: wild type *12/15-LO*$^{loxP/loxP}$.

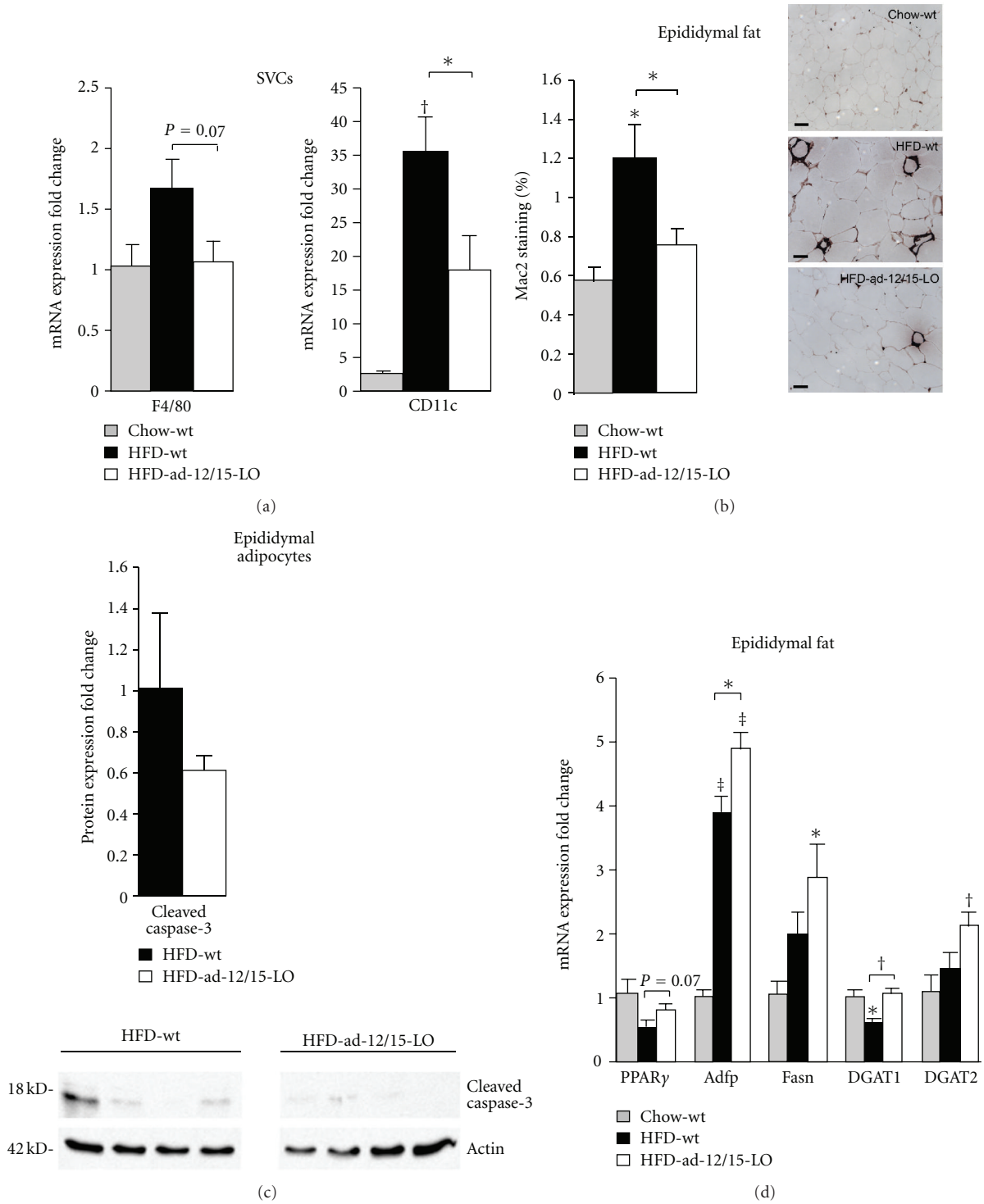

Figure 4: Adipose tissue from fat-specific 12/15-LO-deficient mice exhibits decreased macrophage infiltration and may be more adipogenic and lipogenic. (a) mRNA expression of key macrophage markers was measured by RT-PCR in stromal vascular cells (SVCs) isolated from epididymal adipose tissue. (b) Immunohistochemistry for Mac2 expression was performed in epididymal adipose tissue, and Mac2 staining was quantified. Scale bar = 50 μM. (c) Protein measurements of cleaved caspase-3 were performed from isolated epididymal adipocytes and quantified. Representative western blots are shown, and separate panels for each antibody represent the same exposure from the same gel. (d) mRNA measurements of key adipogenic and lipogenic genes were examined by RT-PCR in epididymal adipose tissue from mice. All mRNA and protein data were normalized to total actin, and the fold changes in expression were calculated relative to control. All data represent the means ± SEM; $n = 3$–6. $^{*}P < 0.05$, $^{\dagger}P < 0.02$, $^{\ddagger}P < 0.001$ versus control, unless otherwise indicated. Chow: chow diet; HFD: high-fat diet; wt: wild type *12/15-LO*$^{loxP/loxP}$.

Adfp and Fasn mRNA expression is higher in the ad-12/15-LO mice compared to wild type mice when fed a high-fat diet (Figure 4(d)). Also, while DGAT1 mRNA expression is significantly decreased when wild type mice are fed a high-fat diet, its expression remains unchanged in the high-fat diet-fed ad-12/15-LO mice, and DGAT2 mRNA expression is significantly increased in the high-fat diet-fed ad-12/15-LO but not wild type mice (Figure 4(d)).

3.6. 12/15-LO Deficiency in Fat Alters Serum Lipid Levels. Given the reduced inflammation in adipose tissue in the context of fat-specific 12/15-LO deficiency when mice are fed a high-fat diet, we examined serum levels of lipids that can be highly affected by adipocyte health. In particular, total cholesterol and nonesterified free fatty acids were similarly significantly increased in both wild type and ad-12/15-LO mice fed a high-fat diet; however, large changes in triglycerides were not observed (data not shown).

