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HIPERTROFIA DE ADIPÓCITOS VISCERAIS: UM EVENTO INICIAL SUBJACENTE A DISTÚRBIOS METABÓLICOS EM RATAS OVARIECTOMIZADAS É PREVENIDO POR AGNOSÍDEO, UM FITOESTRÓGENO DO *Vitex agnus-castus*

Maringá,
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Orientadora: Prof.ª Dr.ª Clairce Luzia Salgueiro-Pagadigorria

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HIPERTROFIA DE ADIPÓCITOS VISCERAIS: UM EVENTO INICIAL SUBJACENTE A DISTÚRBIOS METABÓLICOS EM RATAS OVARIECTOMIZADA É PREVENIDO POR AGNOSÍDEO, UM FITOESTRÓGENO DO VITEX AGNUS-CASTUS

Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de Doutora.

Aprovada em: 18 de dezembro de 2017

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Franciele Neves Moreno nasceu em Maringá – PR, em 18 de fevereiro de 1986. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2008) e Mestrado em Ciências Biológicas com área de concentração em Biologia Celular e Molecular (2014). Atualmente cursa o doutorado no Programa de Pós-graduação em Ciências Biológicas com área de concentração em Biologia Celular e Molecular na Universidade Estadual de Maringá, desenvolveu seu trabalho no laboratório de Esteatose Experimental e Oxidações Biológicas do Departamento de Bioquímica, atuando principalmente nos seguintes temas: pós-menopausa, ovariectomia, obesidade, esteatose, metabolismo energético e estresse oxidativo em animais.

Dedico

aos meus pais
João e Shirley...

...e ofereço

especialmente, ao meu marido
Carlos Henrique.

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APRESENTAÇÃO

Esta tese é resultado de um trabalho em equipe, realizado principalmente no Laboratório de Oxidações Biológicas e Esteatose Experimental da Universidade Estadual de Maringá, composta por dois artigos científicos. Inicia com o artigo intitulado "**Visceral adipocyte hypertrophy: an early event underlying metabolic disorders in ovariectomized rats is prevented by Agnuside, a phytoestrogen of *Vitex agnus-castus***" e tem continuidade com o artigo de revisão intitulado "**The postmenopausal metabolic syndrome."**. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as normas das revistas especificas a seguir.

Moreno FN, Lucredi NC, Guandalin GF, Gilglioni EH, Ferreira SM, Brito NA, Brito MN, Garcia RF, Salgueiro-Pagadigorria CL (2017). Visceral adipocyte hypertrophy: an early event underlying metabolic disorders in OVX rats is prevented by Agnuside, a phytoestrogen of *Vitex agnus-castus*. **Metabolism (*Submetido*).**

Moreno FN, Salgueiro-Pagadigorria CL (2017). The postmenopausal metabolic syndrome. **Metabolism (*A ser submetido*).**

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS — Na pós-menopausa, há um padrão central de distribuição de gorduras e hipertrofia de adipócitos, ambos os fatores predisponentes da resistência à insulina (RI) e esteatose hepática (*non-alcoholic fatty liver disease*, NAFLD). De fato, a hipertrofia de adipócitos viscerais produz diversas disfunções fisiológicas, incluindo aumento da lipólise, com maior influxo de ácidos graxos diretamente na veia porta, sobrecarregando o fígado e levando ao acúmulo de triacilgliceróis (TAG) no citosol dos hepatócitos. Com o intuito de amenizar as comorbidades associadas à pós-menopausa, novas terapias estão sendo estudadas. Neste contexto, nosso grupo de pesquisa tem investigado o efeito de várias substâncias naturais e sintéticas, dentre elas, o *Vitex agnus-castus* (VAC). Em um trabalho realizado recentemente, ratas ovariectomizadas (OVX) foram tratadas com dois extratos de VAC, bruto ou enriquecido em agnosídeo (AGN), e foi observado que o tratamento com o extrato enriquecido em AGN foi efetivo em reverter várias desordens no metabolismo de lipídios e em reduzir a produção de espécies reativas de oxigênio (EROs). Uma questão que surgiu à época foi a de que estes efeitos poderiam ter sido o resultado de ações sinérgicas de outros compostos ainda presentes neste extrato. Assim, o objetivo deste trabalho foi, primeiramente, realizar uma revisão sobre as correlações existentes entre adiposidade e hipertrofia de adipócitos viscerais, RI, NAFLD e dislipidemias envolvidos na síndrome metabólica (MSyn, *metabolic syndrome*) e de que maneiras a deficiência estrogênica poderia agravar estas desordens metabólicas. Além disso, utilizando o mesmo modelo de ratas OVX, investigamos a possibilidade de atenuação destas alterações metabólicas tratando estes animais com o AGN puro.

MÉTODOS — Para o desenvolvimento do artigo de revisão, realizou-se uma pesquisa bibliográfica da literatura disponível em inglês sobre o objetivo geral deste estudo, usando os bancos de dados PubMed e Web of Science. As palavras-chaves utilizadas foram dislipidemia, hipertrofia de adipócitos, pós-menopausa, síndrome metabólica, NAFLD e microbiota intestinal (GM, *gut microbiota*).

Ratas Wistar OVX foram utilizadas como modelos animais de síndrome metabólica na pós-menopausa e tratadas com AGN. Foram avaliados os tamanhos dos adipócitos viscerais e subcutâneos, a sensibilidade à insulina periférica, a ocorrência de NAFLD e o estado redox do fígado. Os resultados foram comparados com aqueles obtidos de ratas controle (*sham*-operadas; CON) e OVX tratadas com doses diárias de AGN (0.042 mg/Kg de peso corporal (PC); OVX+AGN).

RESULTADOS:

1) Após 13 semanas, as ratas OVX apresentaram maior ganho de peso corporal, aumento da adiposidade com hipertrofia de adipócitos, hiperinsulinemia e NAFLD. Sem alterações na ingestão alimentar.

2) O tratamento com AGN foi efetivo em reduzir os depósitos de gorduras viscerais e o tamanho de seus adipócitos. Entretanto, isto não foi observado no depósito de gordura subcutânea.

3) As ratas tratadas com AGN tiveram uma melhora na tolerância à glicose.

4) As ratas OVX apresentaram aumentados níveis de TAG e VLDL séricos e o tratamento com AGN intensificou este aumento.

5) O tratamento reverteu parcialmente a NAFLD.

6) Ao avaliar a oxidação mitocondrial dos AGs, observou-se uma redução na oxidação do palmitoil-CoA tanto nas ratas OVX não tratadas, quanto tratadas com AGN.

7) Os animais OVX apresentaram maior geração mitocondrial de H_2O_2, maior conteúdo de proteínas carboniladas mitocondriais, redução nos conteúdos de glutationa reduzida (GSH) e nas atividades das enzimas nicotinamida nucleotídeo transidrogenase (NNT) e glutationa

peroxidase 1 (GPx1). O tratamento conseguiu reduzir parcialmente a geração mitocondrial de H_2O_2 bem como o conteúdo de proteínas carboniladas mitocondriais e restaurou a atividade das enzimas NNT e GPx1.
8) O estado redox do fígado de ratas OVX sofreu uma piora considerável, conforme observado pelos baixos níveis citosólicos de GSH, menores atividades das enzimas glicose 6-fosfato desidrogenase (G6PD) e glutationa peroxidase 3 (GPx3) e níveis aumentados de proteínas carboniladas e de peroxidação lipídica. O tratamento foi eficaz em recuperar parcialmente a atividade da G6PD e completamente a atividade da GPx3, além disso, foi eficaz em reduzir os níveis de proteínas carboniladas e peroxidação lipídica.
DISCUSSÃO: Os resultados obtidos neste estudo demonstraram uma forte relação entre o diâmetro dos adipócitos viscerais e a tolerância à glicose, NAFLD e dislipidemia em ratas OVX. Também foi demonstrado que as ações do AGN puro, foram semelhantes ao estrogênio na reversão de vários distúrbios metabólicos, a maioria dos quais poderia resultar de sua ação primária na redução dos diâmetros dos adipócitos viscerais. Além disso, o AGN promoveu uma melhora no estado redox do fígado ao contrário de outros derivados de plantas, utilizados por mulheres no tratamento dos sintomas da pós-menopausa e aos quais os efeitos hepatotóxicos foram atribuídos. Alguns efeitos do AGN, em especial sobre o metabolismo lipídico, foram menos poderosos do que os relatados por nós, para o extrato de VAC enriquecido em AGN, provavelmente porque neste, outros compostos poderiam estar atuando sinergicamente com o AGN.
CONCLUSÕES — Conclui-se que há uma forte relação entre a hipertrofia de adipócitos viscerais com tolerância à glicose, hiperinsulinemia e NAFLD, neste modelo animal de deficiência estrogênica. Os resultados obtidos neste estudo sugerem que os efeitos benéficos do AGN sejam decorrentes de sua ação primária em reduzir diâmetro de adipócitos viscerais.

PALAVRAS-CHAVE: Síndrome metabólica; ratas ovariectomizadas; tamanho dos adipócitos; NAFLD; estado redox; agnosídeo.

GENERAL ABSTRACT

INTRODUCTION AND AIMS — In postmenopausal, there is a central pattern of fat distribution and hypertrophy adipocytes, both predisposing factors of insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD). In fact, the hypertrophy of visceral adipocytes produces several metabolic dysfunctions, including increased lipolysis, with greater amounts of fatty acid being released directly in the portal vein, overloading the liver and leading to the accumulation of triacylglycerols (TAG) in the citosol of hepatocytes. In order to ameliorate postmenopausal comorbidities, new therapies have been studied. In this context, our team has investigated the effect of various substances, natural and synthetic, among them, the *Vitex agnus-castus* (VAC). In a study performed recently, ovariectomized (OVX) rats were treated with crude or AGN-enriched extracts of VAC and we could observe that the last one was effective in reversing several disorders on lipid metabolism and reducing the reactive oxygen species (ROS) production. An issue that arose at that time was that these effects could have been the result of other compounds that could be acting synergistically with AGN. In this way, the purposes of this work was, firstly, to perform a review about the relationship between adiposity and visceral adipocytes hypertrophy, IR, NAFLD and dyslipidemias involved in the metabolic syndrome (MSyn) and how much and what ways the estrogen deficiency could aggravate these metabolic disturbances. In addition, using the same animal model of OVX rats, we investigated the possibility of attenuation of these metabolic alterations by AGN.

METHODS — For the development of the review article, a bibliographical research was conducted on the literature available in English on the purpose of this study, using the PubMed and Web Science databases. The keywords used were dyslipidemia, adipocyte hypertrophy, postmenopausal, MSyn, NAFLD and gut microbiota (GM).

OVX Wistar rats were used as animal models of postmenopausal metabolic syndrome. The visceral and subcutaneous adipocyte sizes, the peripheral insulin sensitivity and the occurrence of NAFLD as well liver oxidative damage, were evaluated. The results were compared with those obtained from control (*sham-operated*; CON) and OVX rats treated with daily doses of AGN (0.042 mg/Kg de BW; OVX+AGN).

RESULTS:

1) After 13 weeks, OVX rats presented higher body weight gain, increased adiposity with hypertrophy adipocyte, Hyperinsulinemia and NAFLD. There were no alterations in food intake.
2) AGN treatment was effective in reducing the visceral deposits fat and the size of its adipocytes. However, this was not observed in the inguinal fat.
3) OVX+AGN rats exhibited an improvement in glucose tolerance.
4) OVX rats presented an increased in the TAG and VLDL levels and the treatment with AGN further increased these levels.
5) The treatment with AGN partially reversed NAFLD.
6) When evaluating the mitochondrial oxidation of FA, a reduction in palmitoyl-CoA oxidation was observed in both OVX and OVX+AGN rats.
7) The OVX animals showed a higher mitochondrial H_2O_2 generation, higher contents of mitochondrial carbonylated proteins, decreases in reduced glutathione (GSH) contents and in the activities of the nicotinamide nucleotide transhydrogenase (NNT) and glutathione peroxidase 1 (GPx1) enzymes. The treatment partially reduced the mitochondrial H_2O_2 generation, as well as, the content of mitochondrial carbonylated proteins and restored the activity of the NNT and GPx1 enzymes.
8) The general liver redox state of OVX rats suffered a considerable worsening, as could be observed by the reducing in the cytosolic GSH levels, in the activity of the glucose 6-

phosphate dehydrogenase (G6PD) and glutathione peroxidase 3 (GPx3) enzymes, and increased levels of carbonylated proteins and lipid peroxidation. Treatment was effective in partially recovering the G6PD activity and completely recovering the GPx3 activity, in addition, it was effective in reducing the levels of carbonylated proteins and lipid peroxidation.

