UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
ÁREA DE CONCENTRAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

DANIELLE APARECIDA MUNHOS HERMOSO

ALTERAÇÕES METABÓLICAS CAUSADAS PELA DEFICIÊNCIA DE ANDRÓGENOS E ESTRÓGENOS:
I – REVISÃO SISTEMÁTICA DA ESTEATOSE HEPÁTICA E DA SÍNDROME METABÓLICA NO HIPOGONADISMO EM HOMENS E MODELOS ANIMAIS
II – EFEITOS DISTINTOS DA ADMINISTRAÇÃO DE MELATONINA EM RATAS NORMOESTROGÊNICAS E DEFICIENTES EM ESTRÓGENOS

Maringá
2019

DANIELLE APARECIDA MUNHOS HERMOSO

ALTERAÇÕES METABÓLICAS CAUSADAS PELA DEFICIÊNCIA DE ANDRÓGENOS E ESTRÓGENOS:
I – REVISÃO SISTEMÁTICA DA ESTEATOSE HEPÁTICA E DA SÍNDROME METABÓLICA NO HIPOGONADISMO EM HOMENS E MODELOS ANIMAIS
II – EFEITOS DISTINTOS DA ADMINISTRAÇÃO DE MELATONINA EM RATAS NORMOESTROGÊNICAS E DEFICIENTES EM ESTRÓGENOS

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutora em Ciências Biológicas.

Orientadora: Profª. Drª. Emy Luiza Ishii-Iwamoto
Coorientador: Prof. Dr. Rodrigo Polimeni Constantin

Maringá
201

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá - PR, Brasil)

Hermoso, Danielle Aparecida Munhos

H556a Alterações metabólicas causadas pela deficiência de andrógenos e estrógenos : I. revisão sistemática da esteatose hepática e da síndrome metabólica no hipogonadismo em homens e modelos animais. II. efeitos distintos da administração de melatonina em ratas normoestrogênicas e deficientes em estrógenos / Danielle Aparecida Munhos Hermoso. -- Maringá, PR, 2019.

133 f.: il. color., figs., tabs.

Orientadora: Profa. Dra. Emy Luiza Ishii-Iwamoto.
Coorientador: Prof. Dr. Rodrigo Polimeni Constantin.
Tese (Doutorado) - Universidade Estadual de Maringá, Centro de Ciências Biológicas, Departamento de Bioquimica. , Programa de Pós-Graduação em Ciências Biológicas (Biologia Celular), 2019.

1. Deficiência de estrógenos. 2. Estresse oxidativo. 3. Deficiência de andrógenos. 4. Melatonina. 5. Síndrome metabólica. I. Ishii-Iwamoto, Emy Luiza, orient. II. Constantin, Rodrigo Polimeni, coorient. III. Universidade Estadual de Maringá. Centro de Ciências Biológicas. , Departamento de Bioquimica. Programa de Pós-Graduação em Ciências Biológicas (Biologia Celular). IV. Título.

CDD 23.ed. 572.4

Rosana de Souza Costa de Oliveira - 9/1366

DANIELLE APARECIDA MUNHOS HERMOSO

ALTERAÇÕES METABÓLICAS CAUSADAS PELA DEFICIÊNCIA DE ANDRÓGENOS E ESTRÓGENOS

I-Revisão sistemática da esteatose hepática e da síndrome metabólica no hipogonadismo em homens e modelos animais

II-Efeitos distintos da administração de melatonina em ratas normoestrogênicas e deficientes em estrógenos

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutora em Ciências Biológicas

Aprovado em: 07/11/19

BANCA EXAMINADORA

Profª Drª. Emy Luiza Ishii-Iwamoto
Profª. Orientadora
Universidade Estadual de Maringá

Prof. Dr. Fábio Erminio Mingatto
Prof. Componente da Banca
Universidade Estadual Paulista

Profª Drª. Lilian Brites Campos Shimada
Prof. Componente da Banca
Centro Universitário Integrado

Profª Drª. Jorgete Constantin
Prof. Componente da Banca
Universidade Estadual de Maringá

Profª Drª. Elismari Rizato Martins Maciel
Prof. Componente da Banca
Universidade Estadual de Maringá

BIOGRAFIA

Danielle Aparecida Munhos Hermoso nasceu em Maringá – PR, em 22 de setembro de 1987. Possui graduação em Educação Física pela Universidade Estadual de Maringá (2011) e Mestrado em Ciências Biológicas pela Universidade Estadual de Maringá (2015). Em agosto de 2015 iniciou o Curso de Doutorado em Biologia Celular e Molecular na Universidade Estadual de Maringá, em Maringá, PR. 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.

AGRADECIMENTOS

A Deus, pela vida, família e oportunidade de realizar o Mestrado e Doutorado;

Aos meus pais, Valdecir e Aparecida por me ajudar, apoiar e acreditar em mim nesta etapa da vida. Ao meu avô Luiz que sempre torceu por mim e a todos familiares que me apoiaram;

Aos professores Rodrigo P. Constantin, Jorgete Constantin, Elismari R. Martins Maciel, Eduardo H. Gilglioni, Karina S. Utsunomiya, Maria Raquel M. Nátali e Tatiana C. dos Santos, pela contribuição e incentivo durante a execução deste trabalho;

Aos meus amigos de laboratório, Eduardo H. Gilglioni que contribuiu imensamente com a elaboração dos artigos. Franciele Moreno, Eduardo Makiyama, e Lenilson, na realização em parte dos experimentos, e aos momentos de descontração vivenciados;

Aos demais amigos do Laboratório de Oxidações Biológicas e Esteatose Experimental da UEM, Juliana, Gislaine, Márcio, Paulo Menezes, Isabela, Ana Luiza, Byanca, Paulo Veiga, Ana Flávia e Karina, pelos momentos vividos, cafés e confraternizações e a todos os outros amigos que a vida me proporcionou e torcem por mim;

A todo apoio técnico dos laboratórios de histotécnica animal e bioquímica, principalmente ao realizado pela Aparecida P. Munhos Hermoso, que foi fundamental na conclusão deste trabalho;

Ao CNPq, CAPES e Fundação Araucária, pelas bolsas de estudo concedidas e projeto aprovado;

Em especial agradeço à professora Emy Luiza Ishii-Iwamoto, pela oportunidade de tê-la como orientadora no Mestrado e Doutorado. Pela paciência, compreensão e muita humildade durante o desenvolvimento dos artigos, e com sua inspiração, contribuir para tornar este trabalho valioso!

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 de dois artigos científicos.

Artigo I: Systematic review of metabolic syndrome in testosterone deficiency: relevance of studies in humans and rodent models

Danielle Aparecida Munhos Hermoso, Eduardo Hideo Gilglioni, Paulo Francisco Veiga Bizerra, Márcio Sigueaki Mito, Rodrigo Polimeni Constantin, Emy Luiza Ishii-Iwamoto**.** A ser submetido ao periódico *The Aging Male.*

Artigo II: Differential melatonin-induced effects on body weight gain, adipocytes morphology and liver lipid metabolism in female rats under normoestrogenic or deficient conditions

Danielle Aparecida Munhos Hermoso, Eduardo Hideo Gilglioni, Lenilson da Fonseca Roza, Aparecida Pinto Munhos Hermoso, Eduardo Makiwama Klosowski, Franciele Neves Moreno, Karina Sayuri Utsunomiya, Elismari Rizato Martins Maciel, Maria Raquel Marçal Natali, Tatiana Carlesso dos Santos, Jorgete Constantin, Rodrigo Polimeni Constantin, Emy Luiza Ishii-Iwamoto. A ser submetido ao periódico *Life Sciences.*

RESUMO GERAL

INTRODUÇÃO – Embora a expectativa de vida humana tenha aumentado nos últimos séculos, ainda existem complicações de saúde na população idosa, muitas relacionadas ao declínio, dependente da idade, na produção dos hormônios gonadais. Nas mulheres, a falência ovariana cessa a produção de hormônios e resulta em redução rápida dos níveis séricos de estradiol (E2), a menstruação cessa, o que define a menopausa. Já nos homens, o principal andrógeno circulante é a testosterona (T). Ao contrário das mulheres, a função reprodutiva permanece comprometida nos homens mais velhos e nem todos eles têm deficiência de T. A T é mais alta na terceira década de vida, declinando com o avanço da idade em aproximadamente 0,4 a 1% ao ano. Na população idosa, a incidência de componentes da síndrome metabólica (SM) aumenta, tais como, ganho de peso corporal, diabetes tipo 2, resistência à insulina, doença hepática gordurosa não alcoólica (DHGNA) e doenças cardiovasculares. A melatonina tem sido sugerida como uma alternativa à terapia de reposição estrogênica para os distúrbios associados à menopausa. No entanto, a capacidade da melatonina de prevenir ou tratar o ganho excessivo de peso corporal e distúrbios metabólicos relacionados ainda é controversa.

OBJETIVOS – Nesta revisão, investigamos a relação entre deficiência de testosterona, esteatose hepática e síndrome metabólica por uma revisão sistemática da literatura. O objetivo do nosso estudo foi analisar se o tratamento preventivo com melatonina é capaz de suprimir as alterações metabólicas induzidas pela deficiência de estrogênio em ratos OVX, com ênfase no ganho de peso e na adiposidade. Considere os efeitos da melatonina em animais normoestrogênicos para fornecer informações de segurança para uso em mulheres na pré-menopausa.

MÉTODOS - No artigo I, foi realizada uma busca sistemática no banco de dados PubMed, incluindo as palavras relacionadas à deficiência de T, os distúrbios relacionados à SM e esteatose hepática. No artigo II, as ratas foram divididas em quatro grupos: ratas controle com operação simulada tratadas com solução salina (CON); ratas controles com operação simulada tratadas com melatonina (CON+MEL), ratas ovariectomizadas tratadas com salina (OVX) e ratas OVX tratadas com doses diárias de melatonina (OVX+MEL). No dia seguinte à cirurgia, uma dose de 10 mg/kg de melatonina foi administrada diariamente às ratas dos grupos CON+MEL e OVX+MEL por sonda esofágica por um período de 16 semanas. Nos dias dos experimentos, os animais foram sacrificados com tiopental sódico (50 mg/kg i.p) para coleta de amostras de sangue, fígado, tecido adiposo, útero e carcaça. Parâmetros biométricos e ingestão de alimentos foram medidos ao longo do período experimental. A carcaça foi utilizada para determinar sua composição; os tecidos adiposos foram utilizados para determinar o índice de adiposidade e para análise histológica. O colesterol total, a lipoproteína de alta densidade (HDL-colesterol), os triglicerídeos (TG) e a glicose foram analisados no soro/plasma por métodos padrão usando kits de ensaio. O conteúdo lipídico total do fígado foi determinado pelo método gravimétrico e o colesterol e triglicerídeos no fígado foram determinados por ensaios específicos em kits. As alterações histológicas no fígado também foram investigadas e a porcentagem de infiltração lipídica foi medida em cortes histológicos do fígado corados com Sudan III. Mitocôndrias do fígado foram isoladas por centrifugação diferencial para avaliação da respiração ligada à β-oxidação de ácidos graxos, respiração acoplada à fosforilação de ADP, atividade de enzimas associadas às membranas e geração de H_2O_2. Parâmetros de estresse oxidativo celular foram avaliados em mitocôndrias e em homogenatos de fígado e tecido adiposo. Foram medidos os teores de glutationa reduzida (GSH), proteínas carboniladas e o conteúdo de malondialdeído (MDA) realizado pela técnica de espécies reativas ao ácido tiobarbitúrico (TBARS). Foram determinadas as atividades das seguintes enzimas antioxidantes: superóxido

dismutase (SOD), catalase (CAT), glutationa reductase (GSSG-red), glutationa peroxidase (GSH-Px) e glicose 6-fosfato desidrogenase (G6PD). O fígado intacto foi perfundido em outro lote de animais, no qual a oxidação dos ácidos graxos e a cetogênese foram medidos.

RESULTADOS E DISCUSSÃO - No artigo de revisão, trinta e nove artigos foram incluídos e a maioria deles mostraram que baixos níveis de testosterona estão associados positivamente a distúrbios na homeostase da glicose, menor sensibilidade à insulina e dislipidemia. A distribuição de gordura e a composição corporal também são frequentemente modificadas sob condições de baixa testosterona. Alguns estudos verificaram a relação causal entre a DHGNA e os níveis séricos totais de T e indicaram que o fígado pode ser mais suscetível a engordar durante a deficiência de T. Quando os efeitos da terapia de reposição de T foram avaliados, os resultados mostraram que ela pode reverter alguns dos sinais clínicos da síndrome metabólica, o que enfatiza a importância da testosterona para a homeostase do metabolismo energético nos homens.

No artigo original, os principais resultados foram os seguintes:

A) O peso corporal de todas as ratas aumentou progressivamente durante as 16 semanas de tratamento. Após a terceira semana, o peso corporal das OVX foi maior que as CON (+26%). A administração de melatonina em ratas OVX+MEL não modificou o padrão de ganho de peso durante todo o período de tratamento, em comparação com OVX não tratadas, enquanto reduziu o ganho de peso em ratas CON+MEL, uma queda estatisticamente significativa após a sétima semana de tratamento. Nessas ratas o ganho de peso no final do tratamento foi 21% menor que nas CON e 55,5% menor em relação às OVX+MEL. O consumo alimentar não foi diferente. A composição da carcaça apresentou 5% menos matéria seca nas ratas OVX em comparação às ratas CON. Ratas OVX exibiram um aumento de 13% no extrato etéreo em relação aos valores das ratas CON e este extrato permaneceu elevado nas ratas OVX+MEL (+21% em relação às ratas CON). A melatonina induziu diminuição de 18% na porcentagem de extrato etéreo na carcaça de ratas CON+MEL. O índice de adiposidade foi 17% maior em ratas OVX em comparação às ratas CON que permaneceu elevado (+18%) nas ratas OVX+MEL.

B) Os níveis médios de estradiol nas CON+MEL diminuíram 53% quando comparados às ratas CON. Não foram observadas diferenças significativas entre os quatro grupos de ratas nos níveis sanguíneos de FSH, glicose, triglicerídeos, colesterol total e suas lipoproteínas. Foi encontrada uma diferença significativa para os marcadores das funções hepáticas: as atividades da aspartato aminotransferase (AST) e alanina aminotranferase (ALT) foram, respectivamente, 46% e 28% maiores no sangue de ratas OVX quando comparadas com ratas CON, e o tratamento com melatonina induziu uma reversão parcial nos níveis de ambas as enzimas. Apesar do aumento substancial no peso total de gordura inguinal nas ratas OVX, a análise histológica deste tecido revelou que o tamanho dos adipócitos era muito semelhante ao das ratas CON. Foi evidenciada prevalência de adipócitos com tamanho reduzido em ratas tratadas com melatonina, não apenas no OVX+MEL, mas também no CON+MEL.

C) O conteúdo de GSH na gordura inguinal foi reduzido em 23% nas ratas OVX, efeito suprimido pela administração de melatonina (ratas OVX+MEL). Nas ratas CON+MEL, a melatonina induziu uma redução de 29% no teor de TBARS na gordura retroperitoneal, e na gordura inguinal, verificou-se uma diminuição de 41% no teor de GSH e um aumento no teor de tióis (+40%) quando comparado com os respectivos grupos não tratados. As atividades GSSG-red e G6PD foram 44% e 41% reduzidas, respectivamente, em OVX quando comparadas com os valores das ratas CON. A melatonina aumentou as atividades das duas enzimas antioxidantes GSSG-red e G6PD em ratas OVX+MEL para níveis

equivalentes aos das ratas CON+MEL. Na gordura inguinal, a melatonina aumentou a atividade da CAT nas ratas OVX+MEL (+37% em relação às OVX).
D) No fígado, o conteúdo de proteína carbonilada aumentou (+39%) e o conteúdo de GSH foi menor (-19%) nas ratas OVX quando comparado às CON. Nas ratas OVX+MEL, essas alterações foram suprimidas. As atividades da SOD e G6PD foram alteradas nas ratas OVX; já a administração de melatonina nas CON+MEL reduziu a atividade de G6PD para um valor médio 37% inferior ao das ratas CON.
E) A quantificação de lipídios mostrou maior teor de gordura no fígado das ratas OVX. Triglicerídeos, mas não colesterol, foram os responsáveis pelo acúmulo de lipídios nas ratas OVX. O tratamento de ratas OVX+MEL reduziu os teores totais de lipídios e TG a valores semelhantes aos das ratas CON. Alterações significativas foram induzidas pelo tratamento com melatonina na respiração controlada por ácidos graxos em mitocôndrias desacopladas. Apesar das taxas semelhantes de respiração induzida por ácidos graxos nas mitocôndrias das ratas CON e OVX, com diversos substratos foi encontrado estímulo em ratas tratadas com melatonina, OVX+MEL e CON+MEL. Já no fígado intacto, a cetogênese foi aproximadamente 55% maior nas ratas CON+MEL do que nas ratas CON durante todo o período de infusão de palmitato.
CONCLUSÃO PRINCIPAL – Na revisão, encontramos evidências da relação direta entre deficiência de T e síndrome metabólica. Programas de rastreamento em pacientes mais velhos ou com sobrepeso são importantes para ajudar a entender e detectar indivíduos com baixos níveis de T sérico para suplementar adequadamente. Deve-se considerar a importância de uma terapia específica para o tratamento da síndrome metabólica, que contemple todos os componentes da síndrome, o estágio da vida e o perfil hormonal como aspectos-chave para uma intervenção clínica assertiva. Finalmente, modificações no estilo de vida e na dieta parecem ser abordagens preventivas eficazes para evitar o acúmulo ectópico de gordura no fígado em homens com diagnóstico de deficiência de testosterona. Quanto ao artigo original, as ratas OVX, em nossa condição experimental, apresentaram características de adiposidade metabolicamente saudáveis, uma vez que as ratas não exibiram dislipidemia e sinais de resistência à insulina. A hipertrofia da gordura retroperitoneal e a hiperplasia da gordura inguinal na OVX provavelmente representam uma adaptação dos tecidos adiposos para armazenar maiores quantidades de lipídios e, assim, evitar a deposição ectópica de lipídeos. A administração a longo prazo de melatonina foi eficaz na prevenção do desenvolvimento de esteatose hepática e estresse oxidativo nas ratas OVX e, embora não tenha reduzido o ganho maior de adiposidade, a melatonina foi capaz de alterar a distribuição lipídica e a morfologia dos adipócitos em diferentes depósitos de gordura. A supressão da hipertrofia da gordura retroperitoneal e a preservação da hiperplasia da gordura inguinal sugeriram que a melatonina induziu uma redistribuição de lipídios em diferentes depósitos de gordura, reduzindo a sobrecarga lipídica excessiva e, assim, eliminando a possibilidade de células progredirem para inflamação e anormalidades relacionadas. Em ratas normoestrogênicas, a melatonina reduziu o ganho de peso corporal, reduziu o tamanho de adipócitos da gordura inguinal, reduziu a ingestão de alimentos e aumentou a oxidação hepática dos ácidos graxos. A melatonina também reduziu os níveis plasmáticos de estradiol em ratas controle. Pode-se concluir que a melatonina elimina o risco de desenvolver distúrbios metabólicos relacionados à deficiência de estrogênio, principalmente alterando a distribuição lipídica nos depósitos de gordura e suprimindo o estresse oxidativo dos tecidos. Sob condições normais de estrogênio, a melatonina pode induzir efeitos adversos, incluindo perda de peso corporal e distúrbios nas funções reguladas por estrogênio. Como a melatonina foi proposta como agente farmacológico no tratamento de muitas doenças, sua função em mulheres na pré-menopausa deve ser considerada com cuidado.

GENERAL ABSTRACT

INTRODUCTION – Even though human life expectancy has increased in recent centuries, there are still health complications in older population, many related to age-decline in gonadal hormones. In women, the production of sex steroid hormones by the ovaries stops, the serum estradiol (E2) level decreases rapidly, menstruation ceases, defining menopause. The main circulating androgen in men is testosterone (T). In contrast to women, reproductive function remains intact in older men and not all of them have T deficiency. T is highest in the third decade of life declining with advancing age by approximately 0.4 to 1% per year. In the elderly population, the incidence of metabolic syndrome (MetS) components increases, such as body weight gain, type 2 diabetes, insulin resistance, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease. Melatonin has been suggested as an alternative to estrogen replacement therapy for the menopause-associated disorders. However, the ability of melatonin to prevent or treat excessive body weight gain and related metabolic disorders is still controversial.

AIMS – In this review, we investigate the relationship between testosterone deficiency, hepatic steatosis and metabolic syndrome by a systematic review of the literature. The objective of our study was to analyse whether preventive treatment with melatonin is able to suppress the metabolic changes induced by estrogen deficiency in OVX rats, with emphasis on weight gain and adiposity. Consider the effects of melatonin on normoestrogenic animals to provide safety information for use by premenopausal women.

METHODS – In article I, a systematic search of the PubMed database was performed, including the words related to testosterone deficiency, the disturbs related to metabolic syndrome, metabolic syndrome and hepatic steatosis. In the article II the rats were divided into four groups: untreated control rats, SHAM-operated (CON); control rats treated with daily doses of melatonin (CON+MEL); untreated ovariectomized rats (OVX) and OVX rats treated with daily doses of melatonin (OVX+MEL). The day after surgery, a dose of 10 mg/kg of melatonin was daily administered to rats of the groups CON+MEL and OVX+MEL by esophageal gavage over a period of 16 weeks. On the days of the experiments, the animals were euthanized with thiopental sodium (50 mg/kg *i.p*) for collection of the blood samples, liver, adipose tissues, uterus and carcass. Biometrical parameters and food ingestion were measured along the experimental period. The carcass was used to determine its composition the adipose tissues were used to determine the adiposity index and for histological analysis. Total cholesterol, high-density lipoprotein (HDL-cholesterol), triglycerides (TG) and glucose were analysed in serum/plasma by standard methods using assay kits. The liver total lipid content was determined using the gravimetric method and cholesterol and triglycerides in the livers were determined by specific kit assays. Histological changes in the liver were also investigated and the percentage of lipid infiltration was measured in histological slices of the liver stained with Sudan III. Mitochondria were isolated from livers by differential centrifugation for evaluation of respiration linked to β-fatty acid oxidation, respiration coupled to ADP phosphorylation, activity of enzymes associated to membranes and H_2O_2 generation. Parameters of cellular oxidative stress were evaluated in mitochondria and in liver and adipose tissue homogenates. The contents of reduced glutathione (GSH), carbonyl proteins and malondialdeyde (MDA) performed by the thiobarbituric acid reactive species (TBARS) technique were measured. The activities of the following antioxidant enzymes were determined: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSSG-red), glutathione peroxidase (GSH-Px) and glucose 6-phosphate dehydrogenase (G6PD). The intact liver was perfused in another batch of animals, in which the fatty acids oxidation and ketogenesis was measured.

RESULTS AND DISCUSSION In the review article**, t**hirty-nine articles were included and most of them show that low testosterone levels are positively associated with disturbs on glucose homeostasis, lower insulin sensitivity and dyslipidaemia. The fat distribution and body composition are also frequently modified under low testosterone condition. Some studies verify the causal relationship between NAFLD and total serum T levels and have indicated that the liver may be more susceptible to becoming fatty during T deficiency. When the effects of T replacement therapy were evaluated, the results showed that it can reverse some of the clinical signs of metabolic syndrome, which emphasizes the importance of the testosterone for the homeostasis of the energy metabolism in men. Screening programs in older or overweight patients are necessary to understand and detect individuals with low serum T and supplement accordingly. The importance of a specific therapy to treat MetS should be considered, which contemplate every component of the syndrome, the stage of life and the hormonal profile as key aspects for an assertive clinical intervention. Finally, lifestyle and diet modifications can be adopted as preventive approaches to avoid the ectopic accumulation of fat in the liver in men with diagnose of testosterone deficiency.

In the original article, the main results were the following:

A) The body weight (BW) of rats increased progressively during the 16 weeks of treatment. After the 3rd week the body weight of OVX rats was higher than the CON rats (+26%). Melatonin administration to OVX+MEL rats did not modify the pattern of body weight gain during all periods of treatment when compared with that one of untreated-OVX rats and reduced the body weight gain of CON+MEL rats which was significant after the 7th week of the treatment. The effective body weight gain at the terminus of treatment was 21 % lower than the CON rats and 55.5 % relative to OVX+MEL. The food consumption was not different among them. The composition of the carcass had 5% lower dry matter in OVX rats compared with the CON rats. OVX rats exhibited a 13% increase in ethereal extract relative to values of CON rats and this extract remained elevated in OVX+MEL rats (+21% relative to CON rats). Melatonin induced a decrease of 18 % in the percentage of ethereal extract in CON+MEL rats. The adiposity index was 17% higher OVX rats compared to CON rats that remained elevated (+18%) in OVX+MEL rats.

B) The mean levels of estradiol in CON+MEL reduced by 53% when compared to CON rats. No significant differences were observed among the four groups of rats in the blood levels of FSH, glucose, triglycerides, total cholesterol and its lipoproteins. A significant difference was found for markers of hepatic functions: the activities of AST and ALT were, respectively, 46% and 28% higher in the blood of OVX rats when compared with CON rats, and melatonin treatment induced a partial reversion in the levels of both enzymes. Despite substantial increase in the total weight of inguinal fat in OVX rats, histological analysis of this tissue revealed that the size of adipocytes was very similar to that of the CON rats. In the retroperitoneal tissue the analysis of the distribution of adipocyte according to their areas revealed hypertrophy of adipocytes in OVX rats, which melatonin was able to prevent.

C) The content of GSH in inguinal fat that was 23% reduced in OVX rats, an effect that was suppressed by melatonin administration (OVX+MEL rats). In CON+MEL rats melatonin induced a 29% reduction in the TBARS content in retroperitoneal fat and in inguinal fat it was found a decrease of 41% in the GSH content and an increased protein thiols content (+40%) when compared with their respective untreated groups. The GSSG-red and G6PD activities were 44% and 41% reduced, respectively, in OVX when compared with the values of the CON rats. Melatonin increased the activities of the two antioxidant enzymes GSSG-red and G6PD in OVX+MEL rats to equivalent levels of

those of CON rats. In inguinal fat, melatonin increased the CAT activity in OVX+MEL (+37% than OVX).

D) In the liver, the protein carbonyl groups content increased (+39%) and the GSH content was lower (-19%) in OVX rats when compared to CON. In OVX+MEL rats these alterations were suppressed. The activity of SOD and G6PD were altered in OVX and the administration of melatonin in CON+MEL rats reduced the activity of G6PD to a mean value 37% above of that of CON rats.

E) The quantification of lipids showed high content of fat in livers from OVX rats. Triglycerides and not total cholesterol accounted to lipid accumulation in OVX rats. The treatment of OVX rats with melatonin reduced both total lipid and TG contents to values very similar to those of CON rats. Significant changes were induced by melatonin treatment in the FA-driven respiration in uncoupled mitochondria. Despite the similar rates of FA-induced respiration in mitochondria from the CON and OVX rats, irrespective of fatty acid oxidized, a stimulus was found in melatonin-treated rats in both ovariectomized OVX+MEL and CON+MEL rats. In the intact liver the ketogenesis in was approximately 55% higher in CON+MEL rats than in CON rats in the whole period of palmitate infusion.

MAIN CONCLUSION - In the review, we found evidences of the direct relationship between T deficiency and metabolic syndrome. Screening programs in older or overweight patients are important to help the understanding and detection of individuals with low serum T levels to supplement adequately. It should be considered the importance of a specific therapy for the treatment of metabolic syndrome, which contemplates all the components of the syndrome, the stage of life and the hormonal profile as key aspects for an assertive clinical intervention. Finally, changes in lifestyle and diet seem to be effective preventive approaches to avoid the ectopic accumulation of fat in the liver in men with a diagnosis of testosterone deficiency. Regarding the original article, OVX rats, under our experimental condition, exhibit features of metabolically healthy adiposity, since the rats did not exhibit dyslipidemia and signs of insulin resistance. Retroperitoneal fat hypertrophy and inguinal fat hyperplasia in OVX probably represent an adaptation of adipose tissues to store larger amounts of lipids and, thus, to avoid ectopic lipid deposition. Long-term administration of melatonin was effective in preventing the development of hepatic steatosis and oxidative stress in OVX rats, and although it did not reduce increased adiposity, melatonin was able to alter lipid distribution and adipocytes morphology in different fat depots. The suppression of retroperitoneal fat hypertrophy and preservation of inguinal fat hyperplasia suggested that melatonin induced a redistribution of lipid in different fat depots, reducing excessive lipid overload and, thus, eliminating the possibility of cells progressing to inflammation and related abnormalities. In normoestrogenic rats, melatonin reduced body weight gain, reduced inguinal fat adipocyte size, reduced food intake, and increased fatty acid hepatic oxidation. Melatonin also reduced plasma estradiol levels in control rats. It can be concluded that melatonin eliminates the risk to develop estrogen deficiency-related metabolic disorders, mainly by altering the lipid distribution in fat depots and suppressing tissues oxidative stress. Under normal estrogen condition, melatonin may induce adverse effects, including body weight loss and disturbance in estrogen-regulated functions. Because melatonin has been proposed as a pharmacological agent in the treatment of many diseases, its function in premenopausal women should be regarded with care.

LIST OF ABBREVIATIONS

ACC-1	Acetyl-CoA carboxilase
ACACA	Acetyl-CoA carboxylase alpha
AMS	Aging male symptoms
AACE	American Association of Clinical Endocrinologists
AHA	American Heart Association
AR	Androgen receptor
BI	Blood insulin
BP	Blood pressure
BFP	Body fat percentage
BMI	Body mass index
BW	Body weight
CVD	Cardiovascular diseases
CPT-1	Carnitine palmitoyltransferase I
CAT	Catalase
CNS	Central nervous system
CAC	Citric acid cycle
CP	Crude protein
DHT	Dihydrotestosterone
DM	Dry matter
ED	Erectile dysfunction
E2	Estradiol
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERT	Estrogen replacement therapy
HRT	Estrogen/progestogen replacement therapy
EE	Ether extract
EGIR	European Group for the Study of Insulin Resistance
FGB	Fasting blood glucose levels
FAS	Fatty acid synthase
FSH	Follicle stimulating hormone
FT	Free testosterone

HbA1C	Glucated hemoglobin
G6PD	Glucose-6-phosphate dehydrogenase
GSH-Px	Glutathione peroxidase
GSSG-red	Glutathione reductase
HFD	High fat diet
GnRH	Hormone gonadotropin releaser
HRP	Hormone replacement therapy
SHBG	Hormone sex binding globulin
IR	Insulin resistance
IDF	International Diabetes Federation
LOH	Late-onset hypogonadism
LH	Luteinizing hormone
MDA	Malondialdeyde
MetS	Metabolic syndrome
MTP	Microssomal TG transfer protein
MM	Mineral matter
UCP-2	Mitochondrial uncoupling protein 2
NCEP	National Cholesterol Education Program's Adult
CONCEA	National Council for Control of Animal Experimentation
NAFLD	Non-alcoholic fatty liver disease
NAFLD	Non-alcoholic steatohepatitis
PPAR-α	Peroxissome proliferator-activated receptor alpha
SHBG	Sex hormone binding globulin
NASH	Steatohepatitis
SREBP	Sterol regulatory element-binding protein
SAT	Subcutaneous white adipose tissue
SOD	Superoxide dismutase
Tfm	Testicular feminized
T	Testosterone
TDS	Testosterone deficiency syndrome
TRT	Testosterone replacement therapy
TU	Testosterone undecanoate
TBARS	Thiobarbituric acid reactive species

T2DM	Type 2 diabetes mellitus
VAT	Visceral white adipose tissue
WC	Waist circumference
WHR	Waist hip-ratio
WAT	White adipose tissue
WHO	World Health Organization

Systematic review of metabolic syndrome in testosterone deficiency: relevance of studies in humans and rodent models

Danielle Aparecida Munhos Hermoso[1], Eduardo Hideo Gilglioni[1], Paulo Francisco Veiga Bizerra[1], Márcio Sigueaki Mito[1], Rodrigo Polimeni Constantin[1], Emy Luiza Ishii-Iwamoto[1].

[1]Universidade Estadual de Maringá (UEM), Maringá, PR, Brazil.

Abstract

Steroids hormones regulate carbohydrate, fat, and protein metabolism. Testosterone is the predominant steroid in men and the lack of it can be a trigger to the development of metabolic syndrome. In this review, we investigate the relationship between testosterone deficiency, hepatic steatosis and metabolic syndrome by a systematic review of the literature. Thirty-nine articles were included and most of them show that low testosterone levels are positively associated with disturbs on glucose homeostasis, lower insulin sensitivity and dyslipidaemia. The fat distribution and body composition are also frequently modified under low testosterone condition. In both men with hypoandrogenism and castrated rats, the low testosterone levels lead to higher accumulation of visceral fat, while increase in subcutaneous fat is reported by some studies with castrated rats. Despite some contradictions, the overall association between the components of the metabolic syndrome with low testosterone levels is a common point between the studies. The hepatic steatosis was evaluated by some authors by ultrasonography and some found association with hypogonadism. Many studies with rodents have associated the castration with high fat diet to study metabolic syndrome

components. When the effects of testosterone replacement therapy was evaluated, the results showed that it can reverse some of the clinical signs of metabolic syndrome, by mechanisms that includes the reduction in visceral fat and improving insulin sensitivity, which emphasizes the importance of the testosterone for the homeostasis of the energy metabolism in men.

Keywords: Andropause, metabolic syndrome, hyperglycaemia, obesity, dyslipidemia, insulin resistance, hepatic steatosis.

Introduction

Even though human life expectancy has increased in recent centuries, there are still health complications in older population, many related to age-decline in gonadal hormones. In women, the production of sex steroid hormones by the ovaries stops abruptly at approximately 50 years of age. Consequently, the serum estradiol (E2) level decreases rapidly, menstruation ceases, defining menopause. Menopause are usually associated with climacteric symptoms such as hot flushes, vaginal dryness, decreased libido and mood swings [1]. In contrast to women, reproductive function remains relatively intact in older men. Some of them, however, have symptoms similar to those of climacteric women including hot flushes, decreased libido, erectile dysfunction, tiredness and decreased vigour [2]. As a slight decrease in testosterone levels may be responsible for some of these symptoms, the term andropause has been suggested for men. However, there is no precise definition for this term [3]. Not all elderly men have testosterone deficiency and, thus, the analogy between andropause and menopause may belong to clinical manifestations, but not the nature of the hormonal changes [4]. Therefore, the widespread use of testosterone, in analogy to estrogen/progestogen replacement therapy (HRT) or estrogen replacement therapy (ERT) in menopausal and postmenopausal women, is questioned [3].