3.7. 12/15-LO Deficiency in Fat Restores β-Cell Function. Inflammation in adipose tissue is thought to precede the development of inflammation elsewhere and is thus an early indicator for disease development in peripheral tissues [11, 30]. Given that pancreatic β-cells are an early key target tissue in obesity-induced type 2 diabetes, we evaluated whether the beneficial effects of fat-specific 12/15-LO deficiency are manifested in the pancreatic islets. C57BL/6 mice fed a high-fat diet are known to undergo islet hyperplasia as a mechanism to meet increased demands for insulin production given the rise in insulin resistance. Interestingly, while high-fat diet-fed wild type mice exhibit a trend for larger pancreatic islets compared to chow-fed wild type mice, the high-fat diet-fed ad-12/15-LO mice display even larger islets (Figure 5(a)); a similar trend is observed for total islet area per pancreas area (Figure 5(a)). In addition, the percentage of insulin-stained β-cells per islet slightly increases in the high-fat diet-fed wild type mice compared to chow-fed wild type mice, and this is further augmented in the high-fat diet-fed ad-12/15-LO mice (Figure 5(a)). In a separate study where wild type C57BL/6J and ad-12/15-LO mice were fed a high-fat diet for 16 weeks, islet gene expression was measured for proinflammatory cytokines. 12/15-LO and IL-6 mRNA expression was significantly reduced, and IL-12p40 and INFγ mRNA expression was reduced in islets from ad-12/15-LO mice compared to wild type mice fed a high-fat diet (Figure 5(b)). Islets from chow-fed wild type and ad-12/15-LO age-matched control mice were analyzed for 12/15-LO mRNA expression, and no differences were observed (data not shown), suggesting the *aP2-Cre* transgene is specific to fat and the exhibited changes in 12/15-LO mRNA expression in islets from mice on a high-fat diet were a consequence of inflammation. Thus, it appears that deletion of 12/15-LO in adipose tissue is able to confer protection to β-cell function.