DISCUSSION: The results obtained in the current work demonstrated a strong relation between the diameter of visceral adipocytes and glucose tolerance, NAFLD and dyslipidemia in OVX rats. There were also demonstrated that actions of pure AGN are similar to estrogen action in the reversal of various metabolic disorders, most of them was a consequence of its primary action in reducing the diameters of visceral adipocytes. Besides, AGN promoted an overall improvement on the liver redox state, on the contrary of other plant derivatives, used by women in the treatment of menopausal symptoms, and to which the hepatotoxic effects have been attributed. Some effects of AGN, especially on lipid metabolism, were less powerful than those reported by us for AGN-enriched VAC extract, probably because other compounds could be acting synergistically with AGN.

CONCLUSION — It is concluded that there was a strong relationship between visceral adipocyte hypertrophy with glucose tolerance, hyperinsulinemia and NAFLD in this animal model of estrogen deficiency. The results obtained in this work suggest that the beneficial effects of AGN could be mainly due its primary action in reducing the visceral adipocytes size.

KEYWORS: Metabolic syndrome; ovariectomized rats; adipocyte size; NAFLD, liver redox state, agnuside.

Visceral adipocyte hypertrophy: an early event underlying metabolic disorders in ovariectomized rats is prevented by Agnuside, a phytoestrogen of *Vitex agnus-castus*

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ABSTRACT

Background: This study aimed to investigate the existence of a correlation between adipocyte sizes and the metabolic dysfunctions commonly observed in ovariectomized (OVX) rats and the possibility of improvement by agnuside (AGN), a phytoestrogen of *Vitex agnus-castus* (VAC).

Methods: OVX Wistar rats were used as animal models of postmenopausal metabolic syndrome. The visceral and subcutaneous adipocyte sizes, the peripheral insulin sensitivity and the occurrence of non-alcoholic fatty liver disease (NAFLD) as well liver oxidative damage, were evaluated. The results were compared with those obtained from control (*sham-operated*) and OVX rats treated with daily doses of AGN.

Results: Obese OVX rats exhibited visceral adipocyte hypertrophy and developed glucose intolerance, hyperinsulinemia, NAFLD and dyslidipemia. AGN exerted several estrogen-like beneficial effects, including reductions in the visceral fat depots, in visceral adipocyte sizes and an improvement in glucose tolerance. A partial reversion of NAFLD and a general improvement in the redox state of the liver was also observed.

Conclusions: There was a strong relationship between mesenteric adipocyte hypertrophy, in special, with glucose tolerance, hyperinsulinemia and NAFLD in OVX rats. The beneficial effects of AGN appeared be mediated mainly by a primary action reducing the mesenteric adipocyte size. Besides, AGN promoted a general improvement in the liver redox state.

KEYWORDS: Metabolic syndrome; postmenopausal; adipocyte size; NAFLD, liver redox state, alternative therapy.

Abbreviations: MSyn, metabolic syndrome; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; TNF-α, tumor necrosis factor-alpha; FA, fatty acids; GM, gut microbiota; VLDL, low density lipoproteins; LPL, lipoprotein lipase; TAG, triacylglycerol; CD36, cluster of differentiation 36; ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; HSL, hormone-sensitive lipase; IL, interleukin; MCP-1, monocyte chemotaxis protein-1; FFA, free fatty acids; OVX, ovariectomized; TLR4, toll-like receptor-4; ER (α or β), estrogen receptors (alpha or beta); SREBP, Sterol regulatory element binding protein; PPARα, peroxisome proliferator-activated receptors-alpha; apoB100; apolipoprotein B-100; PI, phosphatidylinositide; PIP3, phosphatidylinositide-3,4,5 triphosphate; MTP, microsomal triglyceride transfer protein; PLTP, phospholipid transfer protein; SCD-1, stearoyl coenzyme desaturase-1; SCFAs, short-chain FA; B/F, Bacteroidetes/Firmicutes; GPR, G-protein-coupled receptors; Fiaf, fasting-induced adipocyte factor; ME, metabolic endotoxemia; LPS, bacterial lipopolysaccharide

1. Introduction

In postmenopausal obesity, there is a central pattern of fat distribution and hypertrophy of adipocytes [1], both predisposing factors of insulin resistance (IR) [2] and non-alcoholic fatty liver disease (NAFLD) [3,4]. In fact, the altered metabolic activity of visceral fat has been considered the triggering factor of NAFLD, since the excessive lipolysis by hypertrophied adipocytes and increased releasing of fatty acids (FA) directly in the portal venous system, overloads the liver leading to accumulation of triacylglycerols (TAG) in the cytosol of hepatocytes. [5,6].

Besides, by genomic actions, estrogen positively influences the liver lipid metabolism in such way that, in conditions of estrogen deficiency, the incidence of NAFLD is considerably higher [7]. The fat liver accumulation, in turn, leads to oxidative cell damage [8−11].

Ovariectomized (OVX) rats are very suitable animal models of estrogen deficiency and has been extensively used by us [9−11] and others [7,12,13] in studies carried out aiming to clarify the mechanisms involved in the metabolic disturbances associated with menopause and in the search of ways to treat this condition.

About 6 weeks after the surgical removal of the ovaries, OVX rats develop several metabolic disturbances characteristic of the postmenopausal metabolic syndrome, including increased adiposity, with a central pattern of fat distribution, IR, NAFLD and dyslipidemia [7,9−11]. Using this animal model, our team has investigated the effects of several substances, such as synthetic estrogen and natural compounds, on the liver lipid metabolism as well their hepatotoxic potentials.

Recently we investigated the effects of a plant extract, *Vitex agnus-castus* (VAC), commonly used by postmenopausal women, as alternative therapy [14]. The crude extracts of VAC contains considerable quantities of an iridoid glycoside, agnuside (AGN) to which some of the beneficial effects of VAC has been attributed, by its estrogenic and antioxidant actions [15,16]. Our results revealed that, in OVX rats, the crude extract of VAC and its butanolic fraction (enriched in AGN), were effective in reversing several disorders in lipid metabolism, including NAFLD and adiposity [11]. Besides, the AGN-enriched extract was effective in suppressing the reactive oxygen species (ROS) generation by liver mitochondria. Despite these results were very promising, an issue that arose at that time was the possibility that other active compounds in the extracts, besides AGN could also have a role on the observed effects.

Therefore, this study aimed to investigate whether there is a correlation between NAFLD, IR and visceral adipocyte hypertrophy in OVX rats and the possibility of improvement of these disturbances by pure AGN.

2. Materials and methods

2.1. Materials

The following substrates and reagents were purchased from Sigma Chemical Co. (St. Louis, USA): AGN, adenosine diphosphate (ADP), phenylmethylsulfonyl fluoride (PMSF), reduced glutathione (GSH), 2',7'-dichlorofluroscein diacetate (DCFH-DA), 2',7'-dichlorofluorescein (DCF), o-phthalaldehyde (OPT), sodium dodecyl sulfate, Brij® L23 solution, 1-palmitoyl-sn-glycero-3-phosphocholine, oxidized 3-acetylpyridine adenine dinucleotide (APAD), 2,4-dinitrophenylhydrazine (DNPH), streptomycin sulfate salt, guanidine, β-nicotinamide adenine dinucleotide (phosphate) and reduced dipotassium salt (NAD[P]H). Gold Analisa® (Belo Horizonte, Brazil) metering kits of glucose and lipids were used. Sodium heparin was obtained from Roche. The other reagents used were from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

2.2. Animals

Female Wistar rats (45 days old, 130-160 g body weight (BW), 152 total animals) were provided by central biotery of the University of Maringá and were randomly assigned for: *sham*-operation (Control; CON) or bilateral ovariectomy (OVX), in a proportion of 1:2. Animals undergoing OVX were anaesthetized (10 mg xylazine + ketamine 50 mg/Kg BW, i.p.) and their ovaries were removed. The CON rats were submitted to the same procedures, but without removing the ovaries.

Food consumption and body weight (BW) of the animals were measured throughout the entire experimental period (13 weeks). During this period, the rats were maintained in polypropylene cages (maximum of 4 animals per cage) at controlled temperature (23 °C) and a light/dark cycle of 12 h. The animals were fed with standard diet and water *ad libitum*. All experiments were conducted in strict adherence with the

guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (Certificate nº 149/2014).

2.3. Animal treatment and material collection

Ten weeks after the surgical procedures the OVX rats were randomly subdivided into two groups: untreated rats (OVX) and rats treated with daily doses of AGN (0.042 mg/Kg of BW; OVX+AGN) suspended in arabic gum (1%). The AGN dose used in this study was equivalent to those present in the doses of crude extracts of VAC recommended for postmenopausal women [14]. The OVX and CON received arabic gum (1%). The treatment was performed by gavage during a period of 21 days. In the day of the experiments, the animals were anesthetized with thiopental sodium 50 mg/Kg BW, i.p. [9,11] associated with lidocaine (4 mg/Kg) [17] to collect samples of blood and to remove the liver, adipose tissues and the uterus.

Adipose tissues were collected from three visceral deposits (retroperitoneal, mesenteric and uterine) and one subcutaneous (inguinal). These fats were weighed and expressed in g per 100 g of BW. The adiposity index was calculated by adding the weights of these tissues. The uterus was also collected, weighed and expressed in g per 100 g BW.

2.4. Morphometric analysis of adipocytes

The adipocytes fixed overnight at 4°C in Carnoy´s solution (ethanol-chloroform-glacial acetic acid 6:3:1), dehydrated in a graded series of ethanol, diaphanized in xylol and embedded in histological paraffin. Transverse semi-serial sections of 5 µm thicknesses were obtained using a Leica RM 2145 microtome (Leica Microsystems, Wechsler, Germany) with a steel knife. The histological sections were stained with hematoxylin and eosin. Morphometric analyses were carried out using digital images captured by a high resolution camera (Q Color 3 Olympus American, Burnaby, BC, Canada) coupled to an Olympus BX 41 light microscope at × 40 objectives (Olympus, Tokyo, Japan); subsequently, these images were transmitted to a computer using Q Capture Pro 5.1 and Image-Pro Plus 4.5 (Media Cybernetics, Silver Springs, MD, USA). Adipocyte sizes were obtained by area measurements using Image-

Pro Plus 4.5 software, counting 400 cells in different microscopic fields per tissue section.

2.5. Serum biochemical analysis

The blood was collected by cardiac puncture from fasted rats. Total cholesterol, high-density lipoprotein (HDL-cholesterol) and TAG were analyzed in serum, by standard methods using assay kits (Gold Analisa®). Very-low-density lipoprotein (VLDL-cholesterol) levels were calculated using the Friedewald equation, and low-density lipoprotein (LDL-cholesterol) levels were determined by subtracting HDL and VLDL from total cholesterol. These levels were expressed as mg/dL.

2.6. Intravenous glucose tolerance test (ivGTT)

For ivGTT tests, the 12 h-fasting animals were anesthetized (10 mg xylazine + ketamine 50 mg/kg BW, i.p.) and a cannula was implanted into the jugular vein for administration of a glucose load (1 g/kg BW) and blood collection during the experiment, which started 24 h later of implantation of the cannula [18]. The animals were kept awake in individual cages and blood was collected just before (time 0) and 5, 15 and 30 min after the injection of the bolus of glucose.

The plasma glucose was measured using assay kits (Gold Analisa®) and insulin by radioimmunoassay. The fasting insulinemia and glicemia were used to calculate IR, expressed in terms of the homeostasis model assessment-insulin resistance (HOMA-IR) index [19].

2.7. Liver lipid content determination

The total liver lipid content was determined by gravimetry [20] and the lipid contents were expressed in percentage terms (g/100 g liver wet weight). The liver total cholesterol and TAG were determined after the suspension of fat in 2% Triton, followed by vortexing and heating at 55 °C. The liver lipid content was measured using assay kits (Gold Analisa®).

2.8. Isolation of liver fractions

Liver mitochondria were isolated by differential centrifugation in a mannitol-sucrose medium [21]. Intact mitochondria were used to measure β-oxidation capacity, ROS generation, protein carbonyl content and nicotinamide nucleotide transhydrogenase (NNT) activity. Freeze-thawing disrupted mitochondria were used to measure GSH contents and sonication disrupted mitochondria were used to measure the activity of glutathione peroxidase 1 (GPx1) [22].

The liver cytosolic fractions were obtained by centrifugation of intact mitochondrial suspensions at 15,000 × *g* for 15 min and were used to measure the activity of the GPx3.

To measure the activity of the glucose 6-phosphate dehydrogenase (G6PD), livers from fed animals were homogenized in a medium containing 0.1 M Tris/HCl buffer and 1 mM EDTA (pH 7.6) and centrifuged at 30,000 × *g* for 15 min. The activity of this enzyme was determined in the supernatant by using classical methods as described below.

Homogenates obtained from freeze-clamped liver of overnight fasted rats were used to measure GSH contents and thiobarbituric acid reactive substances (TBARS).

