

Valter Tadeu Boldarine

**Avaliação dos efeitos da ovariectomia sobre parâmetros metabólicos e comportamentais
em ratas**

Tese apresentada à Universidade Federal de São Paulo - Escola Paulista de Medicina, para obtenção do Título de Doutor em Ciências.

SÃO PAULO

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VALTER TADEU BOLDARINE

**AVALIAÇÃO DOS EFEITOS DA OVARIECTOMIA SOBRE PARÂMETROS
METABÓLICOS E COMPORTAMENTAIS EM RATAS**

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“A Ciência atua na fronteira entre o conhecimento e a ignorância sem medo de admitir que não sabemos. Não há nenhuma vergonha nisso. A única vergonha é fingir que temos todas as respostas”.

(Neil deGrasse Tyson)

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Dedico esta tese àquela que me preparou, mesmo sem saber, para atravessar de cabeça erguida a um momento tão nefasto que a Ciência e a Educação de modo geral enfrentam nos dias atuais.

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RESUMO

A menopausa é frequentemente acompanhada de obesidade visceral, bem como de alterações metabólicas e comportamentais ainda não totalmente esclarecidas. Além disso, a ingestão de alto teor de gordura pode influenciar os efeitos da menopausa. Utilizamos um modelo de ratas ovariectomizadas a fim de explorar esses aspectos.

Inicialmente, estudamos os efeitos da ovariectomia, aliada ou não a uma dieta rica em gordura e à reposição de estradiol, nos parâmetros hormonais, metabólicos e comportamentais, para explorar a conexão entre obesidade e depressão após a menopausa. Ratas *Wistar* foram submetidas à ovariectomia ou falsa cirurgia e alimentadas com dieta controle ou rica em banha de porco por doze semanas. Subgrupos de ratas ovariectomizadas receberam reposição de estradiol. Os comportamentos de tipo depressivo foram avaliados pelo teste de natação forçada e a atividade locomotora foi avaliada pelo teste de labirinto em cruz elevado. A ovariectomia aumentou o ganho de peso corporal e a eficiência alimentar e induziu hiperleptinemia e intolerância à glicose, enquanto aumentou a ingestão calórica e a adiposidade corporal apenas marginalmente. A ingestão de dieta rica em gordura induziu obesidade e, quando administrada aos animais ovariectomizados, acentuou as alterações causadas pela ovariectomia. A reposição de estradiol atenuou as alterações hormonais apenas em ratas alimentadas com dieta controle. Em conclusão, a ovariectomia combinada à ingestão de dieta hiperlipídica induziu comportamentos de tipo depressivo, que foram atenuados marginalmente pelo estradiol. Estes comportamentos foram associados a parâmetros metabólicos e de composição corporal e ao *status* do estrogênio. Os dados indicam que a vulnerabilidade ao desenvolvimento de depressão após a menopausa é influenciada pela ingestão de alto teor de gordura.

Com o objetivo de explorar as consequências da insuficiência ovariana sobre a gordura visceral, avaliamos os efeitos da ovariectomia e reposição de estrogênio no proteoma/fosfoproteoma e no perfil de ácidos graxos do tecido adiposo retroperitoneal (RET) de ratas. Para esta investigação, foram analisadas 18 ratas alimentadas com dieta controle, sendo 6 falso-ovariectomizadas, 6 ovariectomizadas e 6 com reposição hormonal. As amostras de RET foram analisadas por cromatografia líquida acoplada a espectrômetro de massas em

tandem (LC-MS/MS) e as proteínas diferencialmente expressas/fosforiladas foram submetidas à análise de vias metabólicas. O perfil lipídico do RET foi analisado por cromatografia gasosa.

A ovariectomia induziu alta adiposidade e resistência à insulina e alterou o padrão de expressão proteica, promovendo sub-expressão de 42 proteínas e super-expressão de 49 proteínas. Noventa e seis proteínas (106 peptídeos) foram diferencialmente fosforiladas, com diminuição da fosforilação de 39 peptídeos e aumento da fosforilação de 67 peptídeos. A análise de vias mostrou que 5 vias foram afetadas significantemente pela ovariectomia, a saber, metabolismo de lipídios (incluindo metabolismo de ácidos graxos e β-oxidação mitocondrial de ácidos graxos), biossíntese de acil-CoA graxa, sistema imunológico inato (incluindo degranulação de neutrófilos), metabolismo de vitaminas e cofatores e integração do metabolismo energético (incluindo expressão de genes do metabolismo ativada por ChREBP). A análise do perfil lipídico mostrou aumento do teor dos ácidos palmítico e palmitoleico. A análise dos dados indicou que a ovariectomia favoreceu a lipogênese enquanto prejudicou a oxidação dos ácidos graxos e induziu um estado pró-inflamatório no tecido adiposo visceral. Esses efeitos são consistentes com os achados de alta adiposidade, hiperleptinemia e diminuição da sensibilidade à insulina. As alterações observadas foram parcialmente atenuadas pela reposição de estradiol. Os dados indicam que distúrbios do metabolismo lipídico no tecido adiposo visceral desempenham um papel relevante na gênese da obesidade após a menopausa.