4. Discussion

Obesity is marked by an increased demand for intracellular lipid storage of excess circulating free fatty acids, leading to adipocyte stress and ensuing chronic inflammation and macrophage infiltration [2]. Production of proinflammatory cytokines by adipose tissue further leads to local and systemic insulin resistance. The 12/15-lipoxygenase (12/15-LO) enzyme generates proinflammatory lipids, such as 12(S)-HETE, which promote local dysfunction in various tissues such as adipose, pancreatic, and vascular tissues (reviewed in [4]). We show here that local chronic activation of 12/15-LO in adipose tissue by a high-fat diet is sufficient to drive local adipocyte dysfunction with ensuing systemic decline.

Inflammation in adipose tissue induced by a high-fat diet is characterized by increased expression of proinflammatory cytokines, such as TNFα. TNFα decreases insulin sensitivity, exacerbates inflammation, and inhibits adipogenesis [2]. TNFα is generated predominantly by infiltrating macrophages and promotes increased lipolysis [2, 25]. These in effect lead to increased circulating free fatty acids that promote ectopic lipid accumulation and insulin resistance. Indeed, we observed that ad-12/15-LO mice fed a high-fat diet did not demonstrate increased TNFα expression, consistent with improved insulin sensitivity and overall decreased inflammation. Interestingly, low expression of Twist-1 in human adipose tissue is correlated with high levels of TNFα expression, consistent with our results, and Twist-1 expression in Th1 lymphocytes limits TNFα expression [22, 31]. Furthermore, IL-10 overexpression in mice is able to prevent diet-induced insulin resistance [32], and indeed we see increased IL-10 expression in the ad-12/15-LO mice fed a high-fat diet with concomitant improvements in insulin sensitivity. Thus the absence of chronic 12/15-LO activation in adipose tissue in obese conditions is able to prevent the phenotypic proinflammatory state induced by a high-fat diet.

The complex inflammatory cascade and cell death elicited in adipose tissue by obesity are responsible for the characteristic feature of macrophage infiltration into the fat pad [33]. MCP-1 is thought to be one of the key adipokines in this process. We observed that macrophage infiltration into the epididymal fat pad of high-fat diet-fed ad-12/15-LO mice is significantly reduced compared to the wild type mice in agreement with the decreased TNFα expression (likely secreted by the macrophages); however, while MCP-1 mRNA expression was high in the high-fat diet-fed groups, no difference was observed between the groups. This may suggest that another key chemoattractant player is important for eliciting macrophage infiltration given the absence of macrophages in the high MCP-1-expressing ad-12/15-LO fat, a possibility supported by the observation that MCP-1 knockout mice still exhibit obesity-induced macrophage infiltration into adipose tissue [34]. This additional chemoattractant would be dependent upon chronic 12/15-LO activation whereby specific lipid metabolites, such as 12(S)-HETE, may be responsible for eliciting the inflammatory cascade and subsequent macrophage infiltration. Alternatively, the decreased macrophage infiltration could be explained by the role of 12/15-LO to induce endothelial expression of intercellular adhesion molecule-1 (ICAM-1) [35]. ICAM-1 mediates monocyte adhesion to the vascular wall endothelium and is required for transendothelial migration of monocytes into the surrounding tissue. Therefore, the absence of