Protein concentrations in the sub-cellular fractions were determined using bovine serum albumin as standard [23].

2.9. Liver mitochondrial β-oxidation capacity

The amount of oxygen consumed by intact mitochondria oxidizing FA was measurements polographically at 37 ºC using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) [24]. The reactions were initiated by the addition of: (a) 20 mM octanoyl-CoA + 2.0 mM L-carnitine, (b) 20 mM palmitoyl-CoA + 2.0 mM L-carnitine or (c) 20 mM palmitoyl-L-carnitine. The rate of oxygen consumption was expressed as nmol/min × mg protein.

2.10. Mitochondrial ROS generation

ROS production by the mitochondrial fraction was evaluated using DCFH-DA oxidation assays as previously described [25]. After a 3 min incubation period of the

mitochondrial suspensions (1 mg protein/mL), in a medium containing 250 mM mannitol, 5 mM malate, 5 mM glutamate, 15 µM DCFH-DA and 2 mM TRIS (pH 7.2), the reaction was initiated by the addition of 600 µM of ADP. Mitochondrial ROS generation was estimated by measuring the linear increase of fluorescence (excitation, 495 nm; emission, 529 nm) recorded over a period of 5 min and expressed as pmol DCF produced/min × mg protein.

2.11. Determination of GSH contents in liver homogenates and isolated mitochondria

GSH contents were measured fluorimetrically using OPT [26]. The fluorescent product of this reaction, GSH-OPT, was measured fluorimetrically (350 nm excitation and 420 nm emission). The results were expressed as µg GSH/mg protein.

2.12. Measurements of antioxidant enzyme activities

The activities of the two isoforms of GPx were assessed in the cytosolic (GPx3) fractions and in mitochondrial matrix (GPx1) according to their ability to oxidize GSH in the presence of H_2O_2 [27]. The activity of these enzymes was expressed as nmol of NADPH oxidized/min × mg protein (ε, 6,220 $M^{-1}\times cm^{-1}$).

The activity of G6PD was determined spectrophotometrically by measuring the rate of increasing in absorbance [28] at 340 nm, due the conversion of $NADP^+$ to NADPH by G6PD. The activity of this enzyme was expressed in nmol of NADPH produced/min × mg protein (ε, 6,220 $M^{-1}\times cm^{-1}$).

The activity of the NNT enzyme was measured in isolated liver mitochondria by direct spectrophotometry using a combination of previously described methods with modifications [29,30]. The enzyme activity was evaluated by the rate of increasing in the absorbance at 375 nm, recorded by 3 min and was expressed as mmol APADH produced/min × mg protein (ε, 5.1 $mM^{-1}\times cm^{-1}$).

2.13. Determination of protein carbonyl content and lipid peroxidation levels

The protein carbonyl contents were determined in liver homogenates and freshly isolated mitochondria using the DNPH method as described by Guarnier *et al.*

[31] with modifications [32,33]. The protein carbonyl groups levels were evaluated spectrophotometrically at 370 nm and expressed in nmol carbonyl/mg protein (ε, 22,000 $M^{-1}\times cm^{-1}$).

The TBARS contents were measured by direct spectrophotometry [34] and used as biomarkers of oxidative stress and lipid peroxidation. The results were expressed as nmol of malondialdehyde (MDA)/mg protein (ε, $1.56 \times 10^5\ M^{-1}\times cm^{-1}$).

2.14. Treatment of data

The data in the figures and tables are presented as means ± standard error (SE). The data were analyzed using one-way analysis of variance (One-way ANOVA), followed by Newman-Keuls post-test. The compared values are provided in the text as probability values (*p*), and the minimum criterion of significance was $p<0.05$. Statistical analyses were performed with Prism GraphPad 5.0 software (GraphPad Software, Inc.).

3. Results

3.1. General Features

As shown in Table 1, the OVX and OVX+AGN rats exhibited pronounced uterine atrophy, a characteristic of the estrogen deficiency. Besides, these groups exhibited higher BW gain than CON groups, in approximately 58% and 69%, respectively, and this could not be attributed to differences in food ingestion. Despite the similar BW gain, only the OVX rats exhibited an adiposity index significantly higher than CON rats (+29%). In these animals, with the exception of retroperitoneal fat, all other deposits were increased, compared to CON: mesenteric in 55%, uterine in 25% and inguinal fat in 35%. The treatment with AGN was effective in reducing the mesenteric and uterine fat deposits, which became similar to those from CON rats, but it failed in reducing the inguinal fat (+32%). In the OVX+AGN rats, the adiposity index reached intermediate values, between those found in CON and OVX rats but did not differed significantly from them.

3.2. Morphometric analysis of adipocytes

Fig. 1 shows the morphometric analysis of white adipose tissue (WAT). Panels 1a, 1b, 1c and 1d show the average adipocyte sizes of retroperitoneal, mesenteric, uterine and inguinal fats, respectively. The adipocyte sizes were significantly higher in OVX rats, except in retroperitoneal fat. The treatment with AGN reduced the adipocyte sizes in mesenteric and uterine fats, which became similar to the CON rats. However, the treatment was not effective in reducing the adipocyte sizes of the inguinal fat. The representative photomicrography of these fats are shown in Fig. 1, panels A, B and C (retroperitoneal), D, E and F (mesenteric), G, H and I (uterine) and J, K and L (inguinal), from CON, OVX and OVX+AGN rats, respectively.

3.3. Serum biochemical analysis

Table 2 shows the lipid profile. As can be seen, the OVX rats presented higher TAG and VLDL levels (about 29% in both) when compared with CON rats. The treatment with AGN exerted the undesirable effect of further increasing the TAG and VLDL serum levels, both 53% higher than those of CON. The total, HDL-, and LDL-cholesterol levels did not differ between the groups, neither the HDL/LDL ratio.

3.4. Intravenous glucose tolerance test (ivGTT)

Fig. 2 shows the time course of glycemia (panel A) and insulinemia (panel B). Although the glycemic response of the animals did not differ from each other, the insulin peak at 5 min was significantly higher in OVX rats, compared to CON and OVX+AGN rats. These results were corroborated by the histograms of the areas under the curves (AUC) of glycemia (panel C) and insulinemia (panel D). The HOMA index (panel E) did not differed between the groups.

3.5. Liver lipid content determination

In order to investigate the occurrence of NAFLD the total liver lipid contents were measured (Fig. 3A) and revealed that the livers from CON rats presented normal total lipid content (4.392 ± 0.266 g/100 g liver wet weight), while the livers of OVX rats

exhibited significantly higher amount of total lipids (+38%), characterizing extensive NAFLD. This condition was partially reversed by the treatment with AGN and in OVX+AGN rats the liver lipid contents reached intermediate values to those found in OVX and CON rats, but differing significantly from them.

The amount of TAG (Fig. 3B) and total cholesterol (Fig. 3C) in the liver were also quantified. The TAG, were higher in OVX rats comparatively to CON rats (+40%) and the treatment reduced these values to close to those found in CON. The amount of total cholesterol in the liver was not different between groups.

3.6. Liver mitochondrial β-oxidation capacity

The ability of isolated liver mitochondria to oxidize octanoyl- and palmitoyl-CoA (in the presence of L-carnitine) and palmitoyl-L-carnitine was measured and the results are shown in Fig. 4. As can be seen, the oxidation of octanoyl-CoA and palmitoyl-L-carnitine in isolated liver mitochondria was not different between the groups, but the oxidation of palmitoyl-CoA was smaller in OVX and OVX+AGN, when compared with CON rats.

3.7. Liver mitochondrial redox state

Mitochondrial ROS generation and the activity of mitochondrial antioxidant enzymes were also evaluated in this work and the results are shown in Fig 5. As can be seen in panel A, the liver mitochondrial H_2O_2 production from OVX rats was significantly higher than that of CON rats (+238%) and the treatment partially reduced this production in about 30%. The panel B shows the carbonyl protein contents in mitochondria, which were increased in OVX comparatively to CON (+98%) and were completely restored in OVX+AGN rats. The mitochondrial GSH contents, reduced in OVX rats (Panel C), were not significantly restored in OVX+AGN rats. The activities of mitochondrial antioxidant enzymes were also assessed in isolated mitochondria: GPx1 (panel D) and NNT (panel E). Similar results were found for these two enzymes: both were reduced in liver mitochondria from OVX, compared with CON rats (-28% and -29%, respectively). The treatment with AGN completely restored the GPx1 activity, but did not alter significantly the NNT activity, although a clear tendency of

restoration of its activity could be observed and the results in the OVX+AGN rats did not differ significantly from those found for CON or OVX rats.

3.8. Liver redox state

The increased ROS generation found in the liver mitochondria from OVX rats could induce alterations in the general redox state, a possibility that was evaluated in this work, through the measurements of GSH and TBARS. The activities of cytosolic antioxidant enzymes were also measured and the results are presented in Fig. 6 (panels A to E). GSH levels (panel A) were significantly reduced in the OVX rats (-33%) and were not significantly restored by treatment with AGN. The G6PD enzyme, (panel B), which provides the reducing equivalents to glutathione reductase, was reduced (-54%) in the OVX rats and was partially recovered by treatment. The GPx3 activities (panel C), was reduced (-39%) in the OVX rats and were completely restored in OVX+AGN rats. The carbonyl protein content in liver homogenate (panel D) was significantly higher (+33%) in the OVX rats and completely recovered in OVX+AGN rats, as well as the lipid peroxidation levels (panel E).

4. Discussion

The results presented here demonstrated that OVX rats exhibit increases in almost all fat deposits and in adiposity index. The adipocytes of two visceral deposits (mesenteric and uterine) underwent hypertrophy in OVX rats, a phenomenon that was accompanied by glucose tolerance, NAFLD and dyslipidemia. Additionally, the fat liver accumulation conducted to a worsening in the general redox state of this organ. More importantly, the treatment of OVX rats with AGN reduced the visceral adipocyte sizes, ameliorated the glucose tolerance, reduced the fat liver accumulation and improved the liver redox state. All these actions of AGN appeared to result of its interaction with estrogen receptor and reinforce the relationship between visceral adipocyte size and metabolic dysfunctions in this animal model of estrogen deficiency [16].

Among all, mesenteric adipocytes, exhibited the smallest diameters in CON rats, and were the ones that exhibited the higher increases in size, in OVX rats. The largest adipocytes of CON rats, on the other hand, did not hypertrophy (retroperitoneal) or exhibited a discrete increase in their sizes (subcutaneous). These same features were

described by comparing lean premenopausal and obese postmenopausal women [1], as well as healthy and unhealthy, severely obese men [2].

Most importantly, although the treatment of OVX rats with AGN did not affected the diameter of the subcutaneous adipocytes, it was able to prevent the hypertrophy of visceral adipocytes, in special the mesenteric ones, and this effect was accompanied by an improvement on glucose tolerance and NAFLD, showing a strong correlation between mesenteric adipocyte hypertrophy and metabolic disorders, similar to what was described for unhealthy, obese men [2] and postmenopausal women [1]. It is worth noting that AGN produced a reduction in the size of mesenteric fat deposits without changing the body adiposity index, clear evidence that body mass index is a poor indicator of metabolic health or degree of fatty liver disease, as already highlighted by O'Connell et al. [2].

The omental/mesenteric adipocytes hypertrophy leads to more detrimental metabolic effects, in part because of close proximity to hepatic and visceral immune cell populations [6]. Besides, the enlarged visceral adipocytes exhibit the most pronounced diameter-related alterations, including high tumor necrosis factor (TNF-α) [35] and low adiponectin productions [36], which was shown to be related to lower responsiveness of adipocytes to insulin [37,38], resulting in increased adipocyte lipolysis [39] and lower glucose uptake [40].

According the "overflow" hypothesis, although subcutaneous adipocytes may not have a direct impact on metabolic dysfunctions, their reduced expansibility and inability of proliferation and/or differentiation limit their capacity to store FA. Thus, a fraction of FA hydrolyzed by lipoprotein lipase (LPL) "spillover" into systemic circulation and can be taken up by distant adipocytes [2,41] by passive (flip-flop) or facilitated diffusion (via CD36 protein) [42]. Our results support this theory, since the subcutaneous adipocytes in the OVX rats exhibited the smallest increases in diameter when compared with other fat deposits evaluated in this study.

Although the alterations observed in OVX rats occurred in the absence of significant changes in insulin sensitivity as judged by HOMA index, under conditions of glucose overload, they presented higher insulin peaks, suggesting reduced insulin sensitivity. In adipocytes, insulin stimulates LPL and inhibits hormone-sensitive lipase (HSL) [43]. In this regard, hyperinsulinemia and IR are associated with a higher LPL activity. However, the suppression of HSL activity by insulin is impaired in this condition [39] and thus, the enlarged visceral adipocytes accumulate more TAG and

release more FA, as already demonstrated for postmenopausal women [44]. The greater daytime insulinemia in OVX rats may also have impacted the activities of the enzymes acyl-CoA synthetase (ACS) [45] and diacylglycerol acyl transferase (DGAT) [46], promoting FA storage in visceral fat deposits.