Os dados obtidos nos presentes estudos sugerem que o controle do peso é uma questão crucial em mulheres na pós-menopausa, provavelmente tendo um papel benéfico na prevenção de problemas de saúde mental e as consequências deletérias do acúmulo de gordura visceral

Palavras-chave: Menopausa; Obesidade; Depressão; Gordura visceral;
Proteoma/Fosfoproteoma

ABSTRACT

Menopause is often accompanied by visceral obesity, as well as metabolic and behavioral alterations not completely understood. Additionally, high-fat intake may influence the menopause effects. We used a rat model to explore these aspects.

First, we studied the effects of ovariectomy, allied or not to high-fat feeding and estradiol replacement, on hormonal, metabolic and behavioral parameters, to explore the connection of obesity and depression after menopause. Wistar rats were either ovariectomized or sham-operated and fed with either standard chow or lard-enriched diet for twelve weeks. Sub-groups of ovariectomized rats received estradiol replacement. Depressive-like behaviors were assessed by the forced swim test and locomotor activity was assessed by the elevated plus maze test. Ovariectomy alone increased body weight gain and feed efficiency and induced hyperleptinemia and glucose intolerance while it increased caloric intake and body adiposity only marginally. High-fat intake alone induced obesity and, in combination with ovariectomy, accentuated the ovariectomy-induced alterations. Estradiol replacement attenuated the hormonal alterations only in chow-fed rats. Ovariectomy combined with high-fat intake induced depressive-like behaviors, which were marginally attenuated by estradiol. Depressive-like behaviors were associated with metabolic and body composition parameters and with estrogen status. The data indicate that the vulnerability to develop depression after menopause is influenced by high-fat intake.

With the aim of exploring the consequences of ovarian failure on visceral fat, we evaluated the effects of ovariectomy and estrogen replacement on the proteome/phosphoproteome and on the fatty acids profile of the retroperitoneal adipose depot (RET) of rats. Eighteen three months-old female Wistar rats were either ovariectomized or sham-operated and fed with standard chow for three months. A sub-group of ovariectomized rats received estradiol replacement. RET samples were analyzed using data-independent acquisitions by liquid chromatography- tandem mass spectrometry (LC-MS/MS) and pathway analysis was performed with the differentially expressed/phosphorylated proteins. RET lipid profile was analyzed by gas chromatography.

Ovariectomy induced high adiposity and insulin resistance. Ovariectomy promoted down-regulation of 42 and up-regulation of 49 differentially expressed proteins compared to

control group. Ninety-six proteins (106 peptides) were differentially phosphorylated, with decreased phosphorylation of 39 peptides and increased phosphorylation of 67 peptides. Pathway analysis showed that 5 pathways were significantly affected by ovariectomy, namely metabolism of lipids (included fatty acid metabolism and mitochondrial fatty acid β -oxidation), fatty acyl-CoA biosynthesis, innate immune system (included neutrophil degranulation), metabolism of vitamins and cofactors, and integration of energy metabolism (included ChREBP activates metabolic gene expression). Lipid profile analysis showed increased palmitic and palmitoleic acids content. The analysis of the data indicated that ovariectomy favored lipogenesis while it impaired fatty acids oxidation, and induced a pro-inflammatory state in the visceral adipose tissue. These effects are consistent with the findings of high adiposity, hyperleptinemia, and impaired insulin sensitivity. The observed alterations were partially attenuated by estradiol replacement. The data point to a role of disrupted lipid metabolism in adipose tissue in the genesis of obesity after menopause.

The data obtained in the present studies bring the suggestion that weight management is a crucial issue in postmenopausal women, probably having a beneficial role in preventing the appearance of mental health problems and visceral fat accumulation and its consequences.

Keywords: Menopause; Obesity; Depression; Visceral Fat; Proteome/Phosphoproteome

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LISTA DE ABREVIATURAS

AA: ácido araquidônico

ACADL: *long-chain specific Acyl-CoA dehydrogenase*

ACADS: *short-chain specific acyl-CoA dehydrogenase*

ACADVL: *very long-chain specific Acyl-CoA dehydrogenase*

ACC1: acetil-CoA carboxilase tipo 1 (do inglês *acetyl-CoA carboxylase 1*)

ACOT2: *acyl-coenzyme A thioesterase type 2*

ACSL1: *long-chain-fatty-acid-CoA ligase 1*

ACSL5: *long-chain-fatty-acid-CoA ligase 5*

AMPK: proteína quinase ativada por AMP (do inglês *AMP-activated protein kinase*)

ATGL: lipase de triglicerídeos de adipócitos (do inglês *adipose triglyceride lipase*)

BCA: ácido bicinconílico

BHT: hidroxitolueno butilado

CAP1: proteína associada a adenilato-ciclase tipo 1 (do inglês *adenylyl cyclase-associated protein-1*)

CD36: *Platelet glycoprotein 4*

CEUA: comitê de ética em pesquisa

CONCEA: conselho nacional de controle de experimentação animal

DHA: ácido docosahexaenoico

DTT: ditiotreitol (2S,3S)-1,4-Bis-sulfanilbutano-2,3-diol

E2: estradiol

ECI1: *enoyl-CoA delta isomerase-1*

EEF1D: fator de elongação tipo 1- δ (do inglês *elongation factor 1- δ*)