The main circulating androgen in men is testosterone (T). Testosterone is produced in the testicular Leydig cells and is stimulated by the pituitary secretion of luteinizing hormone (LH). In serum, testosterone is bound to albumin (50%), sex hormone binding globulin (SHBG) (44%) and cortisol-binding globulin (4%). Unbounded or free testosterone (FT) represents 2% of total T [5]. In target cells, testosterone is converted to most androgen bioactive dihydrotestosterone (DHT) via 5α-

reductase, both of which bind to the androgen receptor (AR). Testosterone can also be converted to E2 by aromatase, which subsequently activate estrogen receptors ERα and ERβ [6]. The concentration of T is highest in the third decade of life (320 ng/dL or 11 nmol/L) and tends to decline with advancing age by approximately 0.4 to 1% per year [7-9] resulting in a 20 to 30% decrease from 20 to 80 years [10]. In a group of males aged 40 to 79 years, 17% had a total testosterone level below the reference level for young men, 11 nmol/L [11].

The diagnosis of male "andropause" requires the presence of not only low serum T levels, but also clinical symptoms, a condition defined as hypogonadism or late-onset hypogonadism (LOH). Due to popularization of the term andropause, in this review andropause was used as a synonym of hypogonadism or late-onset hypogonadism (LOH). Wu et al. define hypogonadism as T serum levels below 11 nmol/L (320 ng/dL), a FT below 220 pmol/L (640 pg/dL) and three sexual symptoms [11]. Based on these criteria, the overall prevalence of LOH in population was demonstrated to be 2.1% and increase with age from 0.1% for men aged 40 to 49, to 0.6% for those aged 50 to 59, to 3.2% for those aged 60 to 69, and 5.1% for those 70 to 79 years old [11]. The decrease in testosterone levels in LOH syndrome has been suggested to be a decline in Leydig testicular cells and pituitary-hypothalamic axis functions, an increase in hepatic production of SHBG, with consequent lower free testosterone, and also the presence of visceral fat [12].

Several lifestyle factors have been shown to alter testosterone levels, including alcohol consumption [13], vigorous physical activity [14] and body adiposity. The inverse relation between testosterone and body mass index has suggested to be due to inhibition of hepatic SHBG synthesis induced by hyperinsulinemia [15,16] or to a higher conversion to estrogen by aromatase in adipose tissue [15]. An association of many age-related

diseases with low plasma testosterone has been demonstrated, such as heart disease, obesity, metabolic syndrome (MetS) and type 2 diabetes, sarcopenia, osteoporosis, anemia, mood and cognitive disorders [15,16,17,18]. It seems apparent a close association between low testosterone level and components of MetS [19]. It is unclear, however, whether partial androgen deficiency is the main cause of most of this age-related decline in physiological functioning.

The MetS includes a range of cardiometabolic risk factors consisting of an accumulation of visceral adipose tissue, dyslipidaemia, insulin resistance and/or impaired glucose regulation, non-alcoholic fatty liver disease and hypertension [20-21]. The World health organization (WHO) defined MetS by the presence of insulin resistance and two other factors: triglycerides equal to or greater than 150 mg/dL; HDL cholesterol less than 39 mg/dL in men and less than 35 mg/dL in women; Elevated blood pressure (BP) (≥140/90 mmHg or under drug treatment); diabetes, glucose intolerance [22]. Several guidelines to define MetS have been proposed over the years, such as National Cholesterol Education Program's Adult (NCEP), WHO, International Diabetes Federation (IDF), American Association of Clinical Endocrinologists (AACE) and the European Group for the Study of Insulin Resistance (EGIR) [23]. The criteria according to several entities guidelines to define MetS are summarized in Suplemmentary Table 1. Having three or more of the following characteristics: fasting blood glucose levels (FGB) ≥ 100 mg/dL, serum triglyceride (TG) ≥ 150 mg/dL, serum high density cholesterol (HDL) < 40 mg/dL, hypertension or blood pressure (BP) ≥ 130/85 mmHg and waist girth ≥ 102 cm, is defined as Mets according to the American Heart Association (AHA) [24]. The IDF recognizes that central obesity and insulin resistance are important causal factors for the development of MetS, although its pathogenesis and the involvement of each of its components are still not well understood. According to this criterion, central obesity

is a prerequisite for the diagnosis of the metabolic syndrome and is classified as a waist circumference of at least 94 cm for men and equal to or greater than 80 cm for women, both European, with values according to the ethnic groups. In addition to central obesity, two of the following four factors must be present: triglycerides equal to or greater than 150 mg/dL (or in specific treatment for this lipid abnormality); HDL cholesterol less than 40 mg/dL in men and less than 50 mg/dL in women (or in treatment specific for this lipid abnormality); Elevated BP (≥130/85 mmHg or in drug treatment); (≥100 mg/dL or diagnosis of type 2 diabetes) [25].

Despite the slight differences on their guidelines, the entities agree that MetS has been increasing worldwide, and the higher prevalence is in the elderly population [26,27]. Specifically this syndrome is more present in people over 50 years of age, in different populations, [28,29] in the United States the prevalence of MetS is 24-42% in this range, while, one of the highest worldwide "Iran" the prevalence was 30% in Tehran and 45% in Khorasan province [30]. Present in about 25% of the world population, people with MetS are twice as likely to die, accounts for 7% of global mortality and 17% of cardiovascular diseases-related (CVD-related) deaths [31,32].

In general, central obesity and the glucose intolerance are important sings of metabolic disturbances that integrates MetS. The increased adipose tissue mass, the main characteristic of obesity, is a fact commonly reported in studies evaluating men with hypogonadism, which showed significant differences in weight, body mass index (BMI), waist circumference (WC) in subjects with low levels of T [33-38] and the insulin resistance is also a common observation in studies investigating metabolic alterations related to low T levels in men [33,34,36,38,39]. In experimental models, testosterone deficiency causes metabolic changes in young and adult animals. Besides changes in body weight, adiposity index and insulin resistance, the low T levels affect also other metabolic

organs [40-42]. In the case of the liver, in young mice, the surgical induction of T deficiency favoured hepatic steatosis induced by a hyperlipidic diet [41,43]. In adult rats, castration induced increased TG content in the liver [43,44]. Despite of these studies, some authors emphasize that parameters such as body weight, food intake, insulin resistance and fasting glycemia in animal models of T deficiency, mainly caused by gonadectomy, are still controversial and require more investigations and very careful extrapolation of the results found in rodents to humans [42].

The main hypothesis for the mechanism that link low levels of T with obesity are shown in Figure 1. The low T condition can be a consequence of a direct inhibition of T production or the decrease of gonadotropins. The hormone Gonadotropin releaser (GnRH) stimulates a pituitary to release the hormone luteinizing hormone (LH), which stimulates Leyding cells to produce T, and follicle stimulating hormone (FSH). Obesity and increased visceral fat can accentuate the condition of low T levels due to the increased activity of the enzyme aromatase, which converts T into estradiol, and due to the release of proinflammatory cytokines. Estradiol, for its part, acts by negative feedback on the pituitary and hypothalamus, increasing the secretion of leptin. The leptin has inhibitory effects on the testicles, reducing even more T production [45,46]. The higher prevalence of metabolic complications [47] in the older men [48] highlights the importance of conducting studies that seek to improve the health aspects of men in this life period.

In this review, we explore the literature about the MetS on the context of T deficiency and the hypogonadism syndrome aiming to better understand their relationship and summarize the new findings to help provide a complete base for more assertive therapeutic interventions. Particular emphasis was given to the hepatic manifestation of the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) [49] which is defined as excessive liver lipid accumulation (steatosis) in the absence of significant alcohol

consumption. This is a component of MetS that is oftentimes neglected by researchers and clinicians as NAFLD may remain a benign condition, qualified only as reversible steatosis [50]. However, the steatotic liver may become vulnerable to secondary insults, contributing to the progression of NAFLD to more severe liver disease, leading to an infiltrative inflammatory process known as NASH (non-alcoholic steatohepatitis), with the development of hepatic fibrosis that may progressively progress to cirrhosis and even hepatocellular carcinoma [51,52].

Literature search

A systematic search of the PubMed database was performed including the following words: “androgen deficiency aging men”, “androgen deficiency and MetS”, “hypogonadism (LOH), androgen deficiency and Mets”, “hypogonadism (LOH) and MetS”, “inflammation and androgen deficiency”, “testosterone deficiency and hepatic steatosis”, “testosterone and NAFLD” “testosterone deficiency and NAFLD”, “testosterone deficiency and dyslipidaemia”, “testosterone deficiency and obesity”, “testosterone deficiency and type 2 diabetes”, “testosterone deficiency and hyperglycaemia”, “testosterone deficiency and hypertension” and “testosterone deficiency and insulin resistance”. All the studies investigating the relationship between testosterone deficiency and the components of MetS were considered and the identification of the relevant studies was performed by two of the authors.

Study selection

We included all originals studies that evaluated MetS, no limit about year of publication has been set, searches started in July 2018 and the final search is updated to June 2019. Only articles in English and Portuguese were included in the analysis and excluded review articles. An initial evaluation was carried out, based on the titles and the summary of the articles. Articles showing the relationship between testosterone deficiency and MS components were selected, the full text was analysed secondarily. Following this procedure, we found 2932 publications and, after applying the selection criteria, and excluded 40 review articles, the total number of relevant publications was reduced to 38. In figure 2 are shown the steps for the search in PubMed data base.

Findings from human studies

Out of 2932 retrieved articles, 39 were included in the study. The characteristics of the retrieved trials and type of outcomes considered are reported in Tables 1 and 2. Over the time, many research groups have tried to better understand the possible relationship between T deficiency and metabolic disturbances in diverse populations of men, of different age, in different regions of the world. Most of these studies with humans focused on metabolic changes that are detectable by easily measurable anthropometric dimensions and biochemical blood tests. Considering that erectile dysfunction can predict T deficiency, many of the studies also investigated the association with sexual symptoms trough specific surveys.

In Australia, a longitudinal study of aging conducted over 8 years with 195 men aged 70 years and over, Chen et al. [33] aimed to investigate the relationship between metabolic status, obesity and the plasma T levels. The percentage of subjects diagnosed with MetS, according to the NCEP (ATPIII) criteria, with the cutoff point for WC set as >102cm, was 17.9%. Among these subjects, 43% had type 2 diabetes mellitus (T2DM) at the beginning of the study and this number raised to 57% at the end their results indicated the risk of incident T2DM in men with MetS is 11.67 times higher when compared to men without MetS and indicated an association between low total T levels and less favorable metabolic profile (higher TG, BMI, WC) particularly in men with age over 70 years [33]. The WC was considered a better indicator of central obesity than BMI and was related to the risk of T2DM and cardiovascular diseases (CVD). These relationships were not observed in men with T2DM and the difference was attributed to the fact that diabetes *per se* prevails all other factors. The logistic regression analysis revealed that FGB is the only factor that independently can predict the risk of incident

diabetes [33]. Even though the metabolic profile of men with relative T deficiency was less favorable, the subjects with T2DM had similar levels of total T to those who were healthy. The authors indicate that T deficiency is a consequence rather than the cause of poor metabolic status and WC was significantly related to variations of total T, indicating the link between the hypogonadism and visceral adiposity, a feature commonly evidenced by other later studies. The authors conclude that even though total T could not be a large predictive to the future development to T2DM, it is certainly linked to poor metabolic status [33].

In another population of men aged 70 years and over, a cross-sectional analysis made by Tang et al. [53] evaluated in Asian men in a veterans´ nursing home the relationship between MetS and T levels by measuring serum free and total T levels. The authors also analysed the correlation of T deficiency with clinical characteristics, metabolic disturbs and chronic diseases. According to the criteria of NECP (ATPIII), 26% of the subjects were classified with MetS carried out with a modification of WC for Asians (>90 cm). The elevated percentage of MetS was attributed to the high incidence of hypertension and hyperglycemia, more than obesity, among that population of elderly men. The total T levels were inversely correlated with the MetS and among the subjects with low total T (0.2-3.5 ng/mL) the percentage of MetS was 35.3%, against 14.4% in the group with high total T levels (over 5 ng/mL). The same correlation failed to be reproduced with the results for free T levels, which were calculated according to Nanjee-Wheeler´s equation, based on the measured levels of total T and Hormone Sex Binding Globulin (SHBG). The authors showed by correlation analysis that the lower T levels were associated to markers of central obesity (BMI, waist hip-ratio (WHR), body fat percentage (BFP)), insulin resistance (FBG, blood insulin (BI), glucated hemoglobin (HbA1C)) and risk for cardiovascular disease (TG and low HDL-c), but multiple linear

regression only showed significantly independent association with serum total T for the variables BMI and FBG. The authors conclude that total T is a better predictor than free T to estimate the risk of the development of MetS in elderly men.

Interestingly, in the population-based observational cohort study of Kupelian et al. [34] 950 randomly selected men aging from 40 to 70 years and they were observed at three time points over a period of 15 years. The study aimed to determine the association between T deficiency and the subsequent development of MetS. It was found that total T levels, as well as SHGB levels are inversely associated with the risk of MetS and this association was dependent on BMI, since stronger association was observed for men with BMI below 25 mg/Kg^2. The authors suggested that elevated adiposity is a dominant risk factor for developing MetS, although non-obese men with low SHGB or low T had 2-to 4-fold increased risk of developing MetS. This study also found that age at baseline was not associated with the incidence of MetS, while a worse self-report health was a good predictive of MetS development.

In Germany, Haring et al. [54] performed a populational-based prospective cohort study with 1004 adults men aged from 20 to 79 years over a follow-up time of 5 years. The aim was to investigate the association between serum T and dehydroepiandrosterone sulphate (an adrenal androgen) levels with the components of the MetS, by assessing sociodemographic and behavioral characteristics by computer-assisted personal interviews and measurements of biometrical and biochemical parameters. In agreement with the previously mentioned studies, these authors found that 47.8% of the men included in the study developed MetS and low T was predictive for MetS, even after adjustment for sociodemographic and behavioral variables, especially among young men aged 20-39 years. On the other hand, the dehydroepiandrosterone sulphate was not related with the prediction of MetS in this study.

In the study of Rotter et al. [55] the dehydroepiandrosterone sulfate showed a negative correlation only with WC among all measured variables. The authors investigated which indicator of accumulation of adipose tissue correlated with low T levels in 455 Poland men aged between 50 and 75 years (mean age 62.83 ± 6.57) and found that 40% of the subjects had T deficiency. Different from dehydroepiandrosterone sulfate, all biometrical and biochemical studied parameters correlated negatively with total T levels with special emphasis to the visceral adiposity index, which was the strongest predictor of T deficiency among these population of non-diabetic aging men.

This relevance of visceral adiposity was also evidenced by a cross-sectional study of Blaya et al. [36] with 143 men older than 40 years in Brazil. The authors investigated the correlation between total T levels and the components of the MetS also in non-diabetic subjects and the analysis have shown that men with low total T has more prevalence of MetS components than men with normal total T levels. The WC, indicator of visceral adiposity, was the individual component that showed strong correlation with low T levels, indicating the complex net linking androgens, abdominal obesity and MetS.

Aiming to better understand the association between hypogonadism and MetS or insulin resistance, Katabami et al. [56] made a cross-sectional survey with 274 Japanese adult males with mean age of 49.0 ± 11 years. They found that 25.5% of the subjects had MetS, among which significant decreased free T levels were detected. The authors concluded that among Japanese males free T levels were associated with MetS, independently of other variables, including age, BMI and WC. The higher incidence of low T levels among the subjects with MetS was partially attributed to a vicious cycle in which serum T levels may be further reduced by MetS and insulin resistance via primary hypogonadism and/or hypogonadotrophic hypogonadism.

An epidemiological study by Jiann et al. [57] focused on the links between T levels and lipid profiles by analyzing blood samples from a total of 856 Taiwanese men with mean age of 40 years. The authors pointed out that T has beneficial effects on cardiovascular system due to its effect on postprandial TGs and HDL-c, despite of its effect increasing LDL-c, independently of other factors investigated, as age, BMI, SHBG, hypertension and T2DM.

Ho et al. [58] made a cross sectional study in Taiwan with men diagnosed with T2DM, including 105 men aged 61.2 ± 6.8 years that were newly diagnosed and 81 men aged 57.8 ± 8.8 years that already had T2DM diagnose for more than 24 months to investigate the prevalence and the risk factors of T deficiency and found that MetS (ATPIII criteria for Asian men), obesity, hypertriglycemia and other risk factors were common among individuals with newly diagnosed or previously known T2DM.

Ugwu et al. [59] made a cross-sectional survey and performed clinical and biochemical analysis in 200 men with age between 32 and 69 years in Nigeria to determine the prevalence the hypogonadism in men with T2DM and its clinical manifestations. They have found that 29.5% of the subjects had hypogonadism, accompanied by clinical manifestations like erectile dysfunction (ED). Older age and higher WC were predictors for the hypogonadism, while LH and FSH levels were significantly lower in the subjects with hypogonadism, which directly relates to the dysfunction of the pituitary gland, the hypothalamus, or both, leading to low gonadotropin secretion and hypogonadism-associated T2DM.

In a cross-sectional study made by Agarwal et al. [60] with 900 Indian males with T2DM aged between 30 to 59 years the prevalence of hypogonadism was investigated by surveys and biochemical measurements. In 20.7% of the individuals had hypogonadism and the prevalence was higher among older subjects (50-59 years old), with significant

correlation between age, IMC and hypogonadism. The authors conclude that the diagnosis for hypogonadism and the supplementation accordingly is crucial for adequate management of diabetes.

In the retrospective cross-sectional study of Souteiro et al. [38] with 150 obese men with mean age of 4.65 ±10.9 years the impact of hyperglycemia, insulin resistance and SHBG on the T levels were investigated. The T deficiency was found in 52% of the subjects when total T was considered and when free T was the criteria the T deficiency was found in only 17.6% of the subjects. This variation was attributed to the low SHBG levels in obese patients as shown by the fact that SHBG levels were negatively correlated with IMC. Total T levels were also negatively correlated with IMC, weight, WC, HOMA-IR, TG and SHBG levels. The insulin resistance was a main determinant of T deficiency and SHBG levels were associated with free T levels independently of insulin resistance.

Regarding the sexual symptoms, Tan et al. [61] made a cross sectional community-based study with 1046 multi-ethnic Asian men aged 40 years and above. By interviewing the subjects and measuring biochemical parameters, the authors aimed to examine the complex association between erectile disfunction, T deficiency syndrome and MetS. The study showed that 31% of the subjects had MetS and the prevalence of moderate to severe ED was significantly higher among them. These findings lead the authors to conclude that there is a strong association between ED, T deficiency and MetS. Besides the implications of the ethnicity on the risk to develop these conditions, most of the components of the MetS were independently associated with T deficiency and some components such as high blood pressure and elevated fasting plasma glucose were found to be good predictors for ED.

A longitudinal study conducted over 5 years with 167 men aged from 42 to 78 years with abdominal obesity in Slovakia by Fillo et al. [62] assessed the incidence of

ED, T deficiency, MetS and the prevalence of morbidity at different levels of circulating T. The authors collected anthropometric, hormonal and urological parameters and the MetS was found in 61.7% of the subjects according to Berlin modification of NCEP-ATP III criteria, while 73.1% had ED and 46.7% had T deficiency syndrome. There was a significantly higher prevalence of T2DM, hypertension, elevated HDL-c and TG levels among the subjects with T levels below 10 nmol/l when compared with those with T levels over 14 nmol/l, suggesting that the level of 14 nmol/l is the minimum required for proper metabolism in men. Among the comorbidities investigated, the three major risk factors for the development of MetS and cardiovascular disease were elevated TG, low HDL-c and obesity.

In a cross-sectional study with men aged 40-79 years Tajar et al. [63] investigated the association between T deficiency and sexual symptoms with pertinent metabolic and physiological alterations. Among the 2966 subjects included in the study 2.1% had T deficiency associated with sexual symptoms. These subjects were among the oldest ones and had the highest BMI, WC, TG and HOMA-IR levels and lowest HDL-c when compared with the eugonadal population. A considerable part of the of men with LOH also had T2DM, cardiovascular disease and MetS. The comparison between the subjects with low T levels with or without the sexual symptoms revealled the importance of the sexual symptoms for the LOH and its relations with metabolic alterations that favors the development of the MetS.

In a multicenter cross-sectional study conducted in Spain by García-Cruz et al. [64] with 999 men (61.2 ± 8.1 years old), anthropometric and biochemical measurements allowed to investigate the association between the presence and the severity of T deficiency symptoms and the MetS. The prevalence of MetS was 69.6% and even higher (75.3%) among the subjects with moderate and severe aging male symptoms (AMS). The

ED was found in 97.4% of the subjects and about half defined it as moderate or severe, while the central obesity was found in 35.5% of the subjects. The AMS were reported by 94.8% of the men, who mainly referred to sexual symptoms. The authors revealed that even though the three factors are strongly associated with the higher likelihood of MetS, after adjusting for the severity of AMS, the central obesity was defined as the main determinant risk factor for MetS, more predictive than severe and moderate ED.

Testosterone replacement therapy (TRT)

In a study done by Saad et al. [65] with 21 subjects aged 54-76 years with low T levels and the diagnose of MetS the authors observed that men with sexual disfunction often suffer from MetS. The authors explored the effects of 12 months administration of testosterone undecanoate (TU) over metabolic parameters and found that despite of the modest decline on body weight, there was positive effects over WC, blood pressure, plasma cholesterol and TGs. Interestingly, the levels of SHBG felt over the first six months of the treatment and raised after, when the hyperinsulinemia was reduced, supporting the SHBG levels as indicator of the degree of hyperinsulinism and of the severity of the MetS. All the effects observed in this study were attributed to the TU treatment and the subjects had no changes on diet or exercise routine.

Some studies have also provided important evidences by investigating the effects of T replacement therapy (TRT). Jeong et al. [35] performed an investigation on the effect of TRT in 200 patients who were diagnosed with testosterone deficiency syndrome (TDS) and were undergoing TRT between August 2006 and August 2009. In this research, 71 patients were diagnosed with MetS and 129 without Mets. The criteria for identified the MetS in these men was NCEP III criteria for Asians. TRT promotes beneficial effects over WC and fasting blood glucose for both groups of patients, while reduction on total

cholesterol and LDL-c were significantly reduced only in the group without MetS, despite of a trend of reduction in the other group as well. The authors conclude that TRT in patients without MetS can improve fewer more serum variables than TRT in patients with MetS, suggesting that the correction of complications as diabetes, obesity and hypertension might be important during TRT.

The study of Singh et al. [66] investigated the blood pressure, anthropometrical, biochemical and hormonal parameters in Indian men. 32 health males with mean age of 34 ± 6.7 years as controls and 63 patients with MetS with mean age of 35.2 ± 8.1 years (according to the classifications of the IDF 2005 for south Asian Indian ethnicity) were included in the study. The authors found that in the patients with MetS parameters as IMC, WC, blood pressure, fasting plasma glucose, insulinemia, HOMA-IR were significantly higher in comparison with the subjects without MetS. Only HDL-c was significantly lower in the subjects with MetS in comparison with those without. Besides, the levels of serum T, free T, bioavailable T and SHGB were all significantly lower in the subjects with MetS. The multiple regression analysis revealed that free T strongly and directly correlated with T levels and inversely correlated with age, HOMA-IR and SHBG levels. The authors also investigated the effects of TRT with oral Testosterone 40 mg twice a day for 3 months on the patients with MetS. For this purpose, the 63 subjects with MetS were subdivided in eugonadal (n=44, 70%) and hypogonadal (n=19, 30%) groups. The comparison of the anthropometrical and hormonal parameters between these groups revealed that besides T, there was differences among the groups for insulin, HOMA-IR and SHBG, which were higher in the eugonadal group. TU was administered for 13 of the subjects in the hypogonadal group twice a day for 3 months and led to beneficial effects, including significant reduction on WC, blood pressure, HOMA-IR, SHBG and TG levels and the authors conclude that restoring T levels to normal by hormonal

replacement can ameliorate some of the alterations that favors insulin resistance with the potential to reverse MetS, especially in hypogonadal males.

Another study that reported beneficial effects of TRT on the components of the MetS was performed by Traish et al. [67] with 255 men with mean age of 58 ± 6.3 years who sought urological consultation due to symptoms of T deficiency. Only 11 patients did not fulfill 3 or more MetS criteria ('reconciled' definition by IDF and AHA). All 255 subjects were followed up for at least 1 year and 116 for 5 years. In this study, the authors found that TRT was able to increase HDL-c, while reduced total and LDL-c, TG, blood glucose, blood pressure, lowering the risk of cardiovascular diseases. Interestingly, the reduction of the TG (-69%) was remarkable on the first year of the treatment and remained low after 60 months. This finding is in accordance with the reports that TRT leads to reduction in body weight and WC as the visceral fat storage is determined by the TG availability for accumulation. Also, the TRT led to a sustained reduction on blood glucose, probably due to improved insulin sensitivity and glucose utilization. The authors concluded suggesting that long-term treatment of T produces important clinical benefits and ameliorates MetS. On the other hand, in a randomized-controlled trial Huang et al. [68] studied the effect of TRT on subjects that were chronically using opioid for pain and who had a morning total T lower than 12nmol/l, characterizing the opioid-induced hypogonadism. In these subjects, the TRT was not associated with worsening or improvements of metabolic and inflammatory markers with the 14 weeks therapy.

Groti et al. [39] focused on the effects of TRT on glycemic control and components of MetS, including IR, visceral obesity in obese hypogonadal men with T2DM. They performed a one-year, double-blind, randomized, placebo-controlled clinical study with a total of 55 men (60 ± 7.23 years) who had hypogonadism (total T below 11nmol/l associated with 3 or more sexual symptoms) and IMC above 30 Kg/m^2

and who were treated with oral antidiabetic medication. Surprisingly, both placebo and treated groups showed significant reduction on body weight and WC, which the authors attributed to a possible general positive effect on lifestyle by the subjects upon the participation on the study. The group that received T showed clinical benefits including improved glycemic control, insulin resistance and endothelial function after one year of TRT.

Besides T replacement, other compounds have been tested as alternatives to treat metabolic disturbances, as the aromatase inhibitor (anastrozole) investigated by Dias et al. [69] in a randomized double-blind, placebo-controlled, parallel-group, proof-of-concept trial with 29 male with low total T levels (< 350ng/dL) older than 65 years (mean 71 ± 3 years). The variables were assed at baseline and after 12 months of treatment. The results showed that while the therapy with T reduced subcutaneous fat, the treatment with aromatase inhibitor reduced subcutaneous and visceral fat compared with placebo, but neither T nor aromatase inhibitor were able to affect fasting glucose and insulin levels after 12 months of treatment. The authors conclude that more trials are required before the aromatase inhibitor is considered as a treatment option for low T levels in older men.

Studies that investigated the influence of T levels on NAFLD in humans

The NAFLD is considered as the hepatic component of the metabolic syndrome. The studies that have focused on the NAFLD in the context of T deficiency in humans were mainly focused on indirect measurements of the liver lipid content to diagnose NAFLD.

The transversal study of Barbonetti et al. [70] was done with 55 Italian male patients, aged 46.6 ± 17.3 years, with chronic spinal cord injury. A total of 49.1% of the

patients had NAFLD, diagnosed by ultrasonography. The patients with NAFLD were older and exhibited higher BMI, insulin, HOMA-IR, TG and gamma-glutamyl transpeptidase and lower levels of total and free T when compared with the patients without fatty liver. Logistical regression analysis have indicated that NAFLD was independently associated with low and free T levels and the authors suggested a direct link between T levels and NAFLD, which persisted even after statistical adjustment for the effect of BMI, insulin resistance and inflammatory status, indicating a direct link between T deficiency and liver fat deposition. A limitation of this work was that although the method for identifying the fat infiltration in the liver the non-invasive, the ultrasonography is not the gold standard for detecting hepatic steatosis [70].

In the study by Hasanain et al. [71] the NAFLD was also diagnosed through ultrasonography, associated with no history of alcohol consumption, exposure to steatogenic medications and no evidence of viral hepatitis B or C. This study was conducted at the Assiut University Hospital in Egypt with 192 male patients with diagnose for NAFLD and the aim was to investigate the association between ED and NAFLD. Low serum T was a predictor to the development of ED, which was found in 45.8% of the patients. The authors pointed the small sample size and the lack of estradiol level estimation to be considered as a draw-back as the main limitations of this study.

Seo et al. [72] also used the ultrasonography to analyse NAFLD and investigate its relationship with T levels. The results revealed that low levels of T is found in patients with NAFLD in cross-sectional analyses, but the baseline level of T did not independently influence the development or regression of fatty liver at the median 4.2 years follow-up. In Korea, ultrasonography associated with computed tomography was also used to analyse the NAFLD in the retrospective observational cross-sectional study of Kim et al. [73]. The study included 495 Korean men who were at least 20 years of age and aimed to

investigate the association between serum total T levels and the NAFLD. After adjusting for the influence of visceral adiposity and insulin resistance, the results showed an elevated risk for NAFLD in men in the low serum T than men with the highest serum T.

Evidences found in studies with rodents

The studies with rodents have provided important insights on the molecular mechanisms related to the changes observed in humans with T deficiency and it also allow to better differentiate between the direct effects of the lack of T and those related with other factors like age and diet. Nevertheless, many of the studies with castrated animals have associated the T deficiency with other challenges, mainly the high fat diet (HFD).

Sprague-Dawley rats with 16 weeks of age and fed either a standard or a high energy diet-induced obesity Christoffersen et al. [42] have shown that the castration affects many biometrical and metabolic parameters. By using computed tomography, the authors demonstrated that subcutaneous fat area increased in the castrated rats but remained unchanged in the sham-operated rats. The total abdominal and the visceral fat areas, on the other hand, were not affected by the castration and increased equally in sham-operated and castrated rats after nine weeks after the surgical procedure. The fasting glucose was also higher in castrated rats, but other biometrical and metabolic parameters were not affected by the castration. Attempting to relate this findings to observations in studies with humans, the authors discuss that if T deficiency or the castration had the same metabolic effects in rats as in humans one would expect increases in visceral fat mass, decreased glucose tolerance and insulin sensitivity, among other typical features of human MetS and the authors conclude that rats are not the ideal model to study the influence of T deficiency on body fat distribution and other components of MetS.

Xia et al. [40] used advanced-age castrated Sprague-Dawley rats to investigate if T deficiency affects metabolism, body weight and glucose kinetic metabolism. The body weight decreased after castration, probably due to the loss of muscle and bone mass. The authors also found that ten weeks after castration the blood glucose and insulin levels were higher in T deficient animals, which also enhanced hepatic gluconeogenesis and decreased extra-hepatic insulin sensitivity.

Dubois et al. [41] used high fat diet and T deficiency models to investigate the combined effects on glucose homeostasis in C57BL/6J male mice. The authors used two models of androgen deficiency, orchidectomy and androgen receptor knockout mice. The findings showed increased adiposity and serum leptin in both models of T deficient mice. The fat accumulation in the orchidectomy accompanied by sedentary behavior, which also showed white adipocyte hypertrophy, decreased mitochondrial content but not function increased lypogenesis and decreases lypolisis. Both models also exacerbated high fat diet induced glucose intolerance impairing hepatic and extra-hepatic insulin sensitivity. The authors concluded that T deficiency in combination with high fat diet, exacerbated adiposity insulin resistance and T, but not dihydrotestosterone supplementation, restored the castration effects on body composition and glucose homeostasis. Despite the fact that T deficiency per se had no deleterious effects on metabolic parameters, it was shown the metabolic alterations induced by high fat diet was exacerbated. When translated to humans these findings reinforce that untreated T deficiency may accelerate the development of MetS in men, although this may be avoided with a healthy diet.

The relation between the higher susceptibly of T deficient animals to the metabolic effects of HFD Harada et al. [74] have shown that castration leads to abdominal obesity in high-fat diet-fed C57BL/6J mice, but not when the animals were treated with

antibiotics that disrupt the intestinal microflora. The castration induced an increase of visceral fat mass only in the absence of antibiotics in HFD-fed mice, whereas subcutaneous fat mass was increased by castration irrespective of antibiotics. The finding that elevated fasting blood glucose levels and increased liver TG levels are only seen in castrated animals fed a HFD lead to the conclusion that T deficiency can alter the intestinal microbiome and induce abdominal obesity, but it depends on the diet.

Borbélyova et al. [44] investigated the metabolic effects of long-term androgen deficiency starting before puberty in middle-aged male rats. The authors found that androgen deficiency does not induce MetS but may affect the liver by increasing the liver triacylglycerol content, which correlated with higher plasma AST and ALT, suggesting partial liver damage. Liver cholesterol content was not affected.

Jia et al. [43] reported that T deficiency enhances high-fat/low carbohydrate diet-induced hepatic steatosis in castrated male rats by modulating the endoplasmic reticulum stress, independently of insulin resistance. Besides, the replacement therapy reduced hepatic macrovesicular steatosis and inflammation. The propose mechanism to explain the T's protective effect on high-fat diet-induced hepatic steatosis, seems to involve the expression of lipid export proteins ApoB100 and microsomal TG transfer protein (MTP), which are suppressed by high-fat diet in castrated and sham operated rats and restored by T replacement. Besides, proteins that are associated with macrovesicular steatosis (perilipin 1 and fat-specific protein 27 FSP27) where also increase by high fat-diet in castrated rats and suppressed by the therapy with T. Also, it was found a higher activation of ER proteins (CHOP, NF-κB, IRE-1α, PERK, JNK) in castrated rats fed high fat-diet and the T replacement therapy suppressed these changes. The authors concludes that high fat-diet reduces lipid export by the suppression of ApoB100/MTP while the castration promotes impairment of hepatic steatosis through the activation of the ER stress and

enhancement or increasement of the expression of proteins related to macrovesicular steatosis, and all these undesirable effects where reverted or by the treatment with T.