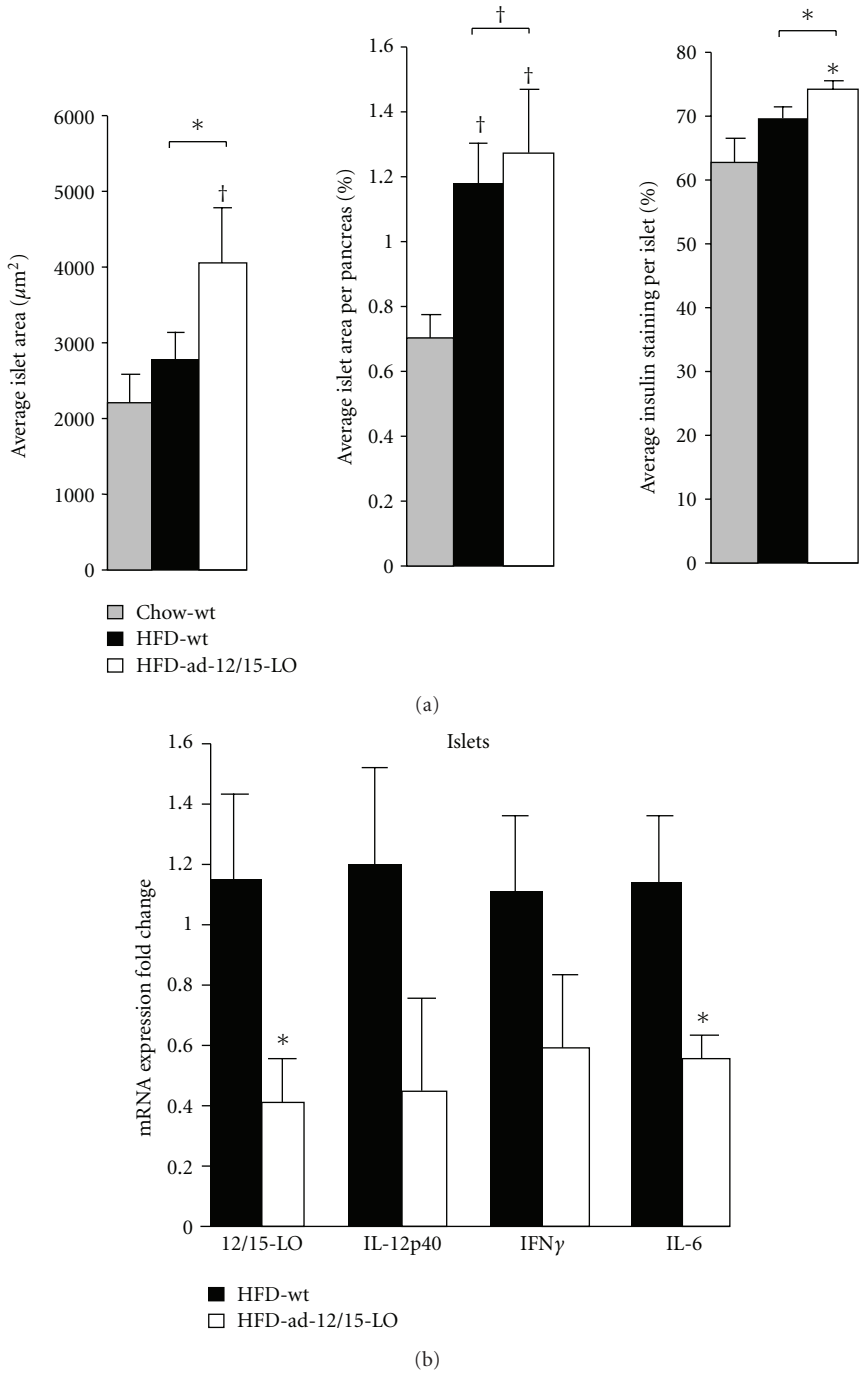

Figure 5: Fat-specific 12/15-LO-deficient mice exhibit improvements in pancreatic islet health. (a) Immunohistochemistry for insulin expression on pancreatic sections from mice after the 16-week feeding regimen was analyzed for average islet area, average islet area per pancreas area, and average insulin staining per islet; $n = 3$–5; Chow: chow diet; HFD: high-fat diet; wt: wild type *12/15-LO*$^{loxP/loxP}$. (b) mRNA measurements of key proinflammatory genes were examined by RT-PCR in isolated pancreatic islets from mice after the 16-week feeding regimen; $n = 6$–8; HFD: high-fat diet; wt: wild type C57BL/6J. All mRNA data were normalized to total actin, and the fold changes in expression were calculated relative to control. All data represent the means ± SEM. $^*P < 0.05$ and $^\dagger P < 0.02$ versus control, unless otherwise indicated.