EPA: ácido eicosapentaenoico

EPM: teste do labirinto em cruz elevado

ESI: electrospray

FAME: ésteres metílicos de ácidos graxos

FAS: ácido-graxo sintase (do inglês *fatty acid synthase*)

FDR: taxa de falsa descoberta (do inglês *false discovery rate*)

FST: teste do nado forçado modificado

HDMS^E: mobilidade iônica acoplada a espectrometria de massas de múltiplos estágios

IL-6: Interleucina-6 (do inglês *Interleucine-6*)

ITB1: Integrina-β tipo 1 (do inglês *integrin- β I*)

LC-MS/MS: cromatografia líquida acoplada a espectrometria de massas em *tandem*

LPL: lipase de lipoproteínas (do inglês *lipoprotein lipase*)

LPS: lipopolissacarídes

mRNA: ácido ribonucleico mensageiro

MUFA: ácido graxo monoinsaturado

NDUS1: NADH-ubiquinona oxidoreductase (do inglês *NADH-ubiquinone oxidoreductase*)

NFκB: fator nuclear kappa B (do inglês *nuclear factor kappa B*)

NLO: nitrogênio livre de oxigênio

NPY: neuropeptídeo Y (do inglês *neuropeptide Y*)

OMS: organização mundial de saúde

Ovx: ovariectomia

PAI-I: inibidor do ativador de plasminogênio tipo 1 (do inglês *plasminogen activator inhibitor type I*)

POMC: pro-opiomelanocortina (do inglês *pro-opiomelanocortin*)

PTM: modificação pós-traducional

PUFA: ácido graxo poliinsaturado

RET: tecido adiposo retroperitoneal

RETSAT: retinol saturase (do inglês *all-trans-retinol 13,14-reductase*)

RT1-Aw2: *Class I histocompatibility antigen, Non-RT1.A alpha-1 chain*

SFA: ácido graxo saturado

TLR-4: receptor do tipo *toll*-4 (do inglês *toll-like receptor-4*)

TNF- α : fator de necrose tumoral - α (do inglês *tumor necrosis factor - α*)

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1. INTRODUÇÃO

A menopausa marca um período na vida das mulheres caracterizado pela perda de função dos hormônios ovarianos, em que o risco de desenvolver obesidade central é maior do que em qualquer outro (Donato et al, 2006; Georgakis et al, 2016; Kozakawski et al, 2017). A obesidade, por sua vez, pode acarretar uma série de comorbidades, como diabetes tipo 2, doenças cardíacas e psicopatologias, incluindo depressão leve a moderada (Andersson et al, 2007; Jantaratnotai et al, 2017). Dados epidemiológicos e experimentais têm estabelecido a existência de uma associação bidirecional entre obesidade e depressão (McElroy et al, 2004; Bornstein et al, 2006).

Os estrogênios, principalmente na sua forma biológica mais ativa, o estradiol, participam da regulação da homeostase energética, demonstrando afetar, dentre outros mecanismos, acúmulo e distribuição de gordura, sensibilidade à leptina e tolerância à glicose (Lizcano & Guzmán, 2014; Riant et al, 2009; Li et al, 2016). Além disso, alguns dados sugerem que o estradiol pode atuar no sistema nervoso central e assim atenuar as alterações de humor e regulação alimentar causadas pela ovariectomia em ratas (Estrada et al, 2018).

A existência de uma ligação direta entre menopausa e depressão ainda carece de total comprovação, visto que estudos em ambos roedores e humanos têm mostrado resultados conflitantes. Em ratos e camundongos, há dados de que a ovariectomia induziu comportamento de tipo depressivo (Sasayama et al, 2017; Li et al, 2014; Lagunas et al, 2010), mas outros estudos não constataram este efeito (Dornellas et al, 2018; Gogos et al, 2018; Estrada-Carmanera et al, 2011). Em humanos, a transição menopausal foi associada a maior risco de depressão (Vivian-Taylor et al, 2014), enquanto correlações tanto diretas quanto inversas entre os estágios da menopausa e a depressão foram descritas em mulheres de meia-idade (Campbell et al, 2006; Naufel et al, 2019).

Nosso grupo de pesquisa demonstrou previamente que a ingestão de dieta hiperlipídica por ratas ovariectomizadas, embora potencializando o ganho de massa corporal e as alterações metabólicas induzidas pela ovariectomia, falhou em afetar o comportamento de tipo depressivo de uma maneira consistente (Dornellas et al, 2018). Dentre mulheres de meia-idade, grupo que frequentemente inclui uma alta participação de mulheres na pós-menopausa, uma relação

positiva entre sintomas depressivos e marcadores de obesidade foi apontada (Everson-Rose et al, 2009; Murabito et al, 2013; Blümel et al, 2015). Essas considerações indicam a importância de um melhor entendimento acerca da associação obesidade/depressão na pós-menopausa.

Em relação ao metabolismo periférico, foram relatadas diversas alterações no metabolismo do tecido adiposo visceral usando como modelos roedores ovariectomizados. Por exemplo, aumento da conversão de cortisona a colesterol, devido ao aumento de expressão da enzima 11 β -hydroxysteroid dehydrogenase tipo 1, foi encontrado nos depósitos de gordura viscerais, porém não nos subcutâneos, o que contribui para a redistribuição de gordura típica da falta de estrogênio (Anderson et al, 2010). Além disso, os estrogênios parecem também desempenhar um papel no controle específico de enzimas antioxidantes no tecido adiposo retroperitoneal, já que ratas ovariectomizadas demonstraram atividade reduzida de enzimas reguladoras de processos oxidativos, bem como diminuição nos níveis de RNA mensageiro de citocinas e adipocinas anti-inflamatórias (Amengual-Cladera et al, 2012).