The condition of estrogen deficiency of OVX rats could also promote the visceral fat accumulation as already described by us [9−11] for OVX rats and by others for postmenopausal women [1,49,50]. Estrogen can modulate LPL activity by suppressing gene transcription [47] and increased activity of LPL has been described in OVX rats [48] and postmenopausal women [1,49,50]. Santosa and Jensen [1] observed that, in postmenopausal women, the abdominal LPL activity increased more from fasted to fed state than in premenopausal women. The authors also found that activities of three other enzymes involved in lipogenesis - CD36 protein, ACS and DGAT - were also increased in postmenopausal women, in special in abdominal adipocytes. All these could be contributing factors to the central pattern of fat distribution associated with female hypogonadism and the more pronounced increases in size of visceral adipocytes in this condition, as observed in our rats and in postmenopausal women [1].

It has been demonstrated that AGN has estrogenic activity, by interacting with estrogen receptor α (ERα) [16]. Analyzing all these facts, we can conclude that abdominal fat is more dependent on the anti-lipogenic action of estrogen than the subcutaneous is, especially in the postprandial periods, in the setting of hyperinsulinemia.

The increased lipolysis by the mesenteric adipocytes and the releasing of FA directly in the portal vein overload the liver and OVX rats developed extensive NAFLD [6]. Besides, insulin robustly promotes liver FA synthesis and decreases FA oxidation [51,52]. Insulin also promotes autophagic degradation of apolipoprotein B100 (apoB100), thus limiting secretion of VLDL from the liver [12]. This is consistent with insulin acting as an anabolic hormone stimulating energy storage in both liver and fat. Because of reduced hepatic secretion of VLDL, there is less competition for lipolysis with intestinal lipoproteins resulting in their preferential clearance.

The estrogen deficiency also promotes the fat liver accumulation. By interacting with ERα, estrogen controls nuclear transcriptional factors that are pivotal in the liver lipid metabolism: SREBP-1c, which controls the expression of lipogenic enzymes, is inhibited by estrogen, and PPARα, which controls the expression of enzymes involved

in FA oxidation, is activated by estrogen [7]. In this way, in a condition of estrogen deficiency, the liver lipid metabolism is diverted from oxidation to synthesis and TAG accumulate in the cytosol of hepatocytes. In fact, in our OVX rats we observed that the mitochondrial FA oxidation was somehow impaired.

The fasting VLDL levels were increased in OVX rats, probably as a consequence of the physiological loss of hepatic insulin regulation of apoB100, by fat liver accumulation [53]. The treatment with AGN further increased these levels and partially reversed the NAFLD. Similar results were found by the treatment of OVX rodents with estrogen [12,13]. The ability of the estrogen treatment to block the insulin-suppression of VLDL secretion involved hepatic ERα, and increased availability of apoB100, via SREBP-1c generation [12].

Although the fasting circulating triglyceride rich-protein, as measured in this work, reflects mainly the liver secretion (VLDL), we could not discharge the possibility that, in OVX rats, the levels of chylomicron-TAG were increased. Elevated postprandial chylomicron-TAG levels were found in postmenopausal women [1] and premenopausal women treated with an inhibitor of estrogen [41]. Accordingly, gut microbiota alterations has been associated with metabolic disorders in postmenopausal women [54,55] and in OVX rats [56] and this could be associated with higher intestinal absorption of TAG and dyslipidemia.

The liver redox state of these animals was also evaluated in this study. OVX rats exhibited a general worsening in the liver redox state. It is known that, the fat liver accumulation, *per se*, lead to oxidative cell damage [8]. Besides, estrogen has antioxidant actions not only because of its phenolic structure [57] but also because of its capacity of reducing the mitochondrial ROS generation [58] and regulating the expression or activity of antioxidant enzymes [9−11,57,58].

Among the other beneficial effects of AGN, stand out the restoration of the activities of GPx1 and GPx3 as well as NNT and G6PD, which supplying the reducing equivalents to glutathione reductase, in mitochondria [29] and cytosol [28], respectively. The recovery of the NNT activity with the treatment with AGN could be a result of the partial reduction of TAG in liver of OVX+AGN rats, since NNT is strongly inhibited by FA, primarily long chain FA (Palmitoyl-CoA) [59,60]. It is also reasonable to consider that the partial recovery of the activities of NNT and G6PD enzymes could have contributed to the partial restoration of the GSH levels. With respect to the activities of two other important antioxidant enzymes, superoxide dismutase and

catalase, in our previous study [11], we have found that their activities were not altered by ovariectomy and so, they were not subject of study in this work.

In summary, our results demonstrated a strong correlation between mesenteric adipocyte diameter and glucose intolerance, NAFLD and dyslipidemia in estrogen deficient female rats. We also demonstrated the estrogen-like actions of pure AGN in reversing several metabolic disorders, most of them were consequences of its primary action in reducing the visceral adipocyte diameters. Most importantly, AGN promoted an overall improvement on the liver redox state, on the contrary of some other plant derivatives, used by women in the treatment of the menopausal symptoms, and to which hepatotoxic effects have been attributed, also shown by us for *Cimicifuga racemosa* [9]. Some effects of AGN, in special on lipid metabolism, were less powerful than those reported by us [11], for AGN-enriched extract of VAC, probably because in this one, other compounds could be acting synergistically with AGN, as is very common in herbal medicine. Further investigation should be done in the future, using this animal model, to better investigate the actions of AGN.

Authors' Contributions

F.N.M. conducted experiments, acquired and analyzed the data and wrote the manuscript, N.C.L. and G.F.G. acquired the adiposity data, E.H.G. acquired the ROS data, S.M.F. acquired the insulin data, N.A.B. and M.N.B. acquired and analyzed the ivGTT data, R.F.G. proof-read the manuscript, C.L.S.P designed the research studies, analyzed data and wrote and proof-read the manuscript. All the authors have read and approved the final version of this manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Table 1. General features.

	CON	OVX	OVX+AGN
Uterine weight	0.2526 ± 0.020	0.0370 ± 0.004[a]	0.0400 ± 0.004[a]
Body weight gain	106.4 ± 5.839	167.9 ± 7.311[a]	180.0 ± 6.537[a]
Food consumption	18.52 ± 0.517	18.24 ± 0.477	18.00 ± 0.390
Adiposity index	7.884 ± 0.467	10.23 ± 0.754[b]	9.394 ± 0.203
Retroperitoneal fat	2.367 ± 0.239	2.279 ± 0.160	2.325 ± 0.102
Mesenteric fat	1.419 ± 0.108	2.203 ± 0.189[c]	1.762 ± 0.067
Uterine fat	2.151 ± 0.144	2.682 ± 0.146[c]	2.140 ± 0.068
Inguinal fat	2.061 ± 0.158	2.780 ± 0.123[d]	2.722 ± 0.139[d]

Notes: The uterine and fat depots weights were expressed in g/100 g BW (n = 6 – 8). The body weight gain was expressed in g (n = 7). Food consumption was expressed in g/day (n = 7). The adiposity index was expressed to g/100 g BW (n = 6). The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ([a]p<0.001 *vs.*CON; [b]p<0.05 *vs.* CON; [c]p<0.05 *vs.* CON and OVX+AGN; [d]p<0.01 *vs.* CON).

Table 2. Serum biochemical analysis.

	CON	OVX	OVX+AGN
Triacylglycerols	31.58 ± 2.286	40.62 ± 2.044[a]	48.29 ± 1.794[b]
Total cholesterol	65.67 ± 3.606	72.12 ± 2.857	70.86 ± 1.623
HDL-cholesterol	32.15 ± 2.881	35.11 ± 1.586	34.14 ± 1.753
LDL-cholesterol	28.50 ± 2.240	30.44 ± 1.974	26.64 ± 1.575
VLDL-cholesterol	6.315 ± 0.457	8.123 ± 0.409[a]	9.657 ± 0.359[b]
HDL/LDL ratio	1.165 ± 0.139	1.178 ± 0.156	1.428 ± 0.162

Notes: Triacylglycerols (mg/dL; $n = 12$), total cholesterol (mg/dL; $n = 7$), high-density lipoprotein (HDL-cholesterol; mg/dL; $n = 7$), low-density lipoprotein (LDL-cholesterol; mg/dL; $n = 6$), very low density lipoprotein (VLDL-cholesterol; mg/dL; $n = 12$) and HDL/LDL ratio ($n = 5$) were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ([a]$p<0.05$ *vs.* CON; [b]$p<0.01$ *vs.* CON and OVX).

Figure Legends

Figure 1. **Adipocyte sizes of retroperitoneal, mesenteric, uterine and inguinal fat depots.** Morphometric analysis and representative photomicrography of the retroperitoneal (panels 1a and A, B and C; *n* = 5), mesenteric (panels 1b and D, E and F; *n* = 4), uterine (panels 1c and G, H and I; *n* = 5) and inguinal (panels 1d and J, K and L; *n* = 5) adipose tissues, from CON, OVX and OVX+AGN, respectively. The scale bar is of 50 µm. The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.01$ *vs.* CON and OVX+AGN; $^{b}p<0.05$ *vs.* CON).

Figure 2. **Plasma glucose and insulin concentrations from intravenous glucose tolerance test (ivGTT).** Time course of glycemia (Panel A, mg/dL; n = 7 – 8), time course of insulinemia (Panel B, ng/mL; n = 5 – 6), the areas under the curves (AUC) of glycemia (panel C) and insulinemia (panel D), and HOMA index (Panel E, n = 5 – 6) are presented. The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.05$ *vs.* CON and OVX+AGN).

Figure 3. **Liver lipid content.** The liver total lipid content (Panel A, g /100 g wet liver weight; *n* = 14), the liver TAG (Panel B, mg/100 g wet liver weight; *n* = 13) and in the liver total cholesterol (Panel C, mg/100 g wet liver weight*; n* = 12) are presented. The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.05$ *vs.* CON and OVX+AGN; $^{b}p<0.05$ *vs.* CON; $^{c}p<0.01$ *vs.* CON and OVX+AGN).

Figure 4. **Liver mitochondrial β-oxidation.** The liver mitochondrial β-oxidation capacity in rats was determined by polarography as described in the Material and methods section. Reactions were initiated by the addition of the octanoyl-CoA + L-carnitine (Oct-CoA), palmitoyl-CoA + L-carnitine (Palm-CoA) or palmitoyl-L-carnitine (Palm-L-Carn). The values are expressed as the means of 3 to 6 individual experiments with different mitochondrial preparations. The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.01$ *vs.* CON).

Figure 5. **Mitochondrial ROS generation and ROS scavenger systems.** The liver mitochondrial oxidative status was evaluated by assessing mitochondrial ROS generation and the ROS scavenging system. Mitochondrial H_2O_2 generation (Panel A, pmol DCF/min × mg protein; *n* = 5); mitochondrial protein carbonyl (Panel B, nmol carbonyl/mg protein; *n* = 8); mitochondrial GSH levels (Panel C, µg GSH/mg protein; *n* = 8); GPx1 activity (Panel D, nmol NADPH oxidized/min × mg protein; *n* = 8) and NNT activity (Panel F, mmol APADH produced/min × mg protein; *n* = 6) were evaluated. The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.001$ *vs.* CON; $^{b}p<0.01$ *vs.* CON and OVX+AGN; $^{c}p<0.001$ *vs.*CON and OVX+AGN; $^{d}p<0.05$ *vs.* CON; $^{e}p<0.05$ *vs.* CON and OVX+AGN).

Figure 6. **Evaluation of the liver redox status.** The liver oxidative status was evaluated by assessing the liver GSH levels (Panel A, µg GSH/mg protein; *n* = 7); G6PD activity (Panel B, nmol NADPH produced/min × mg protein; *n* = 7); GPx3 activity (Panel C, nmol NADPH oxidized/min × mg protein; *n* = 9); protein carbonyl (Panel D, nmol carbonyl/mg protein; *n* = 7) and liver lipid peroxidation using the TBARS method (Panel E, nmol MDA/mg protein; *n* = 8). The results were expressed as the means ± SE. The letters indicate the statistical significances as revealed by one-way ANOVA ($^{a}p<0.05$ *vs.* CON; $^{b}p<0.001$ *vs.* CON and OVX+AGN; $^{c}p<0.01$ *vs.*CON and OVX+AGN; $^{d}p<0.05$ *vs.*CON and OVX+AGN).

