Modulation of adipose tissue biology by compounds with anti-inflammatory properties

Vera Alexandra de Araújo Moura

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MODULATION OF ADIPOSE TISSUE BIOLOGY BY COMPOUNDS WITH ANTI-INFLAMMATORY PROPERTIES

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VERA ALEXANDRA DE ARAÚJO MOURA

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>11βHSDH</td>
<td>11β-Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>ASA</td>
<td>Acetylsalicylic acid</td>
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<tr>
<td>AT</td>
<td>Adipose tissue</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>C</td>
<td>Control</td>
</tr>
<tr>
<td>CCR2</td>
<td>Chemokine receptor 2</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>EtOH</td>
<td>Ethanol solution</td>
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<td>FA</td>
<td>Fatty acid</td>
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<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GOT</td>
<td>Glutamic-oxaloacetic transaminase</td>
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<tr>
<td>GPT</td>
<td>Glutamate pyruvate transaminase</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>IKK</td>
<td>Inhibitor of κB kinase</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IL-10</td>
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<tr>
<td>IRS-1</td>
<td>Insulin receptor-1</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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NF-κB: Nuclear factor-κB
NO: Nitric oxide
NSAID: Non steroid anti-inflammatory drugs
PAI-1: Plasminogen activator inhibitor-1
PKC: Protein kinase C
PPARγ: Peroxisome proliferator-activated receptorγ
RIA: Radioimmunoassay
RT-PCR: Reverse transcriptase-polymerase chain reaction
RW: Red wine
TAG: Triacylglycerols
TNFα: Tumor necrosis factor α
VLDL: Very low density lipoprotein
WHO: World Health Organization
ABSTRACT/RESUMO
ABSTRACT

BACKGROUND: Obesity, or the increased accumulation of adipose tissue (AT), is an important risk factor for the development and worsening of other diseases, in part because of the associated metabolic deregulation and low grade inflammatory state. In order to develop more effective interventions, including health policies and treatment options, the identification of substances capable of preventing/treating obesity may be of great help. In this study we focused on acetylsalicylic acid (ASA), because of its recognized anti-inflammatory properties, and also on red wine (RW), which appears to be an interesting beverage from a nutritional point of view, due to its abundance of polyphenolic compounds, with their antioxidant, anti-inflammatory and cell cycle regulation properties. Also, ethanol (EtOH) has been described to interfere with the anti-inflammatory and metabolic effects of ASA.

AIM: This work was carried out to evaluate the effects of ingestion of anti-inflammatory substances (ASA and RW) on the biology of AT, particularly the size of adipocytes, transcription and differentiation markers of inflammation and evaluation of inflammatory and metabolic plasmatic markers in the rat.

METHODS: The work was divided into two stages. In the first one, 4 groups of male Wistar rats were subjected to different liquid regimens: 1) water (C, control; n = 5), 2) 13% EtOH solution (v/v, n = 5), 3) ASA solution (10 mg/kg of body weight (n = 6), and 4) ASA (10 mg/kg of body weight, n = 6) in 13% EtOH (ASA+EtOH). The second stage involved 3 groups of animals having as sole drinking source: 1) water, 2) 13% EtOH solution (n = 5), and 3) RW (with 13% EtOH; n = 4). The beverages were supplied to the rats ad libitum in dark bottles. Body weight was monitored every 2 to 3 days. Beverages and animal pellet food were renewed every 2 to 3 days. At the end of treatment, animals were sacrificed, and plasma, and epididymal and mesenteric AT were collected. Markers of metabolic and inflammatory status and hepatic function were evaluated in plasma. Mesenteric AT was used to evaluate the size of adipocytes and transcription of markers of adipocyte function and cytokines.

RESULTS: EtOH and ASA+EtOH treatments resulted in lower weight gain although no changes were observed in adipocyte size. Important alterations in plasma lipid profile were observed, namely reduction of triacylglycerols, total cholesterol and cholesterol fractions, and non-esterified fatty acids in EtOH+ASA-treated rats. This combined treatment reversed the EtOH-
induced increase in plasma uric acid levels, and resulted in a striking increase in insulin sensitivity index, associated with a reduction of plasma insulin concentration. This effect seemed more pronounced than the reduction observed with EtOH or ASA when administered alone. In the AT ASA+EtOH stimulated peroxisome proliferator-activated receptor γ (PPARγ) and adiponectin transcription, and reversed the EtOH-promoted increasing trend in plasminogen activator inhibitor-1 (PAI-1) transcription. RW ingestion did also result in decreased weight gain without changing adipocyte size. The EtOH-induced increase in uric acid and interleukin-10 plasma concentrations was not seen with RW intake. Furthermore, RW tended to decrease C-reactive protein levels and did significantly decrease the transcription of PAI-1 in the AT when compared with EtOH alone. A significant reduction in insulin concentration, more pronounced than that induced by EtOH alone was observed in the plasma of RW-treated rats, resulting in a tendency towards a higher insulin sensitivity index.

CONCLUSIONS: The results obtained in this study provide evidence of an amelioration of metabolic parameters upon ASA administration. Remarkably, some of ASA effects seemed to be enhanced by EtOH co-treatment. The metabolic effects of ASA alone or supplied with EtOH may possibly be related to anti-inflammatory actions and include direct effects on the AT. In our opinion, this kind of effect deserves to be further explored. As to the experiment testing prolonged RW consumption, we report important modulatory effects on the metabolic and inflammatory status of the animals. Parts of the observed RW effects were distinguishable from those of EtOH, implying that the non alcoholic components of RW play an important role in the influence of the beverage on health. The effects of ASA and RW treatments upon insulin signaling and inflammatory modulation may share mechanisms, direct effects on the AT having been made evident. Further exploration of the actions of these treatments on the AT is warranted.

KEYWORDS: Acetylsalicylic acid, adipose tissue, ethanol, inflammation, metabolic syndrome, obesity, red wine.
RESUMO

ENQUADRAMENTO: A obesidade, ou acumulação excessiva de tecido adiposo (TA), de gênese multifactorial, constitui um importante factor de risco para o desenvolvimento e agravamento de outras doenças, em parte devido à desregulação e inflamação do TA. Com o intuito de desenvolver intervenções mais eficazes, incluindo políticas de saúde e opções de tratamento, tem-se procurado identificar substâncias com características capazes de prevenir/tratar a obesidade e suas respectivas comorbilidades. Este estudo focou-se sobre o ácido acetilsalicílico (ASA), devido às suas reconhecidas propriedades anti-inflamatórias, e também no vinho tinto (VT), que se apresenta como uma bebida interessante do ponto de vista nutricional, devido à sua abundância em compostos polifenólicos, com propriedades antioxidantes, anti-inflamatórias e de regulação do ciclo celular. Além disso, foi já descrita a interferência do etanol (EtOH) nos efeitos anti-inflamatórios e metabólicos do ASA.

OBJECTIVO: Este trabalho foi realizado com o objectivo de avaliar os efeitos da ingestão prolongada de substâncias anti-inflamatórias (ASA e VT) na biologia do TA, com especial relevo no tamanho de adipócitos, transcrição de marcadores de diferenciação e inflamação e avaliação de marcadores metabólicos e inflamatórios plasmáticos, no Rato.

MÉTODOLOGIA: O trabalho foi dividido em duas fases. Na primeira, 4 grupos de ratos Wistar machos foram sujeitos a diferentes regimes de ingestão de líquidos: 1) água (C, controlo; n = 5), 2) solução de EtOH a 13% (v/v, n = 5), 3) solução de ASA (10 mg/kg do peso corporal, n = 6) e 4) ASA (10 mg/kg do peso corporal, n = 6) em solução de EtOH a 13% (ASA+EtOH). A segunda fase envolveu 3 grupos de animais aos quais foi fornecido como única fonte de bebida: 1) água (C, controlo; n = 5), 2) 13% solução de EtOH (n = 5) e 3) VT (com 13% EtOH, n = 4). As bebidas foram fornecidas ad libitum em recipientes escuros. O peso corporal foi monitorizado a cada 2 a 3 dias. As bebidas, bem como a ração dos animais foram renovadas a cada 2 a 3 dias. No final do tratamento, os animais foram sacrificados e foram colhidas amostras de TA mesentérico e TA epididimal. Foi colhido sangue para obtenção de plasma sanguíneo, com vista à avaliação do estado metabólico e inflamatório, bem como avaliação da função hepática. O TA visceral foi usado para avaliação do tamanho dos adipócitos e da transcrição de marcadores da função dos adipócitos e citocinas.

RESULTADOS: Os grupos tratados com EtOH e ASA+EtOH apresentaram um menor ganho de peso corporal, contudo não foram observadas alterações no tamanho dos adipócitos. Ocorreram alterações importantes no perfil lipídico do plasma, nomeadamente com redução
das concentrações de triacilgliceróis, colesterol total e fracções do colesterol e ácidos gordos não esterificados nos ratos tratados com EtOH+ASA. Este mesmo tratamento reverteru o aumento nos níveis plasmáticos de ácido úrico induzido pelo EtOH e, também, resultou num aumento marcado do índice de sensibilidade à insulina, bem como numa redução da concentração de insulina plasmática. Este último efeito pareceu mais pronunciado do que a redução observada com EtOH ou ASA, quando administrados isoladamente. No TA de ratos tratados com ASA+EtOH verificou-se uma estimulação da transcrição de peroxisome proliferator-activated receptor γ (PPAR γ), bem como da transcrição da adiponectina, revertendo-se a tendência de aumento da transcrição do inibidor da activação de plasminogénio (PAI-1) provocada pelo EtOH. O VT também resultou num menor ganho de peso corporal, sem alterar o tamanho dos adipócitos. Verificou-se que o aumento das concentrações plasmáticas de ácido úrico e da interleucina-10, induzido pelo EtOH, foi impedido pela ingestão de VT. Além disso, o VT diminuiu os níveis de proteína C reactiva, além de diminuir significativamente a transcrição do PAI-1 no TA, em comparação com os ratos tratados com EtOH. No plasma de ratos tratados com VT, foi observada uma redução significativa na concentração de insulina, mais pronunciada do que a induzida pelo tratamento com EtOH, resultando num índice de sensibilidade à insulina tendencialmente superior.

CONCLUSÕES: Os resultados obtidos neste estudo tornaram evidente uma melhoria de parâmetros metabólicos, mediante administração de ASA. De forma notória, estes efeitos foram potenciados com a co-administração de EtOH. Os efeitos metabólicos do ASA isoladamente, ou em conjunto com o EtOH, podem estar relacionados com acções anti-inflamatórias e incluir efeitos directos sobre o TA. Na nossa opinião, estes efeitos merecem ser aprofundados. Na segunda parte do trabalho verificou-se uma importante modulação dos estados inflamatório e metabólico, dos animais, em resultado do consumo prolongado de VT, sendo parte dos efeitos observados distintos dos do EtOH, o que indica que os componentes não alcoólicos do VT desempenham um papel importante na influência da bebida na saúde. Os efeitos na sinalização insulínica e de modulação inflamatória observados após administração de ASA e VT levam a concluir que estes dois produtos podem compartilhar mecanismos, tendo sido evidenciados efeitos directos sobre o TA. Estes resultados justificam mais estudos para o respectivo aprofundamento.

PALAVRAS-CHAVE: Ácido acetilsalicílico, etanol, inflamação, obesidade, síndrome metabólica, tecido adiposo, vinho tinto.
CHAPTER I

BACKGROUND

AIM
The World Health Organization (WHO) estimates that at least 1 in 10 adults worldwide are obese and in some Western countries a much higher percentage of the population (25% or more) is affected\(^1\). Obesity is a serious concern as it increases the cardiovascular risk, of type 2 diabetes and of development of some types of cancer, among other health problems. Obesity can be defined as excessive and abnormal fat accumulation that can be detrimental to health\(^2\). In order to develop effective interventions, including health policies and treatment options, it is necessary to understand the interaction between genetic and environmental factors that favor weight gain and how excessive weight disturbs metabolism.

Understanding the causes of obesity and related metabolic disorders is a major challenge, in part because many body systems are affected. Separating the causes and effects is difficult, because disturbances in one organ or tissue can compromise the function of several others. So, new perspectives have now emerged regarding the subject of obesity. Among these is the notion that metabolic dysfunction associated with obesity arises from exposure of adipocytes to excess nutrients\(^3\). A possible extension of this view is that although the cellular consequences of nutrient excess are similar in different cell types, the diverse nature of the underlying cellular responses can be obscured by the complexity of the starting event. In this context, successfully identifying cellular responses that cause disease requires a comprehensive and integrated approach, which can be translated into more effective therapeutic strategies against obesity\(^4\).