Adicionalmente, perturbações nas funções do tecido adiposo visceral têm sido associadas à sua composição de ácidos graxos em diversas condições, como na obesidade induzida por dieta e diabetes (Bueno et al, 2010; Weijers et al, 2012). Entretanto, os dados acerca dos efeitos da obesidade induzida pela perda de hormônios ovarianos na composição de ácidos graxos do tecido adiposo visceral são escassos. Um estudo reportou níveis aumentados de ácidos graxos de cadeia curta na gordura visceral de mulheres na pós-menopausa (Yamatani et al, 2014). Portanto, sugere-se que mais estudos sejam necessários para maior compreensão do assunto.

Um estudo que realizou análise proteômica do tecido adiposo periováriano verificou que ratas ovariectomizadas mostraram uma modulação diferencial na expressão de proteínas relacionadas à transdução de energia, estrutura celular e sistema imunológico (Amengual-Cladera et al, 2016). O uso de uma técnica de proteômica mais sensível e acurada poderia auxiliar na descoberta de um número expressivo de proteínas e vias metabólicas afetadas na gordura visceral pela perda da função ovariana. Além disso, a identificação de mudanças na fosforilação de proteínas é de alta relevância, visto que as fosforilações desempenham um papel de extrema importância em uma série de funções celulares.

Os dados acima expostos indicam que menopausa, obesidade e depressão se conectam de uma maneira complexa e ainda não completamente entendida. A elucidação desses aspectos é de grande importância tanto em termos de saúde pública quanto para questões terapêuticas.

Assim, um dos objetivos do presente estudo foi avaliar parâmetros metabólicos, hormonais e comportamentais induzidos por ovariectomia, aliada ou não a ingestão de dieta hiperlipídica e reposição de estradiol. Os dados também apontam para a falta de entendimento sobre as consequências da falência ovariana sobre o metabolismo do tecido adiposo, o que nos levou ao segundo grande objetivo, o de expandir nosso conhecimento acerca das consequências da falta dos hormônios ovarianos na gordura visceral, examinando para isso os efeitos da ovariectomia e reposição de estradiol sobre o proteoma/fosfoproteoma, assim como sobre o perfil de ácidos graxos do tecido adiposo retroperitoneal de ratas.

2. REVISÃO DA LITERATURA

2.1 Obesidade e Depressão na pós-menopausa

A obesidade é uma doença crônica multifatorial que se caracteriza pelo acúmulo excessivo de gordura corporal e decorre do desequilíbrio entre a ingestão e o consumo calóricos, associando-se a doenças crônicas, como a síndrome metabólica e diabetes tipo 2. O balanço energético depende de processos neurais, endócrinos, metabólicos e comportamentais (Jantaratnotai et al, 2017). Sobrepeso e obesidade representam um dos maiores fatores de risco de morte no mundo (WHO, 2016). No Brasil, estima-se que cerca de 12,5% dos homens e 16,9% das mulheres sejam obesos (Jaime et al, 2013). Por sua vez, a Organização Mundial da Saúde (OMS) estima que em menos de duas décadas a depressão será a doença mais comum no mundo (WHO, 2016). A doença afeta indivíduos de todas as idades, sendo mais evidenciada em mulheres. Os países em desenvolvimento deverão ser os mais afetados, sofrendo altos custos econômicos para o tratamento da doença, além daqueles decorrentes da perda de produtividade dos indivíduos. Assim, esforços direcionados a aprofundar o entendimento dos distúrbios envolvidos nestas enfermidades são necessários.

A depressão é reconhecidamente um transtorno complexo, cujos mecanismos não estão completamente elucidados. Demonstrou-se ativação inflamatória na depressão, associada a aumento na translocação bacteriana devida a maior permeabilidade intestinal (Maes et al, 2008). A presença de lipopolissacarídios na corrente sanguínea desencadeia um processo inflamatório ao ligar-se ao *toll like receptor-4* (TLR-4) e ativar a cascata de sinalização do fator nuclear NFκB e a transcrição de citocinas proinflamatórias (Takeuchi & Akira, 2001). A administração sistêmica de LPS elevou TNF- α , induzindo neuroinflamação associada a comportamento de tipo depressivo (Qin et al, 2007).

Fatores dietéticos também podem influenciar o humor. Evidências sugerem que a ingestão de peixe e outras fontes de ácidos graxos ômega-3 (ω -3) pode proteger contra depressão (Bourre, 2007; Colangelo et al, 2009; Martins, 2009). Baixas concentrações de ácidos eicosapentanóico (EPA) e docosahexanóico (DHA) foram detectadas no plasma, na membrana de células sanguíneas vermelhas e em tecidos adiposos de pacientes depressivos (Mamalakis et al, 2006; Peet et al, 1998). Por outro lado, a ingestão de uma combinação de dieta hiperlipídica

com alta concentração de frutose foi capaz de induzir comportamentos do tipo depressivo em ratos machos após oito semanas de tratamento (Gancheva et al, 2017). Da mesma maneira, o consumo de dieta hiperlipídica *per se* foi capaz de induzir um fenótipo de tipo depressivo em camundongos também tratados por oito semanas, inclusive diminuindo a expressão do fator orexigênico neuropeptídio Y (NPY) (Hassan et al, 2019).