12/15-LO may simply lead to impaired transendothelial migration and subsequent decreased macrophage infiltration into the adipose tissue. Furthermore, normal recruitment of macrophages to the fat pad occurs when adipocytes undergo lipolysis or cell death and macrophages are activated to uptake the free fatty acids or engulf apoptotic cells to protect local adipocyte and distal tissue health [36, 37]. However, chronic activation of these macrophages in the obese state

promotes a phenotypic proinflammatory switch in the macrophages that is detrimental to local adipocyte health and metabolism [36, 37]. Thus in the ad-12/15-LO mice fed a high-fat diet, less macrophages may be present due to decreased lipolysis and cell death.

Additionally, *de novo* adipogenesis and lipogenesis accompanied by decreased lipolysis are important for insulin sensitivity and longevity as this process promotes the development of healthy, active adipocytes and removes the excess circulating fatty acids to prevent ectopic lipid accumulation and ensue inflammation and insulin resistance in nonadipose tissue [28]. On the contrary, TNFα inhibits adipogenesis and lipogenesis in part through downregulating expression of PPARγ and its lipid droplet protein targets, respectively [2, 25]. Our data suggest that adipocytes from ad-12/15-LO mice versus wild type mice fed a high-fat diet may display increased adipogenic and lipogenic potential given the increased expression of PPARγ, Adfp, Fasn, DGAT1, and DGAT2 with a trend for decreased circulating free fatty acids in the serum. This is of importance given the evidence describing a positive correlation between high lipid-droplet protein expression and insulin sensitivity in patient fat samples [38] and studies demonstrating that mice overexpressing DGAT1 in adipocytes and macrophages were protected from diet-induced inflammation and insulin resistance [39]. While not significant, it is possible that there is a tendency for ad-12/15-LO adipocytes to become larger compared to wild type adipocytes after a high-fat feeding. This may suggest a greater tendency for these adipocytes to increase their lipid load, in agreement with the increased expression of lipogenic genes. Interestingly, Col6 is a component of extracellular matrix and Col6-deficient *ob/ob* mice display larger but healthier adipocytes since they can expand in size in response to increased lipid loading without being subjected to shear stress. Indeed, ad-12/15-LO adipocytes seem to exhibit lower levels of Col6. Furthermore, while white adipose tissue mass is greater in the high-fat diet-fed ad-12/15-LO mice compared to the high-fat diet-fed wild type mice, no changes in overall body weight were observed. This may suggest differences in lean versus fat mass and warrants further investigation to determine the role of 12/15-LO in partitioning of lipids in adipose tissue. Finally, while analysis of fatty acid and triglyceride serum levels was not different between the high-fat diet-fed ad-12/15-LO and wild type nonfasted mice, analysis of serum from fasted mice will likely reveal a greater difference than seen in this study and thereby further decipher the role of 12/15-LO in adipogenesis and lipogenesis. This is important as a 16-week high-fat diet is likely overloading the adipocyte, and thus differences in serum levels of fatty acids and triglycerides between the strains are hard to discern in the nonfasted state.

Adipose tissue has an important role in controlling whole-body glucose homeostasis such that pancreatic islets are highly susceptible to the damaging effects elicited by circulating cytokines and fatty acids released by inflamed adipose tissue [40]. Within the first week of high-fat feeding in mice, insulin resistance primes the islets to respond by becoming hyperplastic to meet the increased demands of insulin production to compensate for insulin resistance and thereby maintain normal blood glucose levels [41]. Furthermore, the islets themselves become inflamed and begin to decline in insulin production and secretion [41]. Indeed, fat-specific 12/15-LO deficiency may protect β-cell function as evident by decreased islet expression of proinflammatory genes, such as IL-12p40, IFNγ, and IL-6. Furthermore, while it is generally accepted that hyperplasia is an adaptive response to overcome insulin resistance, we observed that the ad-12/15-LO mice exhibited even larger islets despite their improvements in glucose metabolism. The decreased inflammatory environment in these mice may allow this hyperplastic response to ensue without facing the normal detrimental consequences of inflamed islets. This is a novel aspect of the study that will certainly be investigated in future studies as an interesting crosstalk clearly exists between 12/15-LO activity in adipose tissue and pancreatic islet inflammation and β-cell function.