In this sense, it is important to understand the physiology and metabolism of adipocytes, the functional units of the AT, due to their involvement in many of the responses at the level of inflammatory and metabolic regulation of the body during obesity, which becomes particularly evident in cases of dysfunction.
THE CONCEPT OF ADIPOSE TISSUE

In a normal individual, the AT represents about 20% (8-18% in men and 14-28% in women) of body weight, being thus one of the largest organs of the human body. It is composed of adipocytes (50%), nervous tissue, collagen fibers, lymph nodes, leukocytes, macrophages, mesenchymal cells, fibroblasts and preadipocytes (adipocyte precursor cells). Anatomically, body fat is distributed mainly between the subcutaneous and visceral compartments. At visceral level, the deposits of tissue are located at the thoracic (mediastinal) and abdominal (omental, mesenteric, perirenal, retroperitoneal, parametrical, epididymal and periovary) spaces. Some adipocytes are also found dispersed in the skin, thymus, lymph nodes, bone marrow, parotid and parathyroid glands, pancreas, among other tissues.

We can distinguish two types of AT, white and brown, mainly composed of white and brown adipocytes, respectively. Body fat distribution is largely determined by genetic factors and dependent on factors such as age, sex, environmental temperature and nutritional status. Brown AT participates in thermoregulation, as it has the ability to dissipate the energy reserves in the form of heat, being more abundant in newborns. Although it was thought that over the years it gradually disappeared and eventually be sparsely dispersed in white AT, becoming a vestigial organ, it was recently described that it is present in adults, especially in cervical and interscapular areas. It was also demonstrated that it is activated by cold in the course of this work, unless otherwise stated, whenever there is reference to AT we will be addressing white AT.

In addition to the important role it plays at the level of mechanical protection of surrounding organs, its contribution to maintain body temperature and serving as a thermal insulator, the AT has traditionally been defined as the principal storage site for large energy reserves.

Currently, we know that beyond these important functions, adipocytes and the surrounding stromal vascular cells produce and secrete autocrine, paracrine and endocrine factors. These factors are able to regulate aspects involved in the development of adipocytes, and exert effects
on peripheral organs important for metabolism, what justifies attributing to these cells functions as metabolic sensors in the body. Regulation of these endocrine factors could lead to new therapeutic approaches targeted at aspects related to adipogenesis, preadipocyte proliferation and differentiation, inflammatory cytokine release and secretion, AT vascularization, and regulation of lipid and glucose metabolism, blood clotting, blood pressure and hormone environment. In this sense, the AT is now considered a true endocrine organ\textsuperscript{7,8}.

**Adipose tissue as an endocrine organ**

AT is a metabolically active tissue that secretes multiple important proteins known as adipokines\textsuperscript{9,10}. Some adipokines play a major role in insulin resistance and cardiovascular complications associated with obesity, especially central or visceral obesity\textsuperscript{11}. Leptin is almost exclusively expressed and produced by adipocytes of the AT\textsuperscript{12}. Among other actions, leptin works as a signaling system of AT to the central nervous system, which acts to regulate the volume of the deposit of fat, controlling the mobilization and utilization of that energy reserves. This hormone plays a role in energy homeostasis by stimulation of energy expenditure and reducing appetite. In obese subjects, contrary to expectations, there is no lack of leptin but, instead, a state of hyperleptinemia. The large amount of circulating leptin in obese people suggests, therefore, a state of leptin resistance, the evidence or clarification of the involved mechanisms remaining yet to decipher, given the multiplicity of conditions that might be involved\textsuperscript{11}.

Leptin works as a sign of efferent and afferent negative feedback and hyperleptinemia assumes an important role in the chronic subinflammatory state in obesity\textsuperscript{11,12}. Leptin improves insulin sensitivity through activation of AMP-activated protein kinase (AMPK)\textsuperscript{13}, however it has a potentially deleterious action once it promotes platelet aggregation\textsuperscript{14,15}, cholesterol accumulation in macrophages, in hyperglycemia\textsuperscript{16} and in angiogenesis\textsuperscript{17}. 
Leptin is highly interesting from the inflammatory point of view, because it is capable of controlling tumor necrosis factor (TNF-α) production and macrophage activation\(^{18}\) and can trigger monocyte chemotactic protein (MCP)-1 expression\(^{19}\), and endothelial cell proliferation and migration\(^{14,15}\).

Unlike other adipokines, adiponectin is decreased in obesity and increases after weight reduction, being highly expressed in AT, more in subcutaneous than in visceral AT\(^{20}\). It has a protective role in atherosclerosis, improves the action of insulin in the liver, reduces hepatic gluconeogenesis and prevents the accumulation of lipids in tissues other than AT\(^{11,21,22}\). In the skeletal muscle, adiponectin increases glucose uptake and oxidation of TAG\(^{11,21,22}\). Additionally, it has anti-inflammatory properties, interfering with the regulation of adhesion molecule expression on vascular endothelial cells\(^{23}\), with the transformation of macrophages into foam cells\(^{24}\), and modulating smooth muscle cell proliferation\(^{25}\). Interfering with macrophages, adiponectin has effects against the action of TNF-α, assuming an important protective role in atherosclerosis\(^{23}\). On the other hand, adiponectin is reduced by elevated levels of TNF-α and interleukin (IL)-6\(^{26}\).

TNF-α is an inflammatory agent produced by adipocytes and by macrophages in the AT, and is associated to adiposity and cardiovascular risk factors\(^{11,21,22}\). After a long period of exposure, it has the ability to stimulate lipolysis, as it decreases the levels of perilipin and insulin-stimulated glucose transport in adipocytes by stimulating the phosphorylation of insulin receptor (IRS)-1 in serine\(^{11,22}\). In rodents, TNF-α is involved in the pathophysiology of insulin resistance and is produced in higher quantities than in humans\(^{27}\). TNF-α is poorly expressed in human AT, although its expression is slightly modified in human obesity, which indicates that AT is indirectly involved in the increased circulating concentrations of TNF-α characteristic of obesity\(^{28}\). TNF-α has the capacity to accelerate atherosclerosis, inducing adhesion molecule expression in endothelial and vascular smooth muscle cells, resulting in altered endothelium-dependent vasodilatation\(^{29,30}\), and promotion of endothelial cell apoptosis\(^{31,32}\).
Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of the plasminogen activators. It is an inhibitor of fibrinolysis, a physiological antithrombotic process that allows fibrin clot breakdown when it is no longer needed for haemostasis. A decreased fibrinolytic capacity, as a result of increased circulating levels of PAI-1, is considered a cardiovascular risk factor\textsuperscript{33}. PAI-1 is mainly produced by endothelial cells, but also by AT, in small quantities\textsuperscript{34}. Increased circulating levels of PAI-1 are mainly correlated with central obesity\textsuperscript{33, 35}, since visceral AT secretes more PAI-1 than subcutaneous AT\textsuperscript{34}. A few studies have shown an association between PAI-1 and cardiovascular and metabolic disorders\textsuperscript{33, 35, 36}.

IL-6 is a cytokine produced by several cells (fibroblasts, endothelial cells, monocytes) including adipocytes, being positively correlated with high levels of AT and body mass index (BMI)\textsuperscript{37}. Most of the IL-6 produced on AT comes from endothelial cells and monocytes/macrophages\textsuperscript{34, 37}. IL-6 is a multifunctional cytokine that targets several tissues and cell types. It controls the hepatic production of inflammatory proteins such as C-reactive protein (CRP), a positive relationship existing between IL-6 levels in AT and circulating CRP levels\textsuperscript{38}, an important cardiovascular risk factor\textsuperscript{39}. Visceral AT produces three times more IL-6 than subcutaneous AT does\textsuperscript{37}. This may explain, in part, the deleterious role of central obesity in cardiovascular diseases. Indeed, IL-6 visceral AT production could have a direct effect on hepatic metabolism as its venous drainage goes directly to the liver through the portal vein. Thus, IL-6 produced by intra-abdominal AT could directly contribute to visceral obesity-related hypertriglyceridaemia by stimulating hepatic secretion of triacylglycerols (TAG) in the form of very low-density lipoprotein (VLDL)\textsuperscript{40}. The secretion of IL-6 is strongly stimulated by β-adrenergic activation and slightly suppressed by glucocorticoids. Fundamentally, it has anti-insulin action and increases lipolysis in subcutaneous AT\textsuperscript{41}. Recent studies suggest that IL-6 could be implicated in insulin resistance and its complications\textsuperscript{35, 42, 43}. 
HYPERPLASTIC AND HYPERTROPHIC OBESITY

When energy intake exceeds energy expenditure, the resulting state of excess energy can trigger responses in many cell types, including endothelial cells, hepatocytes, myocytes, adipocytes, monocytes and macrophages, which can lead to metabolic dysfunction\(^1\). In the AT there is an increase in energy storage in adipocytes, in the form of intracellular TAG. Consequently, the increase in fat mass is reflected both by increasing the intracellular lipid content with increasing adipocyte size (hypertrophy) but also by the increased number of adipocytes (hyperplasia)\(^{44}\). The factors that regulate hypertrophy and hyperplasia of adipocytes are not well understood, but high concentrations of insulin and glucocorticoids in circulation appear to stimulate differentiation of preadipocytes. Substances such as insulin-like growth factor 1 (IGF-1), released by hypertrophic adipocytes, stimulate hyperplasia of adipocytes in a paracrine manner\(^{46}\). Whether in response to energy imbalance AT growth occurs by hypertrophy or hyperplasia may vary with the anatomical location of AT (visceral or subcutaneous), and may therefore be associated with more or less deleterious metabolic disorders. In this regard, recent studies suggest that changes associated with hypertrophy of adipocytes seem to be the initial steps towards the adipocyte cell dysfunction\(^{21}\).

The process of hypertrophy, especially if accompanied by insufficient blood supply, induces hypoxia of adipocytes with consequent production of reactive pro-oxidant species. These molecules are generated by mitochondrial oxidation of nutrients and other metabolic processes within the cell. If there is excessive production of these molecules, it creates a situation of oxidative stress, which can damage cellular structures and trigger an inflammatory response\(^{44-46}\). Hypertrophy also affects the functioning of the endoplasmic reticulum (ER, that is involved in the processing of newly synthesized proteins, resulting in mature proteins) also providing the excessive production of reactive pro-oxidant species and activating a cellular response that can result in inflammation and insulin resistance\(^{1,47}\).
From the perspective of cellular homeostasis, this response to block insulin signaling can make sense, in that it limits the uptake of nutrients. However, neighboring cells and distant tissues that remain insulin-sensitive are placed at greater risk. When insulin resistance progresses and inflammation worsens, a vicious cycle may be generated, with an accumulation of pro-inflammatory factors\(^{1,44,47}\).

Macrophage infiltration in white AT is an important feature of the low-grade inflammation of obesity, and is the principal source of IL-6 and TNF-\(\alpha\), which induce insulin resistance in adipocytes. Moreover, body mass index (BMI) is well correlated with the number of macrophages in AT. However, the cellular and molecular mechanisms involved in such infiltration are poorly understood. It seems that chemokine receptor 2 (CCR2) and its ligand MCP-1 are necessary for accumulation of inflammatory macrophages\(^{21, 48-51}\). MCP-1 and CCR2 knockout mice have fewer macrophages in AT and low inflammatory gene expression\(^{52}\). Cinti et al\(^{49}\) showed that the accumulation of macrophages in AT occurs mainly in the vicinity of dead adipocytes, where the inflammatory cells sequester residual lipid droplets. The authors demonstrated a clear sequence of events resulting from the death of adipocytes, leading to a chronic inflammatory state underlying the complications of obesity. However, although the authors showed that there is a significant positive correlation of these events with the size of adipocytes, no explanation was proposed for the association between the size of adipocytes and the predisposition to cell death. In another study, Monteiro et al\(^{53}\) showed that larger adipocytes are more fragile and susceptible to rupture when submitted to common physical forces. These considerations support the fact that the location of fat strongly influences its effects on health, visceral obesity being the most pathogenic, since adipocytes in the abdominal cavity are subject to sudden variations in intra-abdominal pressure, for example during coughing, exercise, and intra-abdominal pathologies. Furthermore, the intra-abdominal pressure is higher in obese individuals. Thus, the visceral adipocytes in the abdominal cavity are more exposed to mechanical stress than adipocytes in subcutaneous AT. In addition, the accumulation of pro-inflammatory factors and stress of the ER affect the recruitment of
macrophages to AT, whose main function is to remove the products resulting from lysis of adipocytes\textsuperscript{46,49}.