Diversos estudos apontam para a existência de forte ligação entre obesidade e depressão, dois fatores crônicos complexos dos quais não se conhecem as reais causas e que representam graves problemas de saúde pública que causam prejuízos à qualidade de vida (Bornstein et al, 2006). Um estudo prospectivo de 5,5 anos reportou que uma grande parte dos indivíduos eutróficos portadores de depressão grave desenvolveram obesidade, atribuindo-se um papel preditivo da depressão para o desenvolvimento da obesidade (Lasserre et al, 2014). Uma revisão sobre os aspectos metabólicos envolvidos na associação obesidade/depressão concluiu que, em humanos, um estado emocional negativo favorece a ingestão de alimentos ricos em gorduras, o que, por sua vez, contribui para o desenvolvimento da depressão, agindo por múltiplos mecanismos incluindo glicocorticoides, adipocinas, insulina e sinalização inflamatória (Hryhorczuk, Sharma, Fulton, 2013).

Em adolescentes obesos de ambos os性os que apresentavam depressão, observou-se que, após uma terapia multidisciplinar capaz de reduzir estas alterações, a diminuição dos níveis de leptina (hormônio produzido principalmente por adipócitos com papel pró-inflamatório) e o aumento dos níveis de adiponectina (hormônio também de origem adipocitária, com ação anti-inflamatória), correlacionaram-se com a atenuação dos sintomas depressivos (de Carvalho-Ferreira et al, 2015). De fato, há ainda evidências de que um aumento dos níveis de leptina, modulado pelo aumento do tecido adiposo visceral, se mostra como fator de risco para o desenvolvimento da depressão (Milaneschi et al, 2014). Estas evidências sugerem que aspectos inflamatórios ligados à obesidade podem ser relevantes para o desenvolvimento de depressão. A relevância destes fatores para o desenvolvimento de depressão após a menopausa não está esclarecida.

O período que compreende a pós-menopausa representa a fase da vida da mulher na qual a associação obesidade/depressão apresenta-se bastante expressa (Everson-Rose et al, 2009). Na pós-menopausa, as mulheres apresentam acúmulo de gordura visceral e há evidências de que a deficiência de estrogênios tenha papel relevante nesta distribuição do tecido adiposo,

afetando também o perfil de lipoproteínas plasmáticas e associando-se a doenças crônicas, como a síndrome metabólica e diabetes tipo 2 (Carr 2003, Ferrara et al, 2002; Milewicz et al, 1996). Essa redistribuição de gordura está associada a maiores índices de hipertensão, resistência insulínica e intolerância à glicose, dentre outros fatores (Carr, 2003). A prevalência de obesidade e o risco de alterações metabólicas aumentam na pós-menopausa, podendo atingir até 50% das mulheres de 50 a 59 anos (Wildman & Sowers, 2011). A redução do gasto energético contribui para este aumento (Lovejoy et al, 2008). Ratas submetidas à ovariectomia podem apresentar hiperfagia e aumento de massa e adiposidade corporais, efeitos que podem ser atenuados pela reposição de estrogênios ou fitoestrogênios (Rogers et al, 2009). A exposição à dieta hiperlipídica exacerbou o aumento de massa corporal e a resistência insulínica induzidos por ovariectomia e a reposição estrogênica foi benéfica em atenuar estes efeitos (Yonezawa et al, 2012).

O estrogênio participa da regulação do consumo de alimento por vias anorexígenas do sistema nervoso central, visto que o aumento de níveis estrogênicos leva a um importante aumento no número de impulsos excitatórios nos neurônios de pro-opiomelanocotina (POMC) do núcleo arqueado do hipotálamo (Gao et al, 2007). Dados da literatura mostram que ratas ovariectomizadas apresentaram resistência central à leptina e alto teor de NPY, o que predispõe a ganho de massa e gordura corporais (Ainslie et al, 2001). Além disso, um estudo em mulheres de meia idade observou que os níveis da proteína do sistema complemento C3 foram elevados naquelas menopausadas e se correlacionaram a níveis mais altos do inibidor do ativador de plasminogênio tipo 1 (PAI-I) (Khoudary et al, 2013).

Doenças associadas ao estresse, que incluem ansiedade e depressão, afetam a qualidade de vida de mais de um bilhão de pessoas no mundo (Schmidt et al, 2011). Durante a fase reprodutiva, os períodos de menor produção de estrógeno associam-se a maiores taxas de aparecimento de sintomas depressivos em mulheres e a depressão pós-parto também está relacionada à queda dos níveis estrogênicos. Estas observações concordam com as evidências de que mulheres menopausadas apresentaram maior risco de manifestarem quadros de depressão (Andreatini et al, 2002; Walf & Frye, 2006; Elavsky & McAuley, 2007). Esta maior vulnerabilidade foi relacionada às alterações hormonais e também ao ganho de massa corporal (Ancelin et al, 2007). Os hormônios estrogênicos têm a capacidade de aumentar a síntese e a atividade de neurotransmissores relacionados ao humor, como serotonina e dopamina

(Epperson et al, 2012; Wharton et al, 2012). Tem sido reportado que, modulando circuitos cerebrais reguladores das emoções, estes hormônios exercem um efeito estabilizador sobre as respostas cognitivas e emociais a fatores estressores (Newhouse & Albert, 2015).