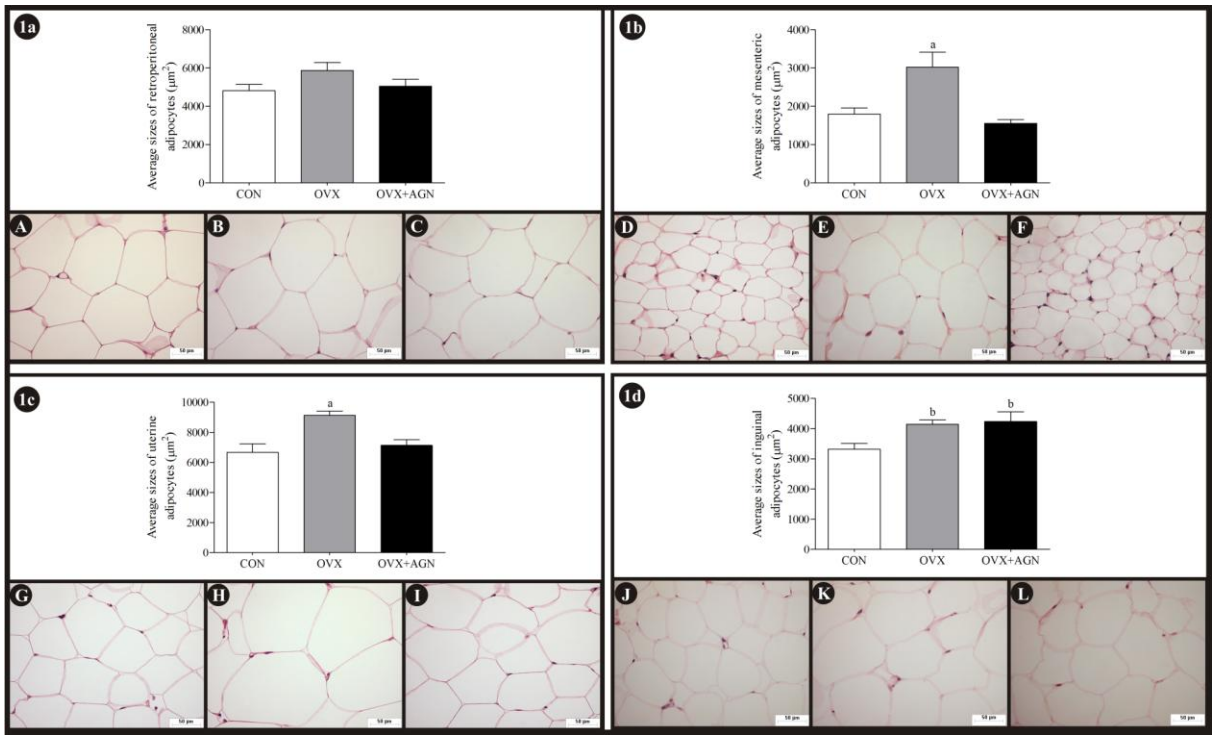

Figure 1. **Adipocyte sizes of retroperitoneal, mesenteric, uterine and inguinal fat depots.**

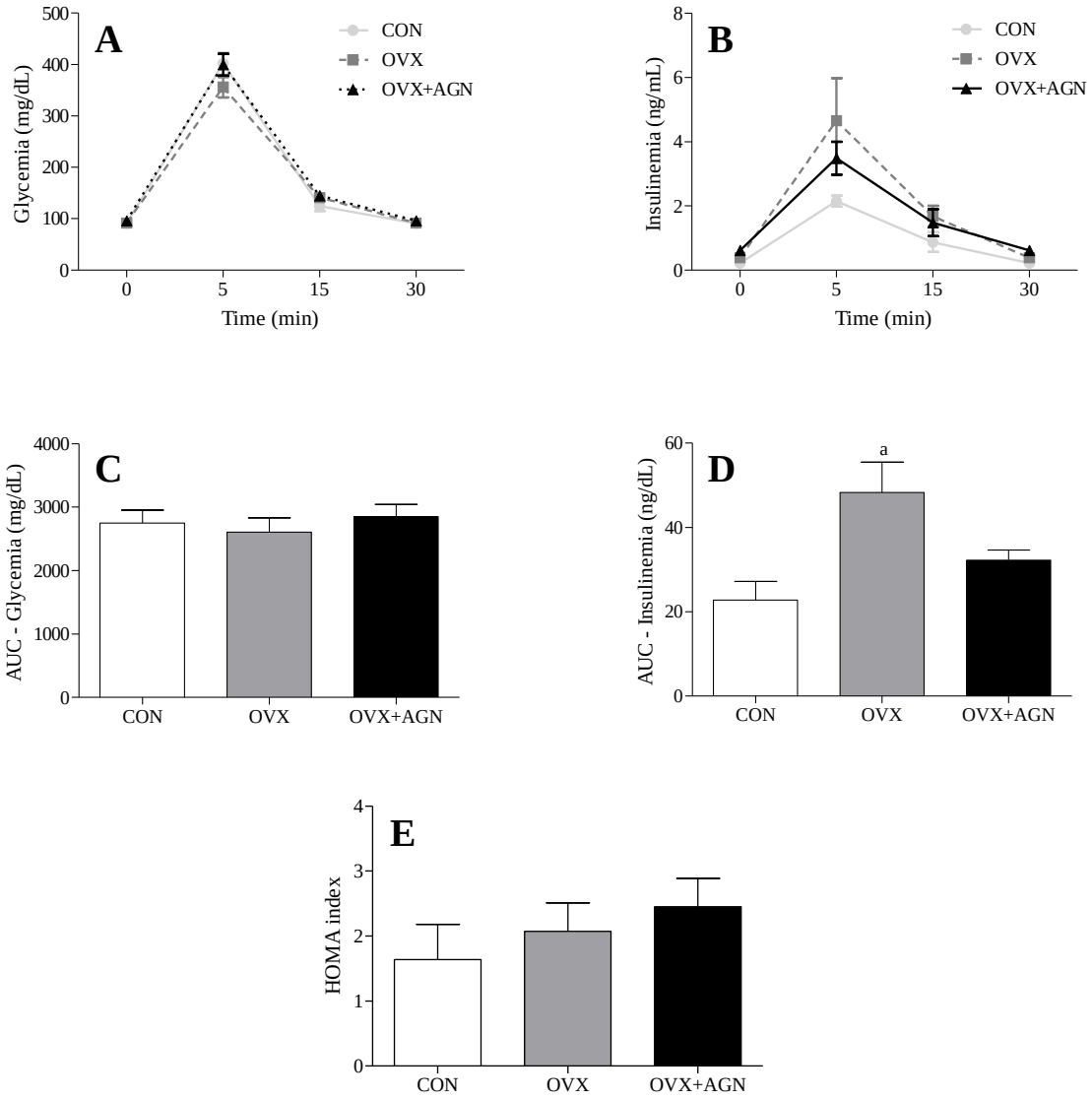

Figure 2. **Plasma glucose and insulin concentrations from intravenous glucose tolerance test (ivGTT).**