Given the inflammatory changes induced by overweight and obesity and their associated comorbidities, it is important to consider the important inflammatory changes induced by weight loss. Cancelo et al\textsuperscript{21} demonstrated that the loss of body weight is accompanied by the reduction of the size of adipocytes, with consequent increase in plasmatic anti-inflammatory molecules, specifically IL-10. A reduction in chemotactic molecules for monocytes and macrophages (MCP-1), and lower expression of TNF-\(\alpha\), inversely proportional to the activity of lipoprotein lipase (LPL), with improved lipid profile, are also observed. Interestingly, weight loss not only leads to an improvement of the inflammation in obesity and its comorbidities, but also to a decrease in inflammatory gene expression\textsuperscript{50}. Three months after gastric bypass surgery, a reduction of macrophages in the AT of obese patients was observed, with a decrease in immune-response gene expression, especially those involved in macrophage aggregation\textsuperscript{21}.

Peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) is a member of the ligand-activated nuclear receptor family which is highly expressed in the AT, where it is critically required for adipocyte differentiation and lipid storage\textsuperscript{54}. Accordingly, it plays a crucial role in adipogenesis and its modulation can affect the capacity of AT to expand. Thiazolidinediones, PPAR\(\gamma\) ligands, the thiazolidinediones, have beneficial effects in hyperlipidemia, insulin resistance, the metabolic syndrome and a variety of diseases, such as type 2 diabetes, cancer and atherosclerosis\textsuperscript{55} and are likely to reduce inflammatory gene expression in AT\textsuperscript{56}.

**METABOLIC SYNDROME**

The topography of the anatomical distribution of AT plays an important role in the development of some risk factors for disease\textsuperscript{11}. Abdominal fat, particularly visceral, is associated with features of metabolic syndrome. The syndrome combines a complex set of interrelated risk factors for cardiovascular disease and diabetes. These factors include dysglycemia, raised blood pressure, elevated TAG levels, decreased high density lipoprotein (HDL) cholesterol levels, and
obesity (particularly central adiposity)\cite{57, 58}. With these risk factors, it has been demonstrated clearly that the syndrome is common and that it has a rising prevalence worldwide, which relates largely to increasing obesity and sedentary lifestyles. As a result, the metabolic syndrome is now both a public health and a clinical problem\cite{58-60}. Several clinical definitions of the metabolic syndrome have been proposed. This has led to some confusion on the part of clinicians regarding how to identify patients with the syndrome. Some controversy also exists about whether the metabolic syndrome is a true syndrome or a mixture of unrelated phenotypes.

Recently, International Diabetes Federation and American Heart Association/National Heart, Lung, and Blood Institute representatives held discussions to attempt to resolve the remaining differences between definitions of metabolic syndrome. An agreement was achieved, that abdominal obesity should not be a prerequisite for diagnosis but that it is 1 of the 5 criteria, so that the presence of any 3 of 5 risk factors constitutes a diagnosis of the metabolic syndrome\cite{61}. This resulted in the common definition shown in Table 1.

<table>
<thead>
<tr>
<th><strong>Measure</strong></th>
<th><strong>Categorical Cut Points</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated waist circumference</td>
<td>Population and country-specific definitions</td>
</tr>
<tr>
<td>Elevated TAG</td>
<td>≥ 150 mg/dL</td>
</tr>
<tr>
<td>Reduced HDL-c</td>
<td>≤ 40 mg/dL in males</td>
</tr>
<tr>
<td></td>
<td>≤ 50 mg/dL in females</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>Systolic &gt; 130 mmHg and/or</td>
</tr>
<tr>
<td></td>
<td>Diastolic &gt; 85 mmHg</td>
</tr>
<tr>
<td>Elevated fasting glucose</td>
<td>&gt; 100 mg/dL</td>
</tr>
</tbody>
</table>

Table 1 – Criteria for clinical diagnosis of the metabolic syndrome. Based on Alberti, 2009\cite{61}.

Several reasons have been proposed to explain the stronger association between visceral, rather than subcutaneous, AT with the predisposition to develop obesity complications and
among them is the differential expression of proteins, between the two depots. Just to name a few examples, in visceral AT there is a greater expression of β1-2 adrenergic receptors compared to α2, which makes it especially sensitive to lipolysis induced by catecholamines. There is also a smaller effect of insulin signaling with a reduction of its anti-lipolytic effect, due to the decreased IRS-1 expression\textsuperscript{49}. Expression of 11β-hydroxysteroid dehydrogenase (11βHSDH) that converts cortisone to cortisol and which predisposes to an increased responsiveness to glucocorticoids and an accumulation of fat is also more pronounced in visceral obesity\textsuperscript{11,46}.

Metabolic syndrome and obesity also have in common a redox imbalance favoring a pro-oxidant status. Oxidative stress is increased in obese subjects\textsuperscript{62,63} and therefore is considered as an early finding and one of the important causes of obesity-associated metabolic syndrome\textsuperscript{64}. Fat accumulation increases the expression of NADPH oxidase and decreases expression of antioxidant enzymes, being also correlated with markers of systemic oxidative stress\textsuperscript{65}. In cultured adipocytes, fatty acids increase oxidative stress via NADPH oxidase activation, provoking dysregulated production of adipokines, including adiponectin, PAI-1, IL-6, and MCP-1\textsuperscript{64}. The close association between redox and inflammatory signaling favour the establishment of vicious cycles between promiscuous pathways of both phenomena, resulting in self-perpetuation of cellular dysfunction and disease.

In short, the size of adipocytes appears to be, in itself, a risk for inflammation with a subsequent series of cardiovascular and metabolic deleterious consequences. It also appears that the overwhelming production of proinflammatory cytokines by the AT of obese individuals, along with a failing production of anti-inflammatory cytokines, may be involved in the pathophysiology of obesity-related pathologies.
Taking into consideration the growing obesity epidemic, along with the recognition that the inflammatory activation that associates with this condition has a special pathological value in determining the increased risk for developing obesity-associated co-morbidities, we aimed to evaluate the effects of substances with anti-inflammatory properties [acetylsalicylic acid (ASA) and red wine (RW)] on the biology of AT, namely the size of adipocytes, AT transcription of differentiation and inflammation markers and plasma inflammatory and metabolic factors, in the rat.
CHAPTER II

EFFECTS OF PROLONGED ADMINISTRATION OF ACETYLSALICYLIC ACID AND ETHANOL
Acetylsalicylic acid (ASA) belongs to the group of nonsteroidal anti-inflammatory drugs (NSAID), used as anti-inflammatory, anti-pyretic, analgesic and also as anti-platelet agents\textsuperscript{66}. It is the active principle of aspirin\textsuperscript{b}, one of the most consumed drugs worldwide\textsuperscript{66}. Like all NSAID, ASA is a nonspecific inhibitor of the enzyme cyclooxygenase (COX or prostaglandin H synthase). It irreversibly acetylates the enzyme at serine 530 which impedes the access of its substrates to the active site\textsuperscript{67}. COX transforms arachidonic acid, or the corresponding n-3 fatty acid (eicosapentaenoic acid) present in membrane phospholipids, into the cyclic prostanoids which include prostaglandins and thromboxanes\textsuperscript{66}. These molecules are inflammatory mediators interfering with vasodynamic function and platelet activity. There are two types of COX enzymes: COX-1 and COX-2. The former is constitutively present in almost all tissues and the latter is induced by cytokines produced locally mainly by leukocytes in response to injury or microbial invasion. COX-2 plays an important role in the generation of inflammation\textsuperscript{67}.

As a result of COX-1 inhibition, ASA has a key anti-platelet effect since it inhibits the synthesis of thromboxane A2, a potent platelet agonist and platelet aggregator. Because platelets lack the capacity to synthesize more COX and the inhibition of the enzyme is irreversible, the aggregant properties of platelets are lost. As to other cells with capacity to synthesize the enzyme, namely endothelial cells, the effects are not as marked and the production of prostacyclins is maintained\textsuperscript{67}. Much owing to this property, the use of ASA for secondary prevention and, to a lesser degree, primary prevention of cardiovascular events is a well-established standard of care\textsuperscript{67}, due to the coagulation, hemodynamic, and metabolic abnormalities seen in these conditions\textsuperscript{67}.
In many cardiovascular disease settings, obesity is a common and frequent finding. Paralleling the rise in obesity, the metabolic syndrome is a rapidly growing health problem around the world. This condition is associated with a three-fold increase in the prevalence of coronary heart disease, myocardial infarction, and stroke\textsuperscript{68}, and the low grade inflammatory state that accompanies both obesity and the metabolic syndrome has been proposed to play a pivotal role\textsuperscript{69}. Studies have proposed that ASA benefits on cardiovascular disease go beyond its effects on platelet aggregation, and some of the mechanisms by which ASA may favorably alter the course of atherosclerosis include effects on endothelial function and glycemia\textsuperscript{69, 70}. Considering diabetes, despite the fact that it constitutes a prothrombotic state, the low-grade inflammation that is present in this condition also plays a pivotal role in atherogenesis development and might be modulated by ASA treatment. In this regard, in a randomized study, Hovens et al\textsuperscript{71} determined the effects of aspirin\textsuperscript{®} on inflammation represented by CRP and IL-6 in type 2 diabetic subjects without cardiovascular disease. It was concluded that although there were no statistically significant results, the values of CRP and IL-6 tended to decrease.

It is also necessary to bear in mind that part of the effects of ASA may be ascribed to its antioxidant properties\textsuperscript{72, 73}. One of the mechanisms involves the decrease in the generation of reactive oxygen species in prostaglandin metabolism through inhibition of COX\textsuperscript{74}. Furthermore, in the liver, ASA is mostly hydrolyzed to the salicylate form, this being the main metabolite present in plasma\textsuperscript{73}. Like other simple phenols, salicylate is an anti-oxidant\textsuperscript{75}. In what concerns the metabolic syndrome, chronic ASA administration prevents oxidative stress, as well as reverses completely glucose intolerance and decreases oxidative status\textsuperscript{73}.

These observations suggest that interventions with anti-inflammatory drugs such as aspirin\textsuperscript{®} and statins might constitute a novel approach to the treatment of obesity and type 2 diabetes mellitus, by increasing the likelihood of weight loss in type 2 diabetes mellitus patients\textsuperscript{76}. A study in mice showed that ASA may be a valuable tool to delay or prevent the development of type 2 diabetes from a pre-diabetic condition\textsuperscript{77}. Some changes on this respect may be associated with improved insulin sensitization\textsuperscript{78}.
Despite these evidences, few studies have proposed that the decrease in metabolic and cardiovascular complications associated with ASA may include direct effects on the AT[79, 80], an important instigator of the inflammatory status underlying metabolic syndrome[46]. In the same line, and because the knowledge of the effects of the prolonged treatment with ASA on this field is still incipient, we proposed to study the effects of exposure to ASA in metabolic and inflammatory markers, focusing on the effects on AT.

Curiously, some results regarding metabolic effects of prolonged aspirin treatment have been demonstrated having ethanol (EtOH) intoxication as an inducer of changes in blood and hepatic lipids[81, 82]. These studies show that ethanol-induced alterations are reversed by treatment with ASA. Two other important studies show that ASA increases alcohol dehydrogenase activity[83] and that EtOH enhances ASA absorption from the gastrointestinal tract[84]. Having this into account, the effects of ASA on the same parameters were also studied in a group of rats treated simultaneously with EtOH and the appropriate EtOH-drinking control was used for comparison.
**MATERIALS AND METHODS**

**CHEMICALS AND REAGENTS.** ASA, ethidium bromide and sodium pentobarbital were obtained from Sigma-Aldrich Chemicals (Saint Louis, MO, USA). Tripure Isolation Reagent was obtained from Roche (Indianapolis, IN, USA). Deoxyribonucleotide triphosphate (dNTP), random hexamers, RNase inhibitor (RNase OUTTM), RNase H, SuperScript-II reverse transcriptase and Taq DNA polymerase were obtained from Invitrogen (Carlsbad, United Kingdom). Primers for RT-PCR analysis were purchased from Metabion International (Martinsried, Germany). The commercial kits used to measure hormones, adipokines and cytokines were acquired from ImmuChemTM Double Antibody kit (Biomedicals, Orangeburg, NY, USA) for corticosterone, Rat Adiponectin Immunoassay Kit (Invitrogen, Camarillo, CA, USA) for adiponectin, Ultrassensitive Rat Insulin Kit (Mercodia Uppsala, Sweden) for insulin and Milliplex Rat Cytokine/Chemokine kit (Millipore, Billerica, MA, USA) for TNF-α, IL-6, IL-10, leptin and MCP-1. NEFA-HR(2) kit used to measure NEFA was purchased from Wako Chemicals GmbH (Neuss, Germany).