Tomados em conjunto, os dados apontam para a existência de uma relação entre a depressão e o aumento da adiposidade e que esta associação pode estar presente na menopausa. Os mecanismos responsáveis não estão elucidados. Estas reflexões conduzem à sugestão de que a adoção de abordagens que permitam o exame de possíveis mediadores apresenta grande potencial de contribuição para o entendimento dos mecanismos bioquímicos envolvidos na associação entre a menopausa e a obesidade e depressão.

2.2 Tecido adiposo branco e perfil lipídico

O tecido adiposo branco é o tipo predominante de tecido adiposo nos mamíferos, sendo composto primariamente por adipócitos envoltos por tecido conjuntivo altamente vascularizado e inervado, contendo fibroblastos, macrófagos, dentre vários outros tipos celulares (Ahima, 2006). Enquanto sua participação no processo de termogênese é mínima, o tecido adiposo branco é um excelente isolador térmico e tem papel importante na conservação da temperatura corporal (Fonseca-Alaniz et al, 2007). Além de sua importante função como órgão armazenador de triglicerídeos, também possui uma excepcional atividade secretora, podendo liberar fatores parácrinos e endócrinos chamados de citocinas. Suas ações são importantes para a dinâmica do controle do metabolismo energético, influenciando tecidos responsáveis pelo controle metabólico e de gasto energético, como fígado e músculo esquelético, sistema nervoso central, além de adipócitos vizinhos (Galic et al, 2010).

Dentre as citocinas do tecido adiposo, as adipocinas leptina e adiponectina (produzidas pelo próprio adipócito) tem relevante papel fisiológico. A leptina tem produção proporcional à massa adiposa e desempenha papel importante como sinalizadora das reservas de gordura para o sistema nervoso central, mas possui ação pró-inflamatória. Outras citocinas inflamatórias incluem a resistina (uma adipocina), interleucina 6 (IL-6) e TNF- α , estes produzidos principalmente por macrófagos do tecido adiposo. A adiponectina eleva a sensibilidade à insulina e modula a atividade de mediadores inflamatórios, principalmente inibindo a ação de citocinas pró-inflamatórias. Demonstrou-se correlação direta entre leptina circulante e de risco

de obesidade, resistência insulínica e doenças cardiovasculares, enquanto essa correlação se mostra inversa quanto aos níveis de adiponectina (Havel et al, 1996; Galic et al, 2010; Trujilo and Scherer, 2006).

O tecido adiposo branco se encontra distribuído em diferentes depósitos, classificados anatomicamente como subcutâneo e visceral. O tecido adiposo subcutâneo localiza-se primariamente em depósitos sob a pele nas áreas abdominais, glúteas e femurais, enquanto o visceral inclui os depósitos de gordura próximos ou dentro das vísceras da cavidade abdominal, tendo como exemplo as gorduras mesentérica, omental e retroperitoneal (Fonseca-Alaniz et al, 2007). Estudos apontam que há diferenças depósito-específicas com relação à expressão de diversos genes, sendo que o excesso de tecido adiposo visceral favorece a produção de citocinas inflamatórias (Gesta et al, 2006; Trujilo and Scherer, 2006). É de importância fisiopatológica o fato de que o tecido visceral tem ligação anatômica direta com a circulação portal, liberando tanto adipocinas quanto ácidos graxos livres diretamente no fígado. Além disso, os depósitos viscerais sofrem maior infiltração de macrófagos durante o desenvolvimento da obesidade, os quais também constituem importante fonte de fatores inflamatórios (Hajer et al, 2008; Cancello et al, 2006).

Na menopausa, ocorre redistribuição dos depósitos de gordura nas mulheres, com aumento dos depósitos viscerais em detrimento da diminuição dos depósitos subcutâneos, e se atribui à falta de estrogênios um papel importante nesta alteração, embora os mecanismos responsáveis não estejam totalmente compreendidos (Mayes and Watson, 2004).

Os estrogênios são derivados dos precursores androgênicos androstenediona e testosterona por meio de aromatização. Estes precursores androgênicos estão disponíveis livremente na circulação, prontos para serem convertidos em estrogênio de maneira local em múltiplos tecidos extragonadais, incluindo cérebro, tecido adiposo, ossos, fígado e pele, de modo que a expressão da enzima aromatase é geralmente considerada evidência de produção local de estrogênio. Depois das gônadas, o tecido adiposo é a principal fonte de estrogênios, tanto em mulheres quanto em homens, e a contribuição deste tecido para os estrogênios circulantes aumenta com o envelhecimento (Bracht et al, 2019).