Animal studies with focus on steatosis

An imbalance of lipid production and turnover is responsible to hepatic lipid accumulation, in this way many studies in animals revealed this condition associated with low T levels, in this context, Sakr et al. [75] used Sprague Dawley male rats to investigate the effects of T deficiency and the replacement therapy on the development of hepatic steatosis associated with peripheral insulin resistance. Data revealed increasing final body weight, visceral and subcutaneous fat, and liver weight. Besides, it was observed increases in fasting plasma glucose and insulin levels and HOMA-IR. Different from Borbélyova et al. [44] in which hepatic cholesterol content was not affected, in the study of Sakr et al. [75] the castration increased not only the hepatic TG content, but also, the cholesterol content and both were restored to normal levels after treatment of T. In the source of mechanisms, the authors found increases in the transcript levels of Sterol regulatory element-binding protein (SREBP) - *Srebp-1*, *Srepb-2*, acetyl-CoA carboxylase *(Acc-1),* fatty acid synthase *(Fas),* and also increases in the protein levels of SREBP-1, SREBP-2, Peroxisome proliferator-activated receptor alpha (PPAR-α), p-PPAR-α, Carnitine palmitoyltransferase I (CPT-1), Mitochondrial uncoupling protein 2 (UCP-2), as well as lower protein levels of phospho-AMP-Activated Protein Kinase (p-AMPK) and p-ACC-1 in the livers of T deficiency rats. Interesting, T administration to the castrated rats improved all the biochemical parameters that were affected by T deficiency, also on mRNA and protein levels leading to the conclusion that T deficiency could be an

independent risk factor for the development of fatty liver, while the T therapy is a protective strategy.

Kelly et al. [19] reported the role of T against hepatic steatosis suppressing the expression of regulatory enzymes of fatty acid synthesis in cholesterol-fed androgen deficient mice. The authors found that T reduces the expression of key regulatory lipogenic enzymes to protect against hepatic steatosis in two animal models of severe T deficiency (Testicular feminised (Tfm) mice, which have a non-functional androgen receptor (AR) (Tfm) and orchidectomised XY littermate). The authors observed strongly suggest that allow T state promotes the development hepatic steatosis and potentially progression to NAFLD. Furthermore, T replacement decreased the amount of hepatic lipid deposition beyond the levels seen in T replacement Tfm mice and placebo-treated wild-types. In the present study, T acts with independent mechanism of the claasic AR, changing the hepatic lipid deposition in this model of cholesterol-fed mice. T exert protective effects through the increased expression of fatty acid synthase (FAS) and acetyl-CoA carboxylase alpha (ACACA) in Tfm mice this actin influencing de novo lipogenesis and decrease hepatic steatosis. The authors concluded that T deficiency could be a risk factor for the development and progression of hepatic steatosis.

Another study of Nikolaenko et al. [76] performed an investigation in castrated Young male Sprague Dawley rats with NAFLD (8 weeks) about the effects of T replacement. The results show the NAFLD is caused by high-fat, low-carbohydrate liquid in androgen deficient adult male rats. T replacement improved pathological changes in the liver and hepatocyte apoptosis also lowered percentage body fat in castrated rats. The authors supposed a hypothesis that T protects development of NAFLD and provides evidence that androgen deficiency could be a risk factor for increasing the severity of hepatic steatosis and inflammation.

Jia et al. [43] showed in castrated male rats with a high-fat/low-carbohydrate diet-induced NAFLD that T is able to reduce the hepatic macrovesicular fat accumulation and inflammation. After 15 weeks of treatment, the castration does not changes the de novo synthesis and fatty acid β-oxidation, on the other hand the expression of lipid export proteins ApoB100 and microssomal TG transfer protein (MTP) was supressed in intact and castrated rats, T replacement was able to restore. The progression to steatohepatitis through activation of the ER stress pathway happened due to castration procedure, such as an enhancement of macrovesicular droplet protein expression. T replacement has been shown to be a suppressing agent to endoplasmic reticulum stress (ER), promotes lipid export, inhibits the formation of macrovesicular lipid droplets, ameliorates steatohepatitis induced by HFD and castration. The authors concluded that T replacement it's a good agent to protect the hepatic tissue against hepatic steatosis induced by HFD with androgen deficiency, reversing a series of actions, resulting in subsequent amelioration in lipid accumulation and consequently the inflammation and apoptosis.

Conclusion

In conclusion, MetS is associated with low T in men and animals (figure 3). T itself seems modulated the body fat distribution and the lipid metabolism in men and animals, thus, a link between fat distribution, MetS and androgens. Our literature review shows that older age and insulin resistance are important factor associated with lower T levels. Several indicators of impairment of metabolic function can be detected as loss of lean body mass, increase in visceral fatt, WC, dyslipidemia, insulin resistance and obesity. Considering the proven relationships between adiposity and hypogonadism, it is crucial to suspect and investigate T deficiency in aging men at any sign of important

changes on body composition and weight. Significant correlation was found between age, BMI, and hypogonadism and many studies indicate that there is an inverse relationship between WC and serum T levels in men with T2DM.

The MetS is a complex mixture of symptoms and usually the criteria for diagnose requires that the patient present 3 or more of those symptoms. Few studies have investigated the role of T deficiency on the development of the NAFLD. The studies that have investigated this question did not use gold standard methods for diagnosing NAFLD. This may be related to the fact that biopsies are invasive and only recommended for patients with signs of severe liver diseases. Many studies verify the causal relationship between NAFLD and total serum T levels, and well-designed randomized clinical trials could define whether T replacement therapy can improve NAFLD and reported indicate that indeed the liver may be more susceptible to becoming fatty during T deficiency.

This was also pointed by the studies made with rodents, which have shown evidences that the androgen deficiency *per se* has some deleterious effects on metabolic parameters, but it can potentiate the HFD-induced metabolic alterations, including increased adiposity, impaired glucose tolerance, and decreased insulin secretion and sensitivity. Castration-induced T deficiency primarily affects fasting blood glucose and leads to decrease extra-hepatic insulin sensitivity and can lead to higher TG accumulation in liver.

Conversely, studies have consistently shown that long-term T treatment can lead to significant weight loss and improvements in other metabolic outcomes [77,78]. This underlines the importance of the treatment with T for a normalization of a metabolic disease state. This is of special concern among middle and late age subjects and it is important to spread the evidences that low total T can be a significant marker to predict the development of MetS. Therefore, a more assertive therapeutic approach should

always include the assessment of the male hormone profile to serve as an (early) indicator for future metabolic risk. The maintenance of physiological levels of T has the potential improve the quality of life and prolong the life. Gaining a clear understanding of the interlinked pathways that mediate the high prevalence of T deficiency in patients with obesity could help us to develop effective strategies in the management of hypogonadism. Hence, implementation of screening programs in older or overweight patients is necessary to understand and detect individuals with low serum total T at any early stage and to supplement T accordingly. In this way, we emphasize the importance of a specific medicine to treat MetS, which contemplate every component of the syndrome, the stage of life and the hormonal profile as key aspects for an assertive clinical intervention. Finally, for those with diagnose of T deficiency, the NAFDL should be a concern and adaptations on the diet and lifestyle may represent important preventive approaches to avoid the ectopic accumulation of fat in the liver.

References

[1] Schindler AE. Climacteric symptoms and hormones. Gynecol Endocrinol. 2006 Mar;22(3):151-4.

[2] Solstad K, Garde K. Middle-aged Danish men's ideas of a male climacteric Dand of the female climacteric.Maturitas. 1992;15:7-16.

[3] Juul A, Skakkebaek NE. Androgens and the ageing male. Human Reproduction Update. 2002;8(5):423-433.

[4] Turnover JC. Androgen administration to aging men. Endocrinol Metab Clin North Am. 1994;23:877-892.

[5] Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid binding globulin in human plasma. J Clin Endocrinol Metab. 1981;53:58–68.

[6] Pardridge WM. Serum bioavailability of sex steroid hormones. Clin Endocrinol Metab. 1986;15:259–78.

[7] Lapauw B, Goemaere S, Zmierczak H, et al. The decline of serum testosterone levels in community-dwelling men over 70 years of age: descriptive data and predictors of longitudinal changes. Eur J Endocrinol. 2008 Oct;159(4):459-68.

[8] Wu FC, Tajar A, Pye SR, et al. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study. J Clin Endocrinol Metab. 2008;93:2737-45.

[9] McBride JA, Carson CC, Coward RM. Testosterone deficiency in the aging male. Ther Adv Urol. 2016;8(1):47–60.

[10] Samaras N, Samaras D, Lang PO, et al. A view of geriatrics through hormones. What is the relation between andropause and well-known geriatric syndromes? Maturitas. 2013;74(3):213–219.

[11] Wu FC, Tajar A, Beynon JM et al. Identification of late-onset hypogonadism in middle-aged and elderly men. N Engl J Med. 2010;363:123–35.

[12] Wylie K, Rees M, Hackett G, et al. Androgens, health and sexuality in women and men. Maturitas. 2010 Nov;67(3):275-89.

[13] Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. Indian J Physiol Pharmacol. 2006;50:291–6.

[14] Shiels MS, Rohrmann S, Menke A, Selvin E, Crespo CJ, Rifai N, et al. Association of cigarette smoking, alcohol consumption, and physical activity with sex steroid hormone levels in US men. Cancer Causes Control. 2009;20:877–86.

[15] Suzuki R, Allen NE, Appleby PN, Key TJ, Dossus L, Tjonneland A, et al. Lifestyle factors and serum androgens among 636 middle-aged men from seven countries in the European Prospective Investigation into Cancer and Nutrition (EPIC). Cancer Causes Control. 2009;20:811–21.

[16] Rohrmann S, Shiels MS, Lopez DS, Rifai N, Nelson WG, Kanarek N, et al. Body fatness and sex steroid hormone concentrations in US men: results from NHANES III. Cancer Causes Control. 2011;22:1141–51.

[17] Emmelot-Vonk MH, Verhaar HJ, Nakhai-Pour HR, Aleman A, Lock TM, Bosch JL, et al. Effect of testosterone supplementation on functional mobility, cognition, and other parameters in older men: a randomized controlled trial. JAMA. 2008;299:39–52.

[18] Li C, Ford ES, Li B, Giles WH, Liu S. Association of testosterone and sex hormone binding globulin with metabolic syndrome and insulin resistance in men. Diabetes Care. 2010;33:1618–24.

[19] Kelly DM, Nettleship JE, Akhtar S, et al. Testosterone suppresses the expression of regulatory enzymes of fatty acid synthesis and protects against hepatic steatosis in cholesterol-fed androgen deficient mice. Life Sci. 2014 Jul 30;109(2):95-103.

[20] Grundy SM. Metabolic syndrome pandemic. Arterioscler Thromb Vasc Biol. 2008;28(4):629–36.

[21] Kaur J. A comprehensive review on metabolic syndrome. Cardiol Res Pract. 2014;2014:943162.

[22] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med. 1998 Jul;15(7):539-53.

[23] Santos CE, Schrank Y, Kupfer R. Análise crítica dos critérios da OMS, IDF e NCEP para síndrome metabólica em pacientes portadores de diabetes melito tipo 1. Arq Bras Endocrinol Metab. 2009;53(9):1096-1102.

[24] Grundy SM, Cleeman JI, Daniels SR et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation. 2005;112:2735–52.

[25] International Diabetes Federation (IDF). The IDF consensus worldwide definition of the metabolic syndrome. Brussels: IDF; 2005.

[26] Grover S, Malhotra N, Chakrabarti S, et al. Metabolic syndrome in bipolar disorders. Indian J Psychol Medicine. 2012; 34:110–118.

[27] O'Neill s, O'Driscoll L. Etiology and Pathophysiology/Obesity Comorbidities. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obes Rev. 2015;16:1-12.

[28] Albuquerque GA, Leite MF, Belém JM, Nunes JFC, Oliveira MA, Adami F. The man in primary healthcare: perceptions of nurses about the implications of gender in

health. Esc Anna Nery [Internet]. 2014 [cited 2019 Out]; 18(4):607-14. Available from: http://www.scielo.br/pdf/ean/v18n4/1414-8145-ean-18-04-0607.

[29] Paula JAT, Moreira OC, Silva CD, Silva DS, Amorim PRS. Metabolic syndrome prevalence in elderly of urban and rural communities participants in the HIPERDIA in the city of Coimbra/MG, Brazil. Invest Educ Enferm. [Internet] 2015 [cited 2019 Out 24]; 33(2):325-33. Available from: http://www.scielo.org.co/pdf/iee/v33n2/ v33n2a15.

[30] Berenjy S, Bt rahmat A, Hanachi P, Sunn LM, Yassin ZB, Sahebjamee F. Metabolic Syndrome in Iran. Global Journal of Health Science . 2010;2(2):117-122.

[31] International Diabetes Federation (IDF). The IDF consensus worldwide definition of the metabolic syndrome. Brussels: IDF; 2006.

[32] Reaven GM. The metabolic syndrome: time to get off the merry-go-round? J Int Med [Internet]. 2010 [cited 2019 Out]; 269:127-36. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2796.2010.02325.x/epdf.

[33] Chen RYT, Wittert GA, Andrews GR. Relative androgen deficiency in relation to obesity and metabolic status in older men. Diabetes, Obesity and Metabolism. 2006;8:429-435.

[34] Kupelian V, Page ST, Araujo AB, et al. Low Sex Hormone-Binding Globulin, Total Testosterone, and Symptomatic Androgen Deficiency Are Associated with Development

of the Metabolic Syndrome in Nonobese Men. J Clin Endocrinol Metab. 2006 Mar;91(3):843-50.

[35] Jeong SM, Ham BK, Park MG, et al. Effect of testosterone replacement treatment in testosterone deficiency syndrome patientswith metabolic syndrome. Korean J Urol. 2011;Aug;52(8):566-71.

[36] Blaya R, Thomaz LDGR, Guilhermano F, et al. Total testosterone levels are correlated tometabolic syndrome components. The Aging Male. 2016;1–5.

[37] Angelova P, Kamenov Z, Tsakova A, et al. Interleukin-18 and testosterone levels in men withmetabolic syndrome. The Aging Male.2017;1-8.

[38] Souteiro P, Belo S, Oliveira SC, Neves JS, Magalhães D, Pedro J, Bettencourt-Silva R, et al. Insulin resistance and sex hormone-binding globulin are independently correlated with low free testosterone levels in obese males. Andrologia. 2018 Sep;50(7):e13035.

[39] Groti K, Žuran I, Antonič B, et al. The impact of testosterone replacement therapy on glycemic control, vascular function, and components of the metabolic syndrome in obese hypogonadal men with type 2 diabetes. The Aging Male. 2018 Sep;21(3):158-169.

[40] Xia F, Xu X, Zhai H et al. Castration-induced testosterone deficiency increases fasting glucose associated with hepatic and extra-hepatic insulin resistance in adult male rats. Reproductive Biology and Endocrinology. 2013,11:106.

[41] Dubois, V, Laurent MR, Jardi F, et al. Androgen Deficiency Exacerbates High-Fat Diet-Induced Metabolic Alterations in Male Mice. Endocrinology. 2016; 157(2):648–665.

[42] Christoffersen B, Raun K Svendsenl O et al.Evalution of the castrated male Sprague–Dawley rat as a model of the metabolic syndrome and type 2 diabetes. International Journal of Obesity. 2006;30:1288–1297.

[43] Jia Y, Yee JK, Wang C, et al. Testosterone protects high-fat/low-carbohydrate diet-induced nonalcoholic fatty liver disease in castrated male rats mainly via modulating endoplasmic reticulum stress. Am J Physiol Endocrinol Metab. 2018 Apr 1;314(4):E366-E376.

[44] Borbélyová V, Domonkos E, Bábíčková J, Tóthová Ľ, Kačmárová M, Uličná O, et al. Does long-term androgen deficiency lead to metabolic syndrome in middle-aged rats?. Exp Gerontol. 2017 Nov;98:38-46.

[45] Medeiros R, Almeida BC, Costa LM, Francesconi A, Follador F, Neves I, Rocio J. Etiopatogenia da acne vulgar: uma revisão prática para o dia a dia do consultório de dermatologia Surgical & Cosmetic Dermatology. 2015;7(3) 2015:20-26.

[46] Dwyer AA, Quinton R. The Metabolic Syndrome in Central Hypogonadotrophic Hypogonadism. Front Horm Res. 2018;49:156-169.

[47] Kirby RCB. Psoriasis is a systemic disease with multiple cardiovascular and metabolic comorbidities. Dermatol Clin. 2015;33:41-55.

[48] Shelton JB, Rajfer J. Androgen Deficiency in Aging and Metabolically Challenged Men. UrolClin N Am. 2012,39(1):63–75.

[49] Rupérez, AI, Gil A, Aguilera CM. Genetics of oxidative stress in obesity. Int J Mol Sci. 2014;15(2):3118-3144.

[50] Moore JB. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. Proceedings of the Nutrition Society. 2010;69(02):211-220.

[51] Day CP, James OF. Steatohepatitis: a tale of two “hits"? Gastroenterology. 1998;114:842–845.

[52] Zou Y, Li J, Lu C, Wang J, Ge J, Huang Y, Zhang L, Wang Y. High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. Life Sci. 2006 Aug 8;79(11):1100-7.

[53] Tang YJ1, Lee WJ, Chen YT, Liu PH, Lee MC, Sheu WH. Serum testosterone level and related metabolic factors in men over 70 years old. J Endocrinol Invest. 2007 Jun;30(6):451-8.

[54] Haring R, Vo¨lzke H, Felix SB, et al. Prediction of Metabolic Syndrome by Low Serum Testosterone Levels in Men. Results From the Study of Health in Pomerania.Diabetes. 2009;58:2027-2031.

[55] Rotter I, Rył A, Grzesiak K, Szylińska A, Pawlukowska W, Lubkowska A, et al. Cross-Sectional Inverse Associations of Obesity and Fat Accumulation Indicators with Testosterone in Non-Diabetic Aging Men. Int J Environ Res Public Health. 2018 Jun 8;15(6). pii: E1207.

[56] Katabami T, Kato H, Asahina T, et al. Serum free testosterone and metabolic syndrome in Japanese men. Endocr J. 2010;57(6):533-9.

[57] Jiann BP, Hsieh JT, Liu SP, et al. Associations of endogenous testosterone and lipid profiles in middle-aged to older Taiwanese men. Int J Impot Res. 2011 Mar-Apr;23(2):62-9.

[58] Ho C, Jaw F, Wu C, et al. The Prevalence and the Risk Factors of Testosterone Deficiency in Newly Diagnosed and Previously Known Type 2 Diabetic Men. J Sex Med. 2015;12(2):389-97.

[59] Ugwu TE, Ikem RT, Kolawole BA, Ezeani IU. Clinicopathologic assessment of hypogonadism in men with type 2 diabetes mellitus. Indian J Endocrinol Metab. 2018 Sep-Oct;20(5):667-673.

[60] Agarwal PK, Singh P, Chowdhury S, Sharma SK, Majumdar A, Shah P, et al. A study to evaluate the prevalence of hypogonadism in Indian males with Type-2 diabetes mellitus. Indian J Endocrinol Metab. 2017 Jan-Feb;21(1):64-70.

[61] Tan WS, Ng CJ, Khoo EM, Low WY, Tan HM. The triad of erectile dysfunction, testosterone deficiency syndrome and metabolic syndrome: findings from a multi-ethnic Asian men study (The Subang Men's Health Study). Aging Male. 2011 Dec;14(4):231-

[62] Fillo J, Breza J, Levcˇ´ıkovaM, et al. Occurrence of erectile dysfunction, testosterone deficiency syndrome and metabolic syndrome in patients with abdominal obesity. Where is a sufficient level of testosterone?. Int Urol. Nephrol. 2012; 44:1113–1120.

[63] Tajar A, Huhtaniemi IT, O'Neill TW, et al. EMAS Group. Characteristics of androgen deficiency in late-onset hypogonadism: results from the European Male Aging Study (EMAS). J Clin Endocrinol Metab. 2012 May;97(5):1508-16.

[64] García-Cruz E, Leibar-Tamayo A, Romero-Otero J, et al. Marked Testosterone Deficiency-Related Symptoms May be Associated to Higher Metabolic Risk in Men with Low Testosterone Levels. J Sex Med. 2014;11:2292–2301.

[65] Saad F, Gooren L, Haider A, Yassin A. An exploratory study of the effects of 12 months administration of the novel long-acting testosterone undecanoate on measures of sexual function and the metabolic syndrome. Arch Androl. 2007 Nov-Dec;53(6):353-7.

[66] Singh SK, Goyal R, Pratyush DD. Is hypoandrogenemia a component of metabolic syndrome in males? Exp Clin Endocrinol Diabetes. 2011 Jan;119(1):30-5.

[67] Traish AM, Haider A, Doros G, Saad F. Long-term testosterone therapy in hypogonadal men ameliorates elements of the metabolic syndrome: an observational, long-term registry study. Int J Clin Pract. 2014 Mar;68(3):314-29.

[68] Huang G, Travison T, Maggio M, et al. Effects of testosterone replacement on metabolic and inflammatory markers in men with opioid-induced androgen deficiency. Clin Endocrinol (Oxf). 2016;Aug;85(2):232-8.

[69] Dias JP, Shardell MD, Carlson OD, et al. Testosterone vs. aromatase inhibitor in older men with low testosterone: effects on cardiometabolic parameters. Andrology. 2017 Jan;5(1):31-40.

[70] Barbonetti A, Caterina Vassallo MR, Cotugno M, Felzani G, Francavilla S, Francavilla F. Low testosterone and non-alcoholic fatty liver disease: Evidence for their independent association in men with chronic spinal cord injury. J Spinal Cord Med. 2016 Jul;39(4):443-9.

[71] Hasanain AFA, Mahdy RE, Mahran AMA, Safwat ASM, Mohamed AO, Abdel-Aal SM.Arab J Gastroenterol. Erectile dysfunction in patients with nonalcoholic fatty liver disease. 2017 Mar;18(1):21-24.

[72] Seo NK, Koo HS, Haam JH, et al. Prediction of prevalent but not incident non-alcoholic fatty liver disease by levels of serum testosterone. J Gastroenterol Hepatol. 2015 Jul;30(7):1211-6.

[73] Kim S, Kwon H, Park JH, et al. A low level of serum total testosterone is independently associated with nonalcoholic fatty liver disease. BMC Gastroenterol. 2012 Jun 12;12:69.

[74] Harada N, Hanaoka R, Horiuchi H, et al. Castration influences intestinal microflora and induces abdominal obesity in high-fat diet-fed mice. 2016;6:23001.

[75] Sakr HF, Hussein AM, Eid EA, AlKhateeb M. Possible mechanisms underlying fatty liver in a rat model of male hypogonadism: A protective role for testosterone. Steroids. 2018 Jul;135:21-30.

[76] Nikolaenko L, Jia Y, Wang C, et al. Testosterone replacement ameliorates nonalcoholic fatty liver disease in castrated male rats. Endocrinology. 2014 Feb;155(2):417-28.

[77] Francomano D, Bruzziches R, Barbaro G, Lenzi A, Aversa A.J. Effects of testosterone undecanoate replacement and withdrawal on cardio-metabolic, hormonal and body composition outcomes in severely obese hypogonadal men: a pilot study. Endocrinol Invest. 2014 Apr;37(4):401-11.

[78] Saad, Yassin, Doros e Haider. Effects of long-term treatment with testosterone on weight and waist size in 411 hypogonadal men with obesity classes I-III: observational data from two registry studies. Int J Obes (Lond). 2016 Jan;40(1):162-70.

Supplementary Table 1 – Criteria for the clinical diagnosis of the MetS according to different entities.

Entitie	IR	Obesity	FBG (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	BP (mm/Hg)	Others
WHO	+ 2 or more criteria	BMC > 30 or WC > 90	DM or altered FBG	>150	< 35	<140/90	MA (>20 mcg/min)
NCEP/ ATP III	Did not evaluate – 2 or more criteria	WC > 102	>110	>150	< 40	<130/85	
IDF		WC > 94	>100	>150	< 40	<130/85	
EGIR	Insulin >75% + 2 criteria	WC > 94	Altered FBG	>150	< 39	<140/90	
AACE	Altered glycemia more 1 criterion	BMC > 25		>150	< 40	<130/85	IR evidence

WHO: World Health Organization; NCEP/ATP III: National Cholesterol Education Program - Adult Treatment Panel III; IDF: International Diabetes Federation; EGIR: Group for the Study of Insulin Resistance; AACE: American Association of Clinical Endocrinologists; IR: Insulin Resistance; FBG: Fasting Blood Glucose; TG: TGs; HDL-C: High Cholesterol Density; BP: Blood Pressure; BMC: Body Mass Composition; WC: Waist Circunference; DM: Diabetes Mellitus; MA: Microalbominuria

Figures

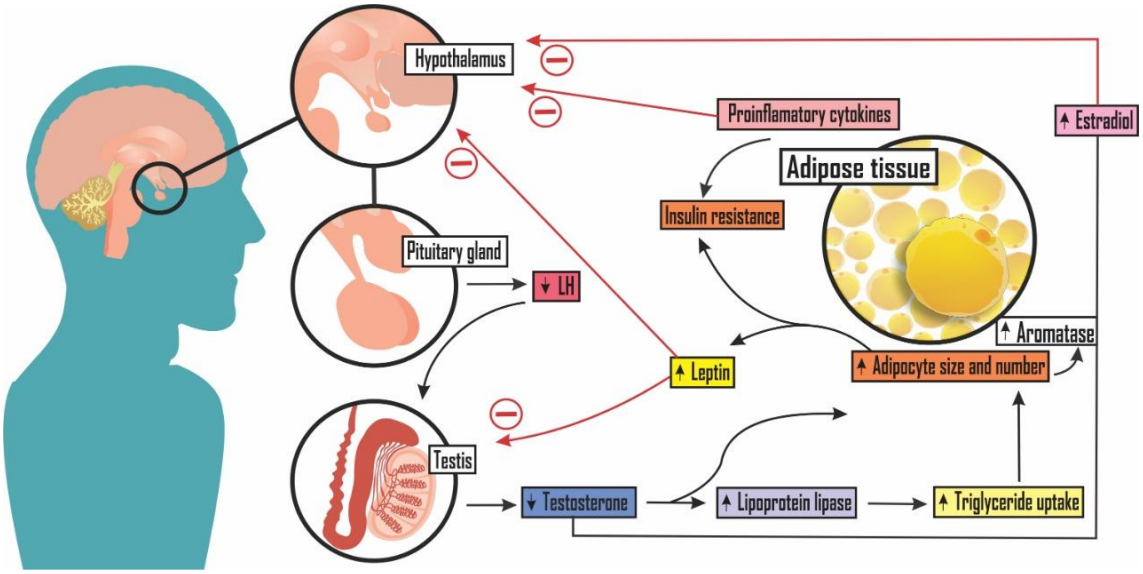

Fig. 1. Metabolic pathways of T. Luteinizing hormone (LH) released by the pituitary gland stimulates Leyding cells to produce testosterone and follicle stimulating hormone (FSH). Obesity and increased visceral fat may accentuate the condition of low testosterone levels due to increased aromatase enzyme activity, which converts testosterone to estradiol, and due to the release of proinflammatory cytokines. Estradiol, in turn, acts by negative feedback in the pituitary and hypothalamus, increasing leptin secretion. Leptin has inhibitory effects on the testes, further reducing the production of T.

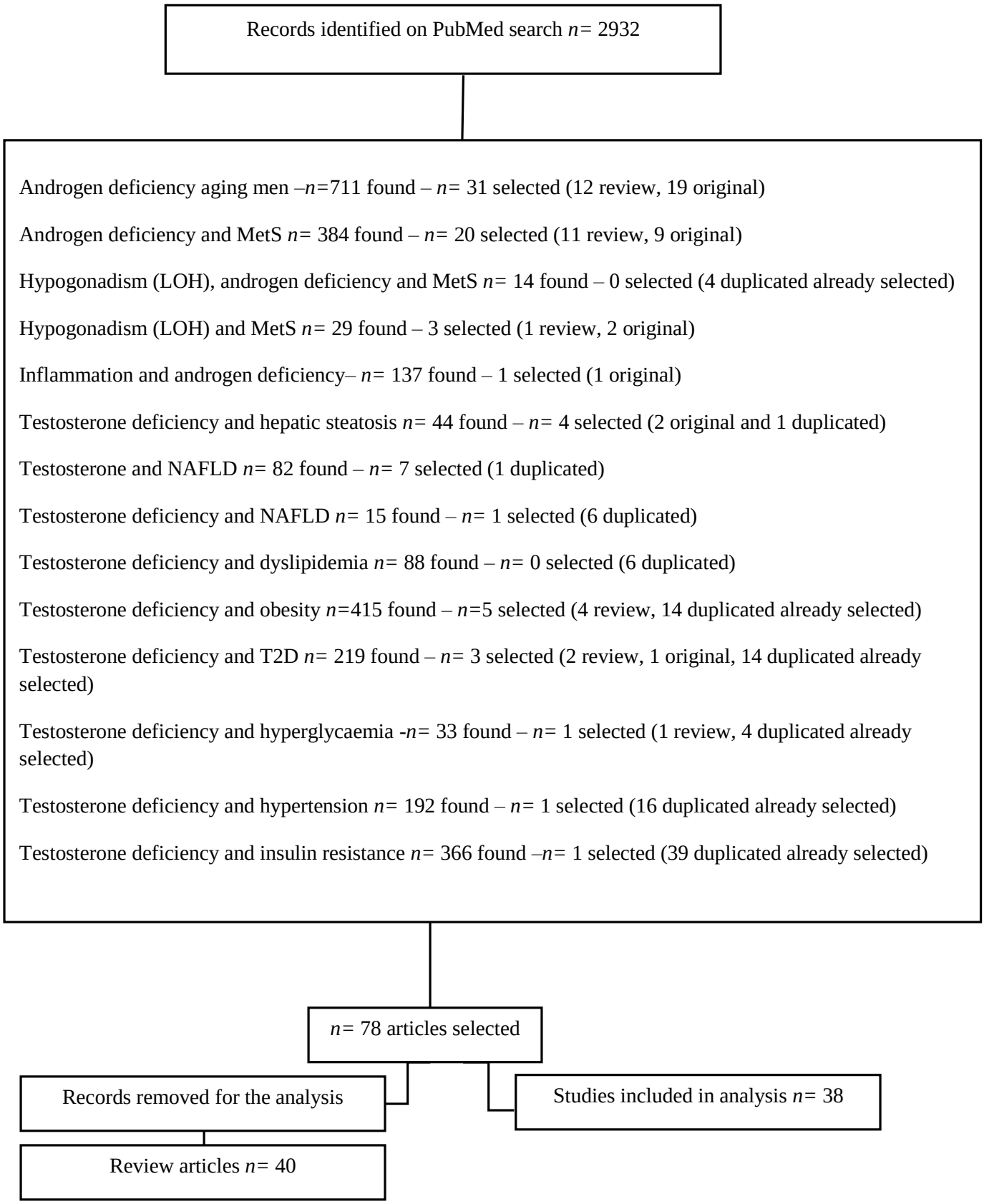

Fig 2. Trial flow diagram. *n* = number.

Tables

Table 1

Reference (y)	Group Charactheristcs	T Levels	T Treatment	MainOutcomes
Katabami et al. (2010)	Japanese men (n=274, mean age: 46.0 ± 11 y)	14.0 ± 4.6 pg/mL	NA	↓FT, ↑MetS components. MetS, IR may ↓serum T via induction of HH, ↓T can cause obesity and IR.
García-Cruz et al. (2014)	Spanish men (n=999, mean age: 61.2.0 ± 8.2 y)	6.6 ± 2.2 nmol/L	NA	↓TT is associated with MetS and ED. Severity of TDS symptoms may indicate ↑CVD risk in men with ↓TT.
Fillo et al. (2012)	Men with AO Fhar(n=167, mean age: 58.4 ± 6.6 y)	<10 e 10-14 nmol/L	NA	TT ↓10 nmol/l was associated with MetS, morbidity and difference in circulating HDL-C-C and TG.
Ugwu et al. (2018)	Men with T2DM (n=200, mean age: 57.99 ± 8.76 y).	Low < 8; Borderline: 8-12 and Normal > 12 nmol/L	NA	↑WC and ED in hypogonadal subjects. ↓TT, ↑VA. Negative correlation between WC and serum TT.
Ho et al. (2015)	Men with T2DM (n=105, mean age=61.2 ± 6.8 y)	<300 and free < 6 ng/dL	NA	↑DBP, FBG, the glycemic control HbA1c was associated with a ↑TT and a ↓risk of ↓TT. A risk factors of ↓T how high HbA1c, ↓SHGB, obesity, HU, ↑TG, MetS.
Kupelian et al. (2006)	Men without MetS, TT, SHBG were predictive of MetS (n=950, age=40-70 y)	T1 17.9; MetS at T1 15.6 and analytic sample 1.4 nmol/L.	NA	↓SHBG, ↓T and AD were associated with ↑risk of developing MetS.
Souteiro et al. (2018)	Obese men (n=150, mean age: 41.5 ± 10.9 y)	3.07 ± 1.29 ng/mL	NA	↓TT was associated with obesity. The main determinant of ↓TT in obese males was IR and weight. SHBG levels were correlated with ↓FT even after HOMA-IR adjustment.