**ANIMALS AND DIETS.** Twenty two male Wistar rats (Harlan Iberica, Barcelona, Spain) with mean body weight of 250.30 ± 8.59 g were maintained under standard temperature and light conditions (20–22°C, 12-h light/dark cycle). Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals.

The animals were divided into four groups that were subjected to different treatments: 1) water (C, control; n = 5), 2) 13% (v/v) EtOH solution ( n = 5); and 3) ASA [10 mg/kg of body weight (average ASA ingestion 11.17 ± 0.34 mg/kg)] solution ( n = 6), and 4) ASA [10 mg/kg of body weight (average ASA ingestion 10.47 ± 0.40 mg/kg)] in 13% EtOH ( n = 6). The beverages were
supplied to the rats *ad libitum* in dark bottles. Body weight gain was monitored every 2 to 3 days. Beverages and animal pellet food were renewed every 2 to 3 days, with beverage intake being assessed every 2 to 3 days.

After 6 weeks of treatment, the animals were anesthetized with sodium pentobarbital (60 mg/kg of body weight, intraperitoneally), and blood was drawn to K$_2$-EDTA-containing tubes to extract plasma. Two samples of visceral (mesenteric) AT were removed. One sample was frozen in liquid nitrogen and stored at -80°C for protein extraction, while the other was formalin-fixed, paraffin-embedded, and sectioned for histological analysis. Epididymal AT pads from both sides and the livers were removed and weighed.

**Plasma measurements.** Plasma samples were used to measure uric acid, ferritin and glutamic-oxaloacetic (GOT) and glutamate-pyruvate (GPT) transaminases as markers of hepatic function. Circulating markers of metabolic status such as glucose, urea, creatinine, TAG, cholesterol, HDL, VLDL and low-density lipoprotein (LDL) were also measured. Analysis of plasma biochemical markers was performed in the Department of Clinical Pathology of the Hospital São João, Porto, Portugal, according to the Nordic Recommendation$^{(83)}$.

Corticosterone was measured by RIA using antiserum against corticosterone and $^{125}$I-corticosterone. The commercial kit used to measure corticosterone was specific for this hormone and had very low reactivity with either estrone or androstenedione. The detection limit was 7.7 ng/mL. Adiponectin and insulin were measured by ELISA using a specific polyclonal antibody for rat adiponectin or monoclonal antibody for insulin, respectively. The commercial kit used to measure adiponectin had a detection limit of 50.00 pg/mL and was specific for this hormone with very low reactivity with either leptin or resistin. The detection limit was 13 μg/mL for the test measuring insulin. NEFA were measured using an enzymatic colorimetric method assay NEFA-HR(2). TNF-α, IL-6, IL-10, leptin and MCP-1 were measured with
Milliplex Rat Cytokine/Chemokine kit. The detection limits for these measurements were 4.44, 9.80, 5.41, 21.50 and 3.81 pg/mL, respectively.

**Measurement of adipocyte size.** Hematoxylin- and eosin-stained 4-μm-thick sections of AT were observed under identity occultation and adipocyte measurement was performed as described previously\(^{[96]}\). Photographs of all adipocytes were made at a final magnification of 200×. The largest diameter of each cell was measured. One hundred to 150 adipocytes from five randomly-selected different optical fields were measured per animal.

**RNA extraction.** Adipose tissue RNA extraction was performed using Tripure Isolation Reagent, according to the manufacturer’s directions. In short, 500-600 mg of AT was dissolved in 2 mL of Tripure and homogenized on a Polytron homogenizer (Kinematica, Luzern, Switzerland). Samples were transferred to Falcon tubes and centrifuged at 12 000 g for 10 min at 4 °C, to remove the excess of lipids. After that, 0.4 mL of chloroform was added to the homogenates, and samples were vigorously shaken and were allowed to sit for 15 min after which were centrifuged at 12 000 g for 15 min at 4°C. The upper aqueous phase was transferred to a new tube and mixed with 1 mL of isopropanol. Then samples were centrifuged at 12 000 g for 10 min at 4 °C, and the resulting pellet was washed with 2 mL of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water and vigorously shaken. After centrifugation at 7 500 g for 5 min, pellets were re-dissolved in DEPC-treated water and samples were stored at -20°C until being used for RT-PCR.

**RT-PCR — Reverse transcriptase-polymerase chain reaction.** Five micrograms of RNA were used as template for cDNA production through the incubation with SuperScript-II reverse transcriptase for 50 min at 42°C with 50 ng/μL random hexamers, 10 mM per dNTP, and 40 U/μL
RNase inhibitor. Samples were incubated for 20 min at 37°C with 1 μL RNase H. PCR amplification was performed in the presence of 2.3 mM MgCl₂, 0.5 mM per primer, 0.2 mM dNTPs, 2 U Taq DNA polymerase and 2 μL of RT product in a final volume of 25 μL. Simultaneous amplification of the target gene with the invariant housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. Primers, termocycling conditions and predicted size of PCR products are shown in Table 2. A denaturation period of 5 min at 94°C preceded amplification cycles after which there was a final extension period of 10 min at 72°C. PCR products were visualized on a 1.6% (v/v) agarose gel and visualized with an ultraviolet transiluminator (Vilber Lourmat, Marne La Vallée, France) using ethidium bromide staining. The expression of each transcript was determined using Visionworks LS Software (UVP Inc, USA), normalized to the expression of GAPDH in the same sample, and compared.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin (390 bp)</td>
<td>ATT ACT GCA ACC GAA GGG CC&lt;br&gt;GCC AGT GCT GCC GTC ATA AT</td>
<td>94°C; 45 s</td>
<td>58.5°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>FAS (324 bp)</td>
<td>TGG GCC CAT TTT GCT GTC AA&lt;br&gt;TGC TGG TCC TGT TGC AAG GC</td>
<td>94°C; 45 s</td>
<td>57.5°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>MCP-1 (394 bp)</td>
<td>CA GGC CAG AAA CCA GCC AAC T&lt;br&gt;GCT GAA GTC CTT AGG GTT GAT GCA</td>
<td>94°C; 45 s</td>
<td>57.5°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>PAI-1 (335 bp)</td>
<td>TCC TGG CCT CCG AAA GCT CT&lt;br&gt;TGC TGG CTT CTA AGA AGG GG</td>
<td>94°C; 45 s</td>
<td>58.2°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>PPARγ (423 bp)</td>
<td>GGG AGA TCC TCC TGT TGA CCC A&lt;br&gt;GCT TTA TCC CCA CAG ACT CGG C</td>
<td>94°C; 45 s</td>
<td>57.6°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>TNFα (295 bp)</td>
<td>TAC TGA ACT TCG GGG TGA TTT GTC C&lt;br&gt;CAG CCT TGT CCC TTT AGG AGA ACC</td>
<td>94°C; 45 s</td>
<td>58.9°C; 45 s</td>
<td>72°C; 45 s</td>
<td>30</td>
</tr>
<tr>
<td>GAPDH (682 bp)</td>
<td>ACT GGC GTC TCC ACC ACC AT&lt;br&gt;TCC ACC ACC CTG TTT CTG TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 – Primer sequences, thermocycling conditions and predicted size of PCR products. Adiponectin, fatty acid synthase (FAS), monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), peroxisome proliferator-activated receptor γ (PPARγ) and tumor necrosis factor α (TNF-α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
CALCULATIONS AND STATISTICAL ANALYSIS. Results are expressed as mean ± standard error of mean (SEM). The differences between groups were evaluated by unpaired Student’s t or One-way ANOVA followed by Newman-Keuls Multiple Comparison Test on GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when P < 0.05.
RESULTS

**Body weight.** During the 6 weeks of treatment the body weight of all animals was monitored and recorded. Mean body weight was similar among groups at the beginning of treatment (Table 3). All animals showed an increase in their body weight during the course of treatment ($P < 0.05$) (Figure 1a), although the increase was not similar among groups ($P < 0.05$) (Figure 1b). At the end of treatment, animals receiving ethanol (EtOH and ASA+EtOH) had lower body weight gains than control rats. The body weight gain in ASA+EtOH group was also significantly lower than that observed in ASA-treated rats. Regarding the final body weights of the animals, although the $P$ value obtained from ANOVA was 0.016, the post-hoc Newman-Keuls test did not reveal statistical significant differences on pair-wise comparisons among groups.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C</th>
<th>EtOH</th>
<th>ASA</th>
<th>ASA+EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>240.2 ± 13.1</td>
<td>244.8 ± 12.0</td>
<td>258.7 ± 6.5</td>
<td>257.5 ± 2.7</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>414.0 ± 9.0</td>
<td>367.8 ± 12.4</td>
<td>414.5 ± 17.5</td>
<td>369.8 ± 6.8</td>
</tr>
</tbody>
</table>

Table 3 – Body weight variation in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, $n = 6$) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, $n = 6$). Results are expressed as mean ± SEM.

**Liver and adipose tissue weight and adipocyte size.** Regarding liver weight, there were no differences among groups when the organ weight was compared before or after the correction of its mass by the body weight of the animal (Table 4). However, regarding epididymal AT, a reduction of its weight was observed in EtOH and ASA+EtOH groups when compared with control ($P < 0.05$ for both), but after correction for total body weight, this difference was dissipated. Adipocytes from
mesenteric AT of control animals measured 149.10 ± 8.05 μm of diameter and no differences were detected among treatment groups (Figure 2).

**Figure 1** – Body weight evolution (a) and body weight gain (b) during treatment in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) and acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). The body weight of animals from all groups varied significantly (P < 0.05) with time of treatment. Results are expressed as mean ± SEM. *P < 0.05 vs C. §P < 0.05 vs C, ASA.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C</th>
<th>EtOH</th>
<th>ASA</th>
<th>ASA+EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>13.72 ± 0.26</td>
<td>12.11 ± 0.37</td>
<td>13.45 ± 0.97</td>
<td>12.56 ± 0.47</td>
</tr>
<tr>
<td>Liver weight/body weight ratio</td>
<td>0.0332 ± 0.0003</td>
<td>0.0330 ± 0.0013</td>
<td>0.0324 ± 0.0016</td>
<td>0.0339 ± 0.0010</td>
</tr>
<tr>
<td>Epididymal AT weight (g)</td>
<td>11.28 ± 1.38</td>
<td>7.12 ± 0.22*</td>
<td>8.70 ± 0.72</td>
<td>7.74 ± 0.89*</td>
</tr>
<tr>
<td>Epididymal AT weight/body weight ratio</td>
<td>0.0269 ± 0.0027</td>
<td>0.0194 ± 0.0007</td>
<td>0.0213 ± 0.0020</td>
<td>0.0211 ± 0.0026</td>
</tr>
</tbody>
</table>

**Table 4** – Liver and adipose tissue weight in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). AT, adipose tissue. Results are expressed as mean ± SEM. *P < 0.05 vs C.
**Biochemical markers of hepatic function.** In order to evaluate the inflammatory status of the liver, we measured plasma markers of liver function, in particular glutamic-oxaloacetic (GOT) and glutamate-pyruvate (GPT) transaminases, which are presented on Table 5. The values of GOT tended to be higher in EtOH-treated group when compared with control, however no significant differences were achieved ($P > 0.05$). Consequently, the GOT/GPT ratio also tended to increase in EtOH-treated animals.