Os estrogênios exercem importantes ações sobre o tecido adiposo, afetando acúmulo e distribuição de gordura corporal bem como afetando a resposta imune inata exercendo ação anti-inflamatória (Eaton & Sethi, 2019). Em ratas, a ovariectomia promoveu acúmulo de

gordura visceral, aumento dos níveis circulantes de ácidos graxos não esterificados e do teor tecidual da enzima lipase de triglicerídos do adipócito (ATGL), além de diminuição dos níveis de perilipina, indicando um desbalanço no controle do metabolismo do tecido (Wohlers et al, 2010). Dados apontam um aumento tanto de ácidos graxos saturados quanto de seus metabólitos na gordura visceral de mulheres na pós-menopausa. Dentre os ácidos graxos que tiveram maior aumento de conteúdo, o estudo destaca o ácido palmítico, um dos principais componentes dos ácidos graxos livres e que tem papel estimulatório na secreção de citocinas pró-inflamatórias pelos macrófagos (Yamatani et al, 2013).

Os estrogênios podem inibir diretamente a deposição de gordura por reduzir a síntese de triglycerídos pela diminuição da atividade da lipase de lipoproteínas (LPL), uma enzima que regula a captação de lipídios pelos adipócitos (Cooke & Naaz, 2004). Dados mostraram que a ovariectomia aumentou os níveis de LPL nos adipócitos, enquanto que a administração de doses fisiológicas de estradiol foi capaz de reverter o processo (Hamosh & Hamosh, 1975). Mais recentemente, verificou-se que acúmulo de gordura visceral também tem relação com o aumento do processo de lipogênese *de novo*, através do qual carboidratos são convertidos em ácidos graxos, que são então esterificados e se tornam triglicerídeos para fins de armazenamento (Song et al, 2018). Os estrogênios foram apontados como tendo uma ação no controle da lipogênese *de novo* tanto indireta (através do controle de acúmulo de gordura visceral) quanto direta, já que têm papel na regulação da proteína quinase ativada por AMP (AMPK). Esta última, por sua vez, é uma das proteínas responsáveis por modular a atividade de enzimas presentes no processo da lipogênese, como a acetil-Coa carboxilase (ACC) e a ácido graxo sintase (FAS) (Lopez & Tena-Sempere, 2017).

Além da contribuição dos estrogênios ao controle da síntese lipídica, estudos em camundongos apontaram que estes hormônios também influenciam processos de degradação lipídica, em especial a β -oxidação mitocondrial de ácidos graxos (Misso et al, 2003; Oliveira et al, 2018). Dados apontam a ocorrência de diminuição da oxidação lipídica em mulheres na pós-menopausa, onde os níveis séricos de estradiol se correlacionaram inversamente com a oxidação lipídica e positivamente com o conteúdo de ácidos graxos livres não esterificados (O'Sullivan et al, 2001).

Levando-se em consideração a importância dos estrogênios sobre as funções metabólicas do tecido adiposo branco e a modulação do conteúdo do perfil lipídico, faz-se necessário o

entendimento mais completo de como essa trama de mecanismos pode se alterar no período pós-menopausa e quais suas consequências.

2.3 Terapia de reposição hormonal

Embora os hormônios ovarianos tenham um importante papel no metabolismo lipídico e deposição de gordura nos tecidos adiposos, estes hormônios ou seus derivados não têm sido usados de forma extensiva no combate ou prevenção da obesidade, muito porque para trazer os efeitos necessários geralmente encontram reações adversas em outras áreas do metabolismo, como aumento de risco de eventos coronários, tromboembolismo venoso, derrame e inclusive com indícios de promover um ambiente fisiológico mais propício ao surgimento de carcinomas (Marjoribanks et al, 2017). Apesar disso, uma área em que os hormônios estrogênicos parecem ter mais efeitos positivos é na terapia de reposição hormonal em mulheres mais velhas (acima dos 60 anos), onde a terapia se mostrou com os melhores resultados em diminuir a obesidade central, prevendo assim alguns dos problemas causados pela falta destes hormônios (Sites et al, 2001).

Por sua vez, a terapia de reposição de estrogênio mostrou alguma melhora na qualidade de vida de mulheres eutróficas, mas seus possíveis efeitos antidepressivos permanecem pouco entendidos (Bello et al, 2010). Uma das questões a serem corretamente respondidas é a janela do período de menopausa em que a terapia de reposição hormonal teria maiores chances de remissão dos sintomas depressivos, já que, ao contrário dos efeitos benéficos da terapia no metabolismo periférico em mulheres mais velhas, o uso de estrogênio transdermal durante oito semanas em mulheres acima de 60 anos não foi eficaz em diminuir os efeitos da depressão leve ou moderada (Morrison et al, 2004). Por outro lado, resultados positivos são descritos com relação ao uso da terapia em mulheres em transição menopausal, não apenas mostrando efeitos anti-depressivos, mas também melhorando outros aspectos, como sintomas vasomotores e distúrbios do sono (Soares & Frey, 2010). Em modelos animais, dados apontam sucesso no uso de estradiol em atenuar os comportamentos do tipo depressivo decorrentes da ovarietomia (Estrada et al, 2018).

Não encontramos na literatura dados sobre o efeito da reposição hormonal sobre o estado depressivo em condições de falência ovariana concomitante a ingestão hiperlipídica.

Considerando que este tipo de padrão dietético pode ser comum nas mulheres menopausadas, é de interesse explorar estes aspectos.