Blaya et al. (2016)	Non-diabetic men (n=143, mean age: 61.49 ± 8.61y)	<300ng/dL or <11nmol/L	NA	Moderated correlation between TT and MetS components, especially the central feature of WC in TT.
Chen et al. (2006)	Men followed up for 8 y (n=195, mean age: 76.2 ± 0.3 y)	Whole cohort: 13.8 ± 0.4; in diabetic men: 12.1 ± 0.7; in non-diabetic men: 14.2 ± 0.4 nmol/L	NA	↓T was associated with T2DM, ↑BMI, ↑TG, ↑LDL-C-C, ↑BP, ↑FGP, ↑WC and MetS. T levels were inversely related to VA.
Jiann et al. (2011)	Midlle-aged Taiwanese men (n=856, mean age: 60.6 ± 11.8 y)	FG: 552.8; NFG: 471.1 ng/dL	NA	↑TT levels were associated with ↓postprandial TGs, ↑postprandial HDL-C-C and ↑LDL-C-C independently of age, BMI, SHBG and DM.
Tajar et al. (2012)	European male aging (n=2966, men age: 60 ± 11y)	All: 16.60 ± 5.91; EG: 16.78 ± 5.83; LOH-M: 9.63 ± 0.79, LOH-S: 5.90 ± 2.02 nmol/L	NA	LOH was associated with ↓TT and the reduced androgen action promoting, LOH ↓muscle mass, BMD, ↓Hb. LOH-S ↑WC, IR and MetS.
Tan et al. (2011)	Malaysian's Men (n=1046, mean age 55.8 ± 8.4 y)	<10.4 nmol/L (300 ng/dL).	NA	MetS ↑age and is associated with ↓TT. TDS was associated moderate to severe ED. ED associated with MetS, ↑WC, ↑BP, ↑FBG, ↓HDL-C-C.
Tang et al. (2007)	Eldery men (n=381, mean age: 78.8 ± 4.1 y)	All: 4.49 ± 1.91; MetS: 3.84 ± 0.17, no MetS 4.67 ± 0.11 ng/dL	NA	TT is inversely associated with BMI, WC, BP, insulin, HbA1c, TG, AI, FBG, and positively related to Hb concentration and HDL-C-C. ↓T may be a indicator for development of MetS.
Haring et al. (2009)	Adults (n=1004, mean age: 51.3 ± 16.6 y)	Without MetS: 17.7; Incident MetS: 15.5 nmol/L	NA	After a median follow-up time of 5 y, 47,8% of men developed MetS and MetS components were associated with ↓TT.

Huang et al. (2016)	Nondiabetic men using opioid analgesics for noncancer pain	T group: 7.7 ± 3.0; placebo group: 8.2 ± 3.4 nmol/L	T gel or placebo gel < 12 nmol/l for 14 w, transdermal	TRT in men under the effect of opioids also contributes to attenuate CVD risk due to improvement in body composition.
Groti et al. (2018)	Obese hypogonadal diabetic men on oral hypoglycaemic treatment (n=55, mean age 60.15 ± 7.23 y)	Placebo group: 7.96 ± 1.34, after 1 y 9.83 ± 1.51; T group: 7.24 ± 1.97, after 1 y 17.04 ± 3.07 nmol/L	TU (1000mg) every 10 w, *i.m.*	TRT ↓HOMA-IR, HbA1c. TRT normalized serum TT, improved glycaemic control and endothelial function.
Saad et al. (2007)	Men with sexual dysfunction (n=28, age= 54-76 y)	0 m: 2.2 ± 0.6, 3 m 3.8 ± 0.9, 6 m: 4.9 ± 1.0, 9 m: 5.3 ± 0.7 and 12 m: 5.6 ± 0.7 ng/mL	TU (1000mg) for 6w	TRT improve MetS, with ↑in plasma SHBG, and BP.
Dias et al. (2017)	Aged men wih low TT (n=29, aged=71 ± 3 y)	Placebo group: 10.5 ± 3.3; Transdermal T gel group: 10.4 ± 2.9; Anastrozole and placebo gel group 10.6 ± 2.9 nmol/L	T gel (5g/d), for 1 y, transdermal	All outcomes were assessed at baseline and 12 m. After 12 m, absolute changes in HOMA-IR, CRP and fasting lipid levels. Adiponectin levels significantly ↓with T treatment and abdominal subcutaneous fat.
Jeong et al. (2011)	Men with TDS and were undergoing TRT (n=200, mean age=57.7 ± 9.85 y)	TDS patients with MetS 2.31 ± 0.82; TDS patients without MetS: 2.52 ± 1.12 ng/mL	TU (1000mg) for 10-14 w, *i.m.*	In patients with MetS it was observed ↑TT levels and ↓WC, ↓FPG and ↓Hb. In patients without MetS was observed ↑TT levels, ↓WC, ↓BMI, ↓TC, ↓LDL-C, ↓FPG and ↑Hb.
Singh et al. (2010)	Healthy men as controls (n=32, mean age= 34.645 ± 7.46 y)	Case group 9.77 ± 2.19, Control group 16.06 ± 4.77; Hypogonadal 7.87 ± 1.35, Eugonadal 10.59 ± 2.	TU (40mg) twice a day for 3m, oral	Hypogonadism was seen in 30% of the subjects with MetS and associated with ↑HOMA-IR than eugonadal subjects. T treatment led to improvement of HOMA-IR.
Traish et al. (2014)	Hypogonadal men (n=255, mean age: 58.02 ± 6.30 y)	9.93 ± 1.38; range: 5.89 -	TU (1000mg), at baseline, at	TU therapy restored physiological T levels and resulted in

		12.13 nmol/L.	6w and every 12w for up to 60m, parenteral	reductions in TC, LDL-C, TG and ↑d HDL-C, ↓the HbA1c, C-reactive protein, AST and ALT.
Agarwal et al. (2017)	Indian men with T2DM (n=900, mean age=45.2 ± 8.14 y)	<0.255 nmol/L	NA	↑age, there is more chance of getting hypogonadism in T2DM patients as compared to T2DM patients not suffering from hypogonadism. ↑BMI.
Rotter et al. (2018)	Non-diabetic aging men (n=455, mean age= 62.83 ± 6.57)	4.05 ± 1.71 ng/mL	NA	↑Fat, TT negatively correlated with blood serum, VAI, BMI, WC and LAP
Barbonetti et al. (2016)	Italian male patients (n=55, mean age 46.6 ± 17.3 y), with chronic spinal cord injury	261.6 ± 159.5 ng/dL	NA	The patients with NAFLD were older and exhibited higher BMI, insulin, HOMA-IR, TG and gamma-glutamyl transpeptidase and lower levels of total and free testosterone.
Hasanain et al. (2017)	Male patients with NAFLD at the Assiut University Hospital in Egypt (n=192, mean age = 42.4 ± 7.7y)	3.17 ± 1.94 ng/mL	NA	Low serum testosterone as a predictor to development of ED and ED is a common disturb in male patients with NAFLD, in this search the patients with NAFLD that had ED were 45.8%.
Kim et al. (2012)	Men who were at least 20 years of age (n=495)	No NAFLD 4.83 ng/mL and NAFLD 3.94 ng/mL		Healthy Korean men inverse association between T and NAFLD persisted even after controlling for the effect of VAT, insulin resistance (as HOMA-IR), and low-grade inflammation (as hs-CRP). After the

				adjusting for VAT and HOMA-IR, the authors show the attenuation of the association between NAFLD and serum testosterone.
Seo et al. (2015)	Korean men (n=1944, mean age:44y) normal and with NAFLD	Normal 17.2 nmol/L and NAFLD 14.5 nmol/L	NA	Low levels of testosterone in patients with NAFLD. Baseline level of testosterone did not independently influence the development or regression of fatty liver at the median 4.2 years follow-up.

AO: Abdominal obesity; d: day; DBP: Diastolic blood pressure; ED: Erectile dysfunction; EG: eugonadal group; FT: free testosterone; HC: hip circumference; HH: Hypogonadal hypogonadism; HTG: Hypertriglycemia; HU: hyperuricemia; LAP: lipid accumulation product; LOH-M: Low moderate hypogonadism; TU: testosterone undecanoate; TST: testosterone supplementation therapy; TT: Testeosterone levels; VA: visceral adiposity; VAI: Visceral adiposity index; VLED: very low diet; w: weeks; WHR: waist to hip-ratio; y: years

Table 2

Reference (y)	Animal Model	T Levels	T treatment	MainFindings
Dubois et al. (2016)	Male mice with androgen deficiency ORX and androgen receptor *k.o.* mice (n=13-15 per group, age: 3w, 10-20 w and 20 w)	NA	NA	↓TT, ↑adiposity, leptin,↑lipogeneses , the glucose intolerance was exacerbated with a HFD. TG ↑and ↓glycogen content. ↑serum IL-1β levels. TRT restored the castration effects on body composition and glucose homeostasis.
Xia et al. (2016)	Castration-induced T deficiency in Sprague-Dawley rats (n=30, age10 w old)	Control group: 2.96 ± 0.55; T deficiency group: 1.45 ± 0.26; castrated rats + T 2.07 ± 0.53 ng/mL.	T propionate (25 mg/kg/d) to supplement androgen (TD+TP) group, for 10w, via intraperitoneal injection	↑hepatic gluconeogenesis, extra-hepatic IR, BW and subcutaneous fat in advanced-aged male rats. The glucose and HbA1c ↑, revealed a clear IR both at the hepatic and extra-hepatic levels
Christoffersen et al. (2006)	Male Sprague-Dawley rats castrated, or sham operated (n=10-12, age:16 w)	Sham-operated: 2.2 ± 1.5 and castrated: < 0.1 ng/mL (10 w)	NA	The castration lead to ↓BW, ↑subcutaneous fat, ↑FBG, ↑HbA1c, ↓FFA, ↓glycerol.
Borbélyová et al. (2017)	Lewis rats, control, GDX (n=64, 18 m)		LET, 1 mg/kg, for 14 d, *i.m.*	↓TT levels, ↑liver enzymes in plasma and ↑TG in liver.
Donner et al. (2015)	Wistar rats fed standard chow (control) or a high fat/high sugar/low protein "obesogenic" diet (OGD) sub-divided into sham-operated or ORX, with and without diet-induced obesity (n=24, age:28w)	Control:168 ± 18; OGD: 136 ± 14; ORX: 24 ± 5 and OGD + ORX: 10 ± 1 ng/dL	NA	OGD-fed animals had ↑in fat mass, TC, TG and FPG compared to controls. The ORX animals had reduced serum T.

Jia et al. (2017)	Adult male rats: intact rats on regular chow diet or HFD, and castrated rats on HFD with or without T replacement (n=28, age:15 w)	NA	T Silastic implants with release rate estimated to be 30 μg.cm^{-1} day^{-1} and lasted for at least 6 m.	ApoB100 and MTP were suppressed by HFD in both intact and castrated rats and restored by T replacement. Macrovesicular lipid droplet-related PLIN1 and *FSP-27* were ↑ by HFD in castrated rats and suppressed by T replacement.
Sakr et al. (2018)	Male Sprague-Dawley rats were divided into 3 groups as follows: 1-sham-operated group (n=11), 2- ORX-induced group (n=9) exposed to ORX and 3-ORX+T treated group (n=10) (n=30, age:9 w)	Sham: 7.1 ± 1.3; ORX: 1.1 ± 0.21; ORX + T: 6.2 ± 2.1 ng/mL	T propionate (11ng/mL) for 3x/ w *i.m.*	↑BW, ↑hepatic fat, ↑visceral fat, ↑subcutaneous fats, ↑FPG, ↑insulin levels, ↑glucose and insulin levels during OGTT, ↑glucose during ITT, ↑serum and hepatic TG, ↑ serum TC and LDL-C-C. The T treatment was able to revert all these features.
Harada et al. (2016)	C57BL/6J mice (7 weeks old) are sensitive to high-fat diet-induced obesity and have glucose intolerance.	NA	NA	↑visceral fat mass by castration only in the absence of antibiotics in HFD-fed mice, ↑subcutaneous fat mass by castration irrespective of antibiotics. ↑fasting blood glucose levels, ↑liver triglyceride levels in a HFD-dependent, testosterone deficiency can alter the intestinal microbiome and induce abdominal obesity, but it depends on the diet.
Jia et al. (2019)	Adult castrated male rats with a high-fat/low-carbohydrate	T levels in castrated animals were undetectable	T from the Silastic implants was estimated to be ~30 μg·cm	T reduced hepatic macrovesicular fat accumulation and inflammation, do not changes the de novo synthesis and

	diet-induced NAFLD		·day and lasted for at least 6 mo	fatty acid β-oxidation, the expression of lipid export proteins ApoB100 and microssomal triglyceride transfer protein (MTP) was supressed in intact and castrated rats, T replacement restored and suppressed agent to endoplasmic reticulum stress (ER), promotes lipid export, inhibits the formation of macrovesicular lipid droplets, ameliorates steatohepatitis induced by HFD and castration.
Kelly et al. (2014)	Androgen deficient mice in two animal models of severe testosterone deficiency(Testicular feminised (Tfm – n=32) mice, which have a non-functional androgen receptor (AR) (Tfm) and orchidectomised XY littermate – n= 16). (8 weeks)	Tfm mice (2.2 ± 1.2 nM) and orchidectomised littermates (2.5 ± 0.6 nM	Tfm intramuscular injection of 10 µl of 100 mg/ml testosterone (Sustanon100 ® Cambridge, UK) Tfm mice receiving once fortnightly intramuscular injection of 10 µl 250 mg/ml testosterone (Sustanon 250®)	Low T promotes hepatic steatosis. T replacement decreased the amount of hepatic lipid deposition beyond the levels seen in testosterone replacement Tfm mice and placebo-treated wild-types. Testosterone exert protective effects through the increased expression of fatty acid synthase(FASN) and acetyl-CoA carboxylase alpha (ACACA) in Tfm mice this actin influencing de novo lipogenesis and decrease hepatic steatosis.
Nikolaenko et al. (2014)	Castrated Young male Sprague Dawley rats with	1.49 ± 0.26 ng/mL and 1.40 ± 0.23 ng/mL Castrated rats	T SILASTIC implants (Dow Corning)	NAFLD is caused by high-fat, low-carbohydrate liquid in androgen

	NAFLD (8 weeks)	had undetectable serum T. Castrated rats with T pellets implanted had significantly higher levels (2.49 ± 0.24 ng/mL	deficient adult male rats. T replacement improved pathological changes in the liver and hepatocyte apoptosis also lowered percentage body fat in castrated rats. The authors supposed an hypothesis that T protects development of NAFLD and provides evidence that androgen deficiency could be a risk factor for increasing the severity of hepatic steatosis and inflammation.

AI: Adiposity index; BW: Body weight; BP: Body pressure; CTRL: Rats chow; CT: total cholesterol; FBG: Fasting blood glucose; FFA: Free fat acid; HbA1c: glycated haemoglobin; HFD: High fat diet; IR: Insulin resistance; LET: Letrozole; MetS: Metabolic Syndrome; MTP: microssomal triglyceride transfer protein; mo:months; ORCD: exposed to orchidectomy; ORX: Orchidectomy; TP: Testosterone propionate; TT: total testosterone; y:year; w:weeks.

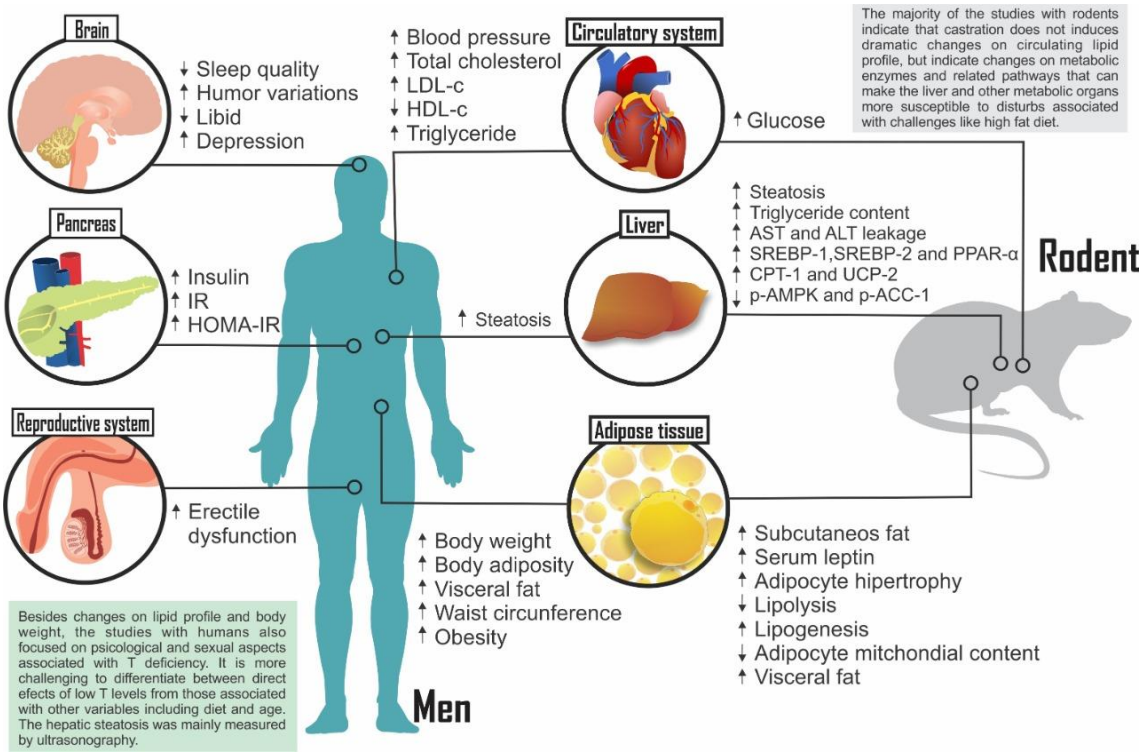

Fig 3. Main findings from human and animal studies. The main findings made in studies that used human and rodents as subjects of studies on the effects of testosterone deficiency on Metabolic Syndrome.

Differential melatonin-induced effects on body weight gain, adipocytes morphology and liver lipid metabolism in female rats under normoestrogenic or deficient conditions

Danielle Aparecida Munhos Hermoso[1], Eduardo Hideo Gilglioni[1], Lenilson da Fonseca Roza[2], Aparecida Pinto Munhos Hermoso[1], Eduardo Makiwama Klosowski[1], Franciele Neves Moreno[1], Karina Sayuri Utsunomiya[1], Elismari Rizato Martins Maciel[1], Maria Raquel Marçal Natali[3], Tatiana Carlesso dos Santos[2], Jorgete Constantin[1], Rodrigo Polimeni Constantin[1], Emy Luiza Ishii-Iwamoto[1*]

[1]Departament of Biochemistry, Laboratory of Biological Oxidation and Laboratory of Experimental Steatosis, Universidade of Maringá, Maringá 87020900, Brazil.
[2]Department of Animal Sciences, University of Maringá, Maringá 87020900, Brazil.
[3]Department of Morphological Sciences, University of Maringá, Maringá 87020900, Brazil.

*Corresponding author
Affiliation: Laboratory of Biological Oxidations,
Department of Biochemistry,
University of Maringá, 87020900 Maringá, Brazil.
E-mail: eliiwamoto@uem.br
Tel: 55-44-30114712
Fax: 55-44-30114896

Word count: 14490

Abstract

Melatonin has been suggested as alternative to estrogen replacement therapy for some menopause-associated disorders. The melatonin effectiveness in reducing body adiposity in estrogen deficiency conditions is still unclear. Regardless of body mass, regional fat deposition and adipocytes morphology seems to be important predictors for the risk of obesity-related metabolic disturbances. Here, this issue was investigated by measuring the melatonin effects on various parameters related to body weight gain, including body protein, minerals and lipids, contribution of white and brown adipose fats, adipocytes morphology, hepatic and adipose tissue antioxidant systems and liver lipid metabolism. 10 mg/kg/day of melatonin were administered orally over a period of 16 weeks in ovariectomized (OVX) and control (CON) rats. Melatonin did not suppress body adiposity in OVX rats, but prevented retroperitoneal and inguinal fat hypertrophy, and hepatic oxidative stress and hepatic steatosis. In CON rats, melatonin reduced body weight gain, inguinal adipocyte size, food intake and plasma estradiol. It can be concluded that melatonin reduces the risk to develop estrogen deficiency-related metabolic disorders, mainly by altering the lipid distribution in fat depots and suppressing tissues oxidative stress. Under normal estrogen condition, melatonin can induce adverse effects, including body weight loss and disturbance in estrogen regulated functions.

Key words**:** melatonin, liver oxidative status, steatosis, adipose tissue.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic compound derived from L-tryptophan, first discovered in 1958 as a product of the bovine pineal gland [1]. Its secretion by the pineal glands is regulated by circadian cycle, modulating functions of target cells as a hormone [2]. Melatonin has been shown to be synthesized by other cells and organs, including the ovaries, placenta, gastrointestinal tract, immune system cells, and retina [3-8]. In these organs, melatonin synthesis is not regulated by circadian cycle, but respond to other signals, exerting paracrine or autocrine effect [9].

The physiological functions of melatonin include regulation of the neuroendocrine reproductive axis, sexual behavior, mood, body temperature, immune responses, food intake, energy balance, and aging [10-16]. Most of the known actions of melatonin are mediated by specific high affinity receptors, namely MT1/MT2/MT3, coupled to G proteins on the plasma membrane [13,17].

Melatonin is a potent antioxidant, acting directly by eliminating different free radicals [18] and indirectly by stimulating antioxidant defense enzymes [19-21]. Melatonin crosses the blood – brain barrier [22] and is therefore designed as a pharmacological agent to treat neurological, psychiatric and neurodegenerative disorders, including Alzheimer, Parkinson and Huntington diseases [23-26]. The beneficial effect of melatonin on other diseases in which the generation of free radicals plays a pivotal role in their pathogenesis, such as atherosclerosis, hypertension, ischemia, obesity, NAFLD, diabetes, cancer and aging, has also been demonstrated in human clinical trials or studies in animal [27-37,16].

The estrogen deficiency in the postmenopause condition is associated with several manifestations of metabolic syndrome (MetS), such as body weight gain, diabetes type 2, insulin resistance, NAFLD and cardiovascular diseases [38,39]. Melatonin has been suggested to play a role in the pathogenesis of these estrogen deficiency-related metabolic disorders, as the levels of melatonin or its metabolite decrease in perimenopausal women and estrogen-deficient experimental animals [40-42]. Melatonin involvement is further corroborated by the observation that pinealectomized rats develop increased resistance to insulin, glucose intolerance and weight gain [43].

Melatonin supplementation therefore emerges as a potential therapeutic method for the treatment of menopause-related disorders, especially in women for whom

hormone replacement therapy (HRP) is not indicated due to potential to increase the formation of blood clots and embolism, strokes or tumors [44-47]. In fact, administration of melatonin in women has been shown to have positive effects on climacteric symptoms in perimenopausal and postmenopausal women [40,48,49].

The therapeutic potential of melatonin in suppressing the consequences of an estrogen deficiency has been intensively studied in ovariectomized (OVX) rodent [50-53]. However, it is still controversial the evidences on the ability of melatonin to prevent or treat excessive body weight gain and related metabolic disorders in OVX rats. Baxi et al. (2010) [51] reported that rats submitted to ovariectomy exhibit, after 6 weeks, increased body weight gain, insulin resistance, glucose intolerance and higher serum and tissue lipid content. The administration of melatonin for a period of 15 days (i.p) beginning 20 days after ovariectomy surgery reverses all ovariectomy-induced changes. Sanchez-Mateos et al., (2007) [54] did not observe significant changes in the blood levels of glucose, LDL, HDL, triglycerides and leptin in OVX rats, and only partial prevention of body weight gain and serum level of cholesterol was observed after the melatonin administration in drinking solution (20µg/ml) for 7 weeks.

In our previous study with OVX rats, we demonstrated, after 13 weeks of ovariectomy surgery, increased body weight gain, increased adiposity, liver steatosis, hyperglycemia, but not dyslipidaemia [55]. Subsequent oral administration of 10 mg/kg per day of melatonin for 3 weeks reverses liver steatosis and also liver damage induced by oxidative stress. However, there was not suppression of body weight gain, abdominal fat accumulation and hyperglycemia [55].

Several factors could have contributed to different responses of OVX rats to melatonin treatment, including age of rats, dose and duration of treatment, and mode of melatonin administration. In our previous study [55] melatonin was administered to OVX rats after the development of metabolic disturbances, similar to the protocol used by Baxi et al. (2010), [51] and melatonin was administered orally, rather by intraperitoneal injection or in beverage solution as used in other studies [50,54].

The most intriguing result revealed in our previous work is the ability of melatonin reduces liver steatosis and oxidative stress without reducing the body weight gain and adiposity. Regardless of body mass, it has been proposed that regional depositions of adipose tissue (visceral and/or subcutaneous) and adipocyte morphology (cell size; hypertrophy and/or hyperplasia) are the most important predictors of the risks for metabolic disturbances associated with obesity [56-59]. Another question arises as to

whether preventive administration of melatonin could be more effective in preventing the metabolic disturbances resulting from loss of estrogen.

Therefore, there are still many questions to be clarified in order to support the effectiveness of melatonin in replace the HRP, with regard to the ability of melatonin to correct or protect estrogen deficiency-related metabolic disorders, particularly the apparent lack of beneficial effects on body adiposity.

Thus, in the present work we extended our investigation on the protective effects of melatonin in OVX rats, by administering melatonin in a preventive manner, i.e., starting on the day of ovariectomy surgery and measuring several other factors related to body weight gain, including the contribution of total protein, mineral and lipids to body weight gain, the relative contribution of white and brown adipose tissues to body adiposity, the morphology of adipose tissues, hepatic and adipose tissue antioxidant system parameters, and liver lipid metabolism. Melatonin was administered orally at a dose of 10 mg/kg/day over a period of 16 weeks in OVX rats and also in SHAM-operated (CON rats). The study in CON rats will provide more information on the safety of melatonin for premenopausal women, considering the propositions of the utilization of melatonin for many therapeutic purposes.

Material and Methods

Material

Melatonin, adenosine diphosphate (ADP), 2,4-dinitrophenol (2,4-DNP), phenylmethylsulfonylfluoride (PMSF), reduced glutathione (GSH), sodium dodecyl sulfate (SDS), o-phthalaldehyde (OPT), 2',7 '-diclorofluorescein diacetate (DCFH-DA), 2',7'-dichlorofluorescein (DCF), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), succinate, L-malate, *N*,*N*,*N′*,*N′*-Tetramethyl-*p*-phenylenediamine (TMPD), β-Nicotinamide adenine dinucleotide (phosphate), reduced dipotassium salt (NAD[P]H) were purchased from Sigma Chemical Co. (St. Louis, USA). Kits from Gold Analisa® (Belo Horizonte, Brazil) were used to measure blood lipids and glucose levels. Sodium heparin was obtained from Roche (São Paulo, Brazil). RNAHolder® (BioAgency Biotechnology, Brazil). The liver perfusion apparatus and the rapid sampling apparatus for multiple-indicator dilution (MID) experiments were built in the workshops of the University of Maringá. Enzymes and coenzymes were purchased from the Sigma Chemical Company (St. Louis, MO, USA) as reagent-grade chemicals, salts, buffers, and substrates. [1-14C] palmitate acid

(25 mCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). The biodegradable counting scintillant solution (BCS®) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Animals

Three hundred and twelve female Wistar rats, weighing 130–160 g, were provided by the Central Biotery of the University of Maringa and were randomly assigned to one of two surgical procedures: sham-operated (CON) or bilateral ovariectomy (OVX). Animals undergoing OVX were anaesthetised with 10 mg xylazine + ketamine 50 mg/kg i.p. [55,60-62]. Thiopental injection was performed in combination with lidocaine (4 mg/kg) [63,64] to minimize local discomfort. After confirmation of general anesthesia through the absence of movement in response to a standardized tail-tightening stimulus, surgery was initiated. A trichotomy was performed followed by asepsis (10% iodopolividone) of the abdominal region for subsequent incision of 1 to 1.5 cm in the skin between the last rib and thigh, 1 cm from the midline, followed by an incision in the muscle layer, opening the peritoneal cavity for later removal of the ovaries, and ligation of the uterine tube. After removal of the ovaries, suture of the musculature and skin will be performed. The same process were performed on the opposite side. The CON and CON+MEL rats were undergo the same procedures; their ovaries were exposed but not removed. Immediately after completion of the surgical procedure, a single dose of Pentabiotic® (6000 IU/kg Benzylpenicillin Procaine, 12000 IU/kg Benzylpenicillin Benzatin, 6000 IU/kg Potassium Benzylpenicillin, 5 mg/kg Streptomycin Sulphate and 5mg/kg Dihydrostreptomycin) was administrated. The use of broad spectrum antibacterial soon after surgery is necessary as a prophylactic measure, since bacterial infections cause temperature changes that could directly interfere with our research data. We used an injection volume of 0.1 mL/kg. The corresponding doses of each of the active ingredients are listed above. Also administered as a single dose (im) is the injectable drug Banamine® (2.5 mg/kg), a non-steroidal anti-inflammatory drug that also has analgesic and antipyretic actions [65,66]. The animals were kept in clean boxes and observed until they are fully awake and able to return to the maintenance vivarium (2 hours of observation). The CON rats were subjected to the same procedures, but their ovaries were only exposed without removal. Throughout the experimental period (16 weeks), rats were housed in polypropylene cages (maximum of four animals per cage), fed a standard diet

and water *ad libitum*, and were kept in a sectorial biotery at a controlled temperature (25 °C) and 12 h light/dark cycle. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (CEUA no. 6631250815).

Treatment of the animals and collection of tissues and blood

The rats were divided into four groups: untreated, sham-operated rats (CON); sham-operated rats treated with daily doses of melatonin (CON+MEL); untreated, OVX rats (OVX); and OVX rats treated with daily doses of melatonin (OVX+MEL). The day after surgery, a dose of 10 mg/kg of melatonin dissolved in 0.9% saline solution was administered daily to CON+MEL and OVX+MEL rats by oesophageal gavage (final volume of 400 µL), over a period of 16 weeks. The rats in the CON and OVX groups received the same volume of 0.9% saline solution. Food intake and body weight (BW) were recorded throughout the trial period. On the day of the experiments, the animals were anaesthetised for euthanasia procedure for blood collection and removal of the liver, adipose tissues and uterus. In part of the experiments, the animals were euthanized by methods approved by CONCEA - National Council for Control of Animal Experimentation – [64]. According to these guidelines, barbiturates such as thiopental are one of the classes of injectable agents recommended for such procedure as they have the following advantages: fast, mild effect and minimal discomfort for animals. They are potent central nervous system (CNS) depressants whose effects are widely known and predictable. These characteristics reinforce the recommendation of barbiturates to be the best option for euthanizing terrestrial life animals, including rodents and small mammals. Thus, the animals were received an intraperitoneal dose of thiopental starting from three times the required dose (150 mg/kg) for anesthesia in rats (50 mg/kg), increasing the dose if necessary until death observation [55, 60-62,64]. Thiopental injection was performed in combination with lidocaine (4 mg/kg) [63,64] to minimize local discomfort. All animals were fasted for 12 hours prior to avoid regurgitation and aspiration of gastric contents. After euthanasia of the animals, the liver, uterus and adipose tissues will be collected for further experimental analysis.

In other experiments, animals were previously anesthetized with thiopental (50 mg/kg) associated with lidocaine (4 mg/kg) [55, 60-64] After confirmation of general anesthesia through the absence of movement in response to a standardized tail-tightening

stimulus, they were undergo cardiac puncture exsanguination [64]. Cardiac puncture is used to obtain large volumes of blood. After anesthesia, the animal should be placed on a flat surface in the right lateral position and the needle inserted at an angle of 10 to 30° above the abdomen, lateral to the xiphoid process [67]. It is a final collection because the animal is usually sacrificed after this type of procedure (hypovolemic shock). Thus, after this procedure, the liver, uterus and adipose tissues will be collected for further experimental analysis. The retroperitoneal, uterine, mesenteric and inguinal fat depots were weighed and expressed in g per 100 g of BW. The adiposity index was defined as the ratio of the sum of the weights of these fat depots per 100 g of BW [68]. To confirm the success of OVX, the uterus was also collected, weighed and expressed in g per 100 g of BW. Blood was collected from fasted rats by cardiac puncture to obtain serum and plasma. After blood collection, liver samples were removed, clamped in liquid nitrogen and then stored at -80 °C for subsequent measurements of the lipid content.

Carcass composition

Body composition was determined in the samples, included the content of the dry matter (DM), crude protein (CP), ether extract (EE) and mineral matter (MM) [69]. The frozen carcasses (n = 6 / treatment) were milled in an industrial meat mill with feathers, viscera, feet and head. The samples were homogenized and an average aliquot of 60g was weighed and taken to the lyophilizer for 36 hours for the determination of DM. Afterwards, they were ground in a ball 28 mill, to perform the analyzes of MM, CP and EE.

For determination of MM, aliquots of lyophilized samples were weighed in porcelain crucibles and oven-dried at 105 °C for 24 hours, and then taken to the muffle at 550 °C for hours, and by incineration the value of ashes were obtained. CP was obtained using the Kjeldahl nitrogen method (crude protein = nitrogen x 6.25). EE was obtained by extraction in Soxhlet extractor. The methodologies used for the analyzes were described in detail by [70].

Serum and plasma biochemical analysis

Estradiol and FSH were determined by chemiluminescence and electrochemiluminescence methods respectively in Laboratório Veterinário São Camilo,

Maringá PR, Brazil. For the procedure, the estrous cycle of the rats was evaluated in the period before the collection of the blood samples and on the day of collection. If the phase of the estral cycle identified was the right one the day before, blood was collected the next day (proestrus phase). The vaginal smear technique was performed to evaluate the estrous cycle by instilling 0.3 ml of saline (0.9% NaCl) and then the vaginal aspirate was collected. The fresh, uncolored slides were read for the differentiation of the cell types (epithelial cells, cornified and leucocytes) and, according to the proposition of each of the cell types, the phase of the cycle was determined, "Proestrus" is the one with the highest prevalence of epithelial cells [71], as well as the stage in which the highest secretion of sex steroid hormones occurs [72]. Glucose, triglycerides (TG), total cholesterol and high-density lipoprotein (HDL-cholesterol), were analysed in serum and was analysed in plasma by standard methods using assay kits (Gold Analisa®). Very-low-density lipoprotein (VLDL-cholesterol) levels were calculated using the Friedewald's equation [73], and low-density lipoprotein (LDL-cholesterol) levels were determined by subtracting HDL- and VLDL-cholesterol from total cholesterol. Plasma samples were assayed for the hepatic marker enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The results were expressed as international units (U) per liter, we used diagnostic kits from Gold Analisa Diagnóstica Ltda.