![Adipocyte diameter graph](image)

**Figure 2** – Adipocyte diameter from mesenteric adipose tissue in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, $n = 6$) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, $n = 6$). Results are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C (U/L)</th>
<th>EtOH (U/L)</th>
<th>ASA (U/L)</th>
<th>ASA+EtOH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (U/L)</td>
<td>95.40 ± 12.52</td>
<td>149.70 ± 58.85</td>
<td>118.80 ± 26.47</td>
<td>89.67 ± 19.26</td>
</tr>
<tr>
<td>GPT (U/L)</td>
<td>39.20 ± 2.62</td>
<td>39.00 ± 2.49</td>
<td>40.83 ± 2.56</td>
<td>42.33 ± 2.85</td>
</tr>
<tr>
<td>GOT/GPT</td>
<td>2.50 ± 0.41</td>
<td>3.61 ± 1.09</td>
<td>2.97 ± 0.75</td>
<td>2.09 ± 0.35</td>
</tr>
</tbody>
</table>

**Table 5** – Biochemical markers of hepatic function in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, $n = 6$) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, $n = 6$). GOT, glutamic-oxaloacetic transaminase; GPT, glutamate-pyruvate transaminase. Results are expressed as mean ± SEM.
CIRCULATING MARKERS OF METABOLIC STATUS. In order to evaluate the metabolic status of animals under study, we measured plasma markers of metabolic function. To this end, we quantified glucose, urea, creatinine, TAG, total cholesterol and the respective fractions VLDL, HDL, LDL, uric acid, CRP and NEFA (Table 6).

The plasma glucose concentrations were similar among all groups, being slightly higher in the group treated with EtOH. Urea and creatinine presented similar values in all groups studied. There was a significant decrease on TAG and VLDL values in animals treated with ASA+EtOH, comparing to animals treated with EtOH, ASA and control group (P < 0.05). Cholesterol and HDL concentrations decreased significantly after ASA+EtOH treatment in comparison to control and ASA groups (P < 0.05). NEFA concentrations also tended to decrease in all treatment groups although the difference was only significant in ASA+EtOH group (P < 0.05). Furthermore, with regard to the ratio between total cholesterol and HDL, the treatments with EtOH increased this parameter significantly (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>EtOH</th>
<th>ASA</th>
<th>ASA+EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g/L)</td>
<td>1.69 ± 0.08</td>
<td>1.80 ± 0.17</td>
<td>1.55 ± 0.08</td>
<td>1.53 ± 0.12</td>
</tr>
<tr>
<td>Urea (g/L)</td>
<td>0.39 ± 0.02</td>
<td>0.40 ± 0.02</td>
<td>0.37 ± 0.01</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>5.38 ± 0.12</td>
<td>5.26 ± 0.32</td>
<td>5.17 ± 0.15</td>
<td>4.97 ± 0.08</td>
</tr>
<tr>
<td>TAG (g/L)</td>
<td>1.34 ± 0.22</td>
<td>1.31 ± 0.20</td>
<td>1.11 ± 0.09</td>
<td>0.61 ± 0.05*</td>
</tr>
<tr>
<td>Cholesterol (g/L)</td>
<td>0.75 ± 0.05</td>
<td>0.68 ± 0.03</td>
<td>0.76 ± 0.05</td>
<td>0.55 ± 0.05*</td>
</tr>
<tr>
<td>HDL (g/L)</td>
<td>0.43 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.42 ± 0.03</td>
<td>0.32 ± 0.03*</td>
</tr>
<tr>
<td>LDL (direct) (g/L)</td>
<td>0.09 ± 0.01</td>
<td>0.17 ± 0.01**</td>
<td>0.15 ± 0.02**</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>VLDL (g/L)</td>
<td>0.28 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>0.24 ± 0.02</td>
<td>0.13 ± 0.03*</td>
</tr>
<tr>
<td>NEFA (μmol/L)</td>
<td>454.0 ± 30.72</td>
<td>350.0 ± 69.25</td>
<td>311.0 ± 23.35</td>
<td>228.0 ± 55.92*</td>
</tr>
<tr>
<td>Cholesterol/HDL</td>
<td>1.74 ± 0.06</td>
<td>2.05 ± 0.12*</td>
<td>1.84 ± 0.05</td>
<td>1.76 ± 0.03</td>
</tr>
<tr>
<td>TAG/HDL</td>
<td>2.08 ± 0.47</td>
<td>1.97 ± 0.38</td>
<td>1.51 ± 0.18</td>
<td>1.52 ± 0.39</td>
</tr>
</tbody>
</table>

Table 6 – Circulating markers of metabolic status in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-sterified fatty acids; TAG, triacylglycerols; VLDL, very-low-density lipoprotein. Results are expressed as mean ± SEM. *P < 0.05 vs C, EtOH, ASA. †P < 0.05 vs C, ASA. **P < 0.05 vs C, ASA+EtOH. ‡P < 0.05 vs C. §P < 0.05 vs C, ASA, ASA+EtOH.
CRP and uric acid, markers involved in inflammatory phenomena, were also measured and the results are presented in Figure 3. CRP displays a trend to increase slightly in EtOH- and ASA+EtOH-treated groups, comparing to control and ASA groups respectively (Figure 3a). However, with regard to uric acid, its concentration was significantly increased in the group treated with EtOH compared with all the others groups ($P < 0.05$) (Figure 3b).

![Figure 3](image_url)

**Figure 3** – Plasma levels of (a) C reactive protein (PCR) and (b) uric acid in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$) acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, $n = 6$) and acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, $n = 6$). Results are expressed as mean ± SEM. *$P < 0.05$ vs C, ASA, ASA+EtOH.

Regarding the metabolic hormones corticosterone and insulin and the insulin sensitivity index (calculated by dividing the plasma concentration of glucose by that of insulin, for each animal), results are presented in Figure 4. Relatively to corticosterone, no significant differences were detected on the plasma concentrations of this hormone among groups (Figure 4a). There was a significant decrease in plasma insulin ($P < 0.05$) among treatment groups, compared to the control group. Interestingly, treatment with ASA+EtOH led to a substantial (more than 3-fold) increase in the insulin sensitivity index.
**Figure 4** – Plasma levels of (a) corticosterone, (b) insulin and (c) insulin sensitivity index in control (C, n = 5) animals, after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n=6) and acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n=6). Results are expressed as mean ± SEM. *P < 0.05 vs C. **P < 0.05 vs C, EtOH, ASA.

**Plasma levels of inflammatory cytokines and adipokines.** Overlaid with particular interest is the evaluation of the plasma concentration of some cytokines (IL-6, IL-10, MCP-1, TNF-α) and adipokines (adiponectin and leptin) in view of its known biological properties. Results regarding these measurements are presented in Figures 5 and 6. Plasma adiponectin and leptin concentrations remained unchanged after the treatments (Figure 5 a and b).

**Figure 5** – Plasma levels of (a) adiponectin and (a) leptin in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). Results are expressed as mean ± SEM.
Plasma concentrations of IL-10 were significantly lower in the group treated with EtOH, compared with group C and the same tendency was observed regarding MCP-1 (Figure 6a and c). IL-6 showed a trend to decrease in the group treated with ASA+EtOH (Figure 6b). Relatively to TNF-α, plasma levels were below the detection limit (4.44 pg/mL) of the kit used in samples analyzed from all groups.

**Figure 6** – Plasma levels of monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-6 and IL-10 in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). Results are expressed as mean ± SEM. *P < 0.05 vs C.

**VISCERAL AT TRANSCRIPTION OF SELECTED PROTEINS.** Analysis of transcription of specific genes on visceral AT collected from the mesenteric depot was performed by RT-PCR and the results are presented in Figure 7. Amplification of the PPARγ gene in AT samples from all groups revealed that although the expression of this nuclear receptor tended to increase after all treatments, ASA+EtOH resulted in a significant increase of its transcription in comparison to control, and also to other remaining groups (P < 0.05) (Figure 7a). The transcription of the enzyme fatty acid synthase (FAS) also tended to increase in the AT of ASA+EtOH and ASA-treated groups while the opposite was observed after EtOH treatment (Figure 7b). Regarding adiponectin, although treatments with EtOH and ASA seemed to decrease the transcription of this adipokine in the AT, treatment with ASA+EtOH resulted in a significant increase of adiponectin transcription, comparing with the other three groups (P < 0.05) (Figure 7c). There was no significant effect of the treatments on TNF-α and MCP-1.
transcription, although the transcription levels of these cytokines were slightly higher in ASA+EtOH-treated animals, compared with the control group (Figure 7 d and f). Curiously, MCP-1 and adiponectin showed a similar pattern of variation. Finally, a significant difference in the levels of transcription of PAI-1 after the treatment ASA+EtOH, in comparison to the EtOH group was observed (P < 0.05) (Figure 7e).

Figure 7 – Visceral (mesenteric) adipose tissue transcription of selected proteins in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5), acetylsalicylic acid solution (ASA, 10 mg/kg of weight body, n = 6) or acetylsalicylic acid in 13% EtOH aqueous solution (ASA+EtOH, 10 mg/kg of weight body, n = 6). FAS, fatty acid synthase; MCP-1, monocyte chemotactic protein-1; PAI-1, plasminogen activator inhibitor-1; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor α. Results are expressed as mean ± SEM. *P < 0.05 vs C, EtOH, ASA. §P < 0.05 vs EtOH.
In the present study we observed a reduction in body weight gain in animals treated with EtOH and ASA+EtOH solutions throughout the six weeks of treatment. The effect of the prolonged treatment with EtOH on body weight has been described previously (87). It has also been reported, that treatment with ethanol results in a decrease in pellet food ingestion, but taking into consideration the energy supplied by the ethanol solution, the energy intake remained unaltered, suggesting that the decrease in body weight gain is independent of energy intake. In regard to ASA, it has been shown that treatment with ASA in diabetic rats (in a concentration 7.9 fold higher than that used in the present study) (88) or in a mouse model of obesity (8.0 g/kg of pellet food ingested) had no influence on energy balance, energy intake or body weight gain (89), which is in agreement with our results on body weight gain. In the latter study, it is demonstrated that ASA treatment does not change the amount of body fat. In the present study we excised epididymal AT from the animals and weighed this depot. The weight of this compartment of AT has been shown to correlate with total AT and is commonly used to estimate body composition (90). The weight of epididymal AT was not significantly altered in ASA-treated rats, which is compatible with the observations previously mentioned. However, after EtOH and ASA+EtOH treatments epididymal AT was decreased. Since there was no difference between those two groups, the effect was most likely due to ethanol. Furthermore, as the effect was no longer observed after correction of epididymal AT weight for final body weight, it seemed that EtOH treatment affects overall body weight gain by changing the percentage of body fat mass. As to the size of adipocytes, no effect was detected for any treatment, including that of ethanol solutions.
Liver mass varied according to animal size, resulting in no differences in relative organ mass. Increased liver weight predicts hepatic damage. This parameter suffered no change in EtOH-treated rats, although the levels of the plasma hepatic function marker GOT, and the GOT/GPT ratio, tended to increase. However, it was interesting to notice that when ASA was administered simultaneously with EtOH, this tendency was completely reversed and both markers were lower than those found in controls. In agreement with this data Zentella de Pinã et al. (81) have demonstrated that ASA reverted the indicators of liver damage (protein carbonyls and lipid peroxidation) promoted by a single dose of ethanol (5 mg/kg body weight).

Results from the same group have also related NSAID treatment in EtOH exposed rats to reverse the changes in circulating lipids induced by EtOH (91). In our study, we observed several modifications of plasma lipids and other metabolic markers on animals receiving ASA+EtOH solution. Interestingly, ASA alone did not modify these parameters. The same absence of effect of ASA treatment in comparison to control animals has been reported in several other studies, although ASA treatment induces impressive metabolic ameliorations in models of metabolic challenge namely chronic treatment with fructose (73) or glucose (92) and induction of diabetes by streptozotocin (93). Two different mechanisms may be involved in the interaction of ASA with ethanol. On one hand, enzymatic activity of alcohol dehydrogenase in the presence of EtOH as substrate has been shown to be enhanced by ASA (83). On the other hand the co-administration of ASA with ethanol results in a higher concentration of salicylic acid in the plasma which was suggested to occur through increased absorption (84).