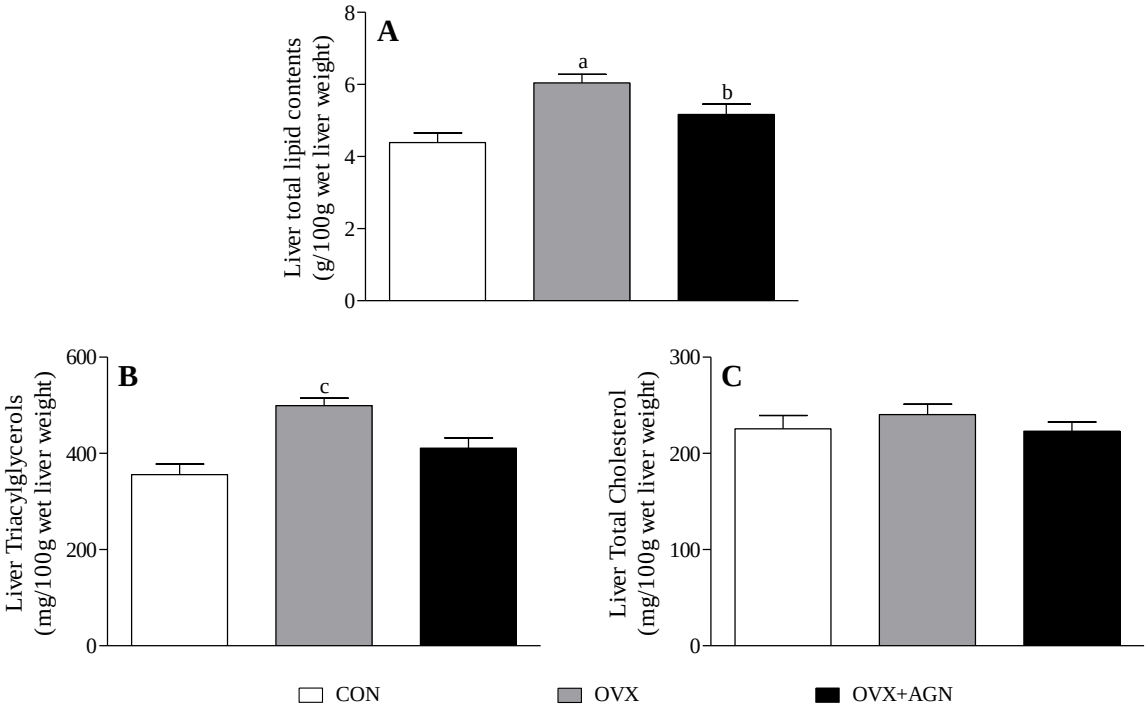

Figure 3. **Liver lipid content.**

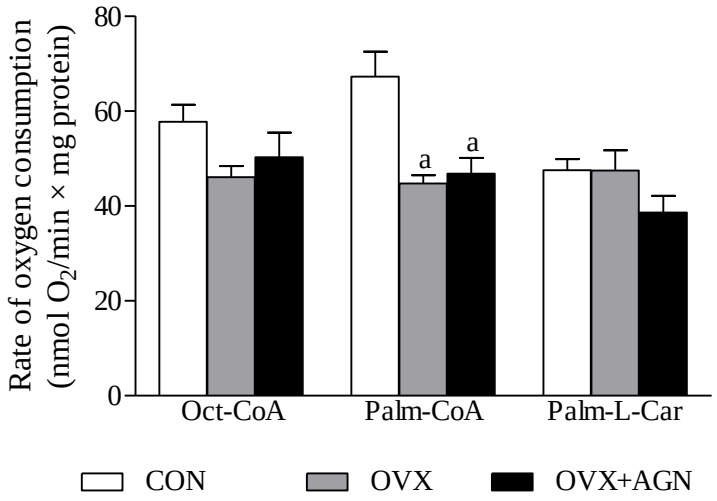

Figure 4. **Liver mitochondrial β-oxidation.**

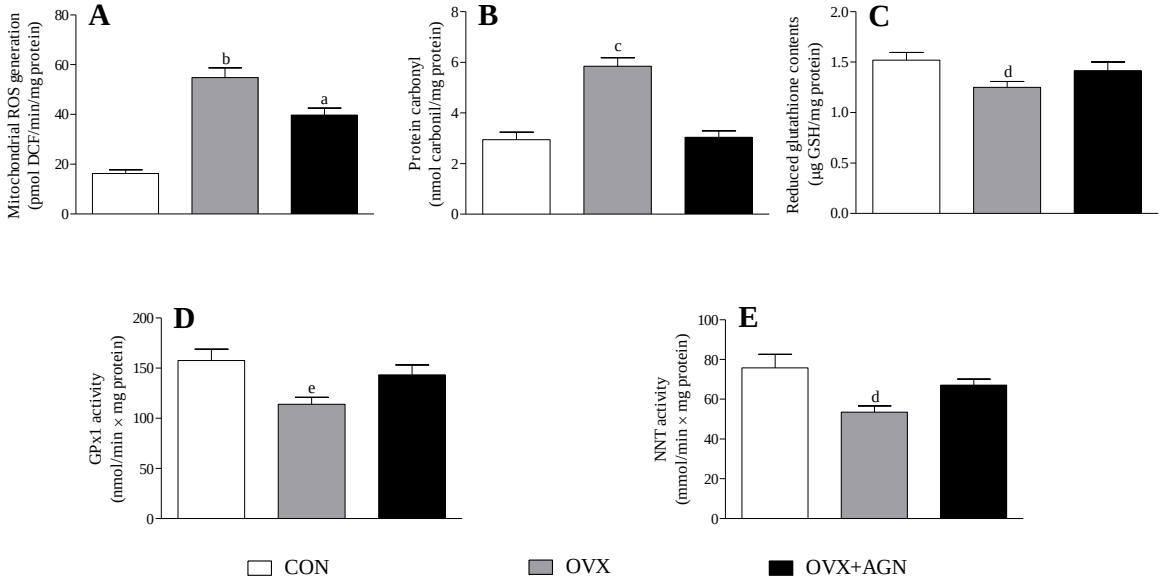

Figure 5. **Mitochondrial H_2O_2 generation and ROS scavenger systems.**

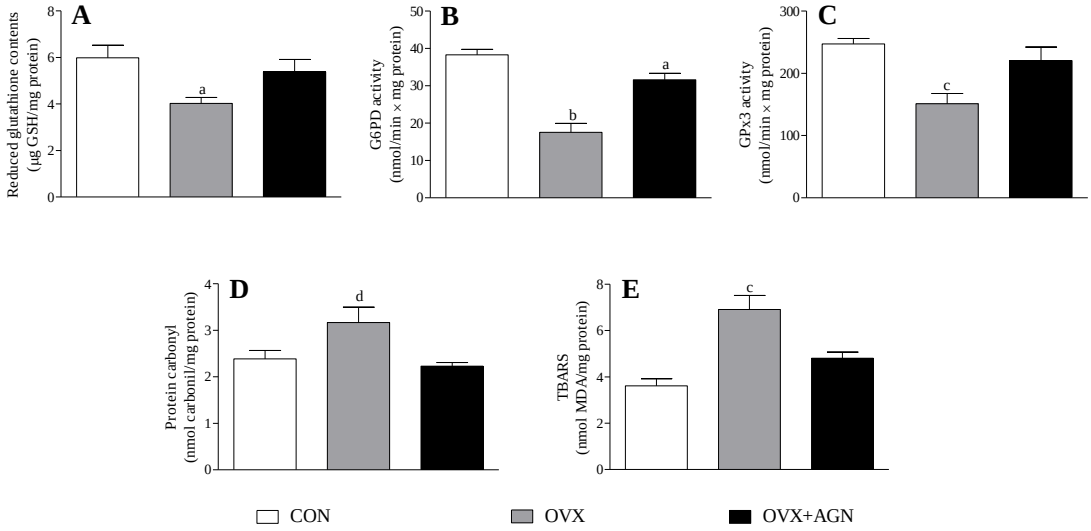

Figure 6. **Evaluation of the liver redox status.**

THE POSTMENOPAUSAL METABOLIC SYNDROME

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ABSTRACT: This review addresses the correlations between obesity, visceral versus subcutaneous adipocyte hypertrophy, inflammation, insulin resistance, steatosis and dyslipidemias, involved in the metabolic syndrome (MSyn), and how much these disturbances could be aggravated by the estrogen deficiency, in postmenopausal women. Although obesity has been mainly related with perturbations between food intake and energy expenditure, other factor considered in this review is the role of alterations in composition and diversity of gut microbiota in the development of metabolic disturbances associated with MSyn. There is a reciprocal influence between gut microbiota and estrogen deficiency and this could contribute to aggravate disturbances of MSyn in postmenopausal women. In this way, a bibliographic survey was carried out on these topics using the PubMed and Web Science databases. The key words utilized were dyslipidemia, adipocytes hypertrophy, postmenopausal, metabolic syndrome, NAFLD and gut microbiota.

KEYWORDS: Dyslipidemia; adipocytes hypertrophy; estrogen deficiency; cardiometabolic disturbances; NAFLD; gut microbiota.

Abbreviations: MSyn, metabolic syndrome; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; TNF-α, tumor necrosis factor-alpha; FA, fatty acids; GM, gut microbiota; VLDL, low density lipoproteins; LPL, lipoprotein lipase; TAG, triacylglycerol; CD36, cluster of differentiation 36; ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; HSL, hormone-sensitive lipase; IL, interleukin; MCP-1, monocyte chemotaxis protein-1; FFA, free fatty acids; OVX, ovariectomized; TLR4, toll-like receptor-4; ER (α or β), estrogen receptors (alpha or beta); SREBP, Sterol regulatory element binding protein; PPARα, peroxisome proliferator-activated receptors-alpha; apoB100; apolipoprotein B-100; PI, phosphatidylinositide; PIP3, phosphatidylinositide-3,4,5 triphosphate; MTP, microsomal triglyceride transfer protein; PLTP, phospholipid transfer protein; SCD-1, stearoyl coenzyme desaturase-1; SCFAs, short-chain FA; B/F, Bacteroidetes/Firmicutes; GPR, G-protein-coupled receptors; Fiaf, fasting-induced adipocyte factor; ME, metabolic endotoxemia; LPS, bacterial lipopolysaccharide

1. Introduction

With the increase in the life expectancy in the last years, women have spent more time in their lives in the postmenopausal period, in a condition of estrogen deficiency, therefore. [1]. This period is accompanied by increased incidence of metabolic syndrome (MSyn), a multifactorial condition characterized by the combinations of three or more of the following cardiometabolic disturbances: visceral obesity, hyperglycemia, insulin resistance (IR), dyslipidemia [2] blood hypertension [3] and steatosis or non-alcoholic fatty liver disease (NAFLD) [4].

The frequent association of these comorbidities suggests that it could not occur merely by chance and that one or a reduced number of factors should underlie such association [5].

In this regard, visceral adiposity has been considered as an independent, determinant factor in the development of the most complications associated with obesity [5]. Over 4 decades ago, adipocyte size was shown to vary inversely with adipocyte insulin sensitivity [6]. More recently, studies have shown functional differences between large and small adipocytes [7-10]. In general, the hypertrophied visceral adipocytes exhibit the more remarkable physiological dysfunctions and leads to more detrimental metabolic effects than subcutaneous [11-15]. For instance, enlarged visceral adipocytes secrete greater amounts of tumor necrosis factor-alpha (TNF-α), which induces IR in both adipocytes and myocytes [16].

The IR, in turn, represents the "first-level" event, responsible by the development of several obesity-associated complications, the so-called "IR-syndromes", such as NAFLD and dyslipidemia [2]. In fact, NAFLD has been considered the hepatic component of MSyn and one of the first complications to be associated with IR [4,17]. This is because the increased lipolysis displayed by insulin-resistant visceral adipocytes, releases fatty acids (FA) directly into the portal vein, overloading the liver and leading to the fat liver accumulation [18].

In addition, adipocytes secrete other cytokines, which similarly to TNF-α are pro-inflammatory [19]. In this way, the development of a chronic low-grade inflammatory state represents the "second-level" contributing event for the pathogenesis of NAFLD, IR and other metabolic and cardiovascular complications of obesity [20-23].

In postmenopausal obesity, there is an increased in the adiposity, with a central pattern of fat distribution and adipocyte hypertrophy [24,25] and as expected, a higher incidence of MSyn [2,3]. Some postmenopausal metabolic disorders, in particular NAFLD and dyslipidemia, very often affect women even if they are slightly overweight, and this has reinforced the idea that estrogen could directly or indirectly exerts some role in the aggravation of several cardiometabolic disturbances of MSyn, acting at various levels.

In this way, the purpose of this review is to perform a general evaluation on the possible mechanisms underlying the pathogenesis of adiposity and hypertrophy of visceral adipocytes, IR, NAFLD and dyslipidemias, involved in the MSyn and how much these disturbances could be aggravated in a condition of estrogen deficiency, in postmenopausal women. For this, a bibliographic survey was carried out of the available English language literature on the topic using the PubMed and Web Science databases. The key words that were utilized were dyslipidemia, adipocytes hypertrophy, postmenopausal, MSyn, NAFLD and gut microbiota (GM).

2. Fat storage, adipogenesis and adipocyte hypertrophy

The major function of adipose tissue is to store and release FA in response to changes in energy balance. The adipose tissue is composed primarily of adipocytes, which represent the majority of the adipose tissue cellular content. But adipose tissue also contains the “stromal vascular fraction” cells, which include mesenchymal stem cells, fibroblasts, pre-adipocytes, endothelial precursor cells and immune cells [26]. Contrary to the previous belief that adipogenesis ceases early in the life, fat cells experience a dynamic turnover, by which mesenchymal cells undergo lineage commitment, pre-adipocyte proliferation and terminal differentiation into mature adipocytes [27]. Approximately 10% of fat cells are renewed annually at the adult stage and at all levels of body mass index [28].

The relative and absolute size of the different adipose tissue depots is ultimately determined by the balance between fat storage on one hand and lipolysis on the other. During a positive caloric balance, adipocytes normally undergo initial hypertrophy, which elicits cellular signaling for the recruitment, proliferation and differentiation of new fat cells [26].

As shown in figure 1, the FA stored in adipose tissue originates primarily from triglyceride-rich lipoproteins [*i.e.*, chylomicrons and very low density lipoproteins (VLDL)]. This pathway requires lipoprotein lipase (LPL) to liberate the FA from the glycerol backbone of the triacylglycerol (TAG) molecule. This enzyme is located on the endothelial wall of the vessels in adipose tissue, major site for fatty acid storage, and muscles, major site for fatty acid oxidation [29]. Then, the FA released by hydrolytic cleavage can be taken up by local cells or by distant adipocytes via passive transport (flip-flop) or facilitated diffusion (FA transport protein CD36) [30].

Other proteins involved on FA storage are acyl-CoA synthetase (ACS) and diacylglycerol acyltransferase (DGAT). ACS catalyzes the intracellular activation of the FA to their CoA form [31]. The DGAT, the final step of storage of FA as TAG, is suggested to be a rate limiting enzyme in FA storage [32]. Three enzymes are involved in TAG hydrolysis: adipose TAG lipase, hormone-sensitive lipase (HSL), and monoglyceride lipase [33]. Among them, HSL is the most studied lipolytic enzyme and hydrolyzes an assortment of substrates including TAG, diacylglycerol, and monoacylglycerol [33].

In this way, the FA storage in adipose tissue is proportional to the adipose tissue LPL activity. This enzyme is positively controlled by insulin [34]. Consequently, in the postprandial periods, following insulin peaks, a significant proportion of circulating chylomicron-TAG is stored in the subcutaneous adipose tissue [35]. Insulin also activates other enzymes involved in the FA synthesis, including ACS [36] and DGAT [37]. On the other hand, insulin inhibits HSL [34]. As the density of insulin receptors is higher in subcutaneous adipose tissue than in visceral one, the preferable subcutaneous fat deposition should occur. However, in the setting of hyperinsulinemia and IR, the hypertrophied adipocytes exhibit increased LPL activity, but the suppression of HSL activity by insulin is impaired, and the enlarged adipocytes store more fat and at the same time exhibited increased lipolysis.

3. Fat storage and inflammation

The IR is known to be an important factor underlying the pathogenesis of several metabolic diseases [2,4], but it is also well recognized that adipose tissue acts as an endocrine organ that secretes hormones, such as adiponectin, leptin and a number of cytokines involved in inflammation [7-10,21]. In this way, a state of chronic low-grade

inflammation is a common feature of obesity and another factor underlying the pathogenesis of several metabolic diseases [21,38].

Overnutrition leads to adipose expansion and pre-adipocytes along with hypertrophic adipocytes secrete various pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β, and IL-6. Hypertrophic adipocytes also secrete monocyte chemoattractant protein-1 (MCP-1), which promotes the infiltration of monocytes into adipose tissue, where they differentiate into macrophages and promote inflammation via the secretion of more pro-inflammatory cytokines (Fig. 1). The TNF-α and IL-6, in turn, block insulin action in the liver, adipose tissue, and skeletal muscle [21,38].

Most importantly, once macrophages are present and active into the adipose tissue, they, along with adipocytes and other cell types, perpetuate a vicious cycle of macrophage recruitment, production of inflammatory cytokines, and adipocyte dysfunction [38].

The more detrimental metabolic effects associated with adipocyte hypertrophy are related to visceral adipocyte, in part because of the close proximity to hepatic and visceral immune cell populations [13]. In the liver, for instance, the activation of Kupffer cells, the resident macrophages, contribute to the production of inflammatory mediators that promote IR and NAFLD [39].

Besides, the enlarged visceral adipocytes exhibit the most pronounced diameter-related alterations, including higher TNF-α [7] and lower adiponectin productions [10], which was shown to be related to lower responsiveness of adipocytes to insulin [16,40], resulting in lower glucose uptake [9] and increased lipolysis [18]. Regarding this last one, worth to say that the most aberrant responses in conditions of IR have been related to the suppression of insulin inhibition of HSL activity [41].

4. Fat storage in subcutaneous versus visceral adipose tissue – The “overflow” hypothesis

Although subcutaneous adipocytes may not have a direct impact on metabolic dysfunctions, they still may play a key role as the initiating factor of fat storage in ectopic sites, such as visceral adipose tissue, liver and muscle [11,42]. The “overflow” hypothesis, proposes that as the size of an adipocyte increases, it will eventually reach a limit and be unable to store further lipid. That is, the inability of subcutaneous adipose tissue proliferation and/or differentiation, causes a sizeable fraction of FA hydrolyzed

by LPL are not taken up locally, but instead “spillover” into systemic circulation [11,42]. So, a second pathway of FA storage involves the direct reuptake of free FA (FFA) [11,43] by distant adipocytes.