Adipose tissue histological analysis

Fed rats were euthanized and adipose tissue morphometric analyses samples from retroperitoneal, brown and inguinal adipose tissue were removed and fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform and 10% acetic acid) and embedded in histological paraffin (BIOTEC® Pinhais, PR, Brazil). Nonserial histological sections (5 µm thick) were obtained by using a Leica RM2145 semimotorized rotary microtome (Leica Biosystems, Richmond, USA) and stained with hematoxylin and eosin (H&E). The morphometric analyses were performed using digital images (TIFF 24-bit color, 2560x 1920 pixels) obtained with light microscopy (Olympus BX41, Tokyo, Japan) and a QColor 3 Olympus camera with a 20X objective. An adipocyte area of 1200 cells per group from 6 rats for each experimental group in retroperitoneal and inguinal fat was measured and analysed with the Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). Also, the areas of the adipocytes are determined, a frequency distribution is calculated removing any objects that fall below an area of 350 µm2, as

these cells may be a mixture of stromal vascular cells and adipocytes. Using the frequency function in Excel for calculate the frequency (=frequency (data_array, bins_array)),the distribution from 0 to 15,000 μm^2 in 500 increments is appropriate for most WAT depots, the size of the array bins may vary depending on the animal model. The number of total adipocytes within the distribution is subsequently calculated and used to convert the frequency to a percentage of total adipocytes counted. A comparison between two frequencies is made using a two-way ANOVA followed by a Bonferroni post hoc analysis. Frequency distribution in brown adipose tissue cannot be determined because there were many smaller cells than 350 μm^2. The images after the capture was analysed in the Image-Pro-Plus 4.5 to determine the area occupied by the lipid in the stained with hematoxylin and eosin (H&E) tissue sections which is related to the total area (area of lipid droplets in % of μm × 100−1 × total area of the image−1).

Determination of GSH contents in the liver homogenate and isolated mitochondria, retroperitoneal, inguinal and brown adipose tissue

GSH contents in liver and freeze-thaw disrupted mitochondria, retroperitoneal, inguinal and brown adipose tissue homogenates were measured fluorimetrically using OPT [74]. The samples were added in a medium containing 0.1 M phosphate buffer and 5.0 mM EDTA (pH 8.0). The reaction was started by adding 100 μL of OPT solution (1 mg/mL, in methanol). The fluorescent product GSH-OPT was measured fluorometrically (350 nm excitation and 420 nm emission) after an incubation period of 15 min at room temperature. The results were expressed as μg GSH/mg protein present in the homogenate or mitochondrial suspension.

Determination of TBARS and protein thiols groups in liver, retroperitoneal, inguinal and brown adipose tissue

The lipid peroxidation level was evaluated in the liver, retroperitoneal, inguinal and brown adipose tissue homogenates by thiobarbituric acid reactive substances (TBARS detection), predominantly malondialdehyde (MDA). Aliquots of liver and adipose tissues homogenates (1 mg protein) were added to 4 mL of a solution containing 0.4% SDS (sodium dodecyl sulfate), 7.5% acetic acid and 0.25% TBA (thiobarbituric acid). After 1h of incubation at 95 °C, the MDA-TBA complex was extracted with 4 mL

n-butanol/pyridine 15:1 (v/v) and the absorbance was determined at 532 nm (ε= 1.56 × 10^5 M^{-1} cm^{-1}). The amount of lipoperoxides was calculated from the standard curve prepared with 1,1',3,3'-tetraethoxypropane, and the values were expressed as nmol/mg protein [75] Thiol content was measured spectrophotometrically (412 nm) using DTNB (5'5'-dithiobis 2-nitrobenzoic acid) as previously described [76] and the contents were calculated using the ε value of 1.36×10^4 M^{-1}. cm^{-1} and expressed as nmol (mg protein)$^{-1}$.

Liver histological and histochemical analysis

Fed rats were euthanized and samples of the left liver lobe (n = 6-8) were fixed in Bouin solution (saturated solution of picric acid, 30% formalin and glacial acetic acid) for 24 hours and transferres to 70% ethanol. After dehydration with solutions of successively higher ethanol proportions (80%, 90%, 100%) and an additional 12 hours in xylol, the samples were embedded in histological paraffin (Biotec®, Pinhais, PR, Brazil). These preparations were cut into 5-µm-thick semiserial sections and stained with hematoxylin-eosin (HE) [77]. The semiserial sections were obtained by using a Leica RM2145 semi-motorized rotary microtome (Leica Biosystems, Wetzlar, Germany) and stained with H&E. Images were captured with 20× and 40× objectives (25 images/animal) with an Olympus BX41 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus Q-Color 3 camera coupled to a microcomputer (Q-Capture software). The NAFLD activity score (NAS) based on the extent of steatosis, the number of inflammatory loci per 200× field and the presence of ballooned cells [78]. For histochemical identification of lipid vesicles, Sudan III was prepared according to a standard method [77]. For Sudan III, liver samples were rapidly frozen in liquid nitrogen, stored at −80 °C and cut into 10-µmthick semi-serial histological sections using the Leica CM1850 cryostat (Leica Biosystems, Wetzlar, Germany). Images were captured with a 20× objective (50 images/animal) with the same equipment described for H&E and the Image-Pro-Plus 4.5 software was used to determine the area occupied by the lipid inclusions in the Sudan-stained tissue sections which is related to the total area (area of lipid inclusions in µm × 100−1 × total area of the image−1).

Determination of hepatic lipid content

The liver total lipid content was determined using the gravimetric method [79]. Lipids were extracted from homogenized liver samples (approximately 1.0 g) in a chloroform-methanol mixture (2:1). The results were expressed as g of total fat per 100 g of liver wet weight. The cholesterol and TG in the liver were determined after the suspension of fat in 200 µL of 2% Triton, followed by vortexing and heating at 55 °C. Cholesterol and TG contents in the suspension were measured by specific assay kits from Gold Analisa®.

Carbonylated protein contents in liver homogenates and disrupted mitochondria

The carbonylated protein content was determined by the method described by Guarnier et al. (2010), with modifications [80,81]. Liver samples (0.95 g) were homogenized in a medium containing 50 mM phosphate butter and 1 mM EDTA (pH 7.4), vortexed and centrifuged at 10.000×*g* for 15 min at 4 °C. The supernatant (5 mg protein/mL) was transferred to tubes with 1% streptomycin sulfate in 50 mM HEPES (9:1 v/v; pH 7.2), incubated for 15 min at room temperature and centrifuged at 6,000×*g* for 10 min. Next, 500 µL of the supernatant was transferred to Eppendorf tubes with 2,4-dinitrophenylhydrazine in 2 M HCl (1:1 v/v) and incubated in the dark for 1 h at room temperature with vortexing every 15 min. Blanks with supernatant and 2 M HCl were performed in parallel. Then, 500 µL of 20% trichlorocetic acid was added and centrifuged at 11.000×*g* for 10 min.The supernatant was discarded and the pellets were washed with 1.0 mL ethanol-ethyl acetate (1:1, v/v) and centrifuged 3 times at 10.000×*g* for 10 min to remove free reagent. The precipitated protein was dissolved in 1.0 mL of 6 M guanidine hydrochloride under vortexing, incubated for 15 min at 37 °C, and centrifuged at 10.000×*g* for 10 min without cooling. The carbonyl group content was calculated using the molar absorption coefficient for aliphatic hydrazones at 370 nm of 22.000 $M^{-1}\times cm^{-1}$ and expressed as nmol/mg protein in the homogenate.

Isolation of liver fractions for measurements parameters of cellular oxidative stress and activities of enzymes and determination of antioxidant enzymes in adipose tissues fractions

Liver mitochondria were isolated by differential centrifugation [82]. The livers were homogenized in a medium containing 200 mM mannitol, 76 mM sucrose, 0.2 mM EGTA, 0.1 mM PMSF, 1.0 mM Tris (pH 7.4) and 50 mg/100 mL fatty acid-free bovine serum albumin (BSA, w/v). The homogenate was fractionated via sequential centrifugation at 536×*g* and 7.080×*g* for 10 min each. The sediment of the last centrifugation was washed twice by suspension and centrifugation at 6.392×*g* and the final, mitochondrial pellet was suspended in isolation medium to yield a protein concentration of 60–90 mg/mL. Intact mitochondria were used to reactive oxygen species (ROS) generation. Freeze-thaw disrupted mitochondria were used for reduced glutathione (GSH) content measurements described early.

Mitochondria disrupted by sonication were used to measure the activities of the mitochondrial antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase 1 (GSH-Px), glutathione reductase (GSSG-red), in mitochondrial suspensions (50 mg of protein/mL) were diluted 1:10 (v/v) with 0.1 M KCI and 20 mM Tris-HCI (pH 7.4) and sonicated for 60s at maximum power. Aliquots of these fractions were centrifuged at 6,000×*g* for 10 min, to sediment intact mitochondria. The CAT activity was estimated in liver, retroperitoneal, inguinal and brown adipose tissue by measuring the change in absorbance at 240 nm using H_2O_2 as a substrate. The enzyme activity was expressed as H_2O_2 consumed/min×mg protein, using the molar extinction coefficient of H_2O_2 of 33.33 $M^{-1}\times cm^{-1}$ [83]

The supernatants were utilized as enzyme sources. The superoxide dismutase (SOD) activity was estimated in liver, retroperitoneal, inguinal and brown adipose tissue by its capacity to inhibit the pyrogallol autoxidation in alkaline medium at 420 nm [84]. The amount sufficient to inhibit the enzyme reaction by 50% (IC_{50}) was defined as 1 unit of SOD, and the results were expressed as U/mg protein. In liver and adipose tissues, GSH-Px3 was determined using H_2O_2 as a substrate in the presence of NADPH and GSH, by monitoring the decrease in absorbance due to NADPH oxidation at 340 nm over a period of 90s [85].

Total liver homogenates and adipose tissues (retroperitoneal, inguinal and brown) homogenates obtained from freeze-clamped liver or adipose tissues of overnight fasted rats were used to measure the soluble antioxidant enzyme activities catalase activity (CAT) described early, activity of glutathione peroxidase (GSH-Px) in the cytosolic fraction, the enzyme activity was expressed as nmol of NADPH oxidized/min× mg protein (ε, 6.220 $M^{-1}\times cm^{-1}$) described above, and glutathione reductase (GR) was determined in cytosolic fractions in liver, retroperitoneal, inguinal and brown adipose tissue by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH [86]. The samples were homogenized in ice-cold medium containing 200 mM mannitol, 75 mM sucrose, 2.0 mM Tris, 0.2 mM EGTA, 100 µM PMSF and 50 mg% fatty acid-free BSA (pH 7.4), using a Dounce homogenizer (20 mL of the homogenization buffer per 2.5 g of tissue).

To measure the glucose-6 phosphate dehydrogenase (G6PD) activity, livers and adipose tissues from fed animals were used and the samples were homogenized in a medium containing 0.1 M Tris/HCl buffer (pH 7.6) and 1 mM EDTA. The homogenate was centrifuged at 30.000×*g* for 15 min and the activity of G6PD was determined in the supernatant. The G6PD activity was estimated by the rate of increase in absorbance at 340 nm due to the conversion of $NADP^+$ to NADPH in the presence of glucose-6-phosphate, [87]. For the calculation, the molar extinction coefficient of NADPH (ε, 6.220 $M^{-1}\times cm^{-1}$) was used and the enzyme activity was expressed as nmol of reduced NADPH/min×mg protein.

Measurement of mitochondrial ROS generation

The mitochondrial ROS generation was monitored by the oxidation of DCFH-DA into a fluorescent compound, DCF. Intact mitochondria (1 mg protein/mL) were incubated with 5 mM glutamate, 5 mM malate and 150 µM DCFH-DA. After 3 min, 600 mmol/L ADP was added and fluorescence was recorded for 5 min (503 nm excitation, 529 nm emission). Mitochondrial ROS generation was expressed as ρmol DCF produced/min×mg mitochondrial protein [88].

Isolation of liver fractions for measurements of respiratory activities

Liver mitochondria were isolated by differential centrifugation [82]. Intact mitochondria were used to measure fatty acid β-oxidation capacity, respiration driven by oxidation of β-hydroxybutyrate and succinate. Freeze-thaw disrupted mitochondria were used for respiration driven by oxidation of NADH (NADH oxidase) and succinate (succinate oxidase).

Determination of the respiratory activity of isolated mitochondria

The oxygen consumption by the isolated liver mitochondria was determined by polarography, using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). For measurements of respiration driven by oxidation of Pyruvate, citrate,, β-hydroxybutyrate, α-ketoglutarate, succinate, fumarate and malate, intact mitochondria (0.6 to 1.0 mg/mL) were incubated in 2.0 mL of a reaction medium containing 0.25 M mannitol, 10 mM KCl, 5 mM potassium phosphate monobasic, 10 mM TRIS-HCl (pH 7.4), 0.2 mM EDTA and 50 mg% fatty-acid-free BSA (w/v) [82]. The reaction was initiated by the addition of α-ketoglutarate (12.5 mM), or β-hydroxybutyrate (12.5 mM), or citrate (12.5 mM), or fumarate (12.5 mM), or malate (12.5 mM), or pyruvate (12.5 mM) or succinate (12.5 mM). The rates of oxygen uptake were measured after the addition of 125 µM ADP (state III respiration), [89]. For measurement of NADH oxidase and succinate oxidase, freeze-thaw disrupted mitochondria (0.2 to 0.5 mg protein/mL) were incubated in 2.0 mL of a medium containing 20 mM Tris-HCL buffer (pH 7.4) at 37 °C [90]. The reaction was by addition of 1.0 mM NADH or 10 mM succinate. The rate of oxygen consumption was expressed initiated as nmol O_2 consumed per min per mg of mitochondrial protein (nmol/min×mg).For fatty acid β-oxidation, mitochondria (0.6 to 1.0 mg/mL) were incubated in a closed plexiglass chamber maintained under agitation and warmed at 37 ºC. The incubation medium (2.0 mL) contained 2.0 mM potassium phosphate monobasic, 0.1 mM EGTA, 130 mM potassium chloride, 5 mM magnesium chloride, 0.1 mM 2,4-dinitrophenol (DNP), 2.5 mM L-malate, 10 mM HEPES (pH 7.2) and 50 mg% fatty acid-free BSA [91]. The reactions were initiated by the addition of (a) 20 mM octanoyl-CoA + 2.0 mM L-carnitine, (b) 20 mM octanoyl-L-carnitine; (c) 20 mM palmitoyl-CoA + 2.0 mM L-carnitine, or (d) 20 mM palmitoyl-L-carnitine.

Liver Perfusion Experiments

The experiments were performed at the end of treatment (16 weeks). For the surgical procedure, animals were anesthetized with a mixture of sodium thiopental-lidocaine injection (50–4 mg/kg, intraperitoneally). To perform the hemoglobin-free, non-recirculating perfusion [92], the livers of overnight (12 h) fasted rats were used in all perfusion experiments. After cannulating the portal and cava veins, the liver was positioned in a plexiglass chamber. A constant flow was maintained by a peristaltic pump (Minipuls 3, Gilson, France) and adjusted to 34–36 mL/min, depending on liver weight. The perfusion fluid was the fatty acid (FA)-free bovine serum albumin Krebs/Henseleit-bicarbonate buffer (pH 7.4) saturated with a mixture of oxygen and carbon dioxide (95:5) using a membrane oxygenator with simultaneous temperature adjustment to 37 ◦C. The Krebs/Henseleit-bicarbonate buffer composition was as follows: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2.5 mM $CaCl_2$. Substrates were added to the perfusion fluid according to the experimental protocol. In experiments, the [1-14C] palmitate (0.1 mM) mixture was infused sodium salt complexed with FA-free bovine serum albumin (0.00375 mM). The use of [1-14C]-labelled FAs is effective at measuring the citric acid cycle (CAC) flux via acetyl-CoA labelling [93]. Consequently, $^{14}CO_2$ production can be regarded as a CAC activity indicator. The following exogenous substrates were also infused and solubilized in Krebs/Henseleit-bicarbonate perfusion fluid (pH 7.4) At the end of the perfusion experiments, the livers were removed and weighted to allow precise metabolic calculation.

Analytical Assays of Liver Perfusion Experiments

After stabilization of oxygen consumption, experiments were initiated and the effluent fluid samples were collected at 2 min intervals and analysed for their metabolic contents. Acetoacetate, and β-hydroxybutyrate were assayed by means of standard enzymatic procedures [94]. The oxygen concentration in the outflowing perfusate was continuously monitored by a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the perfusate output [93,95]. The carbon dioxide production from [1-14C] palmitate was measured by trapping $^{14}CO_2$ in phenylethylamine [96]. Radioactivity was measured by liquid scintillation spectroscopy. The following

scintillation solution was used: toluene/ethanol (2/1) containing 5 g/L, 2,5-diphenyloxazole and 0.15 g/L 2,2-p-phenylenebis(5-phenyloxazole). The metabolic rates were calculated from the input and output difference and the total flow rate and analysed in reference to the liver wet weight. All metabolic fluxes rat livers were expressed as µmol × (min × g wet liver)−1.

Protein content

The protein contents of the liver fractions were determined using the method of [97] using BSA as standard, the protein content was assayed in all fractions with the Folin phenol reagent and determined spectrophotometrically at 700 nm.

Statistical analysis

The data in the figures and tables are expressed as the mean ± standard error (SE). The statistical differences between the four experimental groups were evaluated by a two-way analysis of variance. Significant differences between the means were identified using Newman-Keuls test. The results are expressed as probability values (*P*). $P \leq 0.05$ was adopted as a criterion of significance. Statistical analysis was conducted using the GraphPAD Software programs.

Results

Biometrical parameters

The body weight (BW) of rats of four groups of animals increased progressively during the 16 weeks of treatment as shown in Figure 1 A. After the 3rd week the body weight of OVX rats was higher than the CON rats, which reached at the end of treatment a 26% higher body weight gain (Fig. 1A, inserted graph). Melatonin administration to OVX rats (OVX+MEL) did not modify the pattern of body weight gain during all periods of treatment when compared with that one of untreated-OVX rats, so that the body weight gain remained (22.8%) higher than the CON rats at the 16th week. Contrasting with this lack of effect on OVX rats (OVX+MEL), melatonin reduced the body weight gain of control rats (CON+MEL) which was significant after the 7th week of the treatment. The effective body weight gain at the terminus of treatment was 21 % lower than the CON

rats and 55.5 % relative to OVX+MEL rats (Fig. 1A, inserted graph). Fig. 1B revealed that despite OVX and OVX+MEL rats had a higher body weight gain than the CON rats, the cumulative and mean daily food consumption were not different among them. A correlation was found, however, in the CON+MEL rats with a 13% reduction in the mean daily food consumption compared to CON rats.

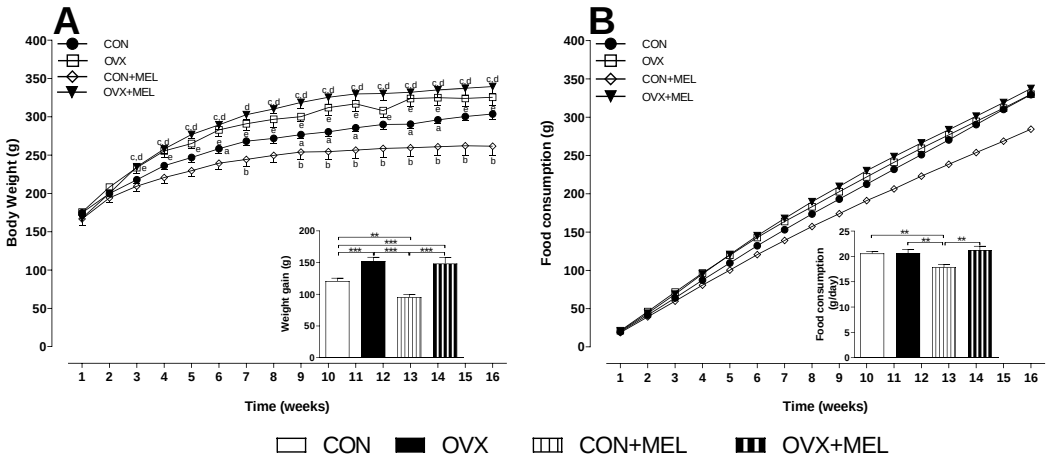

Fig. 01. Biometrical parameters of sham-operated (CON) rats, ovariectomized (OVX) rats, melatonin-treated control rats (CON+MEL) and melatonin-treated OVX rats (OVX+MEL). **(A)** Body weight of the four groups of rats along the experimental period expressed in g (n = 8). The inserted graph in (A) represents the body weight gain over the entire experimental period (16 weeks). **(B)** Cumulative food consumption expressed in g (n = 8). The inserted graph in **(B)** represents the mean daily consumption of food over the (16 weeks). The vertical bars represent the standard errors (SE). The asterisks indicate significant differences between the values as revealed by two-way ANOVA following by Newman Keuls post-test ($P < 0.05$): [a]CON *vs.* OVX; [b]CON *vs.* CON+MEL; [c]CON *vs.* OVX+MEL; [d]CON+MEL *vs.*OVX+MEL and [e]CON+MEL *vs.* OVX.

Carcass composition

Figure 2 shows the composition of the whole body carcass of the rats relatives to their content of the dry matter (DM, panel A), mineral matter (MM, panel B), crude protein (CP, panel C), ether extract (EE, panel D) and the total humidity (water content) expressed as the percentage of the dry matter, which was 5% lower in OVX rats compared with the CON rats. Melatonin treatment did not modify this parameter in both CON+MEL rats and in OVX+MEL rats, the latter ones exhibiting 7% lower values than the CON rats. No significant differences were found in the percentage of mineral matter, among the four groups (B). The percentage of crude protein was also not different in CON and OVX rats

(C), and melatonin treatment increased by 5% the CP in control rats (CON+MEL) compared to the CON group, but without changes in ovariectomized rats (OVX+MEL).

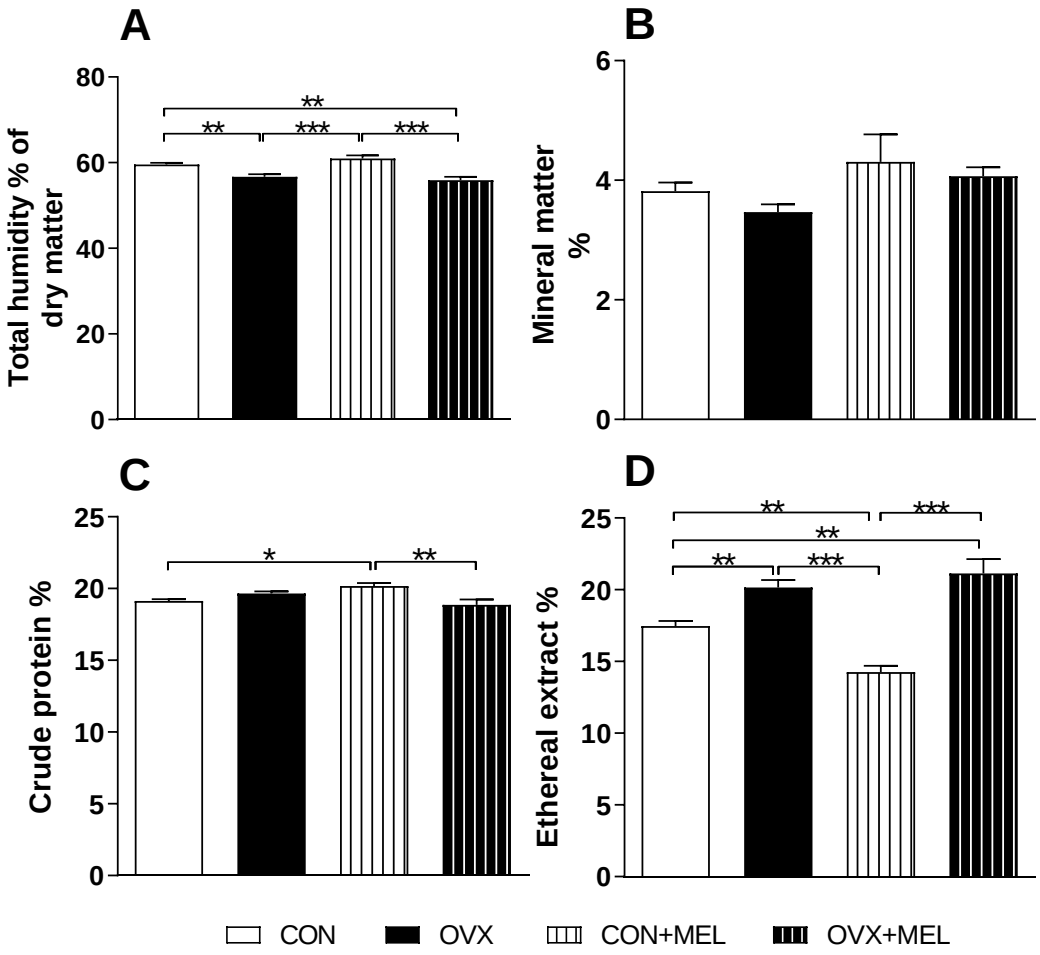

Fig. 02. Carcass composition of sham-operated (CON) rats, ovariectomized (OVX) rats, melatonin-treated control rats (CON+MEL) and melatonin-treated OVX rats (OVX+MEL). The results represent the means for the (A) Total humidity % of dry matter (n = 6), (B) The mineral matter expressed in % (n = 6), (C) The crude protein expressed in % (n = 6), (D) The ethereal extract expressed in % (n = 6). The vertical bars represent the standard errors (SE). The asterisks indicate significant differences between the values as revealed by two-way ANOVA following by Newman Keuls post-test. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. All parameters were measured at the end of treatment (16 weeks).

The most significant differences among the groups were found in the percentage of ethereal extract (EE). OVX rats exhibited a 13% increase in EE relative to values of CON rats (D) and this extract remained elevated in OVX+MEL rats (+21% relative to CON rats). Melatonin induced, however, a decrease of 18 % in the percentage of ethereal extract (EE) in CON+MEL rats. These results in EE suggested that the corporal lipid

contents may account for most of the differences found among the groups in the body weight gain (Figure 1A). Therefore, we have examined separately the adipose tissues located in different regions of the body: the uterine, mesenteric, retroperitoneal and inguinal fat (Figure 3).

The adiposity index calculated as the sum of the retroperitoneal, uterine, mesenteric, and inguinal fat weights relative to 100 g of body weight (Fig 3A) indicated a 17% higher index in OVX rats compared to CON rats that remained elevated (+18%) in melatonin treated rats (OVX+MEL). These results are in accordance with those found in the percentage of corporal EE (Fig. 2D). Such correlation was not found, however, in the CON+MEL rats, as the adiposity index was not reduced by melatonin treatment. The analysis of each adipose tissue, however, revealed that ovariectomy and/or MEL treatment exerted distinct influence on specific adipose tissues. Whereas the weight of uterine (B) and retroperitoneal (C) adipose tissues were not affected by both ovariectomy or MEL treatment, significant modifications were found in the mesenteric (D) and inguinal fat (E).

The weight of mesenteric fat of OVX rats was similar to that of CON rats and melatonin treatment induced a 25% reduction only in the control condition (CON+MEL). The weight of inguinal fat was substantially increased by ovariectomy, with a 86% higher weight in comparison with the CON rats. Melatonin administration did not suppress this increase in inguinal fat in OVX+MEL rats, not affecting also the inguinal fat in CON+MEL rats, which remained in a similar weight of the CON rats.

Figure 3 also shows the influence of ovariectomy and MEL treatment on the uterus weight (F). As expected, a pronounced uterine atrophy was observed in OVX rats, as indicated by a reduction of 85% in uterus weight in comparison with the CON rats. Melatonin also reduced by 17% the weight of uterus in CON+MEL rats when compared with CON rats.

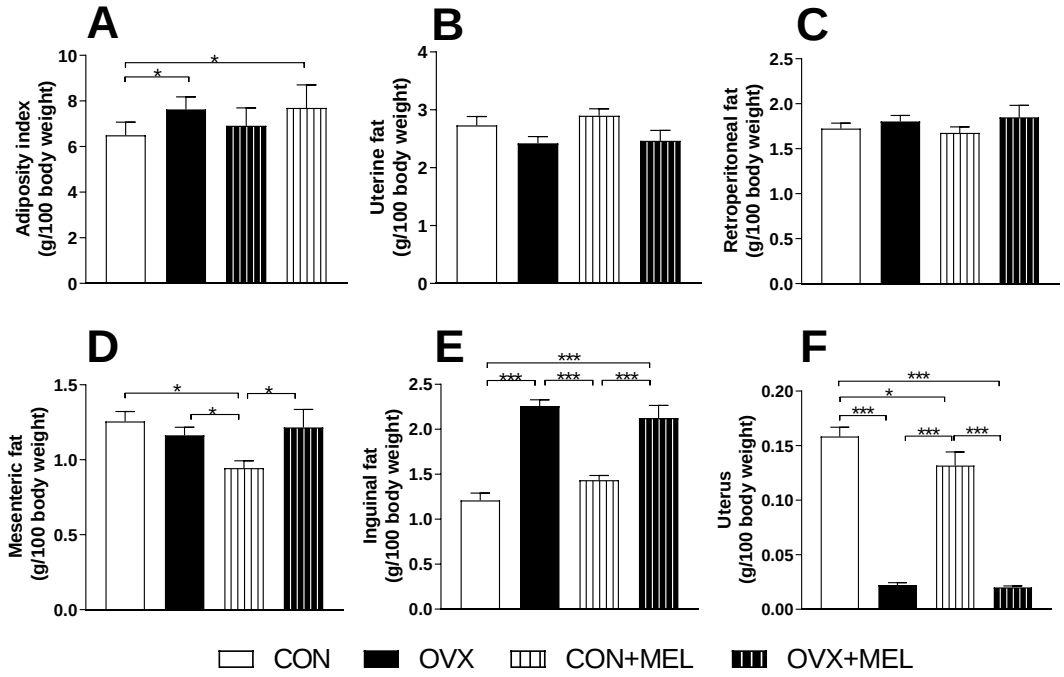

Fig. 03. General features of sham-operated (CON) rats, ovariectomized (OVX) rats, melatonin-treated control rats (CON+MEL) and melatonin-treated OVX rats (OVX+MEL). The results represent the means for the (A) The adiposity index was calculated from the sum of the retroperitoneal, uterine, mesenteric, and inguinal fat weights, which was related to g/100 g BW (n = 6 – 10), (B) The uterine fat was expressed in g/100 g of body weight (BW) (n = 6 – 9), (C) The mesenteric fat was expressed in g/100 g of body weight (BW) (n = 6 – 9), (D) The retroperitoneal fat was expressed in g/100 g of body weight (BW) (n = 8 – 11), (E) The inguinal fat depots was expressed in g/100 g of body weight (BW) (n = 6 – 9), (F) Uterus was expressed in g/100 body weight (BW) (n = 8 – 10). The vertical bars represent the standard errors (SE). The asterisks indicate significant differences between the values as revealed by two-way ANOVA using the Newman-Keuls post-test: * $P < 0.05$; ** $P < 0.01$ and *** $P< 0.001$. All parameters were measured at the end of treatment (16 weeks).

Serum and plasma biochemical analyses

The biochemical analysis of several blood parameters are presented in table 01. The success of the ovariectomy surgery was confirmed by a decrease of 75% in the serum estradiol level compared to CON rats. Melatonin administration did not change significantly the estradiol level in OVX+MEL rats when compared with OVX rats, but a strong reduction was observed in the control rats: the mean levels of estradiol in CON+MEL reduced by 53% when compared to CON rats.

No significant differences were observed among the four groups of rats in the blood levels of FSH, glucose, triglycerides, total cholesterol and its lipoproteins. A significant difference was found for markers of hepatic functions: the activities of AST and ALT were, respectively, 46% and 28% higher in the blood of OVX rats when compared with CON rats, and melatonin treatment induced a partial reversion in the levels of both enzymes. Melatonin did not change the levels of AST and ALT in CON+MEL rats.

Table. 01. Biochemical analysis of blood from sham-operated (CON), ovariectomized (OVX), melatonin-treated control (CON+MEL) and melatonin-treated OVX rats (OVX+MEL). Estradiol (pg/mL), FSH (µUI/mL), plasma glucose, serum triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol expressed as mg/dL, AST and ALT expressed as U/L were measured in blood samples obtained from rats fasted overnight, analysed by standard methods.

	CON	**OVX**	**CON+MEL**	**OVX+MEL**
Estradiol	32.67 ± 7.53	8.000 ± 1.15[a]	14.00 ± 1.53[b]	15.33 ± 3.48[c]
FSH	0.140 ± 0.04	0.140 ± 0.02	0.120 ± 0.02	0.140 ± 0.04
Glucose	90.99 ± 4.39	90.49 ± 3.81	76.65 ± 4.57	87.06 ± 5.78
Triglyceride	31.73 ± .290	33.70 ± 2.57	31.73 ± 4.88	34.36 ± 2.77
Total cholesterol	66.94 ± 4.42	70.66 ± 2.21	64.84 ± 6.09	63.83 ± 5.53
HDL-cholesterol	31.78 ± 2.53	30.66 ± 2.23	22.63 ± 2.82	26.13 ± 3.35
LDL-cholesterol	31.27 ± 2.32	33.30 ± 2.21	37.98 ± 2.96	32.45 ± 2.39
VLDL-cholesterol	5.908 ± 0.42	6.383 ± 0.40	5.738 ± 0.37	6.440 ± 0.42
AST	59.45 ± 3.22	86.68 ± 4.52[a]	69.63 ± 3.94	72.92 ± 4.10[d]
ALT	28.79 ± 2.20	36.76 ± 2.42[a]	32.21 ± 2.86	26.04 ± 1.10[d]

Values are expressed as the mean ± SE of 6-10 animals per group. The letters indicate the statistical significance as revealed by two-way ANOVA following by Newman Keuls post-test ($P < 0.05$): [a]OVX *vs.* CON; [b]CON *vs.* CON+MEL; [c]CON *vs.* OVX+MEL; [d]OVX *vs.* OVX+MEL.

Adipose tissue morphometric analysis

The finding that the accumulation of adipose tissues situated in different regions of the body responded differently to ovariectomy and/or melatonin treatment led us to examine in details the morphology of adipocytes, the redox oxidative status and the

antioxidant enzymes in three representative adipose tissues: a visceral white adipose tissue (VAT): the retroperitoneal fat; a subcutaneous white adipose tissues (SAT): inguinal fat, and a brown adipose tissue (BAT): a interscapular fat.