Here, we show that animals treated with EtOH+ASA had lower plasma TAG and VLDL levels than those of any other group, and lower levels of total and HDL cholesterol than control and ASA-treated rats. Regarding HDL, ethanol seems to play an important role in the effect. Furthermore, ASA+EtOH significantly reduced plasma NEFA levels. Both EtOH and ASA when supplied alone also tended to decrease NEFA in plasma, and the effect observed in ASA+EtOH seems to result from the additive effect of ASA and EtOH. In agreement with our results, basal and catecholamine stimulated lipolysis was inhibited in rats treated chronically with 10% EtOH
solution\textsuperscript{24}. The reduction of adrenaline- and cAMP-induced lipolysis in the AT by ASA has also been described\textsuperscript{22}. The modulation of lipolysis and NEFA release from the AT might explain why rats from EtOH and ASA+EtOH groups had the same average adipocyte size than controls in spite of a lower body weight gain. LDL levels were found to be increased in ASA and EtOH-treated rats and curiously this effect was reversed when the two were supplied concomitantly. The increase in the cholesterol/HDL ratio observed in EtOH rats was also reversed by simultaneous treatment with ASA. These ratios between total cholesterol or TAG and HDL cholesterol are often used in rats to predict cardiovascular outcomes and are believed to be positively associated with risk\textsuperscript{95}. However, interpretation of data regarding rodent plasma lipoproteins must be performed cautiously as their transport functions are not similar to those observed in humans. It is important to note that in murine models, for example, HDL metabolism is different from that found in humans, namely HDL, not LDL, is the principal carrier of circulating cholesterol in mouse plasma and the activity of cholesterol-ester transfer protein is absent\textsuperscript{96}.

No significant differences were observed among treatment groups regarding plasma corticosterone and CRP, although for the later levels tended to be higher in the groups that received EtOH treatment. Uric acid was also measured and it increased in EtOH-treated animals. Interestingly, although low dose aspirin\textsuperscript{\textregistered} is pointed as a medication that can cause hyperuricemia\textsuperscript{97}, ASA did not change uric acid concentration in comparison to control and was even able to prevent part of the increase in plasma uric acid induced by EtOH, as observed in ASA+EtOH. This rise in uric acid plasma levels after EtOH intake is not surprising as ethanol increases purine production and decreases urate excretion\textsuperscript{97}. The effect of ASA in lowering hyperuricemia is potentially indicative of its effects on the metabolic syndrome. Hyperuricemia has been demonstrated to be associated with the metabolic syndrome and each of its individual components. For example, increased BMI is correlated with hyperuricemia\textsuperscript{98}. Although the rise in serum urate levels may be in itself damaging to blood vessels, one should bear in mind that uric acid is an effective anti-oxidant\textsuperscript{99}. 
With respect to the levels of cytokines and adipokines, no significant alterations were observed on adiponectin, leptin, MCP-1 and IL-6 levels. In the case of IL-6, however, ASA and ASA+EtOH treatments tended to decrease the circulating levels of this adipokine. IL-6 is implicated in obesity-associated pathologies, as it can be derived from AT and proinflammatory prostaglandins, which regulate its synthesis elsewhere, are also produced by this tissue\cite{37, 38}. In the AT, COX-2-coupled prostacyclin triggers the release of basal IL-6, which in obese subjects is significantly dampened by ASA treatment, thus offering a novel, modifiable pathway to regulate the potentially pathological component of this cytokine\cite{80}. Addition of ASA to EtOH allowed recovery of the decrease in IL-10 production promoted by EtOH alone. Reports on the increase of IL-10 production have been already made in a model of gastric ulcer healing in diabetic rats\cite{100}. This anti-inflammatory cytokine may be released by the AT where alternatively-activated anti-inflammatory macrophages are supposed to account for most of its production\cite{101, 102}. The production of this cytokine has been reported to be induced by weight loss which establishes a link between IL-10 and metabolic amelioration\cite{21}.

The theory that metabolic control is improved in animals treated with ASA+EtOH is supported by the findings regarding insulin and insulin sensitivity. It was very interesting to find that this treatment resulted in a pronounced and significant increase in insulin sensitivity index. All treatments induced a reduction in insulin concentration and in the case of EtOH and ASA+EtOH the reduction could be due to lower body weight of these animals. However, although EtOH and ASA+EtOH have similar weight gains, the insulin sensitivity is far more pronounced in rats treated both with ASA and EtOH, indicating that, in this case, there should be some effects that are independent of EtOH-related influence on weight gain. ASA and EtOH appear to act in a synergistic manner to achieve the effect on insulin sensitivity. Several previous studies have proposed a role for ASA in glucose metabolism, associating its administration with improvements in several models of impaired insulin sensitivity\cite{73, 92, 93}. Curiously, in all of those studies, the regulation of redox status was implicated. In a model of sepsis-induced insulin resistance, aspirin pretreatment was also able to restore insulin signaling through a mechanism
involving tyrosine phosphorylation of IRS-1 and prevention of c-jun N-terminal kinase (JNK) activation, both in the muscle and the AT\textsuperscript{[79]} . The actions of aspirin on nuclear factor κB (NF-κB) may be implicated as well. ASA and salicylate inhibit both NF-κB and inhibitor of κB kinase (IkB), improving insulin signaling trough IRS\textsuperscript{[69]}. Although the results obtained in the present study and discussed until this stage may result from changes in the AT, only the studies on AT transcription of genes provide definite evidence of what is being modulated locally. In close association with the increase in insulin sensitivity is the increase in the AT PPAR\textgamma and adiponectin transcription in ASA+EtOH-treated animals. The activation of PPAR\textgamma signaling is critical for AT insulin sensitivity\textsuperscript{[103]} . Apart from the control of glucose-handling enzyme machinery, one important result of PPAR\textgamma activation is the synthesis of adiponectin\textsuperscript{[104]} . The circulating concentrations of this adipokine, which has insulin sensitizing effects, inhibits gluconeogenesis and increases lipid oxidation, have been demonstrated to be inversely correlated with the amount of adipose tissue\textsuperscript{[9, 105, 106]} . Higher circulating concentrations of adiponectin are also related to lower insulin resistance and cardiovascular disease\textsuperscript{[106]} . The gene transcription of this anti-inflammatory adipokine\textsuperscript{[105]} was also activated by ASA-EtOH treatment. Although circulating levels of adiponectin were not altered and RNA and not protein levels of adiponectin were quantified in the AT, the result observed raises the possibility of a local increase in adiponectin, possibly influencing AT insulin sensitivity. Of all proinflammatory mediators, only the anti-fibrinolytic cytokine PAI-1 varied inversely with adiponectin, PPAR\textgamma, and insulin sensitivity in ASA-EtOH treated animals, where the EtOH-induced tendency towards PAI-1 induction was abolished. The fact that ASA+EtOH reduces its expression implicates AT effects on the cardiovascular protection associated with ASA use.

The results obtained in this study provide evidence of an amelioration of metabolic parameters upon ASA administration. Remarkably it seems that some of its effects are enhanced by EtOH co-treatment. The metabolic effects of ASA alone or supplied with EtOH may possibly be related to anti-inflammatory actions and include direct effects on the AT. In our opinion, this kind of effect deserves to be further explored.
CHAPTER III

EFFECTS OF PROLONGED RED WINE CONSUMPTION
Wine is an alcoholic beverage produced by fermentation of the grape varieties of *Vitis vinifera* and its history dates back to about 6,000 BC. The fermentation of the grapes is carried out by various types of yeast that use the carbohydrates present in grapes to produce ethanol\(^{[107]}\). During the process, the growing ethanol concentration allows extraction of components from grape seeds, skins and stems resulting in a final complex fluid mixture. Wine, especially RW, is therefore an excellent source of polyphenols such as resveratrol, anthocyanins, flavonols and phenolic acids\(^{[107,108]}\).

Epidemiological studies correlate the intake of RW with low mortality due to cardiovascular problems, despite the presence of a high amount of saturated fat in the diet. This association was first described in 1979 and became known as the *French paradox*\(^{[109]}\). On the other hand, a regular but moderate intake of ethanol (in the form of wine and generally during meals) is an important component of the Mediterranean diet, which among other things, is also characterized by a high intake of fruits, vegetables and whole grain cereals\(^{[110]}\). Despite this evidence, the effects of RW are still controversial\(^{[111,112]}\), since this drink has a significant EtOH concentration. Curiously, it has also been suggested that the presence of ethanol in RW seems to be important for the effect of the drink, as evidenced in some studies that compare the effect of RW with RW without EtOH or grape juice\(^{[113]}\). There has been a decrease in EtOH consumption in industrialized countries\(^{[114]}\) and Portugal has followed the same trend\(^{[115]}\). However, while beer consumption has increased in recent years, as opposite to spirits and wine, the latter is still the beverage showing the highest intake among Portuguese consumers.

Some studies suggest the consumption of alcoholic beverages as a factor favoring the development of obesity and metabolic syndrome, given its high energy density (7 kcal/g of
EtOH), but also because of its appetite stimulating effect\textsuperscript{116-119}. In fact, the relationship between EtOH consumption and the metabolic syndrome is complex: although its consumption is associated with increased risk of hypertension, studies show that moderate consumption of RW and beer seem to reduce the prevalence of the metabolic syndrome while spirits tend to favor the development of this syndrome\textsuperscript{120}.

It is clear that AT deregulation and inflammation, caused by the excess energy, is considered as the instigator of the main metabolic disturbances that constitute the metabolic syndrome and lead to the risk of disease\textsuperscript{44}. While there is a growing amount of research linking RW or EtOH consumption with improvement of metabolic syndrome, it appears that there are few studies considering the possibility that the effects of RW may include direct effects on AT\textsuperscript{121-123}.

Drastic changes have affected the human diet in recent years, leading people to prefer foods with high, instead of low energy density. This new diet often comes accompanied by a sedentary lifestyle, which predisposes to the installation and subsequent worsening of obesity and metabolic syndrome, being considered an obesogenic factor\textsuperscript{124}. In this sense, different sorts of food can either be considered as etiological or prophylactic factors in obesity. For this reason, to elucidate the effects of food and its component nutrients in the metabolism of AT is a matter of indisputable interest.

We considered, thus, interesting to explore the effect of RW, thanks to its composition in polyphenols with antioxidant, anti-inflammatory and cell cycle regulation properties\textsuperscript{125-127}, on AT biology and on systemic and inflammatory markers.
The materials and methods section of this chapter is similar to the one described in Chapter II, section Methodology. Exceptions or changes in the methods performed relate only to treatment of animals and are described below.

**Animals and Diets.** Fourteen male Wistar rats (Harlan Iberica, Barcelona, Spain) with mean body weight of 241.17 ± 12.76 g were maintained under standard temperature and light conditions (20–22°C, 12-h light/dark cycle). Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals.

The animals were divided into three groups that were subjected to different treatments: 1) water (C, control; n = 5), 2) 13% (v/v) ethanol (EtOH; n = 5) solution; and 3) RW (with 13% EtOH; n = 4). The beverages were supplied to the rats *ad libitum* in dark bottles. Body weight gain was monitored every 2 to 3 days. Beverages and animal pellet food were renewed every 2 to 3 days, with beverage intake being assessed every 2 to 3 days.

After 6 weeks of treatment, the animals were anesthetized with sodium pentobarbital (60 mg/kg of body weight, intraperitoneally), and blood was drawn to K$_3$-EDTA-containing tubes to extract plasma. Two samples of visceral (mesenteric) AT were removed and one sample was frozen in liquid nitrogen and stored at -80°C for protein extraction, and the other was formalin-fixed, paraffin-embedded, and sectioned for histological analysis. Epididymal AT pads from both sides and the livers were removed and weighed.
**RESULTS**

**BODY WEIGHT.** Body weight was monitored and recorded, during the treatment. Mean body weight was similar among groups at the beginning of treatment (Table 7). All animals increased their body weight during the course of treatment \((P < 0.05)\) (Figure 8a), although the increase was not similar among groups \((P < 0.05)\) (Figure 8b). At the end of treatment, animals receiving EtOH and RW had lower body weight gains than control rats \((P < 0.05)\) (Table 7).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C</th>
<th>EtOH</th>
<th>RW</th>
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<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>240.2 ± 13.1</td>
<td>244.8 ± 12.0</td>
<td>238.5 ± 13.2</td>
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<tr>
<td>Final body weight (g)</td>
<td>414.0 ± 9.0</td>
<td>367.8 ± 12.4*</td>
<td>360.0 ± 12.4*</td>
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</tbody>
</table>

*Table 7* – Body weight in control \((C, n = 5)\) animals or after treatment with ethanol \((\text{EtOH}, 13\% \text{ aqueous solution}, n = 5)\) or red wine \((\text{RW}, \text{ with } 13\% \text{ EtOH}, n = 4)\). Results are expressed as mean ± SEM. *\(P < 0.05\) vs C.