5. Body fat distribution in postmenopausal women

Women in the postmenopausal period exhibit increased adiposity and a central pattern of fat distribution [24,25], which is associated with higher incidence of MSyn [2,3,44,45]. The increased adiposity could be attributed, at least in part to a decrease in the energy expenditure [46].

Hyperinsulinemia is also a common feature in postmenopausal women [2,47] and this could contribute to increase the fat storage, by stimulating LPL [34] as well ACS [36] and DGAT [37], as commented earlier.

Estrogen can also modulate fat storage by inhibiting the LPL activity [48]. In fact, increased activity of LPL has been described in ovariectomized (OVX) rats [49] and postmenopausal women [24,50,51]. Santosa and Jensen [24] observed that, in postmenopausal women, the visceral LPL activity increased more from fasted to fed state than in premenopausal women. The authors also found that activities of three other enzymes involved in lipogenesis – CD36 protein, ACS and DGAT – were also increased in postmenopausal women, in special in visceral adipocytes. In accordance to this, it has been demonstrated that, in premenopausal women [24] and OVX rats [43,52,53], visceral fat cell size was smaller than the subcutaneous, but visceral adipocytes were increased in postmenopausal women, while subcutaneous not.

The progesterone deficiency could also predispose to postmenopausal visceral fat accumulation: glucocorticoids promote the accumulation of adipose tissue in the intra-abdominal deposits, because of the higher density of glucocorticoid receptors therein [54]. This is suggestive that progesterone in premenopausal women might protect against cortisol-induced intra-abdominal fat accumulation. Collectively, these data support the theory that phenotypic features are involved in the limited ability of subcutaneous adipocytes to hypertrophy, and in promoting the lipid spillover to visceral and other ectopic sites.

Besides, in post-menopausal women, the visceral fat accumulation is also accompanied by a systemic inflammation, with higher circulating levels of several inflammatory cytokines, as TNF-α and IL-1β [55]. In hypertrophied adipocytes, the

lipolytic activity is increased and this lead to increases in the systemic plasma FFA concentrations, as observed in postmenopausal women [47] and then more IR, inflammation and oxidative stress [26]. In fact, it has been demonstrated that FFA can promote IR by activating the toll-like receptor-4 (TLR4), presents in the innate immune system and pro-inflammatory pathways, which also lead to macrophage accumulation [46,56-58].

6. Insulin resistance, estrogen deficiency and NAFLD

Enlarged visceral adipocytes, as those which are often present in postmenopausal women [2,24,44] exhibit greater lipolytic responses to catecholamines [26] and less responsiveness to the anti-lipolytic action of insulin [18]. Consequently, the FA delivered in the portal vein overload the liver and TAG will accumulate in the cytosol of hepatocyte (Fig. 1) [13,14]. Insulin also robustly promotes liver FA synthesis and decreases FA oxidation [52]. Besides, in the setting of hepatic IR, the inhibition of gluconeogenesis by insulin is impaired thus resulting in hyperglycemia, at the same time that put more substrate available to hepatic FA synthesis [59,60].

Estrogen deficiency also promotes fat liver accumulation. By interacting with estrogen receptor alpha (ERα), estrogen controls nuclear transcriptional factors that are pivotal in the liver lipid metabolism: Sterol regulatory element binding protein (SREBP-1c), which controls the expression of lipogenic enzymes, is inhibited by estrogen, and peroxisome proliferator-activated receptors α (PPARα), which controls the expression of enzymes involved in FA oxidation, is activated by estrogen (Fig. 2) [61]. It has also been demonstrated that estrogen blocks the insulin-dependent activation of actyl-CoA carboxilase enzyme [52]. In this way, in a condition of estrogen deficiency, the liver lipid metabolism is diverted from oxidation to synthesis.

The cyclical secretion of insulin, into the portal vein, also inhibit the liver secretion of VLDL in the postprandial periods, allowing for transient TAG storage for future secretion (Fig. 2) [62]. This is consistent with insulin acting as an anabolic hormone stimulating energy storage in both liver and fat. However, in the condition of hyperinsulinemia, the fatty liver accumulation leads to hepatic IR and the suppression of this action on VLDL secretion. In this way, IR lead NAFLD in one side, and hypersecretion of VLDL in other, since insulin induction of *de novo* lipogenesis occurs independent of its effects on apolipoprotein B-100 (apoB100) [62].

7. Liver VLDL secretion and dyslipidemias

In the fasting state the circulating VLDL levels reflects mostly the liver secretion, and then, fasting high levels of VLDL and TAG are common features of conditions of hyperglycemia and insulin resistance [62].

Under physiological conditions, insulin signaling regulates VLDL production by targeting apoB100 for autophagic degradation and limiting their synthesis (Fig. 3) [52,62,63]. The physiological loss of hepatic insulin regulation of apoB100, as result of fat liver accumulation, is the initial step in the development of hypersecretion of VLDL [62] and most likely involves interruption of insulin-stimulated activation of phosphatidylinositide (PI) 3-kinase and the PI-3,4,5 triphosphate (PIP3) generation [62,64,65].

The PIP3 is a highly negatively charged phospholipid, which may interfere either directly or through downstream effectors with the coalescence of VLDL pre-cursors with TAG droplets, thereby reducing the formation of mainly VLDL [62]. ApoB100 secretion is controlled in part by lipidation in endoplasmic reticulum by microsomal triglyceride transfer protein (MTP). Phospholipid transfer protein (PLTP) promotes VLDL secretion by transferring phospholipids onto VLDL particles. In conditions of IR, PLTP and apoB100 are reduced [52].

Estrogen, on the other hand, by interacting with ERα, promotes lipid load of apoB100 and the VLDL secretion in the context of hyperinsulinemia, which would limit hepatic lipid deposition [52]. This action of estrogen appears to depend on the SREBP-1c expression and activation of stearoyl coenzyme desaturase-1 (SCD-1) enzyme, which catalyzes a limiting step in the biosynthesis of monounsaturated fats, required for the synthesis of TAG and production of VLDL [61,66,67]. In fact, it has been demonstrated that, the treatment of OVX rats with estrogen reverse NAFLD and, at the same time, induces increments in the VLDL and TAG plasma levels [53]. The actions of insulin and estrogen on liver lipid metabolism and VLDL secretion are summarized on figure 2.

In this way, changes in the expression of PPARα, SREBP-1c and SCD-1 contribute to hepatic steatosis, which highlights its significant role in hepatic lipid homeostasis and in the lipid profile [61,66,68].

8. Gut microbiota and Metabolic Syndrome

Recent insight suggests that an altered composition and diversity of gut microbiota could play an important role in the development of metabolic disorders. Most of the gut microorganisms reside in the large intestine. The GM includes not only bacteria but also viruses and fungi. There are 100 trillions of different microorganisms, but the main bacteria phyla represented include: Firmicutes (Gram-positive), Bacteroidetes (Gram-negative), and Actinobacteria (Gram-positive) [69]. Firmicutes is found in the highest proportion (60%), with more than 200 genera, the most important of which are: *Mycoplasma*, *Bacillus*, and *Clostridium*; Bacteroidetes and Actinobacteria each comprise about 10% of the GM, with the rest belonging to over 10 minority families [70]. Many different factors influence GM composition, including diet, age and comorbidity conditions. The complex roles played by the GM are central in the development and modulation of the innate and adaptive immune systems both locally facilitating defense against pathogenic invasion and also systemically. Their dietary and homeostatic functions complement those of the liver and include glucose metabolism, bile salts and xyloglucans, the liberation of short-chain FA (SCFAs) from indigestible starches, and the biosynthesis of vitamins many of which the human host cannot perform independently [69,71].

Among the SCFAs produced by GM, butyrate is the main source of nourishment for colonocytes and is thought to help modulate intestinal barrier integrity, via modulation of the expression of tight junction proteins and mucin [72]. Members of Firmicutes phylum are examples of butyrate-producing bacteria. This suggests that encouraging growth of butyrate-producing species could reduce gut permeability, thus reducing systemic inflammation. However, several studies have demonstrated that, the maintenance of the Bacteroidetes/Firmicutes (B/F) ratio is a more important predictive factor of health than its absolute density [69,71].

Studies performed with obese subjects [73] and animal models of obesity [74,75] revealed that obesity is associated with a significant increase in Firmicutes and a correspondent reduction in Bacteroidetes phylum and this could be associated with higher productions of SCFAs and their convertion into TAG in the liver. These SCFAs also are able to bind and activate two G-protein-coupled receptors (GPR41 and GPR43) in gut epitellial cells. The activation of these receptors induces peptide YY secretion, which suppresses gut motility and retards intestinal transit. By this mechanism, the GM

may contribute markedly to increased nutrient uptake and deposition, contributing to the development of metabolic disorders [76].

Moreover, GM also allows the extracted energy to be stored in adipocytes through a pathway that involves inhibition of fasting-induced adipocyte factor (Fiaf) expression by the intestinal cells. Fiaf inhibits LPL activity and thus, by inhibiting Fiaf, the fat storage increases [77].

It is worth to say that within a phylum, not all the genera have the same role, so that bacterial genera have been related with either beneficial or harmful characteristics associated within the same phylum. [69,78].

Several studies have been performed trying to find the relationship between GM, intestinal barrier integrity and metabolic disorders [79-81]. One of them could be the metabolic endotoxemia (ME). The lipopolysacharides (LPS) are endotoxins commonly found in the outer membrane of Gram-negative bacteria that cause ME, characterized by systemic and liver inflammation [81]. Fat-rich diets are associated with changes in microbiota [82,83] and high levels of LPS.

The LPS are absorbed by enterocytes and are conveyed into plasma coupled to chylomicrons [84]. In this way, dietary fats can be associated with increased absorption of LPS, which, in turn could be related with metabolic disturbances. This causal role of LPS on could be demonstrated by infusing LPS in mice with a normal diet inducing hepatic insulin resistance, glucose intolerance and increased adiposity [80]. In fact, it has been recently shown that LPS impairs pancreatic β-cell function, via interaction with TLR4 and suppression of glucose-induced insulin secretion [85].

The LPS could also contribute to the systemic inflammation of obesity, further aggravating IR and diabetes, by interacting with TLR4 receptors and triggering an extensive cascade of events leading to activation of inflammatory pathways (ME).

Estrogen deficiency is other factor contributing to the disruption in the intestinal integrity and inflammation: by interacting with ERβ [86,87], estrogen positively controls the expression of tight junction proteins occludin, members of the claudin family, and junction-associated adhesion molecule 3 in the colon [88-90]. In this way, also because of this, in estrogen deficiency could aggravate the inflammation associated with obesity.

9. Conclusion

In summary, estrogen deficiency strongly favors the deposition of central fat, which is accompanied by hypertrophy and consequent physiological dysfunctions of adipocytes. The role of estrogen in the modulation of regional adiposity can occur through its inhibitory effect on adipose LPL enzyme. Although metabolic disorders have been associated with visceral adipocyte hypertrophy, subcutaneous adipocytes may play a key role as a starter in the fat deposition process at ectopic sites in line with the "overflow" hypothesis. Another factor that has been associated with MSyn is the GM. By influencing the energy harvest and intestinal permeability, alterations in the GM may aggravate several disorders associated with MSyn. Estrogen deficiency also alters the intestinal permeability, further aggravating this condition.

Authors' Contributions

F.N.M. wrote the manuscript, C.L.S.P designed the research studies, and wrote, and proof-read the manuscript. All the authors have read and approved the final version of this manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Legend Figure

Figure 1. **Fat distribution and inflammation in OVX rats.** Fat storage is dependent on the LPL activity, which hydrolyses TAG from chylomicrons and VLDL particles, releasing FA and glycerol. The greater storage of TAG in adipocyte causes hypertrophy; the enlarged adipocytes secrete inflammatory cytokines (TNF-α, IL-6 and IL-1β) and MCP-1. The last one promotes macrophage infiltration which, in turn, secretes more inflammatory cytokines, perpetuating and cycle of macrophage infiltration, systemic inflammation and insulin resistance. In normal conditions, insulin promotes the fat storage preferable on the subcutaneous adipose tissue. When these adipocytes reach their limits of expansibility, increasing amounts of FA released by LPL spillover into systemic circulation, instead of been locally taken up and "overflow" to ectopic sites, including muscle and liver, also leading to peripheral and hepatic insulin resistance, respectively. In conditions of insulin resistance, the HSL activity is increased in subcutaneous and, in special, in visceral adipose tissue. The increased lipolysis causes the circulating FFA levels to increase and these FA, by interacting with TLR4 present in cells of innate immune system, promoting more macrophage infiltration and cytokine release in adipose tissue. The fatty acid released by the visceral adipocytes in the portal vein, in turn, lead to fatty liver accumulation and hepatic insulin resistance. LPL, lipoprotein lipase; TAG, triacylglycerol; VLDL, low density lipoproteins; FA, fatty acid; FFA, free fatty acid; TAG, triacylglycerols; TNF-α, tumor necrose factor α; IL, interleukin; MCP-1, monocyte chemotaxis protein-1; HSL, hormone sensitive lipase; TLR4, toll-like receptor-4.