Figure 4 (panels A to J) shows representative histological images of retroperitoneal and inguinal fat from the four groups of animals. Despite lack of alteration in retroperitoneal fat mass by ovariectomy or melatonin treatment, as revealed in Figure 3C, the images of the retroperitoneal fat show an increase in the size of adipocytes in OVX rats (Fig. 4B) compared to those in the CON rats (Fig. 4A). Melatonin administration in these rats (OVX+MEL) and also in CON rats (CON+MEL) led to histological images very similar to those in the CON rats. The analysis of the distribution of adipocyte according to their areas (Fig. 4E) confirmed the hypertrophy of the retroperitoneal fat in OVX rats and the beneficial effect of melatonin. The curve of frequency of adipocytes in OVX rats was shifted toward the higher area values relative to the curve of the CON rats. The curve of OVX+MEL rats approached to the CON curve. Melatonin did not modify the distribution of adipocyte according with their area in CON+MEL rats when compared with CON rats. The comparison of the mean area of adipocytes in retroperitoneal fat (Fig. 4F) revealed a 26 % increase in OVX rats, which was totally suppressed by melatonin (OVX+MEL rats).

Despite substantial increase in the total weight of inguinal fat in OVX rats (Fig. 3E), the histological image of this tissue (Fig. 4H) revealed that the size of adipocytes was very similar to that of the CON rats (Fig. 4G). A prevalence of the adipocytes with reduced size was evidenced in rats treated with melatonin, not only in OVX+MEL (Fig. 4J) but also in CON+MEL (Fig. 4I), as confirmed by the curves of distribution of the adipocytes according with their areas (Fig. 4K). The curves of distribution of frequency of CON and OVX rats were not significantly different, but the administration of melatonin in both OVX+MEL and CON+MEL rats induced a significant shift of the curves toward the left side of the diagram, a change that was more accentuated in CON+MEL rats. The increase in the frequency of adipocytes with lower areas was confirmed by a reduction of 19% in the mean area of the adipocytes in OVX+MEL and 40% in CON+MEL when compared with their respective untreated controls (Fig. 4L).

The representative histological images of brown adipose tissues (BAT) by using the soluble dye HE (Fig. 5) revealed that OVX (B), CON+MEL (C) and OVX+MEL (D) groups had apparently more lipid inclusions than in the BAT of CON rats (A). The average lipid droplet size shown in Figure 5E revealed a 35% higher lipid inclusion in

OVX rats than in CON rats, but in the groups treated with melatonin (OVX+MEL or CON+MEL) the mean values were not statistically different when compared with their respective untreated controls.

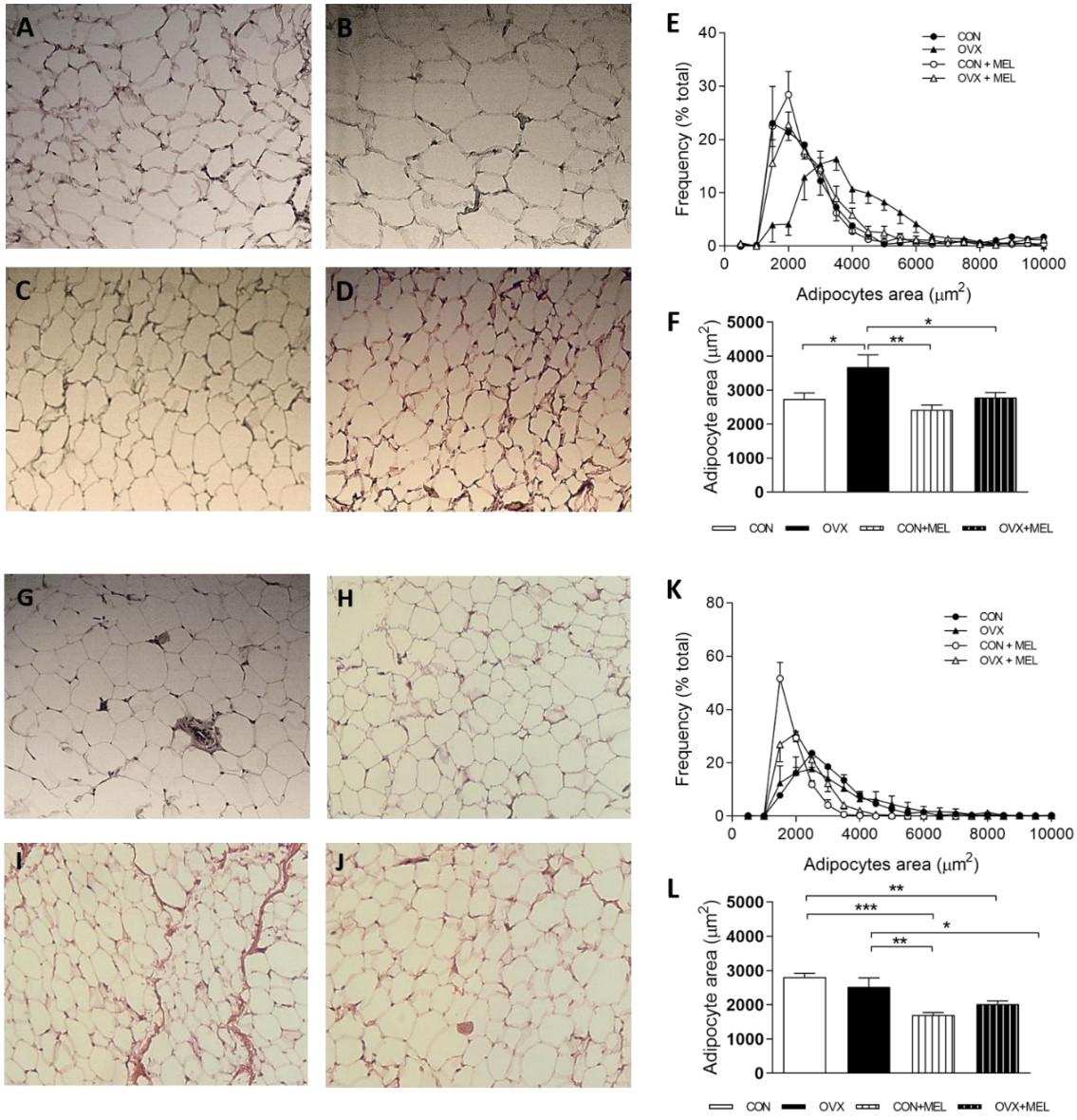

Fig. 04. Representative photomicrography images showing adipose tissue stained with hematoxylin-eosin. Retroperitoneal adipose tissue of CON (A) OVX (B), CON+MEL (C) and OVX+MEL (D) rats and inguinal adipose tissue of CON (G), OVX (H) CON+MEL, (I) and OVX+MEL, (J) rats. (E) Frequency distribution of retroperitoneal adipocyte sizes in percentage of total cells; (F) mean retroperitoneal adipocyte area, expressed as μm^2. Frequency distribution of inguinal adipocyte sizes; (L) inguinal adipocyte area (L) of four groups of rats. All data are expressed as the means SEM from 4-5 rats from each experimental group. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman-Keuls post-test: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

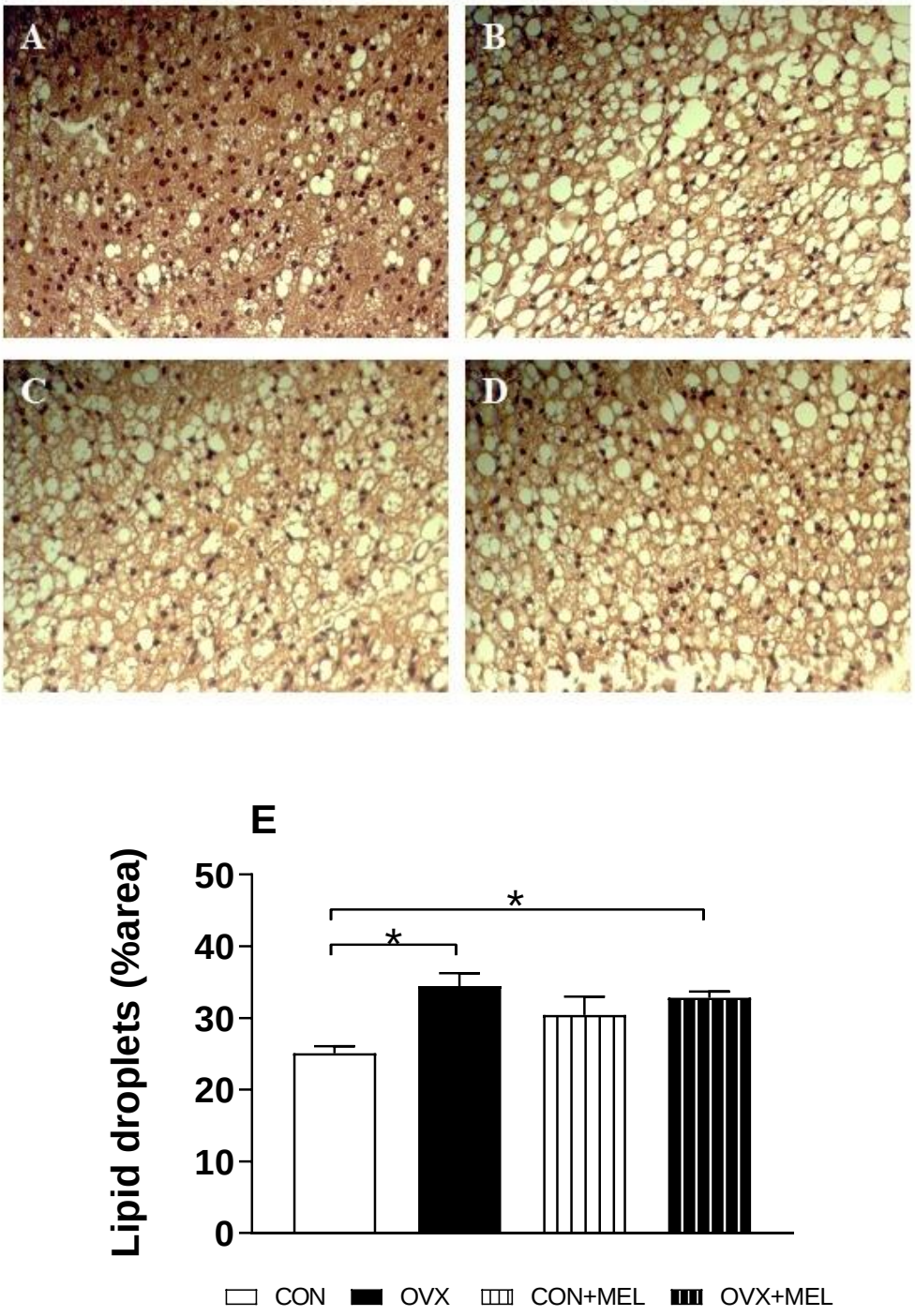

Fig. 05. Photomicrography images of brown adipose tissue stained with hematoxylin-eosin. Adipose tissue morphometric analysis in brown fat of sham-operated (CON), ovariectomized (OVX), melatonin-treated control (CON+MEL) and melatonin-treated OVX (OVX+MEL) rats. Quantitative analyses of adipocyte areas are shown in sequence. All data are expressed as the means SE from 3-4 rats from each experimental group. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman-Keuls post-test: * $P < 0.05$.

Parameters of oxidative stress in retroperitoneal, inguinal and brown fat tissues

The redox status of the adipose tissues was evaluated by measurement the levels of TBARS, GSH and thiol content in the soluble fraction of the cytosol of total homogenate. Figure 06 reveals that the ovariectomy did not induce significant modifications in any of these parameters in retroperitoneal, inguinal or brown fat, as the means values of OVX rats were very similar from those of CON rats. The only exception was the content of GSH in inguinal fat that was 23% reduced in OVX rats (Fig. 6E), an effect that was suppressed by melatonin administration (OVX+MEL rats). No other parameter in the three adipose tissues was modified by melatonin administration in OVX rats (OVX+MEL).

In control rats (CON+MEL), however, melatonin induced a 29% reduction in the TBARS content in retroperitoneal fat (Fig. 6A) and in inguinal fat it was found a decrease of 41% in the GSH content (Fig. 6E) and an increase of 40% in protein thiols content (Fig. 6H) when compared with the values of their respective untreated groups. None of the biomarkers of oxidative stress in the brown adipose tissue was altered neither by ovariectomy nor by melatonin treatment in both CON and OVX rats (Fig. 6 C, F, I).

The activities of antioxidant enzymes in retroperitoneal, inguinal and brown fat tissues are shown in Figure 7. Similar to what was found for oxidative stress indicators, in the ovariectomized rats (OVX) had significant changes only in the inguinal fat: the GR and G6PD activities were 44% (Fig. 7K) and 41% reduced (Fig. 7N), respectively, when compared with the values of the CON rats. These results were in accordance with the reduced content of GSH found in this tissue (Fig. 6E). Also, in agreement with the restoration of the GSH content melatonin increased the activities of the two antioxidant enzymes GR and G6PD in OVX+MEL rats (Figs. 7K, N) to equivalent levels of those of CON rats. In inguinal fat, melatonin increased the CAT activity in OVX+MEL rats to a value 37% higher than that of OVX untreated rats (Fig. 7 E).

Although the activities of GSH-Px (panel G) and GSSG-red (panel J) were not modified by ovariectomy, the administration of melatonin in these rats (OVX+MEL) induced an increment in the activity of both enzymes in approximately 36% (OVX versus OVX+MEL). Melatonin administration in the control rats (CON+MEL) did not exert significant changes in any of the assayed enzymes in the three adipose tissues. Again, none of the assayed enzymes in the brown adipose tissue was affected by ovariectomy or melatonin treatment (panels C, F, I, L, O).

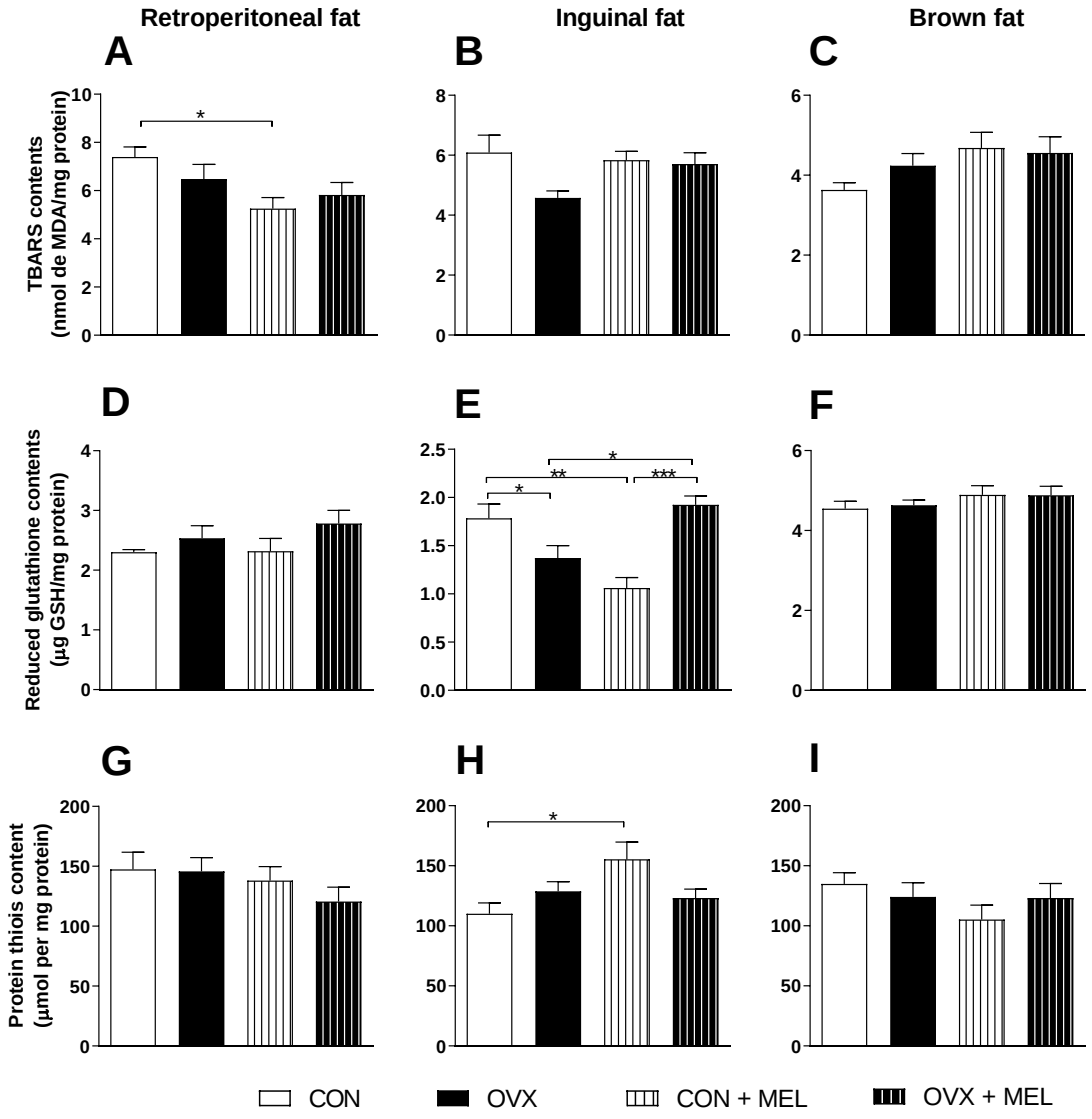

Fig. 06. Effects of melatonin on parameters of adipose tissue oxidative stress condition. Adipose tissue homogenates or cytosolic fractions were prepared as described in the Material and Methods section for the measurements of: TBARS contents in retroperitoneal, inguinal and brown fat; the reduced glutathione content (GSH) in retroperitoneal fat; inguinal and brown fat, and protein thiols content in retroperitoneal, inguinal and brown fat. of 5 to 8 individual experiments. Panels A, D and G show the TBARS content, GSH content and protein thiols content in retroperitoneal fat, panels B, E and H indicated the TBARS content, GSH content and protein thiols content in inguinal fat and panels C, F and I indicated the TBARS content, GSH content and protein thiols content in brown fat. The values are expressed as the mean and the vertical bars represent the SE. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman-Keuls post-test : * $P< 0.05$, ** $P< 0.01$, *** $P< 0.01$.

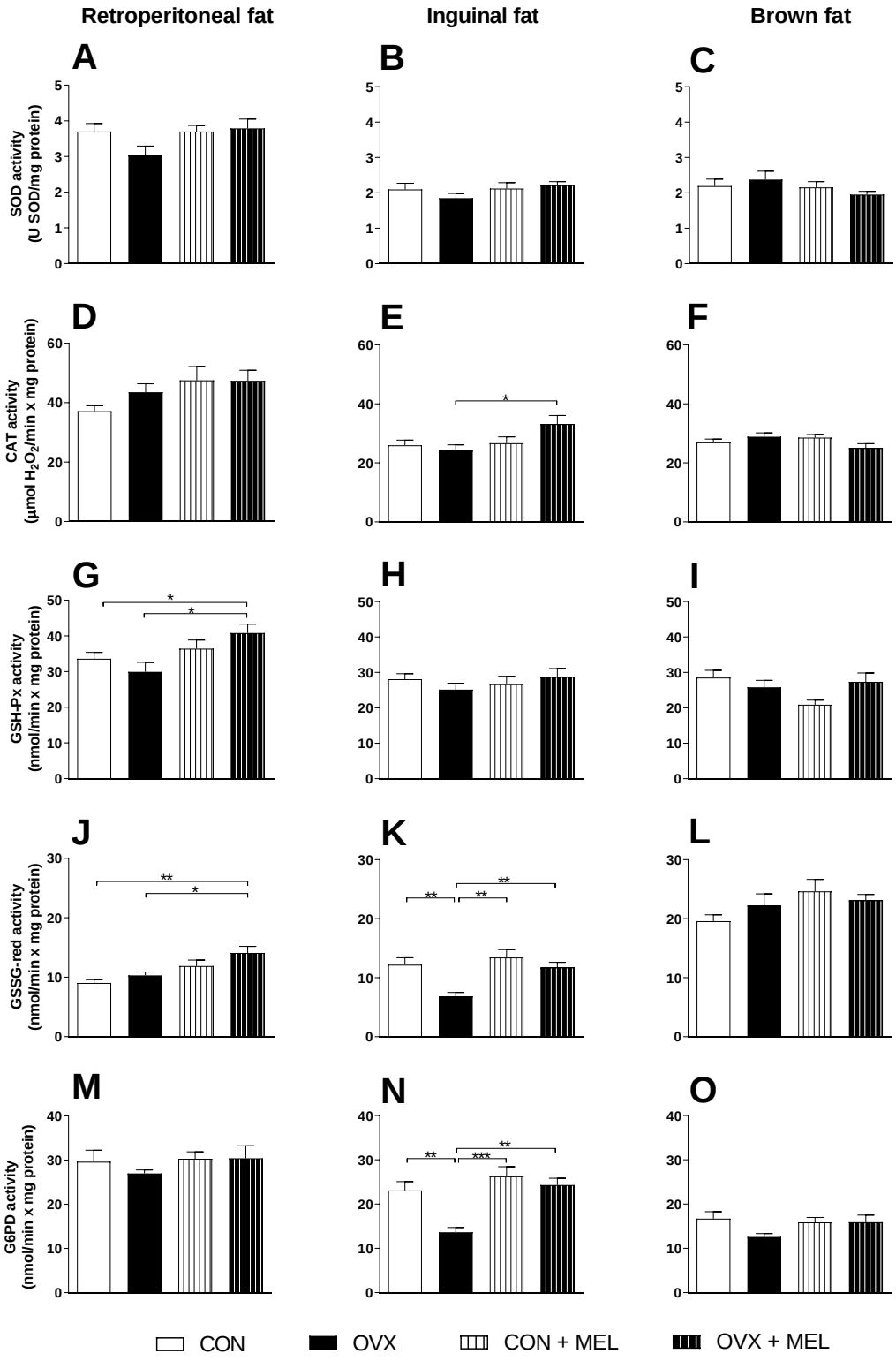

Fig. 07. Effects of melatonin on oxidative stress parameters in retroperitoneal, inguinal and brown fat cytosolic fractions. Panels A, D, G, J and M are respectively the activities of SOD, CAT, GSH-Px1, GSSG-red and G6PD in retroperitoneal fat. Panels B, E, H, K and N are the SOD, CAT, GPx1, GR and G6PD activities in inguinal fat and panels C, F, I, L and O shows the SOD, CAT, GSH-Px1, GSSG-red and G6PD activities in brown adipose tissue. The values are expressed as the means of 4 to 7 individual experiments.

The vertical bars represent the SE. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman Keuls post-test: * $P< 0.05$; ** $P< 0.01$ and *** $P< 0.001$.

Liver histological analysis, h*epatic lipid content and total cholesterol and triglycerides in liver*

H&E staining of liver section from CON (Fig. 8A), OVX (Fig. 8B), CON+MEL (Fig. 8C) and OVX+MEL (Fig. 8D) groups revealed preserved cell organization and shape on, with *chords of hepatocytes* and size and shape apparently intact in all groups. The livers of OVX rats had areas containing hepatocytes with varying degrees of cytoplasmic vacuolation, and in few sections one loci of cellular infiltrate was observed. The hepatocytes of CON, CON+MEL and OVX+MEL groups had rare cytoplasmic vacuolation. Both, the ovariectomy and treatment with melatonin induced a slightly higher prevalence of ballooned cells on the OVX, CON+MEL and OVX+MEL groups, when compared with CON. As a result of these observations, the value for NAS based on Kleiner et al. [78] was 0.92 for CON group and a few higher value on CON+MEL (1.28) OVX (1.31) and OVX+MEL (1.14) groups. According to NAS score that ranges from 0 to 8 and sum occurrence of steatosis, lobular inflammation and hepatocellular ballooning, none of the groups has features of NASH (NAS>or=5).

The supposition that the vacuoles in H&E images were lipid vesicles was confirmed by histological analyses using the fat-soluble dye Sudan III, which were used for calculation of the mean value of the area occupied by lipid inclusions (Fig. 8E). The livers of OVX rats had a lipid area approximately four-fold higher than CON rats. Melatonin treatment suppressed this lipid accumulation, as the area of lipid droplets approached that of CON rats. Melatonin did not modify this parameter in CON+MEL rats.

The quantification of lipids confirmed the high content of fat in livers from OVX rats (Fig. 8F). Among the total lipids, triglycerides and not total cholesterol accounted to lipid accumulation in OVX rats, as indicated by Figs 8G and 8H. The total lipid and TG content was 26 and 35% higher in OVX than in CON rats, respectively, and the treatment of OVX rats with melatonin reduced both total lipid (Fig. 8F) and TG (Fig. 8G) contents to values very similar to those of CON rats. The cholesterol levels were not modified by ovariectomy or melatonin treatment (Fig. 8H).

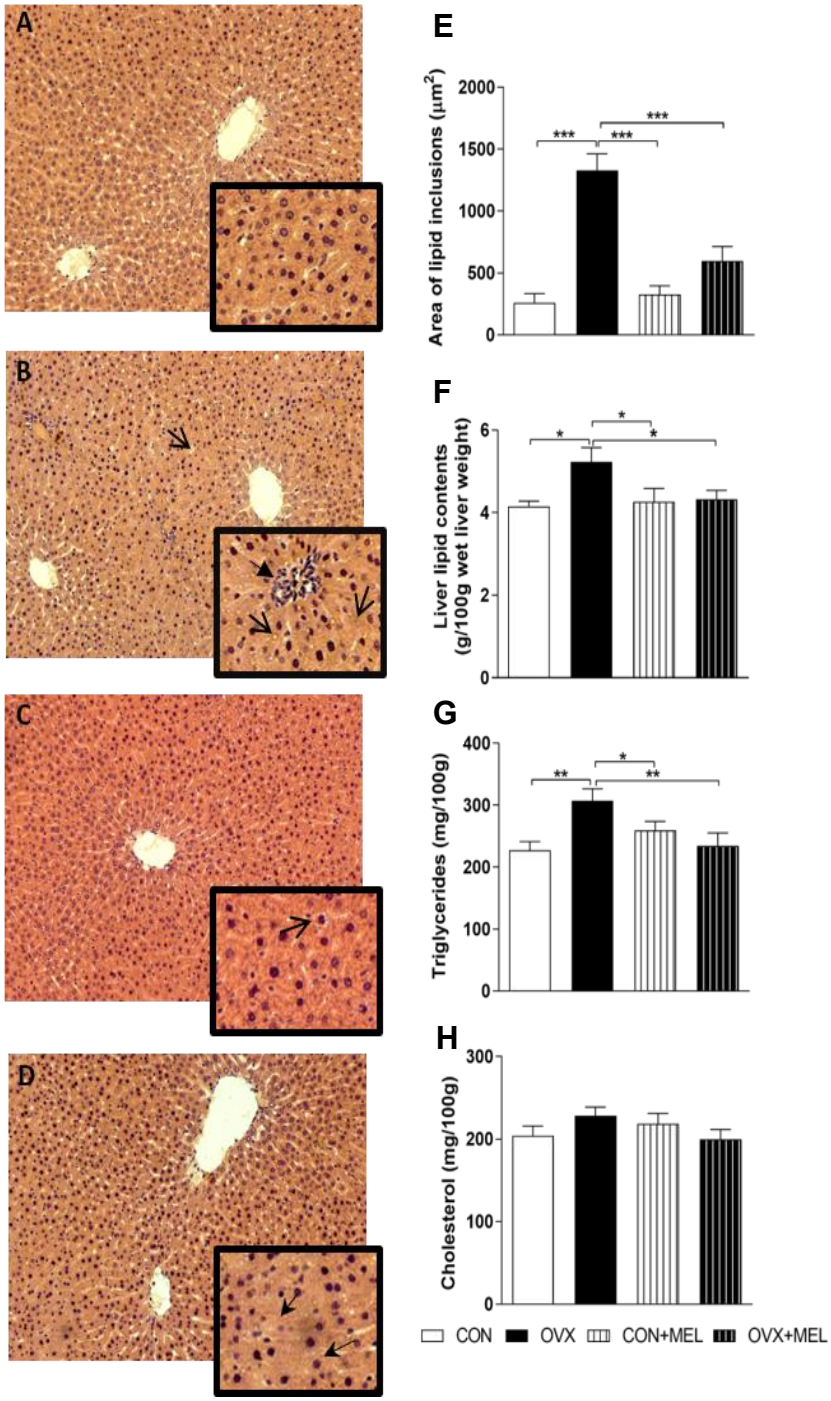

Fig. 08. HE staining of CON (A), OVX (B), CON+MEL (C) and OVX+MEL rats (D), Sudan III (E), total area of the lipid inclusions of CON, CON+MEL, OVX and OVX+MEL rats (F), Contents of total lipids, () cholesterol content, and triacylglycerol (I) in the livers of the four groups of the animals. The results represent the means of 7-The vertical bars represent the standard errors (SE). The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Neuman Keuls post-test. * $P< 0.05$. In the right, images are displayed with 200x magnification. Each value is mean ± SE of 5-6 independent animals. The arrows indicate lipid inclusions (⟶), hepatocellular balloonin (⟶) and lobular infiltration (⟶).

Parameters of oxidative stress and antioxidant enzyme activity in liver homogenates and cytosolic fractions

In the liver homogenates or cytosolic fractions of OVX rats, the biomarkers of oxidative stress TBARS (Fig. 9A) and protein thiols (Fig. 9D) were not modified, but the protein carbonyl groups content (Fig. 9B) was 39% increased and the GSH content (Fig. 9C) was 19% lower when compared to CON rats. In rats treated with melatonin (OVX+MEL) these alterations were suppressed and the values of both parameters were restored to similar values of those found for CON rats. Despite ovariectomy has not altered the livers TBARS content, the administration of melatonin in these rats (OVX+MEL) increase the TBARS content by 47% when compared with the livers of CON rats (Fig. 9A).

Among the assayed antioxidant enzymes, only the activities of SOD (Fig. 9E) and G6PD (Fig. 9I) were altered in OVX rats with a decrease of 25% and 35%, respectively compared to CON rats. Melatonin was not able to suppress the inhibition of both enzymes as demonstrated to values found in OVX+MEL rats with remained above the values of the CON rats and similar to those of OVX rats.

The administration of melatonin in control rats (CON+MEL) reduced the activity of G6PD to a mean value 37% above of that of CON rats (Fig. 9I)

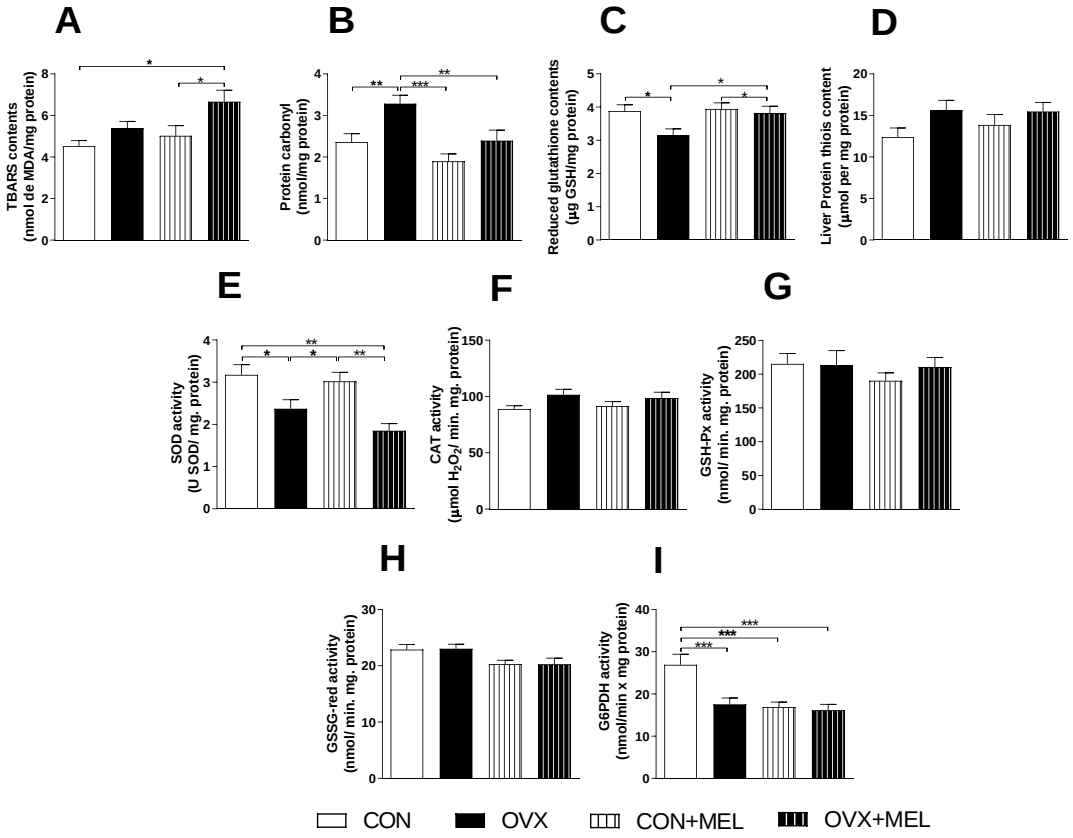

Fig. 09. Effects of melatonin on parameters of liver oxidative status. Liver homogenates or cytosolic fractions were prepared as described in the Material and Methods section for the measurements of: (A) TBARS contents 5 to 7 individual experiments. (B) the protein carbonyl content 5 to 8 individual experiments; (C) the reduced glutathione content 7 to 12 individual experiments and (D) Liver protein thiols content 4 to 6 individual experiments (E) SOD activity; (F) CAT activity; (G) GSH-Px1 activity; (H) GSSG-red activity; (I) G6PD activity 5-8 individual experiments. The values are expressed as the mean and the vertical bars represent the SE. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman Keuls post-test: * $P< 0.05$, ** $P< 0.01$.