![Figure 8](image)

**Figure 8** – Body weight variation (a) and body weight gain (b) in control \((C, n = 5)\) animals or after treatment with ethanol \((\text{EtOH}, 13\% \text{ aqueous solution}, n = 5)\) or red wine \((\text{RW}, \text{ with } 13\% \text{ EtOH}, n = 4)\). Results are expressed as mean ± SEM. The body weight of animals from all groups varied significantly \((P < 0.05)\) with time of treatment. *\(P < 0.05\) vs C.
LIVER AND ADIPOSE TISSUE WEIGHT AND ADIPOCYTE SIZE. There were significant differences between EtOH-treated group and control ($P < 0.05$) in what concerns liver weight. After correction of liver mass by the body weight of the animal, that difference was dissipated (Table 8), but liver/body weight ratio in RW-treated group was significantly different from that of either EtOH or control group ($P < 0.05$). Regarding epididymal AT, a reduction in its weight was observed in EtOH and RW groups when compared with control ($P < 0.05$ for both), but after correction for total body weight no difference was observed. Adipocytes from mesenteric AT of control animals measured $149.10 \pm 8.05$ μm of diameter and no differences were detected among treatment groups (Figure 9).

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<th>Treatment group</th>
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<tr>
<td></td>
<td>C</td>
<td>EtOH</td>
<td>RW</td>
<td></td>
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<tr>
<td>Liver weight (g)</td>
<td>13.72 ± 0.26</td>
<td>12.11 ± 0.37*</td>
<td>13.31 ± 0.56</td>
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<td>Liver weight/body weight ratio</td>
<td>0.0332 ± 0.0003</td>
<td>0.0330 ± 0.0013</td>
<td>0.0370 ± 0.0008*</td>
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<td>Epididymal AT weight (g)</td>
<td>11.28 ± 1.38</td>
<td>7.12 ± 0.22*</td>
<td>7.67 ± 0.9*</td>
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<tr>
<td>Epididymal AT weight/ body weight ratio</td>
<td>0.0269 ± 0.0027</td>
<td>0.0194 ± 0.0007</td>
<td>0.0213 ± 0.0025</td>
<td></td>
</tr>
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</table>

**Table 8** – Liver and adipose tissue weight in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$) or red wine (RW, with 13% EtOH, $n = 4$). AT, adipose tissue. Results are expressed as mean ± SEM. *$P < 0.05$ vs C, 5$P < 0.05$ vs C and EtOH.

![Adipocyte diameter](chart.png)

**Figure 9** – Adipocyte diameter from mesenteric adipose tissue in control (C, $n = 5$) animals or animals after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$) or red wine (RW, with 13% EtOH, $n = 4$). Results are expressed as mean ± SEM. No significant differences were observed among groups.
CHAPTER III

**BIOCHEMICAL MARKERS OF HEPATIC FUNCTION.** We measured plasma glutamic-oxaloacetic (GOT) and glutamate-pyruvate (GPT) transaminases to evaluate the status of the liver (Table 9). Although the values of GOT tended to be higher in EtOH- and RW-treated groups when compared with control, no significant differences were achieved ($P > 0.05$). This also resulted in a tendency towards higher GOT/GPT ratios in EtOH- and RW-treated animals. Concerning GPT, activities in plasma were similar among groups.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C</th>
<th>EtOH</th>
<th>RW</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (U/L)</td>
<td>95.40 ± 12.52</td>
<td>149.7 ± 58.85</td>
<td>136.7 ± 73.69</td>
</tr>
<tr>
<td>GPT (U/L)</td>
<td>39.20 ± 2.62</td>
<td>39.00 ± 2.49</td>
<td>36.50 ± 2.40</td>
</tr>
<tr>
<td>GOT/GPT</td>
<td>2.50 ± 0.41</td>
<td>3.61 ± 1.09</td>
<td>3.48 ± 1.57</td>
</tr>
</tbody>
</table>

Table 9 - Biochemical markers of hepatic function in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$) or red wine (RW, with 13% EtOH, $n = 4$). GOT, glutamic-oxaloacetic transaminase; GPT, glutamate-pyruvate transaminase. Results are expressed as mean ± SEM.

**CIRCULATING MARKERS OF METABOLIC STATUS.** We measured plasma markers of metabolic function, specifically plasma glucose, urea, creatinine, TAG, total cholesterol and respective fractions (VLDL, HDL, LDL), NEFA, insulin, corticosterone, uric acid and CRP (Table 10).

Plasma glucose concentrations were similar in groups C and RW, and tended to be higher in the group treated with EtOH. Urea and creatinine presented similar values in all groups. Furthermore, with regard to total cholesterol and HDL, both treatments led to a downward trend in these two parameters. However, with regard to LDL there was an increasing trend, and this difference was significant between EtOH and C group ($P < 0.05$). The variations in the different plasma cholesterol fractions reflected in a tendency towards a similar increase in the total cholesterol/HDL ratio in the two treated groups compared with group C. Excluding NEFA which showed a tendency to decrease in groups EtOH and RW, the TAG, VLDL and TAG/HDL ratio did not vary among groups.
### Results

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>C</th>
<th>EtOH</th>
<th>RW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g/L)</td>
<td>1.69 ± 0.08</td>
<td>1.80 ± 0.17</td>
<td>1.63 ± 0.12</td>
</tr>
<tr>
<td>Urea (g/L)</td>
<td>0.39 ± 0.02</td>
<td>0.40 ± 0.02</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>5.38 ± 0.12</td>
<td>5.26 ± 0.32</td>
<td>5.03 ± 0.32</td>
</tr>
<tr>
<td>TAG (g/L)</td>
<td>1.34 ± 0.22</td>
<td>1.31 ± 0.20</td>
<td>1.28 ± 0.27</td>
</tr>
<tr>
<td>Cholesterol (g/L)</td>
<td>0.75 ± 0.05</td>
<td>0.68 ± 0.03</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>HDL (g/L)</td>
<td>0.43 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>LDL (direct) (g/L)</td>
<td>0.09 ± 0.01</td>
<td>0.17 ± 0.01*</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>VLDL (g/L)</td>
<td>0.28 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>NEFA (μmol/L)</td>
<td>454.0 ± 30.72</td>
<td>350.0 ± 69.25</td>
<td>317.3 ± 53.98</td>
</tr>
<tr>
<td>Cholesterol/HDL</td>
<td>1.74 ± 0.06</td>
<td>2.05 ± 0.12</td>
<td>2.05 ± 0.04</td>
</tr>
<tr>
<td>TAG/HDL</td>
<td>2.08 ± 0.47</td>
<td>1.97 ± 0.38</td>
<td>2.10 ± 0.29</td>
</tr>
</tbody>
</table>

**Table 10** – Circulating markers of metabolic status in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) or red wine (RW, with 13% EtOH, n = 4). HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; TAG, triacylglycerols; VLDL, very-low-density lipoprotein. Results are expressed as mean ± SEM. *P < 0.05 vs C.

CRP and uric acid, inflammatory markers, were also measured, and the results are presented on Figure 10. It appears that CRP displays a visible decreasing trend in the RW group, opposite to EtOH-treated group (Figure 10a). However, the concentration of uric acid (Figure 10b) tended to be higher in the group treated with RW and increased significantly in the EtOH-treated group compared with the control group (P < 0.05).

Results regarding metabolic hormones, insulin and corticosterone, are presented in Figure 11. Corticosterone (Figure 11a) tended to increase in the group treated with RW relatively to groups C and EtOH, but the effect did not reach a significant difference (P > 0.05). There was a significant decrease in plasma insulin (P < 0.05) in the groups treated with EtOH and RW, compared to the control group (Figure 11b). The insulin sensitivity index (calculated by dividing the plasma concentration of glucose of each animal by that of insulin) followed an inverse
pattern of variation of that observed in plasma insulin, although the differences did not achieve statistical significance when compared to control (Figure 11c).

**Figure 10** – Plasma levels of (a) C-reactive protein (CRP) and (b) uric acid in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) or red wine (RW, with 13% EtOH, n = 4). Results are expressed as mean ± SEM. *P < 0.05 vs C.

**Figure 11** – Plasma levels of (a) corticosterone, (b) insulin and (c) insulin sensitivity index in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) or red wine (RW, with 13% EtOH, n = 4). Results are expressed as mean ± SEM. *P < 0.05 vs C.

**Plasma Levels of Inflammatory Cytokines and Adipokines.** We evaluated the plasma concentration of some cytokines (IL-6, IL-10, MCP-1 and TNF-α) and adipokines (adiponectin and leptin) in view of their known biological properties. As presented in Figure 12, plasma adiponectin tended to decrease in animals treated with EtOH and RW compared to group C (Figure 12a). On the other hand, although no significant variation was detected in plasma leptin levels, it showed a tendency to be higher in RW-treated group whereas EtOH showed slightly lower values than group C (Figure 12b).
RESULTS

Figure 12 – Plasmatic levels of (a) adiponectin and (b) leptin in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) or red wine (RW, with 13% EtOH, n = 4). Results are expressed as mean ± SEM.

Plasma levels of both MCP-1 and IL-10 (Figure 13 a and c) tended to decrease in groups treated with EtOH and RW compared to the control group, and the effect of EtOH treatment on IL-10 achieved significance in comparison with group C. On the other hand, IL-6 (Figure 13b) showed a trend to increase in the groups treated with RW and EtOH, the increase being more marked in the latter. Relatively to the TNF-α, plasma levels were below the detection limit of the kit used (4.44 pg/mL) in all treatment groups.

Figure 13 – Plasmatic levels of (a) monocyte chemotactic protein-1 (MCP-1), (b) interleukin (IL)-6 and (c) IL-10 in control (C, n = 5) animals or after treatment with ethanol (EtOH, 13% aqueous solution, n = 5) or red wine (RW, with 13% EtOH, n = 4). Results are expressed as mean ± SEM. *P < 0.05 vs C.

VISCERAL AT TRANSCRIPTION OF SELECTED PROTEINS. Analysis of transcription of specific genes in mesenteric AT was performed by RT-PCR and the results are presented in Figure 14.
Amplification of the PPARγ, which regulates fatty acid storage and glucose metabolism, did not reveal a significant effect of the treatment in the transcription of the protein although it tended to increase in both treatment groups compared to the control group (Figure 14a). There was a significant decrease ($P < 0.05$) in the transcription of the enzyme FAS in the EtOH group, compared with the RW group, the latter showing a significant increase compared with group C ($P < 0.05$) (Figure 14b). Regarding adiponectin, treatment with either EtOH or RW resulted in a decreasing trend of the transcription of this adipokine in the AT, stronger with EtOH (Figure 14c). No effect was detected on the transcription of TNF-α or MCP-1 in the AT (Figure 14 d and f), although the levels of transcription of these cytokines did also tend to decrease in both treatment groups. There was a marked decrease in the levels of transcription of PAI-1 in the group treated with RW, compared with the EtOH group ($P < 0.05$) (Figure 14e).

**Figure 14** – Visceral (mesenteric) adipose tissue transcription of selected proteins in control (C, $n = 5$) animals or after treatment with ethanol (EtOH, 13% aqueous solution, $n = 5$) or red wine (RW, with 13% EtOH, $n = 4$). FAS, fatty acid synthase; MCP-1, monocyte chemotactic protein-1; PAI-1, plasminogen activator inhibitor-1; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor-α. Results are expressed as mean ± SEM. *$P < 0.05$ vs C. **$P < 0.05$ vs RW. ***$P < 0.05$ vs EtOH.
The fact that diet has a huge impact on the installation and progression of the metabolic syndrome is currently unarguable. The first steps given towards the recognition of wine as an important part of the human diet and linking it to health-promoting effects date back to the description that the Mediterranean diet pattern, albeit originally thought to be poor, was associated with low coronary heart disease and cancer prevalence in the population of Crete, in Greece\textsuperscript{128}. Already at that time, the consumption of wine, and also olive oil and vinegar, were among the suggested dietary items implicated in this inverse relationship between their consumption and disease. Later, the recognition that the French population, despite having a high consumption of saturated fats presented an apparently controversial low mortality by coronary heart disease gave birth to the French Paradox\textsuperscript{107, 109, 129}. This association was soon explained by their high consumption of wine, particularly RW\textsuperscript{129}. Even so, this association was not easy to establish and controversial data are still being generated. Some of the reasons for this difficulty are related to EtOH content in RW. Although EtOH has been demonstrated to possess benefits underlying the reduced coronary heart disease incidence in RW drinker\textsuperscript{130-132}, effects may largely vary with age, gender and genetic background of the population studied\textsuperscript{133}.