Figure 2. **NAFLD and Dyslipidemia in estrogen deficiency condition.** In the liver, by interacting ERα, estrogen controls nuclear transcriptional factors that are fundamental in lipid metabolism: PPARα, which controls the enzymes involved in FA oxidation, is stimulated by estrogen, while SREBP-1c, which positively controls enzymes involved in FA synthesis, is inhibited by estrogen (A). In the absence of estrogen, the lipid metabolism is diverted from oxidation to synthesis of FA. In the liver, insulin promotes apoB100 degradation and TAG storage (B). In the setting of hyperinsulinemia and hepatic IR, there is a reduction of its effect on the degradation of apoB100, resulting in a greater release of VLDL. In addition, in the absence of estrogen the activation of apoB100 and MTP does not occur and this other regulatory pathway of apoB100 is also reduced (B). The peripheral IR and estrogen deficiency cause increases in the FFA release in the portal circulation (C). NAFLD, non-alcoholic fatty liver disease; TAG, triacylglycerols; IR, insulin resistance; apoB100, apolipoprotein B-100; VLDL, low density lipoproteins; FFA, free fatty acids; ERα, estrogen receptors alpha; SREBP-1c, Steroyl regulatory element binding protein-1c; PPARα, peroxisome proliferator-activated receptors α; MTP, microsomal TAG transfer protein.

Figure 3. **Fat storage, adipogenesis and adipocyte hypertrophy.** The greater adiposity in OVX rats could be attributed to the increased of LPL enzyme, by insulin. Estrogen suppress the activation of LPL by insulin, in special in the visceral adipose tissue and, in a condition of estrogen deficiency, the fat storage in these deposits will occur in a more pronounced manner. The hypertrophied adipocytes secrete inflammatory cytokines and MCP-1. The last one causes more macrophage infiltration and inflammation. Estrogen deficiency also causes the disruption of the intestinal barrier and, consequently, greater absorption of LPS, which, along with the circulating FFA released by insulin resistant adipocytes, activate the TLR4 and thus, more macrophages infiltration, cytokines secretion and inflammation. In OVX rats the development of NAFLD could be attributed to at least three factors: (1) increased release of FFA by hypertrophied visceral adipocytes into the portal circulatory system; (2) in the liver, estrogen deficiency favors fatty acid synthesis and deposition and (3) reduces the VLDL exportation. OVX, ovarietomized; LPL, lipoprotein lipase; MCP-1, monocyte chemotaxis protein-1; LPS, bacterial lipopolysaccharide; FFA, Free fatty acid; TLR4, toll-like receptor-4; NAFLD, non-alcoholic fatty liver disease; VLDL, very low density lipoprotein.

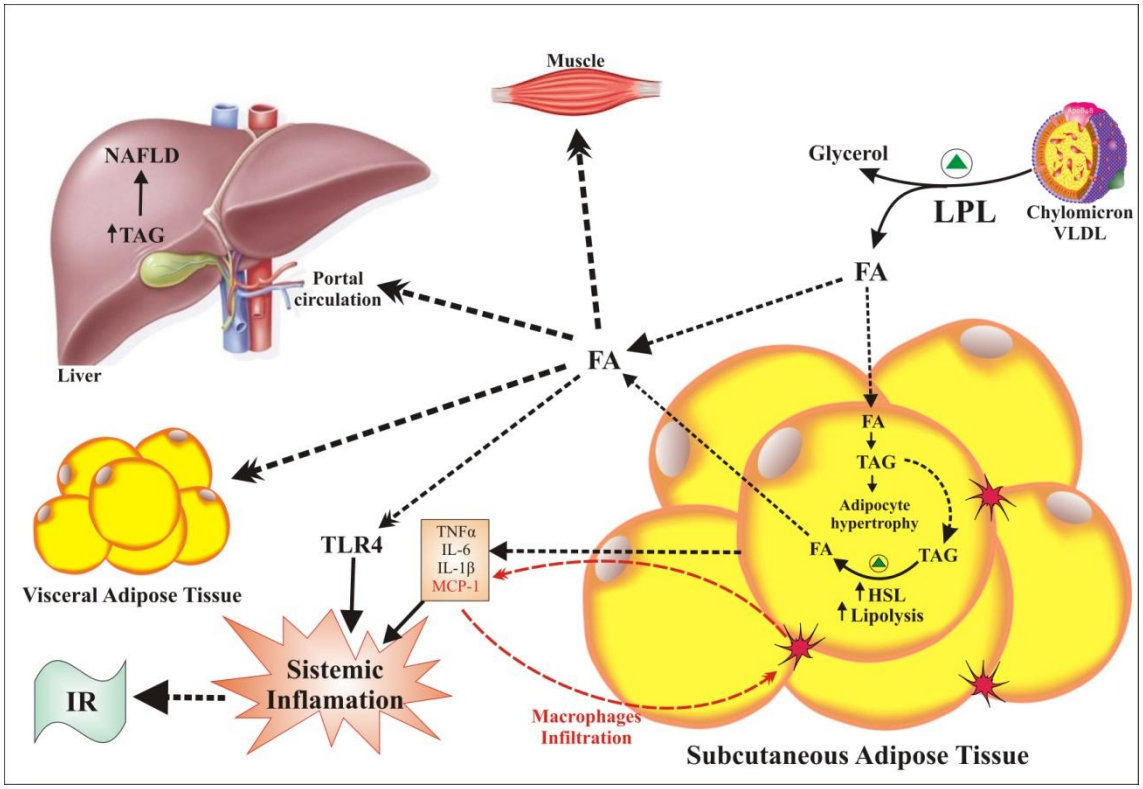

Figure 1. **Fat distribution and inflammation in OVX rats.**

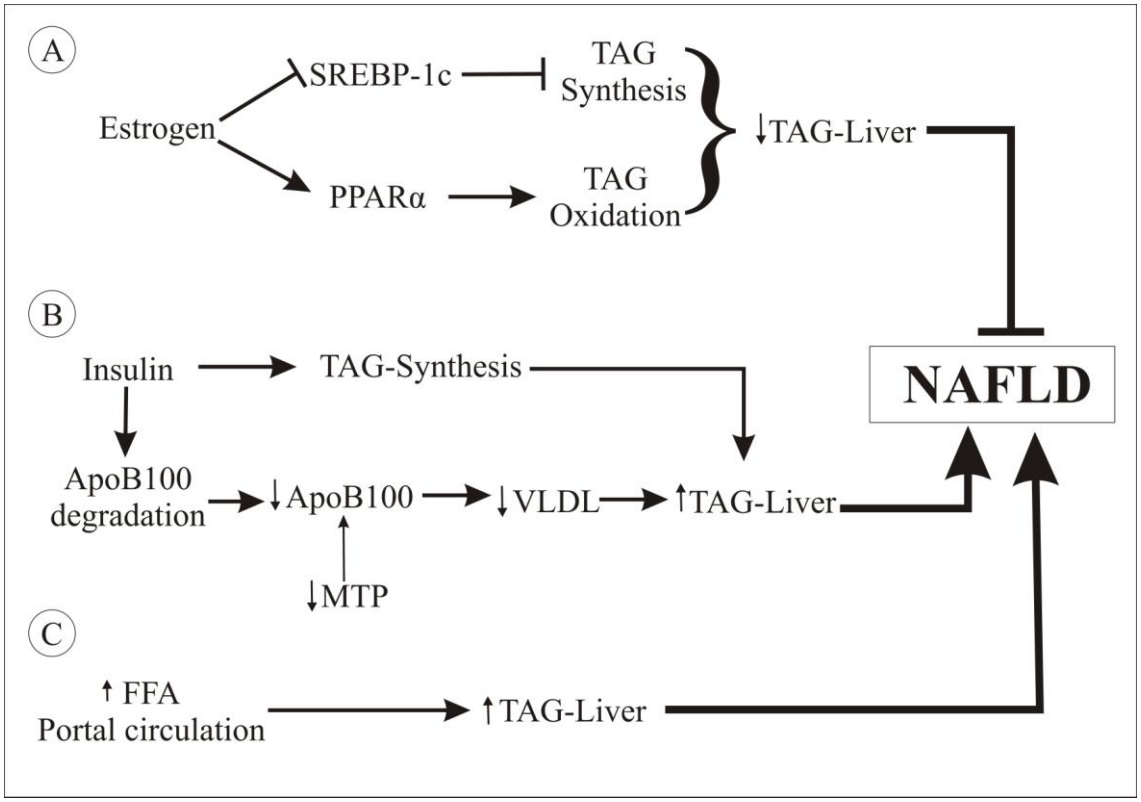

Figure 2. **Dyslipidemia and NAFLD in estrogen deficiency condition.**

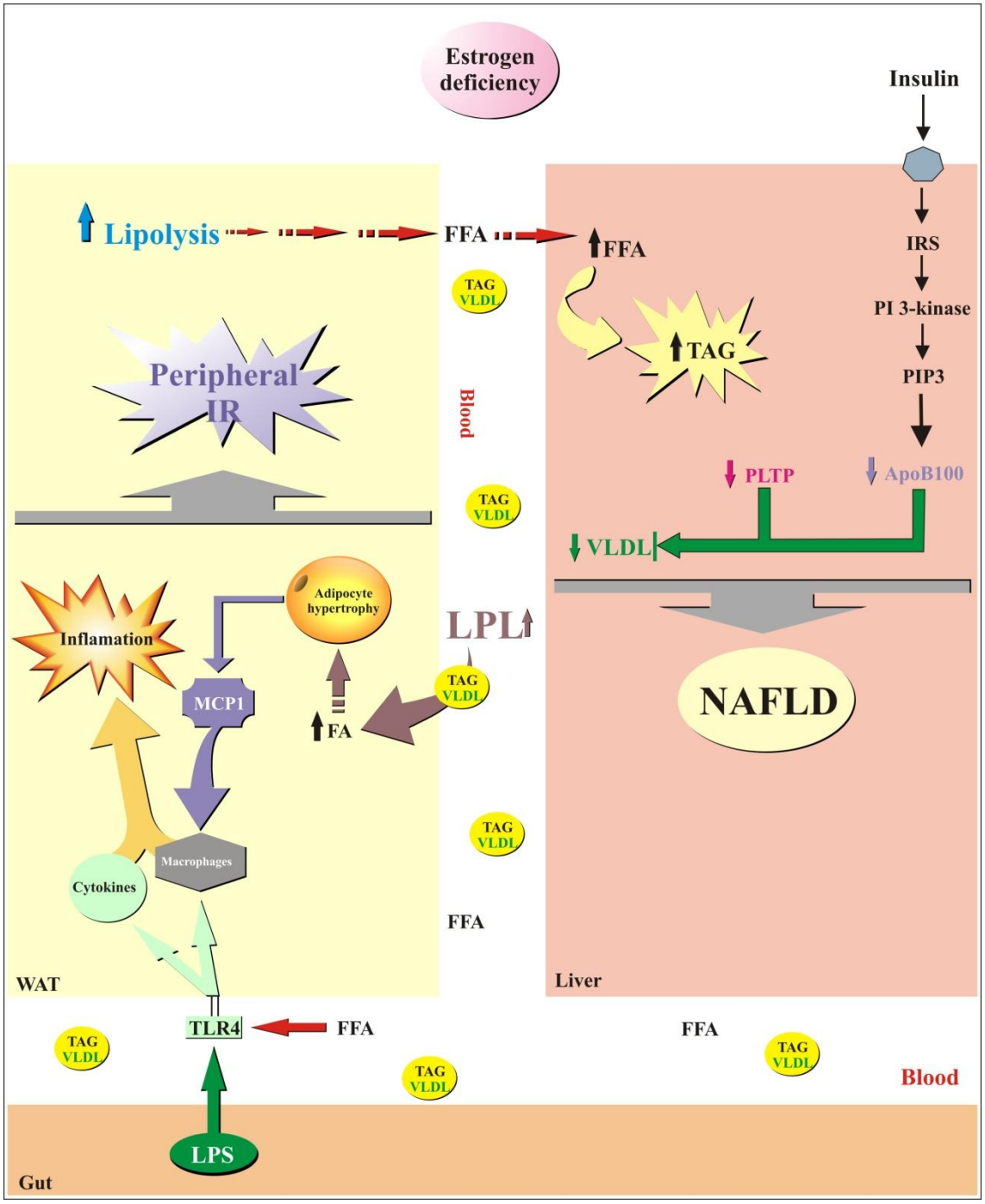

Figure 3. **Fat storage, adipogenesis and adipocyte hypertrophy.**