aP2-Cre can exhibit low levels of expression in macrophages and is of concern in the present study. However, the *aP2-Cre* transgene in our mouse model appears to be specific to adipocytes and not leaky in macrophages as thioglycollate-induced peritoneal macrophages isolated from wild type and ad-12/15-LO mice exhibit similar IL-12 expression (data not shown). This is important as 12/15-LO is a requirement for IL-12 expression in macrophages [42]. Thus this further supports the idea that inflamed adipose tissue driven by 12/15-LO has significant systemic impact apart from macrophages. Future studies comparing isolated macrophages from *aP2-Cre; 12/15-LO*$^{loxP/loxP}$ as well as *adiponectin-Cre; 12/15-LO*$^{loxP/loxP}$ mice (which we are currently generating) will provide further information on the molecular and functional consequences of aP2-Cre in macrophages as adiponectin is expressed only in adipocytes.

Recent data suggests that inflammation in adipose tissue precedes the development of disease progression in peripheral tissues [11, 30]. In particular, Stanton et al. reported that inflammatory gene expression changes in epididymal adipose tissue of C57BL/6 mice in response to a high-fat feeding occur as early as 6 weeks of feeding; by 16 weeks of feeding there is a shift in inflammatory changes in the adipose tissue to the liver. These results suggest that analysis of adipose tissue as early as 6 weeks of high-fat feeding may reveal even more dramatic differences in epididymal adipose tissue of wild type versus ad-12/15-LO mice. Furthermore, careful analysis of liver tissue from 6–16 weeks of high-fat feeding will also reveal whether fat-specific 12/15-LO deficiency can protect mice from obesity-induced fatty liver disease.

If indeed controlling obesity-induced dysfunction of adipose tissue metabolism will protect systemic tissues from the inflammatory adipokines secreted by adipose tissue, then one would suspect to observe significant decreases in circulating cytokine levels in the serum. While serum cytokine levels were not evaluated in this study due to lack of material, we would expect serum cytokine levels to be reduced in the high-fat diet-fed ad-12/15-LO mice compared to wild type. These cytokines likely will reflect the inflammatory expression profile in adipose tissue as this organ is much larger than other cytokine-contributing tissues and thus

adipose tissue adipokine production may more significantly impact circulating systemic cytokine levels.

5. Conclusions

In summary, we report the first study utilizing a targeted tissue-specific deletion of 12/15-LO. The use of *12/15-LO$^{loxP/loxP}$* mice will allow targeted removal of 12/15-LO in various tissues affected by diabetes and cardiovascular disease, including β-cells, liver, and vascular tissue. In addition, our findings suggest that 12/15-LO-mediated pathways in adipocytes play a role in obesity-associated insulin resistance by modulating adipose tissue inflammatory pathways. Inactivation of 12/15-LO in fat was able to relieve local inflammation resulting in positive systemic metabolic consequences. Therefore, deciphering the key molecular players regulating 12/15-LO expression in adipose tissue may have therapeutic value in the treatment of obesity-related disorders, such as diabetes and cardiovascular disease.

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References

[1] H. Zhang and C. Zhang, "Adipose talks to distant organs to regulate insulin sensitivity and vascular function," *Obesity*, vol. 18, no. 11, pp. 2071–2076, 2010.

[2] A. Guilherme, J. V. Virbasius, V. Puri, and M. P. Czech, "Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 5, pp. 367–377, 2008.

[3] G. S. Hotamisligil, "Endoplasmic reticulum stress and the inflammatory basis of metabolic disease," *Cell*, vol. 140, no. 6, pp. 900–917, 2010.

[4] A. D. Dobrian, D. C. Lieb, B. K. Cole, D. A. Taylor-Fishwick, S. K. Chakrabarti, and J. L. Nadler, "Functional and pathological roles of the 12- and 15-lipoxygenases," *Progress in Lipid Research*, vol. 50, no. 1, pp. 115–131, 2011.

[5] S. K. Chakrabarti, B. K. Cole, Y. Wen, S. R. Keller, and J. L. Nadler, "12/15-Lipoxygenase products induce inflammation and impair insulin signaling in 3T3-l1 adipocytes," *Obesity*, vol. 17, no. 9, pp. 1657–1663, 2009.