In our study, the prolonged treatment with EtOH and RW resulted on a decrease in body weight gain in comparison to control. In fact, animals treated for 8 weeks with the same dose of either EtOH or RW reveal a decrease in feed efficiency\textsuperscript{87}, an effect that seems highly dependent on EtOH, as that parameter was not altered by the non alcoholic components of RW. The decrease in feed efficiency means that animals gained less weight per kcal of energy consumed. In the same study, no alteration in total energy consumption has been detected, although the animals
ingested less energy from pellet food, the remaining being supplied by the EtOH contained in treatment solution.

The decrease in body weight gain was correlated with a smaller deposition of AT in the epididymal depot, which reflects the total amount of body fat[60]. Because after correction for body weight no differences were observed, it became evident that the percentage of body fat probably remained unaltered, as well as did the size of adipocytes within the AT. Bargalló et al[134] have previously reported a decrease in energy intake, decreased epididymal fat mass and whole body weight in lean Zucker rats ingesting a high fat diet and voluntarily drinking RW, in comparison to high fat diet-fed controls drinking water. Weight gain reduction may be attributed to the effect of procyanidins in fat absorption due to their inhibition of pancreatic lipase[135]. However, in the present study most of the effects seen on weight gain seemed to be attributable to EtOH. In agreement, Szkudelski et al[94] found decreased body weight gain in rats drinking 10% EtOH solution as the only drinking fluid for 2 weeks, the change being related to effects in plasma lipid and hormone concentrations and modulation of AT lipolysis. Beulens et al[136] also investigated the direct effects of RW in the AT. This study involved healthy men with increased waist circumference, drinking 450 mL of RW (40 g of EtOH) or 450 mL of de-alcoholized RW daily for 4 weeks. No effect of moderate EtOH consumption was observed in subcutaneous and abdominal fat contents and body weight, but liver fat was slightly higher after consumption of RW as compared with de-alcoholized RW.

The significant decrease in liver weight in EtOH-treated rats also seems to be a reflection of lower body size as the liver weight/body weight ratio is similar in those from the remaining groups. The trend displayed by GOT levels in EtOH- and RW-treated rats might suggest that there is hepatic injury, as this enzyme is a marker of hepatic function[137]. In the same way, GOT/GPT ratio presented higher values in these two groups of treatment. Indeed, the measurement of this enzyme is often used in the diagnosis of alcoholic hepatitis and its release from hepatic cells into plasma is increased in alcoholism[138]. Concomitantly with increased plasma GOT, elevated TAG are usually found, and although we did not evaluate the hepatic
content in TAG, their concentration in plasma was unchanged by both treatments. The same occurred with the remaining plasma lipids evaluated, and only an increase in LDL fraction of cholesterol was observed in EtOH-consuming rats. Despite no changes were observed in HDL-cholesterol or TAG, EtOH and RW treatments have been related to simultaneous increase in HDL cholesterol, VLDL and serum total TAG\textsuperscript{139}. However, as mentioned before, the metabolism and transport of lipids, with regard to the different lipoprotein cholesterol fractions, differ between rodents and humans and results cannot be extrapolated\textsuperscript{96}.

The increase in plasma uric acid concentration in EtOH-treated animals is not surprising, given EtOH interference with purine metabolism and urate excretion\textsuperscript{97}, but it was interesting to note that RW prevented this increase mediated by EtOH. Corroborating this finding, epidemiological data report no association of wine consumption with increased risk of gout, contrary to what is observed for other alcoholic beverages\textsuperscript{140}. Regarding CRP, the tendency towards lower levels of this acute phase protein in RW-consuming rats is in the same line with reports from epidemiological studies. In fact, consumption of RW for 4 weeks (supplying 30 g of EtOH per day) led to a significant decrease in the serum concentration of CRP and improvement of endothelial function markers in healthy adult men\textsuperscript{141}. Lower levels of circulating CRP have also been associated to moderate RW consumption in cross-sectional studies\textsuperscript{142, 143}.

The concentrations of plasma adiponectin, leptin, IL-6 and MCP-1 were not significantly changed, although the treatment with RW seems to oppose the pro-inflammatory tendency in the variation of these cytokines in EtOH-treated rats. This is in agreement with the effects observed on the anti-inflammatory cytokine IL-10 after ingestion of RW, that prevented the EtOH-induced decrease of its concentration. This anti-inflammatory tendency of RW treatment in comparison to EtOH fits well with the data observed regarding insulin and insulin sensitivity index. In this regard, RW seems to a have a more pronounced effect in reducing insulin secretion and improving insulin sensitivity. As discussed before, the reduction in body weight gain may justify part of the effect on insulin homeostasis, but the difference between the effects of RW and EtOH suggests that EtOH is not the sole compound involved in the effect of the
alcoholic beverage. From our results, it becomes evident that EtOH may indeed play a role. Furthermore, the decrease in blood insulin has been described earlier in EtOH consuming rats\(^{94}\). A number of investigations have shown that moderate EtOH consumption is associated with enhanced insulin sensitivity\(^{144-147}\) and that moderate drinkers have a lower risk for type 2 diabetes than non-drinkers or heavy drinkers\(^{148}\). Regarding the non-alcoholic components of wine, oral administration of procyanidins to streptozotocin-induced diabetic rats has an anti-hyperglycemic effect and acts synergistically with a low dose of insulin\(^{149}\). The effect of procyanidins is abolished by phosphatidylinositol-3-kinase and p38mitogen-activated protein-kinase inhibitors, showing the involvement of these kinases in procyanidin effects\(^{149}\).

Also in streptozotocin-induced diabetic rats, resveratrol treatment for 14 days decreases plasma glucose concentration compared to vehicle-treated rats\(^{150}\). Furthermore, it results in body weight loss and improved diabetes symptoms such as polyphagia, and polydipsia. The same study included results showing that resveratrol stimulates glucose uptake by hepatocytes, adipocytes and skeletal muscle and hepatic glycogen synthesis. The anti-inflammatory actions of the polyphenol, including inhibition of synthesis and release of pro-inflammatory cytokines, modification of eicosanoid synthesis, inhibition of activated immune cells, or inhibition of inducible nitric oxide synthase and cyclooxygenase-2 via the inhibitory effects on NF\(\kappa\)B or activator protein-1 (AP-1)\(^{151}\) may determine the impact of this polyphenol on glucose homeostasis.

Recently, a study has demonstrated that COX-2 activation is crucially involved in the development of inflammatory response in AT high fat-induced obese rats and that its inhibition by selective COX-2 inhibitors celecoxib or nimesulide is able to suppress obesity-associated increases in gene expressio of COX-2, MCP-1 and TNF-\(\alpha\)\(^{152}\). Other effects of high fat feeding that can be reversed by these COX-2 inhibitors include the attenuated PPAR\(\gamma\) and CCAAT enhancer binding protein \(\alpha\) mRNA expressions in epididymal fat, insulin resistance and hepatic triglyceride content, the reduction in stimulation of whole body glucose uptake, suppression of hepatic glucose production and metabolic clearance rate of insulin\(^{153}\). In this regard, both
preadipose and adipose cells were able to produce prostacyclin upon exposure to arachidonic acid\textsuperscript{154}. This prostanoid has been shown to play a key role in the process of preadipose cell differentiation \textit{in vitro} and treatment with ASA prevents this effect. These observations highlight the importance of COX modulatory actions of RW components in the interference with AT biology.

These effects of EtOH may be related with the trend found in AT relating to PPAR\(\gamma\) that may substantiate the improvement in insulin status. Several previous studies have reported that RW increases the concentration of adiponectin in the plasma of consumers, which might constitute evidence of PPAR\(\gamma\) activation and could explain the trend towards increased insulin sensitivity and the decrease in insulin secretion. Our results suggest that the modulation of adiponectin by RW prevents a possible adiponectin-lowering effect of EtOH on AT transcription of the cytokine. Plasma levels of the cytokine were not altered, opening the possibility of locally mediated effects. The same kind of recovery from EtOH-induced effects was seen in the transcription of FAS, an enzyme required for the synthesis of fatty acids. Conflicting results have been reported on the effects of ethanol consumption in the activity and/or expression of this enzyme, mostly due the differences in models and duration of exposure, but reports have focused on the liver. In this organ, it seems to be one of the possible target genes of sterol regulatory element-binding protein-1. Interestingly, this transcription factor is activated upon ER stress, being involved in hepatic lipid accumulation induced by chronic and excessive ethanol treatment\textsuperscript{155}.

In the AT, the detection of FAS protein can be used as a late marker of adipocyte differentiation and thus its increased levels are in agreement with the tendency towards PPAR\(\gamma\) induction seen in RW-treated rats. However, the opposite relationship was observed in the EtOH group and the meaning of the decrease on FAS transcription deserves to be dissected. Also interesting was the reduction in AT transcript levels of PAI-1, which allows posing the hypothesis that an important part of the anti-thrombotic effects of RW intake could be associated with the modulation of AT PAI-1 production.
In this part of the work, we observed important modulatory effects of prolonged RW consumption on the metabolic and inflammatory status of the animals. As part of the effects observed were distinguishable from those of EtOH, we conclude that the non alcoholic components of RW play an important role in the influence of the beverage on health.
CHAPTER IV

FINAL REMARKS

FUTURE PERSPECTIVES
The recognition that AT is more than just a passive storage for energy, being a true endocrine organ\(^{41,156}\) which also produces inflammation-active molecules\(^{27}\), is allowing us to understand the links between the AT and metabolic syndrome. In this sense, some authors now consider adipocyte dysfunction as the instigator of the main metabolic disturbances that constitute the metabolic syndrome and lead to the risk of disease\(^{6,44,57}\). This hypothesis led us to study the effects of the prolonged treatment with both an anti-inflammatory drug and a beverage with solidly demonstrated anti-inflammatory properties on AT biology.

The effects on insulin signaling and inflammatory modulation observed after ASA and RW treatments may not be as independent from each other as one may think. Indeed, RW shares with ASA some COX inhibitory properties attributed for example to resveratrol\(^{157}\). In addition, the modulation of redox status or interference with redox sensitive cell signaling may be another example, because both ASA and RW have recognized anti-oxidant activity\(^{23,92,93,107}\) and because redox disruption is a common finding in obesity-associated pathologies\(^{64}\).

Given the in vivo nature of this study, it is necessary to bear in mind that, although most of our discussion has been based on AT-related mechanisms it is most likely that at least a part of the effects here reported are related to interference with other body systems. However, direct effects on the AT have been demonstrated and unraveling further the actions of these treatments on the AT is warranted.
FUTURE PERSPECTIVES

It would be interesting to test the effect of these anti-inflammatory substances in a model of diabetes or metabolic syndrome in order to understand if the effects here observed would be maintained or not in a context of a higher metabolic overload. Furthermore, we would like to further deepen this work studying the effects of the treatments on redox status as both treatments have been shown to possess antioxidant properties and redox challenge is a common path to obesity-related pathologies. It would also be within our interests to study the alteration of redox sensitive intracellular signaling pathways (the kinases protein kinase C (PKC), IκB kinase complex (IKK), JNK and the related transcription factors, cAMP response element binding (CREB) protein, AP-1, NF-κB and expression of target molecules, many of which constitute a link between redox and inflammatory signaling. Along the same line, it would be interesting to investigate the influence of the treatments on ER stress and each of the arms of the unfolded-protein response.
REFERENCES
10. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw. 2006; 17(1):4-12


63. Olusi SO. Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans. Int J Obes Relat Metab Disord. 2002; 26(9):1159-64


133. Rosell M, De Faire U, Hellenius ML. Low prevalence of the metabolic syndrome in wine drinkers—is it the alcohol beverage or the lifestyle? Eur J Clin Nutr. 2003; 57(2):227-34


153. Hsieh PS, Jin JS, Chiang CF, Chan PC, Chen CH, Shih KC. COX-2-mediated inflammation in fat is crucial for obesity-linked insulin resistance and fatty liver. Obesity (Silver Spring, Md. 2009; 17(6):1150-7