Parameters of oxidative stress and antioxidant enzyme activity in isolated mitochondria

Mitochondria isolated from OVX rats did not exhibit alterations in the ROS generation (Fig. 10A) and on the GSH content (Fig. 10C) when compared with mitochondria from CON rats. However, similar to that was found in the liver homogenates, the content of carbonylated protein was 56% higher in mitochondria from OVX rats (Fig. 10B). This alteration was completely suppressed by melatonin treatment as mitochondria from OVX+MEL rats had similar values of those of CON rats. The administration of melatonin in control rats (CON+MEL) did not alter the total amount of ROS (Fig. 10A), carbonylated protein (Fig. 10A) or the content of GSH (Fig. 10C).

Differently from cytosolic SOD, the activity of mitochondrial SOD was not altered in OVX rats (Fig. 10D). No change was also found in the activities of mitochondrial CAT (Fig. 10E), GR (Fig. 10F) and GSH-Px (Fig. 10G) when compared to results found in the mitochondria from CON rats.

The treatment with melatonin did not induce changes in enzyme activities in mitochondria from both control (CON+MEL) or ovariectomized rats (OVX+MEL) when compared with their respective control groups. The only exception was a decrease of 22% in the CAT activity in OVX+MEL compared to CON, but without statistical significance compared to OVX rat (Fig. 10E).

Respiratory activity in isolated mitochondria

The dysfunction of mitochondria may play a pivotal role in the liver steatosis as they are involved in β-oxidation of free fatty acids and in the cellular redox homeostasis. To evaluate whether melatonin exerts beneficial effect of mitochondrial functions, several parameters of energy transduction and fatty acid oxidation were evaluated in liver mitochondria isolated from the four groups. Table 1 shows the rate of respiration of intact coupled mitochondria driven by different substrates and in the presence of ADP (state III respiration), the maximal activities of three segments of respiratory chain measured in disrupted mitochondria, and the fatty acid oxidation-driven respiration measured in uncoupled mitochondria using FAs as an acyl-CoA-derivatives (octanoyl-CoA and palmitoyl-CoA) in the presence of carnitine, and also the octanoyl L-carnitina and palmitoyl-L-Carnitine.

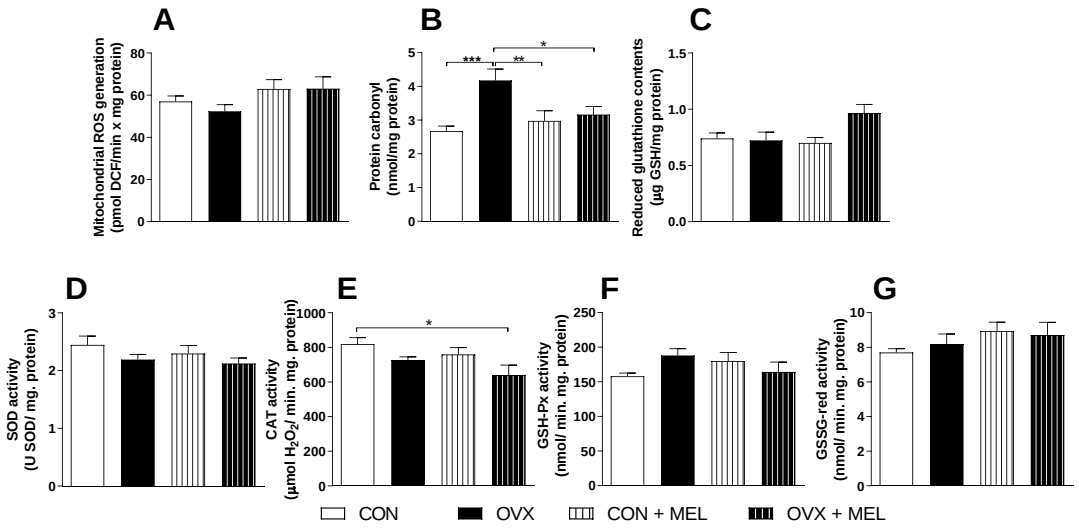

Fig.10. Effects of melatonin on parameters of oxidative stress in isolated mitochondria. Mitochondrial fractions were prepared as described in the section of Material and Methods for the measurements of: (A) mitochondrial H_2O_2 generation, (B) the protein carbonyl content 5 to 11 individual experiments; (C) the reduced glutathione content of 7 to 12 individual experiments, (D) SOD activity; (E) CAT activity; (F) GSH-GPx1 activity; (G) GSSG-red activity. The values are expressed as the mean and the vertical bars represent the SE. The asterisks indicate significant differences between the values as revealed by two-way ANOVA using Newman Keuls post-test: * $P< 0.05$.

The rates of oxygen consumption coupled to ADP phosphorylation (state III respiration) were not different among the mitochondria isolated from the four groups of rats irrespective on the substrate oxidized: citrate, β-hydroxybutyrate, α-cetoglutarate, succinate, fumarate and malate. The activities of NADH oxidase, succinate-oxidase and the respiration coupled to TMPD-ascorbate in freeze-thawing disrupted mitochondria were also not different among the groups.

Significant changes were induced, however, by melatonin treatment in the FA-driven respiration in uncoupled mitochondria. Despite the similar rates of FA-induced respiration in mitochondria from the CON and OVX rats, irrespective of fatty acid oxidized, a stimulus was found in melatonin-treated rats in both ovariectomized (OVX+MEL) and control (CON+MEL) rats. When compared to mitochondria from OVX rats, the rates of fatty acid driven respiration were stimulated in OVX+MEL as follows: Octanoyl-CoA +34%; Octanoyl-L-Carnitine +34%; Palmitoyl-CoA +24%, and Palmitoyl-L-Carnitine +43%. In mitochondria from CON+MEL rats, a significant increase was found in the oxidation of Octanoyl-CoA +21% Octanoyl-L-Carnitine + 21% and Palmitoyl-L-Carnitine +34% relative to rates of CON rats.

Table. 02. Rates of oxygen consumption driven by different substrates in the mitochondria isolated from livers of control (CON), ovariectomized (OVX), melatonin-treated control (CON+MEL) and melatonin-treated OVX (OVX+MEL) rats. Oxygen consumption was measured using a Clark-type oxygen electrode as described in the Materials and Methods section. The rates of fatty acid oxidation, state III, the activities of NADH-oxidase and succinate oxidase, rates of mitochondrial respiration with the Octanoyl-CoA + L-carnitine, the liver Octanoyl lcarnitine, the Palmytoyl-CoA + L-carnitine and the Palmytoylcarnitinewere expressed as nmol O_2 /min× mg protein.

		Intact Mitochondria		
		State III		
	CON	**OVX**	**CON+MEL**	**OVX+MEL**
Pyruvate	11.8 ± 0.78	11.1± 0.57	11.1 ± 0.49	11.5 ± 0.84
Citrate	11.4 ± 0.32	13.1 ± 0.59	13.3 ± 0.97	14.4 ± 0.89[b]
β-hydroxybutyrate	29.9 ± 0.56	27.6 ± 1.92	27.5 ± 2.51	31.1 ± 2.13
α-ketoglutarate	20.6 ± 0.63	20.9 ± 0.64	19.9 ± 0.76	20.5 ± 1.55
Succinate	88.0 ± 3.81	80.2 ± 5.03	82.5 ± 5.54	94.6 ± 7.93
Fumarate	6.73 ± 0.19	6.30 ± 0.13	6.70 ± 0.34	7.00 ± 0.41
Malate	9.08 ± 0.19	9.88 ± 0.29	10.1 ± 0.19	9.90 ± 0.63
	Freeze-thawing disrupted mitochondria			
NADH-oxidase	27.2 ± 3.05	30.5 ± 3.10	29.2 ± 3.25	30.9 ± 2.78
Succinate-oxidase	39.5 ± 3.75	40.0 ± 2.23	36.3 ± 4.87	43.8 ± 3.13
TMPD ascorbate	71.1 ± 3.46	73.0 ± 5.79	73.6 ± 5.29	80.5 ± 5.30
	Uncoupled mitochondria			
Octanoyl-CoA+L-carnitine	31.2 ± 1.83	30.7 ± 1.87	43.2 ± 2.59[a]	39.6 ± 1.18[bc]
Octanoyllcarnitine	37.0 ± 1.32	33.3 ± 2.49	44.9 ± 1.49[a]	44.7 ± 1.11[bc]
Palmytoyl-CoA+L-carnitine	37.5 ± 1.56	38.3 ± 2.16	38.5 ± 0.65[a]	47.7 ± 0.63[bc]
Palmytoyllcarnitine	34.3 ± 1.96	33.6 ± 2.34	46.2 ± 3.24[a]	48.0 ± 2.71[bc]

Values are expressed as the mean ± SEM of 5-8 animals per group. The letters indicate the statistical significances as revealed by two-way ANOVA using Newman-Keuls post-test ($P < 0.05$): [a]CON *vs.* CON+MEL; [b].CON *vs.* OVX+MEL; [c].OVX *vs.* OVX+MEL.

Oxygen consumption, $^{14}CO_2$ production and ketogenesis in intact livers

The rates of fatty acid-driven respiration in isolated mitochondria shown in table 2 reflect the maximal activities of all enzymatic complexes involved in fatty acids oxidation as mitochondria were uncoupled and thus, the rate of reoxidation of NADH and $FADH_2$ generated by fatty acids β-oxidation and cycle of citric acid were not under

control of electrochemical gradient. Since melatonin stimulated this parameter in both CON and OVX rats we performed a series of experiments by using intact perfused rat livers, an experimental model that allow the evaluation of exogenous fatty acid oxidation under the steady-state conditions. The results were shown in Figures 11. A perfusion fluid containing palmitate plus traces of palmitate labeled with the ^{14}C isotope was infused in the livers from fasted rats and the effluent perfusates were collected by measurements of the ketone bodies: acetoacetate and β-hydroxybutyrate, the oxygen consumption and the CO_2 production. The release of $^{14}CO_2$ in the effluent liquid reflects the fraction of fatty acid oxidized by β-oxidation pathway and citric acid cycle; the production of acetoacetate and β-hydroxybutyrate sum indicates the ketogenic activity, and the β-hydroxybutyrate/acetoacetate ratio indicates the mitochondrial redox state [82].

The infusion of the exogenous palmitate (0.1 mM) plus traces of [1-^{14}C[palmitate) in perfused rats livers caused an increment in ketone bodies, β-hydroxybutyrate/acetoacetate ratio, CO_2 production and oxygen consumption in the four groups of animals (Fig. 11). Significant differences among the groups during the whole period of experimental series were observed only in the steady-state rates of ketone bodies production in the control rats. The ketogenesis in livers from CON+MEL (Figure 11B) was approximately 55% higher than in CON rats (Fig. 11 A) in the whole period of palmitate infusion.

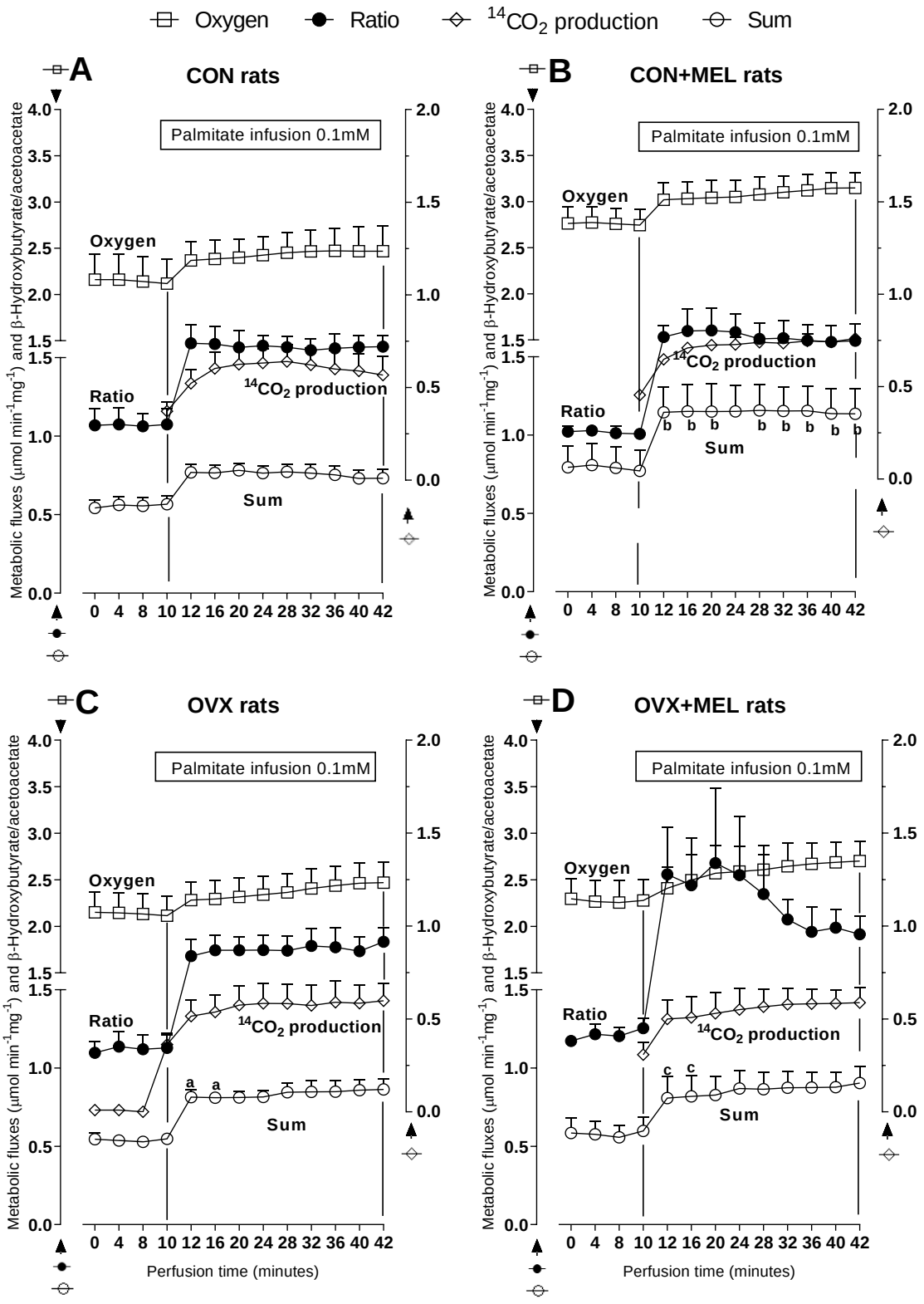

Fig. 11. Time courses of the changes in CO_2 and ketone bodies production: sum, the ratio betweenβ-hydroxybutyrate and acetoacetate and oxygen consumption in the presence of exogenously added substrates in livers of CON and CON+MEL (A,B) and OVX and OVX+MEL (C,D) rats. Livers of overnight (12-h) fasted rats were perfused as described in Materials and methods. Exogenous substrates were infused at the time intervals indicated by horizontal bars. Each experimental point is the mean ± standard error of 4–5

experiments. All parameters were measured at the terminus of treatment (16 weeks). The vertical bars represent the standard errors (SE). The letters indicate significant differences between the values as revealed by two-way ANOVA using Newman-Keuls post-test ($P < 0.05$): [a]CON *vs*. OVX; [b]CON *vs*.CON+MEL; [c]CON *vs*.OVX+MEL.

Discussion

The ovariectomy surgery in 45- day-old rats induced after 16 weeks, as expected, reduction in the serum levels of estradiol, uterus atrophy and some components of the metabolic syndrome (MetS) [98], including increased body weight gain, increased adiposity, hepatic and adipose tissues oxidative stress and hepatic steatosis. OVX rats did not develop dyslipidaemia, hyperglycemia and insulin resistance, found by other authors in OVX rats [51,53,99]. Clinical trials in humans has demonstrated that nearly 20% to 50% of women in menopause and pos-menopause period developed one or more components of MetS including obesity, insulin resistance, NAFLD, diabetes type 2, hypertension or cardiovascular diseases. The other ones remain healthy despite loss of estrogen protection and ageing [100-102]. The apparent resistance of OVX in developing severe metabolic disturbances suggested that OVX rats, under our experimental conditions, can be reproducing the metabolic status of post-menopausal women whom does not develop most of METS components. Alternatively, the OVX rats could be in an early phase of progression to more severe metabolic disturbances. The characterization of the metabolic status of these OVX rats emerge, thus, as a valuable model to understand the pathogenesis of estrogen deficiency-induced diseases and the protective effect of melatonin under this specific metabolic status.

We had previously demonstrated that melatonin administered after 13 weeks of ovariectomy surgery and over a period of 3 weeks suppress liver steatosis but does not protect OVX rats against body weight gain and adiposity [55]. In this study, we have found that even this long-term and preventive treatment, the body weight gain and adiposity index were not suppressed by melatonin, despite preventing liver steatosis. The composition of body carcass relatives to their content of the dry matter, crude protein, ether extract and mineral matter confirmed that the higher body weight gain of OVX rats was due to lipid accumulation, as ether extract percentage was increased in both OVX and OVX+MEL rats compared with CON rats. However, the detailed analysis of the mass and the adipocytes morphology of adipose tissues located in different regions of the body,

among visceral and subcutaneous fat, shown that ovariectomy affected differently these adipose tissues and that melatonin treatment, despite not reducing the whole adiposity induced beneficial effects in adipocytes morphology.

The histological images demonstrated a significant hypertrophy of retroperitoneal adipocytes in OVX rats. There was not increase in retroperitoneal total mass, indicating lack of tissue expansion. The number of cells was probably reduced to accommodate the hypertrophic cells. A distinct phenomenon occurred in inguinal fat. The amount of inguinal fat was substantially increased in OVX rats with adipocytes exhibiting similar size to those of the control rats, thus evidencing tissues hyperplasia. The hypertrophy of retroperitoneal fat and hyperplasia of inguinal fat probably represent an adaptation of adipose tissues to store larger amounts of lipids generated in OVX rats.

Increased adiposity is linked to increased risk of metabolic diseases including insulin resistance, diabetes type 2 and cardiovascular diseases [58,103,104]. Several studies have reported, however, that a subgroup of overweigh individuals are classified as "metabolically healthy obese", as they remain insulin-sensitive and exhibit normal physiology [105,106]. Such healthy overweigh individuals have increased subcutaneous adiposity but reduced expansion of visceral adipose tissue [107]. It has been suggested that the expansion of adipose tissue by enhanced adipogenesis not only distributes excess fat between newly differentiated adipocytes, but also reduces the number of hypertrophic adipocytes that secrete inflammatory cytokines, and thus, the metabolic disturbances initiated by adipose tissues inflammation are minimized [108,109]. Therefore, humans with hyperplastic subcutaneous fat have better blood glucose, insulin and lipid profiles than subjects with hypertrophic fat [56].

OVX rats under our experimental exhibit, thus, features of metabolically healthy adiposity. The finding that adipose tissues redistributed the lipids among different fat depots, avoiding release of fatty acids to circulation was supported by the lack of alterations in the plasmatic levels of triacylglycerol and lipoproteins in OVX rats. Under these conditions, ectopic accumulation of lipid is unlikely. Hence, the hepatic lipid accumulation seen in OVX rats seem not to be consequence of increased lipid supply to liver derived from lipolysis in adipose tissues, neither from the diet, as food intake of OVX rats was not increased.

Liver steatosis in OVX rats was confirmed by histological analysis and the quantifications revealed that lipid into vesicles was mainly triacylglicerides. The H&E images of the livers showed that despite steatosis there was no extensive hepatocyte

ballooning or cell infiltrations and fibrosis was absent in the livers of OVX rats, resulting in a NAS score above 1.0 in OVX rats. Considering that the progression of steatosis to steatohepatitis (NASH) is associated to NAS score above 5.0 [78] the steatosis in OVX rats can be considered benign.

Hepatic steatosis in OVX rats was probably a direct consequence of oestrogen deficiency. The liver is one of the most responsive organs to circulating estrogens effects are mediated by the classic mechanism involving the binding of E2 to steroid nuclear hormone receptors, Estrogen Receptor alpha (ERα) or Estrogen Receptor beta (ERβ) [110]. The most important estrogen-regulated genes in the liver are involved in TG, cholesterol and fatty acid metabolism [111]. Estrogens reduce the expression lipogenic enzymes, such as acetyl-CoA carboxylase 1 (ACC-1) and fatty acid synthase (FAS), by negatively controlling the sterol response element-binding protein-1 (SREBP-1) [112,113]. Regulation of lipogenesis has been shown to be mediated by ERα, since its suppression increases the expression of lipid synthesis genes [114]. Estrogen also promotes the oxidation of FA in the liver by activating peroxisome proliferator-activated receptor-α (PPAR-α) which increases the genes necessary for fatty acid absorption and β-fatty acid oxidation [111,115]. An increased oxygen uptake and ATP production in the liver associated with changes in liver UCP_2 expression has also been proposed [116]. However, in our study, we found no changes in fatty acid oxidation or respiratory activity in OVX rats. The maximum respiratory chain activity and the maximum fatty acid oxidation measured in isolated mitochondria were not altered in OVX rats. In addition, under steady-state condition of perfusion livers, oxygen uptake associated with fatty acid oxidation in OVX rats was also no different from CON rats. These findings indicate that the main factor contributing to the accumulation of TG in OVX rats under our experimental conditions was an increase in lipogenesis without a significant contribution of a reduction in fatty acid oxidation.

Estrogens also act as an antioxidant hormone [50,117] positively modulating the activities of antioxidant enzymes [118,119] and also by their direct antioxidant reaction linked to ring A hydroxyl group of the molecule. Our data confirmed that estrogen deficiency due to ovariectomy results in increased oxidative stress in the liver and adipose tissues. Livers from OVX rats exhibited a reduced content of glutathione, a non-enzymatic reducing agent that protects and prevents oxidative stress, an also an increased content of carbonylated protein in both liver homogenate and isolated mitochondria. The non-enzymatic addition of carbonyl groups in proteins is a consequence of the excess of

ROS which causes irreversible damage to proteins [120]. We have not found in our current study an increase in the levels of ROS in mitochondria from OVX rats. The generation of ROS, however, can also occur in microsomes or associated to other pro-oxidant enzymatic pathways [121] and an imbalance between ROS production and antioxidant defense systems can lead to oxidative damage to cellular macromolecules. The main antioxidant enzymes are the cytosolic and mitochondrial SOD which catalyses the dismutation of superoxide (O_2^-) into hydrogen peroxide (H_2O_2), thereby maintaining a low concentration of (O_2^-), while CAT and GPx remove the H_2O_2 generated from the SOD reaction [122]. GSH-Px catalyses both the reductive detoxification of peroxides and, in some situations, the catalytic transfer of oxidation equivalents to another protein. GSH-Px requires reduced GSH for activity [123] and GSSG-red reduces the oxidised GSSG using the reductive force of NADPH to restore GSH levels [124]. NADPH is mainly generated by the pentose phosphate pathway in the first step catalized by G6PD. Among these enzymes, it was found a decreased in the activities of cytosolic SOD and G6PD in OVX rats when compared to livers from CON rats. Both alterations have contributed to higher content of carbonylated protein and the lower GSH content in the livers of OVX rats.

The evaluation on oxidative stress parameters in adipose tissues, revealed that inguinal fat was more sensitive to estrogen loss than retroperitoneal and brown adipose tissues, as the latter ones showed no signs of cellular oxidative stress.The reduced GSH content found in inguinal fat was probably consequence of reduction in the GSSG-red and G6PD activities [124]. The change in GSH metabolism was not associated with increased TBARS or carbonyl protein content, indicating no extensive oxidative damage.

Oxidative stress in hypertrophic adipocytes plays a key role in the pathogenesis of the inflammatory process, which leads to metabolic complications, especially insulin resistance [125]. The finding that retroperitoneal fat, despite having hypertrophic adipocytes, did not exhibit oxidative stress is in line with our earlier assumption that OVX mice in our experimental condition have a "metabolically healthy obesity". We cannot rule out, however, that the metabolic status of adipose tissues and liver from OVX rats may be a transient phase for the onset of more severe metabolic complications, as mentioned earlier. In the liver, particularly, have been widely demonstrated that oxidative stress and lipid peroxidation are implicated in the pathophysiological mechanisms by which NAFLD can progress to more severe liver diseases, including steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [121,126].

In our search for the elucidation of the mechanisms by which melatonin can protect the postmenopausal women against the development of metabolic diseases, we have found that the preventive administration of melatonin in ovariectomized rats (OVX+MEL) rats did not reduce the body weight gain or adiposity index but modified the morphology of the adipocytes. The hypertrophy of retroperitoneal adipocytes seen in OVX rats was totally suppressed by melatonin. Curiously, melatonin treatment did not modify the inguinal fat mass in OVX + MEL but the frequency of adipocytes with smaller size was increased and the oxidative stress seen in OVX rats was totally suppressed, as indicated by restoration of GSH levels and GSSG-red and G6PD activities. Both effects, suppression of retroperitoneal fat hypertrophy and the preservation of inguinal fat hyperplasia without alteration in total adipose tissues mass indicated that in the presence of melatonin occurred a redistribution of lipid in different fat depots, reducing excessive lipid overload and, thus, eliminating the possibility of cells progressing to inflammation and related abnormalities [58,103,104].

In women during and after menopause, the transition from lower subcutaneous adipose tissues to increased visceral adiposity [127] is known to increase the incidence of overweight, obesity, and related metabolic disorders, including insulin resistance [128]. Our results suggested that these risks are minimized by melatonin treatment, which, demonstrated the ability to reduce visceral adipose tissue hypertrophy and preserve subcutaneous fat hyperplasia.

Beneficial effects of melatonin were also found in livers with complete suppression of steatosis and oxidative stress in OVX+MEL rats. Melatonin was able to suppress ROS-induced protein attack in both liver and mitochondria homogenates as there was a reduction in carbonylated protein. However, melatonin did not prevent inhibition of SOD and G6PD activity observed in OVX rats, clearly contrasting with restoration of GSSG-red and G6PD activities in inguinal fat by melatonin treatment. Probably, melatonin acted by different mechanisms in the liver and inguinal fat.

Melatonin has been shown to directly eliminate free radicals (by neutralizing hydroxyl radicals ($^{\bullet}OH$) or indirectly, by activating glutathione synthesis and antioxidant enzyme activity [19,129]. In the livers, the direct antioxidant effect of melatonin seems to predominate since there was no restoration of SOD and G6PD activities.

To exert direct antioxidant effect, it seems essential that melatonin has access to intracellular compartments. The highest concentration of melatonin after exogenous administration has reported to be in serum, liver and bile, and due its amphiphilic

characteristic, most melatonin accumulates in cell membranes, mitochondria, nucleus and cytosol [6,130,131,132]. Direct free radical detoxification by melatonin is not receptor-dependent, a finding that explains the ability of melatonin to protect different organisms and tissues from oxidative stress, regardless of factors leading to excess of ROS generation, such as drugs, aging, ischemia-reperfusion injury, lipotoxicity [133]. The beneficial effects of melatonin in OVX mice, especially in the liver, were probably due to the replacement of antioxidant action of estrogen.

In adipose tissues, however, melatonin actions appear to be receptor-mediated, as indicated by the modification of GSSG-red and G6PD activities in inguinal fat in OVX+MEL rats and also increased GSH-Px and GSSG-red activities in retroperitoneal fat induced by melatonin in both OVX+MEL and CON+MEL rats, even in absence of cellular oxidative damage.

Peripherical tissues respond to melatonin by interacting with one or more of its receptors, that is MT_1, MT_2 and MT_3, [13,17], nuclear retinoid Z receptors (RZR) [134] and intracellular calmodulin [135]. Alternatively, by acting on MT1 receptors in the suprachiasmatic nucleus neurons, melatonin can activate sympathetic drive in various peripheral tissues, including WAT, BA, liver and adrenal medulla [136].

Song et al. (2017) reported that melatonin inhibits NaF-induced mitochondrial ROS production by increasing the activity of manganese superoxide dismutase (SOD_2) through a Sirt3-mediated deacetylation and also increases SOD2 expression by promoting the transcriptional activity of forkhead box O3 (FoxO3a). The authors demonstrated that melatonin activates MT1-PI3K/AKT-PGC-1α signaling, which is required for ERRα-dependent Sirt3 transcription. It is unlikely that the antioxidant effect of melatonin in OVX rats involved similar signalling responses because mitochondrial SOD activity was not enhanced by melatonin [137].

Melatonin has also been reported to decrease body weight and reduce inflammation in HFD-induced obese mice by modulating the MAPK-JNK/P38 signaling pathway (Sun et al.,2016) [16]. MAPKs are a group of protein serine/threonine kinases that play important roles in cellular programs. The MAPK signaling pathway is involved in the regulation of inflammation and fatty acid metabolism [138] and is sensitive to oxidative stress [139].

In OVX rats, in fact, we found a higher rate of fatty acid oxidation in the mitochondria isolated from both OVX+MEL and CON+MEL rats. However, under the steady-state condition in perfused livers from OVX+MEL rats there were no differences

in the exogenous palmitate when compared with the livers from CON rats. The receptor-mediated signalling by which melatonin can suppress the metabolic disturbances under estrogen deficiency conditions is so far unknown and remains to be elucidated.

Our work has shown that melatonin exerts direct actions on healthy control rats. A decrease in the body weight gain was related to lower gain of corporal lipids as the percentage of body ethereal extract in CON+MEL rats was reduced. Among the adipose tissues measured in the present work, only a decrease in mesenteric fat mass was found in CON+MEL rats when compared with CON rats. We did not evaluate the morphology of mesenteric fat in CON+MEL rats, but inguinal fat adipocytes exhibited a reduced area. A reduction in body weight by melatonin in healthy animals has been previously reported by [28,33,140]. In these studies, melatonin was administered in a beverage solution (25μg/ml) for 9 to 15 weeks, and body weight gain reduction has demonstrated not be related to reduction in food intake. The involvement of leptin in the metabolic effects of melatonin is unclear since a decrease of plasma leptin [33,141] or lack of changes [142] has been reported.

Different from these authors, in our experimental protocol, melatonin induced a reduction in food consumption. Although low energy intake can explain the lower body weight gain in CON rats, we cannot exclude that melatonin affected energy expenditure of the animals by increasing physical activity and/or increasing oxidation of triglyceride and fatty acid. Literature data on the effects of melatonin on locomotor activity are contradictory, with reports of increase [140,143], decrease [144] and no effects [145].

An increase in energy expenditure due to increase brown adipose tissue activity [146,147,148] (BAT) or browning of inguinal white adipose tissue (WAT) has been also suggested as a melatonin mode of action to reduce adiposity [148]. These effects has been attributed to the central and/or peripheral activities of melatonin, mediated by their receptors on SCN neurons [136] and brown adipocytes [149]. In our work, the involvement of BAT in the regulation of energy balance in OVX rats was suggested by higher lipid inclusions than in CON rats, an effect that was prevented by melatonin administration. In CON+MEL rats, however, no significant difference was found in the lipid content of BAT.

It was remarkable that melatonin activated the maximal capacity of fatty acid oxidation in mitochondria isolated from CON+MEL rats. Under steady state conditions of perfusion livers, an increased in the ketone bodies production from palmitate was found when compared with the CON rats. Considering that the $^{14}CO_2$ production and oxygen

consumption was not different from those of the CON rats, the plausible interpretation for the high ketone bodies production is that acetyl-CoA production due to stimulated β-fatty acid oxidation exceeded citric acid cycle capacity and thus, acetyl-CoA was diverted to ketone bodies formation. This effect was observed only in CON+MEL rats and not in OVX+MEL rats suggesting that, under healthy condition, stimulation of hepatic fatty acid oxidation could contribute to reduced body weight gain.

Although fatty acid oxidation in adipose tissues was not evaluated, an increase in fatty oxidation in adipose tissues could also have contributed to the reduction in mesenteric fat mass and size of inguinal fat adipocytes observed in CON+MEL rats. This hypothesis is in agreement with the recent report of Liu et al. (2019) showing that melatonin decreases intramuscular fat in *vastus lateralis* muscle from the mouse hind limb by positively regulating lipolysis and mitochondrial activities [150]. Indeed, it has been suggested that the action of melatonin in SNC increases the sympathetic drive in the adrenal medulla that increases the release of epinephrine into the circulation, promoting lipolysis in WAT [136,151].

Another remarkable effect of melatonin in CON rats was the substantial reduction in the plasma estradiol levels. According to the uterus as the classic estrogen responsive target tissue, the uterine mass of CON rats has also been reduced. This finding is in line with many studies reporting that melatonin inhibits the expression and activity of various enzymes involved in estrogen biosynthesis in peripheral tissues, particularly aromatase [152,153]. Aromatase catalizes the final steps in estrogen biosynthesis by converting 19-carbon steroids (androgens, for example, androstenedione and testosterone) to 18-carbon steroids (estrogens, for example, estrone and estradiol). Aromatase occur in many tissues, including the gonads, brain, adipose tissue, placenta, blood vessels, skin, bone and in breast cancer tissue [154]. The anti-estrogen effect of melatonin has been intensively studied for treatment of breast cancer [155,156].

Melatonin has also been shown to affect estrogen actions by downregulating estrogen receptor (ER) expression, preventing ER-mediated gene activation and inhibiting the gonadotropic axis [153]. Many metabolic consequences due to the anti-estrogen effect of melatonin can be expected, given that liver, fat, thyroid and adrenal glands, all involved in regulating body energy metabolism, are highly responsive to circulating estrogens [157].

In summary, our study demonstrated that long-term administration of melatonin was effective in preventing the development of hepatic steatosis and oxidative stress in

OVX rats, and while not reducing increased adiposity, melatonin was able to alter lipid distribution and adipocytes morphology in different body fat depots, minimizing the risks for the development of metabolic abnormalities often associated with obesity. In CON rats, melatonin reduced body weight gain, reduced inguinal fat adipocyte size, reduced food intake, and increased fatty acid hepatic oxidation. Melatonin also reduced plasma estradiol levels in control rats. It can be concluded that melatonin may be beneficial for menopausal and postmenopausal women, but in premenopausal women melatonin may affect reproductive functions and other estrogen-regulated functions. Because melatonin has been proposed as pharmacological agent in the treatment of many diseases, its function in premenopausal women should be regarded with care.

Acknowledgments

This work was supported by grants from the Fundação Araucária do Estado do Paraná. Danielle Aparecida Munhos Hermoso holds fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

[1] A.B. Lerner, J.D. Case, Y. Takahashi, Isolation of melatonin, a pineal factor that lightens melanocytes. Journal of American Chemical Society. 80 (1958) 2587.