[6] S. K. Chakrabarti, Y. Wen, A. D. Dobrian et al., "Evidence for activation of inflammatory lipoxygenase pathways in visceral adipose tissue of obese Zucker rats," *American Journal of Physiology*, vol. 300, no. 1, pp. E175–E187, 2011.

[7] A. D. Dobrian, D. C. Lieb, Q. Ma et al., "Differential expression and localization of 12/15 lipoxygenases in adipose tissue in human obese subjects," *Biochemical and Biophysical Research Communications*, vol. 403, no. 3-4, pp. 485–490, 2010.

[8] B. K. Cole, N. S. Kuhn, S. M. Green-Mitchell et al., "12/15-lipoxygenase signaling in the endoplasmic reticulum stress response," *American Journal of Physiology*, vol. 302, no. 6, pp. E654–E665, 2012.

[9] C. S. Nunemaker, M. Chen, H. Pei et al., "12-lipoxygenase-knockout mice are resistant to inflammatory effects of obesity induced by western diet," *American Journal of Physiology*, vol. 295, no. 5, pp. E1065–E1075, 2008.

[10] D. D. Sears, P. D. Miles, J. Chapman et al., "12/15-Lipoxygenase is equired for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice," *PLoS ONE*, vol. 4, no. 9, Article ID e7250, 2009.

[11] M. A. Mori, M. Liu, O. Bezy et al., "A systems biology approach identifies inflammatory abnormalities between mouse strains prior to development of metabolic disease," *Diabetes*, vol. 59, no. 11, pp. 2960–2971, 2010.

[12] M. Furuhashi and G. S. Hotamisligil, "Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets," *Nature Reviews Drug Discovery*, vol. 7, no. 6, pp. 489–503, 2008.

[13] W. He, Y. Barak, A. Hevener et al., "Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 26, pp. 15712–15717, 2003.

[14] Z. Yang, M. Chen, J. D. Ellett et al., "Autoimmune diabetes is blocked in Stat4-deficient mice," *Journal of Autoimmunity*, vol. 22, no. 3, pp. 191–200, 2004.

[15] J. D. Carter, S. B. Dula, K. L. Corbin, R. Wu, and C. S. Nunemaker, "A practical guide to rodent islet isolation and assessment," *Biological Procedures Online*, vol. 11, no. 1, pp. 3–31, 2009.

[16] W. T. Cefalu, "Fractalkine: a cellular link between adipose tissue inflammation and vascular pathologies," *Diabetes*, vol. 60, no. 5, pp. 1380–1382, 2011.

[17] R. Shah, C. C. Hinkle, J. F. Ferguson et al., "Fractalkine is a novel human adipochemokine associated with type 2 diabetes," *Diabetes*, vol. 60, no. 5, pp. 1512–1518, 2011.

[18] M. K. Middleton, T. Rubinstein, and E. Puré, "Cellular and molecular mechanisms of the selective regulation of IL-12 production by 12/15-lipoxygenase," *Journal of Immunology*, vol. 176, no. 1, pp. 265–274, 2006.

[19] G. Trinchieri, "Interleukin-12 and the regulation of innate resistance and adaptive immunity," *Nature Reviews Immunology*, vol. 3, no. 2, pp. 133–146, 2003.

[20] Y. Wen, J. Gu, S. K. Chakrabarti et al., "The role of 12/15-lipoxygenase in the expression of interleukin-6 and tumor necrosis factor-α in macrophages," *Endocrinology*, vol. 148, no. 3, pp. 1313–1322, 2007.

[21] M. Saraiva and A. O'Garra, "The regulation of IL-10 production by immune cells," *Nature Reviews Immunology*, vol. 10, no. 3, pp. 170–181, 2010.

[22] A. T. Pettersson, N. Mejhert, M. Jernås et al., "Twist1 in human white adipose tissue and obesity," *Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 1, pp. 133–141, 2011.

[23] T. Kadowaki, T. Yamauchi, N. Kubota, K. Hara, K. Ueki, and K. Tobe, "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1784–1792, 2006.