[2] J. Ferrer, Melatonina: actualidad de una hormona olvidada. Revista CENIC Ciencias Biológicas. 43 (2012) (13 p).

[3] G. Huether, B. Poeggeler, A. Reimer, A. George, Effect of tryptophan administration on circulating melatonin levels in checks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. Life Sci. 52 (1992) 945–53.

[4] M.A. Lopez-Gonzalez, J.R. Calvo, J.J. Segura, J.M. Guerrero, Characterization of melatonin binding sites in human peripheral blood neutrophils. Biotechnol Ther. 4 (1993) 253–62.

[5] Faillace MP, Cutrera R, Keller-Sarmento MI, Rosenstein RE. Evidence for local synthesis of melatonin in golden hamster retina. *Neuroreport.* 1995;6:2093– 5.

[6] Tan DX, Manchester LC, Reiter RJ, et al. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. *Biochim Biophys Acta*. 1999;1472:206–14.

[7] Lardone PJ, Carrillo-Vico A, Molinero P, Rubio A, Guerrero JM. A novel interplay between membrane and nuclear melatonin receptors in human lymphocytes: significance in IL-2 production. *Cell Mol Life Sci*. 2009;66:516– 25.

[8] A. Conti, S. Conconi, E. Hertens, K. Skwarlo-Sonta, M. Markowska, G.J.M Maestroni, Evidence for melatonin synthesis in mouse and human bone marrow cells. J Pineal Res. 28 (2000) 193–202.

[9] F. Radogna, M. Diederich, L. Ghibelli, Melatonin: a pleiotropic molecule regulating inflammation. Biochem Pharmacol. 80 (2010) 1844–1852.

[10] R.J. Reiter, The aging pineal gland and its physiological consequences. BioEssays 14 (1992) 169-175.

[11] K. Angers, N. Haddad, B. Selmaoui, L. Thibault, Effect of melatonin on total food intake and macronutrient choice in rats. Physiology & Behavior. 80 (2003) 9-18.

[12] M.R. Hussein, E.E. Abu-Dief, M.H. Abd el-Reheem, A. Abd-Elrahman, Ultrastructural evaluation of the radioprotective effects of melatonin against X-ray-induced skin damage in Albino rats. Int. J. Exp. Path. 86 (2005) 45–55.

[13] Pandi-Perumal SR, Trakht I, Srinivasan V, et al. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Prog Neurobiol.* 2008;85:335–53.

[14] R. Hardeland, D.P. Cardinali, V. Srinivasan, D.W. Spence, G.M. Brown, S.R. Pandi-Perumal, Melatonin —A pleiotropic, orchestrating regulator molecule. Progress in Neurobiology. 93 (2011) 350-384.

[15] A. Carrillo-Vico, J.M. Guerrero, P.J. Lardone, R.J. Reiter, A review of the multiple actions of melatonin on the immune system. Endocrine. 27 (2005) 189–200.

[16] H. Sun, X. Wang, J. Chen, et al. Melatonin improves non-alcoholic fatty liver disease via MAPK-JNK/P38 signaling in high-fat-diet-induced obese mice. Lipids in Health and Disease. 15 (2016) 202.

[17] S. Reppert, D. Weaver, T. Ebisawa, Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 13 (1994) 1177–85

[18] D.X. Tan, L.D. Chen, B. Poeggeler, L.C. Manchester, R.J. Reiter, Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocr J. 1 (1993) 57–60.

[19] C. Rodriguez, J.C. Mayo, R.M. Sainz, I. Antolín, F. Herrera, V. Martín, R.J. Reiter, Regulation of antioxidant enzymes: a significant role for melatonin. J. Pineal Res. 36 (2004) 1-9.

[20] R.J. Reiter, J.C. Mayo, D.X. Tan, R.M. Sainz, J.M. Alatorre-Jimenez, L. Qin, Melatonin as an antioxidant: under promises but over delivers. J Pineal Res. 61 (2016) 253-78.

[21] N.V. Semenova, I.M. Madaeva, L.I. Kolesnikova, The role of melatonin as a component of the antioxidant defense system in perimenopausal women with insomnia, Zh Nevrol Psikhiatr Im S S Korsakova. 119 (2019) 7-13.

[22] D. Acuña-Castroviejo, G. Escames, C. Venegas, M.E. Díaz-Casado, E. Lima-Cabello, L.C., et al. Extrapineal melatonin: sources, regulation, and potential functions. Cellular and Molecular Life Sciences, 71(2014) 2997–3025.

[23] M. Dahlitz, B. Alvarez, J. Vignau, J. English, J. Arendt, J.D. Parkes, Delayed sleep phase syndrome response to melatonin. Lancet. 337 (1991) 1121–4.

[24] J.E. Jan, M.E. O'onnell, Use of melatonin in the treatment of pediatric sleep disorders. J Pineal Res. 21 (1996) 193–9.

[25] R. Hardeland, S.R. Pandi-Perumal, D.P. Cardinali, Melatonin. Int J Biochem Cell Biol. 38 (2006) 313-6.

[26] A. Carpentieri, G.D. Barboza, V. Areco, M.P. López, N.T. de Talamoni, New perspectives in melatonin uses, Pharmacol. Res. 65 (2012) 437–444.

[27] R.J. Reiter, J.M. Guerrero, J.J. Garcia, D. Acuña-Castroviejo, Reactive oxygen intermediates, molecular damage, and aging. Relation to melatonin. Ann N Y Acad Sci. 854 (1998) 410–24.

[28] T. Wolden-Hanson, D.R. Mitton, R.L. Mccants, Daily Melatonin administration to middle-aged male rats suppresses body weight, intraabdominal adiposity, and plasma

leptin and insulin independent of food intake and total body fat. Endocrinology. 141 (2000).

[29] T. Ravindra, N.K. Lakshmi, Y.R. Ahuja, Melatonin in pathogenesis and therapy of cancer. Indian J Med Sci. 60 (2006) 523–35.

[30] M. Broncel, M. Koziróg-Kołacińska, J. Chojnowska-Jezierska, Melatonin in the treatment of atherosclerosis. Pol Merkur Lekarski. 23 (2007) 124–7.

[31] M.R. Hussein, O.G. Ahmed, A.F. Hassan, M.A. Ahmed, Intake of melatonin is associated with amelioration of physiological changes, both metabolic and morphological pathologies associated with obesity: an animal model, Int. J. Exp. Pathol. 88
(2007) 19–29

[32] R.J. Reiter, D.X. Tan, A. Korkmaz, The circadian melatonin rhythm and its modulation: possible impact on hypertension. J Hypertens Suppl. 27 (2009) S17–20.

[33] M.J. Ríos-Lugo, P. Cano P, V. Jiménez-Ortega, et al. Melatonin effect on plasma adiponectin, leptin, insulin, glucose, triglycerides and cholesterol in normal and high fat–fed rats. *J. Pineal Res.* 2010; 49(4):342–348

[34] A. Agil, M. Alarco, R. Ruiz, S. Abuhamadah, E. Mohamad-Yehia, G.J. Vazquez, Beneficial effects of melatonin on obesity and lipid profile in young Zucker diabetic fatty rats. Pineal Research. 50 (2011) 207–212.

[35] J. Espino, J.A. Pariente, A.B. Rodriguez, Role of melatonin on diabetes-related metabolic disorders. World J Diabetes. 2 (2011) 82–91.

[36] M. Gonciarz, Z. Gonciarz, W. Bielanski, et al. The effects of long-term melatonin treatment on plasma liver enzymes levels and plasma concentrations of lipids and melatonin in patients with nonalcoholic steatohepatitis: a pilot study. Journal of Physiology and Pharmacology. 631 (2012) 35-40.

[37] E. Peschke, I. Bahr, E. Muhlbauer, Melatonin and pancreatic islets: interrelationships between melatonin, insulin and glucagon. Int J Mol Sci. 14 (2013) 6981–7015.

[38] R. Lobo R, Metabolic syndrome after menopause and the role of hormones. Maturitas 60 (2008) 10–18.

[39] S. Ballestri, F. Nascimbeni, E. Baldelli, A. Marrazzo, D. Romagnoli, A. Lonard, NAFLD as a Sexual Dimorphic Disease: Role of Gender and Reproductive Status in the Development and Progression of Nonalcoholic Fatty Liver Disease and Inherent Cardiovascular Risk. Adv Ther. 34 (2017) 1291–1326.

[40] G. Bellipanni, P. Bianchi, W. Pierpaoli,D. Bulian, E. Ilyia, Effects of melatonin in perimenopausal and menopausal women: a randomized and placebo controlled study. Exp Gerontol. 36 (2001) 297–310.

[41] E. Toffol, N. Kalleinen, J. Haukka, O. Vakkuri, T. Partonen, P. Polo -Kantola P, Melatonin in perimenopausal and postmenopausal women: associations with mood, sleep, climacteric symptoms, and quality of life. Menopause. 21 (2014) 493-500.

[42] E. Walecka-Kapica, J. Chojnacki, A. Stępień, P. Wachowska-Kelly, G. Klupińska, C. Chojnacki, Melatonin and female hormone secretion in postmenopausal overweight women. Int J Mol Sci. 5 (2015) 1030-42.

[43] F.B. Lima, U.F. Machado, I. Bartol, et al. Pinealectomy causes glucose intolerance and decreases adipose cell responsiveness to insulin in rats. Am. J. Physiol. 275 (1998) E934–E941.

[44] M. Gambacciani, M. Ciaponi, B. Cappagli B, et al. Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women. J Clin Endocrinol Metab. 82 (1997) 414–7.

[45] H.D. Nelson, L.L. Humphrey, P. Nygren, S.M. Teutsch, J.D. Allan , Postmenopausal hormone replacement therapy: scientific review. JAMA. 288 (2002) 872–81

[46] J.E. Rossouw, R.L. Prentice, J.E. Manson , et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. JAMA 297 (2007) 1465–1477.

[47] D.F. Archer, E. Oger, Estrogen and progestogen effect on venous thromboembolism in menopausal women. Climacteric 15 (2012) 235–240.

[48] A. Wakatsuki, Y. Okatani, N. Ikenoue, C. Kaneda, T. Fukaya, Effects of short-term melatonin administration on lipoprotein metabolism in normolipidemic postmenopausal women. Maturitas. 38 (2001) 171 -177.

[49] C. Chojnacki, A. Kaczka, A. Gasiorowska, J. Fichna, J. Chojnacki, T. Brzozowski, The effect of long-term melatonin supplementation on psychosomatic disorders in postmenopausal women. J Physiol Pharmacol. 69 (2018).

[50] I. Baeza, N.M. De Castro, L. Giménez-Llort, M. De la Fuente, Ovariectomy, a model of menopause in rodents, causes a premature aging of the nervous and immune systems.J Neuroimmunol. 26 (2010) 90-9.

[51] D.B. Baxi, P.K. Singh, K.D. Vachhrajani, A.V. Ramachandran, Melatonin supplementation in rat ameliorates ovariectomy-induced oxidative stress. Climacteric. 16 (2013) 274-83.

[52] L. Xu, L. Zhang, Z. Wang, et al. Melatonin Suppresses Estrogen Deficiency-Induced Osteoporosis and Promotes Osteoblastogenesis by Inactivating the NLRP3 Inflammasome. Calcif Tissue Int. 103 (2018) 400-410.

[53] L. Brites Campos-Shimada, E. H. Gilglioni, D. A. M. Hermoso, A. J. R. Buzzo1, R. F. Garcia, E.R. Martins-Maciel, E.L. Ishii-Iwamoto, C.L. Salgueiro-Pagadigorria, Tibolone Reverses NAFLD in Ovariectomised Rats by Reducing Adiposity and Insulin ResistanceJournal of Pharmacy and Pharmacology. 7 (2019) 148-164

[54] C. Sanchez-Mateos, A. Alonso-Gonzalez A, C.M. Gonzalez , M.D. Martinez-Campa, S. Mediavilla, E.J. Cos, S. Sanchez-Barcelo S, Melatonin and estradiol effects on food intake, body weight, and leptin in ovariectomized rats. Maturitas. 58 (2007) 91–101.

[55] D.A.M. Hermoso, L.B.C. Shimada, E.H. Gilglioni, J. Constantin , M.S. Mito, A.P.M. Hermoso, et al. Melatonin protects female rats against steatosis and liver oxidative stress induced by oestrogen deficiency. Life Sci. 15 (2016) 178-186.

[56] J. Hoffstedt, E. Arner, H. Wahrenberg H, et al. Regional impact of adipose tissue morphology on the metabolic profile in morbid obesity. Diabetologia. 53 (2010) 2496-2503

[57] J. Yang, B. Eliasson, U. Smith, S.W. Cushman, A.S. Sherman, The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. Obesity. (Silver Spring). 20 (2012) 932-938.

[58] M. Zhang, T. Hu, S. Zhang, L. Zhou. Associations of Different Adipose Tissue Depots with Insulin Resistance: A Systematic Review and Meta-analysis of Observational Studies. Sci Rep. 21 (2015) 18495.

[59] L. Fang, F. Guo, L. Zhou, R. Stahl, J. Grams, The cell size and distribution of adipocytes from subcutaneous and visceral fat is associated with type 2 diabetes mellitus in humans. Adipocyte 4 (2015) 273–279.

[60] S.C. Saling, J.F. Comar, M.S. Mito, R.M. Peralta, A. Bracht, Actions of juglone on energy metabolism in the rat liver. Toxicol Appl Pharmacol. 257 (2011) 319-327.

[61] A.A. Soares, A.L. de Oliveira, A.B. Sá-Nakanishi, J.F. Comar, A.P. Rampazzo, F.A. Vicentini, et al. Effects of an Agaricus blazei aqueous extract pretreatment on paracetamol-induced brain and liver injury in rats. Biomed Res Int. (2013) 1-12.

[62] V.R. Vilela , A.L. de Oliveira, J.F. Comar, R.M. Peralta, A. Bracht A, Tadalafil inhibits the cAMP stimulated glucose output in the rat liver. Chem Biol Interact. 220 (2014) 1-11.

[63] P. Sharp , J.S. Villano, The laboratory rat. 2ª ed. CRC Press, Taylor & Francis Group, Boca Raton. 2013.

[64] Conselho Nacional de Controle de Experimentação Animal – CONCEA – Brasília-DF, 2013 (54p.).

[65] R.N. Soriano, M.I. Ravanelli, M.E. Batalhao, E.C. Carnio, L.G. Branco, Propyretic role of the locus coeruleus nitric oxide pathway. Exp Physiol. 95 (2010) 669-677.

[66] A.F. Donatti, R.N. Soriano, J.P. Sabino, L.G. Branco, Involvement of endogenous hydrogen sulfide (H2S) in the rostral ventrolateral medulla (RVLM) in hypoxia-induced hypothermia. Brain Res Bull. 108 (2014) 94-99.

[67] Canadian Council On Animal Care (1998). Manual sobre el cuidado y uso de los animales de experimentación.v.1.Extraídode:http://www.ccac.ca/en/CCAC_Programs/Guidelines_olicies/GUIDES/SPANISH/toc_v1.h tm.

[68] L.B. Campos, E.H. Gilglioni, R.F. Garcia, M.N. Brito, M.R. Natali, E.L. Ishii-Iwamoto, C.L. Salgueiro-Pagadigorria, Cimicifugaracemosa impairs fatty acid β-oxidation and induces oxidative stress in livers of ovariectomized rats with renovascular hypertension, Free Radic. Biol. Med. 15 (2012) 680–689.

[69] AOAC. (1990). Official methods of analysis of the AOAC, 15th ed. Methods 932.06, 925.09, 985.29, 923.03. Association of official analytical chemists. Arlington, VA, USA.

[70] AOAC. (2016). Official methods of analysis of the AOAC, 20th ed. Methods 9780935584875 Association of official analytical chemists. Arlington, VA, USA.

[71] F.K. Marcondes, F.J. Bianchi, A.P. Tanno, Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol. 62 (2002) 609-14.

[72] M.S. Smith, M.E. Freeman, J.D. Neill,The control of progesterone secretion during the estrus cycle and early pseudopregnancy in the rat: Prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96 (1975) 219–226

[73] W.T. Friedewald, R.I. Levy, D.S. Fredrickson, Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem. 18 (1972) 499-502.

[74] P.J. Hissin, R.A. Hilf, Fluorometric method for determination of oxidized and reduced glutathione in tissues, Anal. Biochem. 74 (1976) 214–226.

[75] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358

[76] J. Sedlak, R.H. LINDSAY, Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal. Biochem. 26 (1968) 192-205

[77] J.D. Bancroft, M. Gamble, *Theory and Practice of Histological Techniques*; Churchill Livingstone: London, UK, 2008.

[78] D.E. Kleiner, E.M. Brunt , M. Van Natta, C. Behling, M.J. Contos, O.W. Cummings, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease, Hepatology. 41 (2005) 1313-1321.

[79] J. Folch, M. Lees, G.H. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.

[80] F.A. Guarnier, A.L. Cecchini, A.A. Suzukawa, A.L.G.C. Maragno, A.N.C. Simão, M.D. Gomes, R. Cecchini, Time course of skeletal muscle loss and oxidative stress in rats with walker 256 solid tumor, Muscle Nerve 42 (2010) 950–958.

[81] I. Dalle-donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, Clin. Chim. Acta 329 (2003) 23–38.

[82] A. Bracht, E.L. Ishii-Iwamoto, C.L. Salgueiro-Pagadigorria, 2003. Técnicas de centrifugação e de fracionamento celular, in: Bracht A.; Ishii-Iwamoto, E. L., (eds), Métodos de laboratório em bioquímica. Manole., São Paulo, pp 77-101.

[83] H. Aebi, (1974) Catalase. In: H.U. Bergmeyer, Ed., Methods of Enzymatic Analysis, Verlag Chemie/Academic Press Inc., Weinheim/NewYork, 673-680.

[84] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469-474.

[85] E.D. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutatione peroxidase, J. Lab. Clin. Med. 70 (1967) 158-169.

[86] C.E. Mize, R.G. Langdon, Hepatic glutathione reductase. purification and general kinetic properties, J. Biol. Chem. 237 (1962) 1589-1595.

[87] W.N. Tian, N. Pignatare, R.C. Stanton, Signal transduction proteins that associate with the platelet-derived growth factor (PDGF) receptor mediate the PDGF-induced release of glucose-6-phosphate dehydrogenase from permeabilized cells, J. Biol. Chem. 269 (1994) 14798-14805.

[88] A. Berson, V. De Beco, P. Lettéron, M.A. Robin, C. Moreau, J. El Kahwaji, et al., Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. Gastroenterology. 114 (1998) 764-774.

[89] B. Chance, G.R. Williams, A simple and rapid assay of oxidative phosphorylation, Nature. 175 (1955) 1120-1121.

[90] T.P. Singer, Determination of the activity of succinate, NADH, choline and glycerophosphate dehydrogenase, Method Biochem. Anal. 22 (1974) 125-175.

[91] P.B. Garland, D. Shepherd, D.G. Nicholls, D.W. Yates, P.A. Light, 1969. Interactions between fatty acid oxidation and the tricarboxylic acid cycle, in: Lowestein, J. M., (eds) Citric Acid Cycle. Dekker., New York, pp 163-212.

[92] Scholz, R.; Bücher, T. Hemoglobin-free perfusion of rat liver. In Control of Energy Metabolism; Academic Press: New York, NY, USA, 1965; pp. 393–414.

[93] S. Soboll, H.W. Heldt, R. Scholz, Changes in the subcellular distribution of metabolites due to ethanol oxidation in the perfused rat liver. Hoppe Seylers Z. Physiol. Chem. 362 (1981) 247–260.

[94] Bergmeyer, H.U. *Methods of Enzymatic Analysis*; Verlag Chemie: Weinheim, Germany, 1974

[95] Clark, L.C.J. Monitor and control of blood and tissue oxygenation. Trans. Am. Soc. Artif. Intern. Organ. 1956,
2, 41–45.

[96] R. Scholz, M.S. Olson, A.J. Schwab, U. Schwabe, C. Noell, W. Braun, The effect of fatty acids on the regulation of pyruvate dehydrogenase in perfused rat liver. Eur. J. Biochem. 86 (1978) 86, 519–530.

[97] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[98] K. Kaur, A comprehensive review on metabolic syndrome.Cardiol Res Pract. (2014)943162.

[99] A. Paquette, D. Wang, M. Jankowski, J. Gutkowska, J.M. Lavoie, Effects of ovariectomy on PPAR-α, SREBP-1c and SCD-1 gene expression in the rat liver, Menopause.15 (2008) 1169-1175.

[100] K. Chopra, S. Misra, A. Kuhad, Neurobiological aspects of Alzheimer's disease. Expert Opin. Ther. Targets 15(2011) 535–555.

[101] S.K. Park, D.J. Stefanyshyn, B. Loitz-Ramage, D.A. Hart, J.L. Ronsky, Changing hormone levels during the menstrual cycle affect knee laxity and stiffness in healthy female subjects. Am. J. Sports Med. 37 (2009) 588–598.

[102] D.R. Leblanc, M. Schneider, P. Angele, G. Vollmer, D. Docheva, The effect of estrogen on tendon and ligament metabolism and function. J. Steroid Biochem. Mol. Biol. 172 (2007) 106–116.

[103] J.M. Hughes-Austin, B.A. Larsen, M.A. Allison, Visceral Adipose Tissue and Cardiovascular Disease Risk. Curr. Cardiovasc. Risk Rep. 7 (2013) 95–101.

[104] I. Moreno-Indias, F.J. Tinahones, Impaired adipose tissue expandability and lipogenic capacities as ones of the main causes of metabolic disorders. J. Diabetes Res. 2015(2015) 970375.

[105] J. Rey-López, L. Rezende, M. Pastor-Valero, B. Tess, The prevalence of metabolically healthy obesity: a systematic review an critical evaluation of the definitions used. Obes Rev. 15 (2014) 781–790.

[106] D. Samocha-Bonet, V. Dixit, C. Kahn, et al. Metabolical healthy and unhealthy obese–the 2013 Stock Conference report. Obes. Rev. 15 (2014) 697–708.

[107] M. Longo,, F. Zatterale, J. Naderi, L. Parrillo, P. Formisano, G.A. Raciti, F. Beguinot,C. Miele, Adipose Tissue Dysfunction as Determinant of Obesity-Associated Metabolic Complications.Int J Mol Sci. 2019 May 13;20(9). pii: E2358. doi: 10.3390/ijms20092358.

[108] S. Lemieux , D. Prud'homme , C. Bouchard, A. Tremblay, J.P. Després , Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. Am J Clin Nutr. 1993 Oct;58(4):463-7.

[109] U.A. White, Y.D. Tchoukalova, Sex dimorphism and depot differences in adipose tissue function. Biochim. Biophys. Acta. 184 (2013) 377–392.

[110] C.K. Osborne, R. Schiff, Estrogen-receptor biology: continuing progress and therapeutic implications. J Clin Oncol. 23 (2005) 1616–1622.

[111] B.W. Parks, T. Sallam, M. Mehrabian, N. Psychogios, S.T. Hui, F. Norheim, L.W. Castellani, C.D. Rau, C. Pan, J. Phun,Z. Zhou, W.P. Yang, I. Neuhaus, P.S. Gargalovic, T.G. Kirchgessner, M. Graham, R. Lee, P. Tontonoz, R.E. Gerszten, A.L. Hevener, A.J. Lusis, Genetic architecture of insulin resistance in the mouse. Cell Metab. 21 (2015) 334–346

[112] T.M. D'Eon, S.C. Souza, M. Aronovitz, M.S. Obin, S.K. Fried, A.S. Greenberg, Estrogen regulation of adiposity and fuel partitioning. evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways, J. Biol. Chem. 280 (2005) 35983-35991.

[113] J.J. Zhu, J. Luo, Y.T. Sun, H.B. Shi, J. Li, M. Wu, K. Yu, A.B. Haile, J.J. Loor Short communication: Effect of inhibition of fatty acid synthase on triglyceride accumulation and effect on lipid metabolism genes in goat mammary epithelial cells J. Dairy Sci., 98 (2015), pp. 3485-3491

[114] G. Bryzgalova, H. Gao, B. Ahren, J.R. Zierath, D. Galuska, T.L. Steiler, K. Dahlman-Wright, S. Nilsson, J.A. Gustafsson, S. Efendic, A. Khan, Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. Diabetologia. 49 (2006) 588–597.

[115] W. Fan, R. Evans, PPARs and ERRs: molecular mediators of mitochondrial metabolism, Curr. Opin. Cell Biol. 33 (2015) 49–54.

[116] J.P. Camporez, A. Couvillon, D. Pesta, R.J. Roth Flach, G.I. Shulman, A.M. Bennett, Hepatic mitogen-activated protein kinase phosphatase 1 selectively regulates glucose metabolism and energy homeostasis. Mol Cell Biol. 35 (2015) 26–40.

[117] J.A. Tresguerres, R. Kireev, A.F. Tresguerres, C. Borras, E. Vara, C. Ariznavarreta,Molecular mechanisms involved in the hormonal prevention of aging in the rat.J Steroid Biochem Mol Biol. 108 (2008) 318-26.

[118] K. Moorthy K, D. Sharma, S.F. Basir, N.Z. Baquer NZ. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. Exp Gerontol. 2005 Apr;40(4):295-302.

[119] J. Viña, C. Borrás, J. Gambini, J. Sastre, F.V. Pallardó, Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. FEBS Lett. 2005 May 9;579(12):2541-5. Epub 2005 Apr 14.

[120] A. Höhn, T. Jung, T. Grune, Pathophysiological importance of aggregated damaged proteins. Free Radic. Biol. Med. 71 (2014) 70–89.

[121] J.D. Browning, J.D. Horton, Molecular mediators of hepatic steatosis and liver injury. J Clin Invest. 114 (2004) 147–152.

[122] K. Jarukamjorn, N. Jearapong, C. Pimson, W. Chatuphonprasert, A High-Fat, High-Fructose Diet Induces Antioxidant Imbalance and Increases the Risk and Progression of Nonalcoholic Fatty Liver Disease in Mice. Scientifica (Cairo). (2016)1–10.

[123] C. García-Ruiz, J.C. Fernández-Checa, Mitochondrial Oxidative Stress and Antioxidants Balance in Fatty Liver Disease. Hepatol. Commun. 2 (2018) 1425–1439.

[124] C.G. Miller, A. Holmgren, E.S.J. Arnér, E.E. Schmidt, NADPH-dependent and -independent disulfide reductase systems. Free Radic. Biol. Med. 127 (2018) 248–261.

[125] S. Hurrle, W.H. Hsu, The etiology of oxidative stress in insulin resistance Biomed J. 40 (2017)257–262.

[126] F. Piscaglia, G. Svegliati-Baroni, A. Barchetti, et al. Clinical patterns of hepatocellular carcinoma in nonalcoholic fatty liver disease: a multicentre prospective study. Hepatology. 63 (2016) 827–38.

[127] J.C. Lovejoy, A. Sainsbury, Sex differences in obesity and the regulation of energy homeostasis. Obes. Rev. 10 (2009) 154–167.

[128] M. Rebuffé-Scrive, J. Eldh, L.O. Hafström, P. Björntorp P, Metabolism of mammary, abdominal, and femoral adipocytes in women before and after menopause. Metabolism of mammary, abdominal, and femoral adipocytes in women before and after menopause. Metabolism. 35 (1986) 792-7.

[129] A. Korkmaz, R.J. Reiter, T. Topal, L.C. Manchester, S. Oter, D.X. Tan, Melatonin: an established antioxidant worthy of use in clinical trials. Mol Med. 15 (2009) 43-50.

[130] C. Venegas, J.A. García, G. Escames, F. Ortiz, A. López, C. Doerrier, et al. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. Journal of pineal research. 52 (2012) 217-227.

[131] M. Messner, G. Huether, T. Lorf, G. Ramadori, H. Schwörer, Presence of melatonin in the human hepatobiliary-gastrointestinal tract. Life sciences. 69 (2001) 543-551.

[132] D.K. Lahiri, Y.W. Ge, E.H. Sharman, S.C. Bondy, Age-related changes in serum melatonin in mice: higher levels of combined melatonin and 6-hydroxymelatonin sulfate in the cerebral cortex than serum, heart, liver and kidney tissues. Journal of pineal research. 36 (2004) 217-223.

[133] R.J. Reiter, D.X. Tan, A. Galano, Melatonin: exceeding expectations.Physiology (Bethesda). 29 (2014) 325-33. doi: 10.1152/physiol.00011.2014.

[134] M. Becker-André, I. Wiesenberg, N. Schaeren-Wiemers, et al. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem. 269 (1994) 28531–4

[135] G. Benitez-King, L. Huerto-Delgadillo, F. Anton-Tay, Binding of 3H-melatonin to calmodulin. Life Sci. 53 (1993) 201–7.

[136] T.J. Bartness, G.E. Demas, C.K. Song, Seasonal changes in adiposity: the roles of the photoperiod, melatonin and other hormones, and sympathetic nervous system. Exp Biol Med (Maywood*)*. 227 (2002) 363–376.

[137] C. Song, J. Zhao, B. Fu, D. Li, T. Mao, W. Peng et al. Melatonin-mediated upregulation of Sirt3 attenuates sodium fluoride-induced hepatotoxicity by activating the MT1-PI3K/AKT-PGC-1α signaling pathway. Free Radic. Biol. Med. 112 (2017) 616-630.

[138] A. Lawan, L. Zhang, F. Gatzke, K. Min, M.J. Jurczak, M. Al-Mutairi, et al. Hepatic mitogen-activated protein kinase phosphatase 1 selectively regulates glucose metabolism and energy homeostasis. Mol Cell Biol. 35 (2015) 26–40.

[139] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (1) (2002) 9–18.

[140] Terron, M. P.; Delgado-Adamez, J.; Pariente, J. A.; Barriga, C.; Paredes, S. D.; Rodriguez, A. B. Melatonin reduces body weight gain and increases nocturnal activity in male Wistar rats. Physiology & Behavior. 2013;118:8-13.

[141] D.D. Rasmussen, B.M. Boldt, C.W. Wilkinson, S.M. Yellon, A.M. Matsumoto, Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels. Endocrinology. 140 (1999) 1009–

[142] D.D. Rasmussen, D.R. Mitton, S.A. Larsen, S.M. Yellon, Aging-dependent changes in the effect of daily melatonin supplementation on rat metabolic and behavioral responses. J Pineal Res. 31 (2001) 89–94.

[143] S. Aparicio, C. Garau, M.C. Nicolau, R.V. Rial, S. Esteban, Opposite effects of tryptophan intake on motor activity in ring doves (diurnal) and rats (nocturnal). Comp Biochem Physiol A Mol Integr Physiol. 144 (2006) 173–9.

[144]Y. Isobe, T. Torri, E. Konishi, J. Fujioi, Effects of melatonin injection on running-wheel activity and body temperature differ by the time of day.Pharmacol Biochem Behav. 73 (2002) 805-811.

[145] M.A. Raskind, B.L. Burke, N.J. Crites, A.M. Tapp, D.D. Rasmussen, Olanzapine-induced weight gain and increased visceral adiposity is blocked by melatonin replacement therapy in rats.Neuropsychopharmacology. 32 (2007) 284–288.

[146] G. Heldmaier, K. Hoffmann, Melatonin stimulates growth of brown adipose tissue. Nature.247 (1974) 224–225.

[147] T Tan DX, Manchester LC, Fuentes-Broto L, Paredes SD, Reiter RJ. Significance and application of melatonin in the regulation of brown adipose tissue metabolism: relation to human obesity. Obes Rev. 2011;12:167-88.an et al., 2013

[148] A. Jiménez-Aranda, G. Fernández-Vázquez, D. Campos, M. Tassi, L. Velasco-Perez, D.X. Tan, R.J. Reiter, A. Agil, Melatonin induces browning of inguinal white adipose tissue in Zucker diabetic fatty rats. J Pineal Res. 55 (2013) 416-23. doi: 10.1111/jpi.12089. Epub 2013 Sep 6.

[149] S. Le Gouic, C. Atgié, N. Viguerie-Bascands, N. Hanoun, D. Larrouy, L. Ambid, S. Raimbault, D. Ricquier, P. Delagrange, B. Guardiola-Lemaitre, L. Pénicaud, L. Casteilla, Characterization of a melatonin binding site in Siberian hamster brown adipose tissue. Eur J Pharmacol. 27 (1997) 271-8.

[150] K. Liu, W. Yu, W. Wei, X. Zhang, Y. Tian, M. Sherif, X. Liu, C. Dong, W. Wu, L. Zhang, J. Chen, Melatonin reduces intramuscular fat deposition by promoting lipolysis and increasing mitochondrial function. J Lipid Res. 60 (2019) 767-782.

[151] G.E. Demas, T.J. Bartness, Direct innervation of body fat and adrenal medullary catecholamines mediate photoperiodic changes in body fat. Am J Physiol 281 (2001) 1499-1505.

[152] E.J. Sanchez-Barcelo, M.D. Mediavilla, C. Alonso-Gonzalez, N. Rueda, Breast cancer therapy based on melatonin. Recent Pat. Endocr. Metab. Immune Drug Discov. 6 (2012) 108–116.

[153] S. Chottanapund, M.B. Van Duursen, P. Navasumrit, P. Hunsonti, S. Timtavorn, M. Ruchirawat, M. Van den Berg, Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts. Toxicol In Vitro. 28 (2014) 1215-21.

[154] J. Cui, Y. Shen, R. Li, Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends in molecular medicine, 19 (2013) 197-209.

[155] J. Menéndez-Menéndez J, C. Martínez-Campa, Melatonin: An Anti-Tumor Agent in Hormone-Dependent Cancers. Int J Endocrinol. 2 (2018).

[156] E.C.A. Veiga, R. Simões, V.E. Valenti, J. Cipolla-Neto, L.C. Abreu, E.P.M. Barros, I.C.E. Sorpreso, M.C.P. Baracat, E.C. Baracat, J.M. Soares Junior, Repercussions of melatonin on the risk of breast cancer: a systematic review and meta-analysis. Rev Assoc Med Bras (1992). 3 (2019) 699-705.

[157] D.D. Hutson, R. Gurrala, B.O. Ogola, M.A. Zimmerman, R. Mostany, R. Satou, S.H. Lindsey, Estrogen receptor profiles across tissues from male and female Rattus norvegicus. Biol Sex Differ. 11 (2019).