[24] T. Khan, E. S. Muise, P. Iyengar et al., "Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI," *Molecular and Cellular Biology*, vol. 29, no. 6, pp. 1575–1591, 2009.

[25] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante, "Obesity is associated with

macrophage accumulation in adipose tissue," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, 2003.

[26] A. G. Cristancho and M. A. Lazar, "Forming functional fat: a growing understanding of adipocyte differentiation," *Nature Reviews Molecular Cell Biology*, vol. 12, no. 11, pp. 722–734, 2011.

[27] B. H. J. Chang and L. Chan, "Regulation of triglyceride metabolism. III. Emerging role of lipid droplet protein ADFP in health and disease," *American Journal of Physiology*, vol. 292, no. 6, pp. G1465–G1468, 2007.

[28] I. J. Lodhi, X. Wei, and C. F. Semenkovich, "Lipoexpediency: *de novo* lipogenesis as a metabolic signal transmitter," *Trends in Endocrinology and Metabolism*, vol. 22, no. 1, pp. 1–8, 2011.

[29] C. L. E. Yen, S. J. Stone, S. Koliwad, C. Harris, and R. V. Farese, "Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis," *Journal of Lipid Research*, vol. 49, no. 11, pp. 2283–2301, 2008.

[30] M. C. Stanton, S. C. Chen, J. V. Jackson et al., "Inflammatory Signals shift from adipose to liver during high fat feeding and influence the development of steatohepatitis in mice," *Journal of Inflammation*, vol. 8, article 8, 2011.

[31] U. Niesner, I. Albrecht, M. Janke et al., "Autoregulation of Th1-mediated inflammation by twist1," *Journal of Experimental Medicine*, vol. 205, no. 8, pp. 1889–1901, 2008.

[32] E. G. Hong, H. J. Ko, Y. R. Cho et al., "Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle," *Diabetes*, vol. 58, no. 11, pp. 2525–2535, 2009.

[33] T. Suganami and Y. Ogawa, "Adipose tissue macrophages: their role in adipose tissue remodeling," *Journal of Leukocyte Biology*, vol. 88, no. 1, pp. 33–39, 2010.

[34] K. E. Inouye, H. Shi, J. K. Howard et al., "Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue," *Diabetes*, vol. 56, no. 9, pp. 2242–2250, 2007.

[35] D. T. Bolick, A. W. Orr, A. Whetzel et al., "12/15-Lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-κB," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 11, pp. 2301–2307, 2005.

[36] A. Kosteli, E. Sugaru, G. Haemmerle et al., "Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue," *Journal of Clinical Investigation*, vol. 120, no. 10, pp. 3466–3479, 2010.

[37] P. Fischer-Posovszky, Q. A. Wang, I. W. Asterholm, J. M. Rutkowski, and P. E. Scherer, "Targeted deletion of adipocytes by apoptosis leads to adipose tissue recruitment of alternatively activated M2 macrophages," *Endocrinology*, vol. 152, no. 8, pp. 3074–3081, 2011.

[38] V. Puri, S. Ranjit, S. Konda et al., "Cidea is associated with lipid droplets and insulin sensitivity in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 22, pp. 7833–7838, 2008.

[39] S. K. Koliwad, R. S. Streeper, M. Monetti et al., "DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation," *Journal of Clinical Investigation*, vol. 120, no. 3, pp. 756–767, 2010.

[40] A. J. Guri and J. Bassaganya-Riera, "Systemic effects of white adipose tissue dysregulation and obesity-related inflammation," *Obesity*, vol. 19, no. 4, pp. 689–700, 2011.

[41] M. Prentki and C. J. Nolan, "Islet β cell failure in type 2 diabetes," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1802–1812, 2006.

[42] L. Zhao, C. A. Cuff, E. Moss et al., "Selective interleukin-12 synthesis defect in 12/15-lipoxygenase-deficient macrophages associated with reduced atherosclerosis in a mouse model of familial hypercholesterolemia," *Journal of Biological Chemistry*, vol. 277, no. 38, pp. 35350–35356, 2002.