2.4 Proteômica e fosfoproteômica

O termo proteoma se aplica ao conjunto de proteínas de uma determinada matriz. Este conjunto é dinâmico e seu perfil se altera de acordo com o estado fisiológico e as fases de diferenciação celular. Na investigação dos processos biológicos, o uso de estudo baseado em espectrometria de massas é bastante relevante para a investigação sistemática de proteínas em uma determinada matriz (Jensen, 2004).

A proteômica alia as técnicas de separação de proteínas, que incluem eletroforese bidimensional e cromatografia líquida de alta eficiência, e de detecção, geralmente por espectrometria de massas, a ferramentas de bioinformática, utilizadas para identificação das proteínas bem como sua alocação em processos biológicos. Há grandes expectativas quanto ao avanço das pesquisas na área de proteômica com relação às mais diversas áreas de estudo, como obesidade, diabetes, doenças cardiovasculares, câncer e complicações do sistema nervoso central. Tal avanço facilitará não apenas a descoberta qualitativa, mas a quantificação de proteínas importantes para a regulação de vias metabólicas, e cuja síntese, degradação e modificação são afetadas por distúrbios fisiológicos das mais variadas origens (Wang et al, 2006).

As modificações pós traducionais (PTM - *post-translational modification*) são alterações químicas covalentes que ocorrem nas proteínas após sua síntese nos ribossomos. As PTMs são encontradas em todos os tipos de proteínas e desempenham papel importante em diversos processos celulares, como regulação da atividade enzimática, translocação de proteínas e sua interação com outras moléculas, transdução de sinais, entre outros (Shumyantseva et al, 2014).

Dentre os diversos tipos de modificações pós-traducionais, a fosforilação é a mais comum e tem papel-chave em atividades celulares fundamentais, como regulação do metabolismo energético, regulação do ciclo celular, crescimento e apoptose. A fosforilação e a desfosforilação dos resíduos de serina, treonina e tirosina são os principais reguladores das vias de sinalização (Reinders & Sickmann, 2005). É reconhecida a associação de alterações na

fosforilação de proteínas com diversas doenças, o que faz da análise do fosfoproteoma uma ferramenta muito útil na identificação de novos alvos terapêuticos e biomarcadores (Yil et al, 2013).

Devido à importância da fosforilação em vários processos biológicos, acreditamos que o estudo do fosfoproteoma do tecido adiposo retroperitoneal de animais que apresentam deficiência de hormônios ovarianos pode nos dar indicações relevantes sobre os mecanismos responsáveis pela associação entre obesidade e menopausa.

A elucidação de aspectos moleculares que participam da relação obesidade/menopausa pode contribuir para a prevenção e o tratamento dos efeitos desta associação. Deste modo, no presente trabalho propusemos realizar uma análise ampla de mediadores da relação obesidade/menopausa através da análise de proteínas e perfil lipídico do tecido adiposo retroperitoneal, em modelo animal de deficiência de hormônios ovarianos. A avaliação dos efeitos da reposição hormonal também foi realizada.

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar, em ratas, consequências comportamentais e metabólicas da ausência de hormônios ovarianos e os efeitos da reposição hormonal e da ingestão hiperlipídica.

3.2. Objetivos específicos

Utilizando ratas ovariectomizadas, que receberam ou não reposição de estradiol, avaliar:

- Massa corporal e ingestão de dieta controle ou hiperlipídica (enriquecida com banha de porco), durante 12 semanas desde a ovariectomia ou falsa-ovariectomia.

Ao final das 12 semanas, avaliar:

- Comportamentos de tipo depressivo
- Parâmetros bioquímicos e hormonais séricos
- Perfil de expressão e fosforilação proteicas do RET, nos grupos alimentados com dieta controle
- Perfil de ácidos graxos do RET, nos grupos alimentados com dieta controle

4. MÉTODOS

4.1. Grupos experimentais e procedimentos *in vivo*

Todos os procedimentos foram aprovados e conduzidos de acordo com as diretrizes do Comitê de Ética em Pesquisa da Universidade Federal de São Paulo (CEUA nº: 2172030315/2016), seguindo os protocolos éticos estabelecidos pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA). Ratas Wistar, com doze semanas de idade, foram submetidas à ovariectomia bilateral (Ovx, n = 71) ou falsa-ovariectomia (Sham, n = 35), após anestesia com cetamina/xilazina (66/33mg /kg, ip). Para reposição hormonal, 35 animais Ovx foram implantadas com um pellet subcutâneo de 17 β -estradiol (0,25 mg/pellet, liberação de 90 dias; Innovative Research of America, Sarasota, Flórida, EUA), representando uma dosagem diária de 2,8 μ g. Imediatamente após a cirurgia, todos os animais receberam penicilina (60.000U, i.m.) e ibuprofeno (1 mg/kg de massa corporal, v.o.). Doses adicionais de ibuprofeno foram administradas uma vez ao dia por 2 dias consecutivos. Após a cirurgia, todos os animais foram alojados (dois a três por gaiola) e mantidos sob iluminação (ciclo de 12h claro/escuro, luzes acesas às 6h) e condições de temperatura ($23 \pm 1^\circ\text{C}$) controladas, com livre acesso a comida e água. Na gaiola, os grupos acima foram divididos aleatoriamente de acordo com a dieta oferecida. Os grupos ShamC, OvxC e OvxC+E2 receberam dieta padrão para ratos (2,87 kcal/g, 15% das calorias provenientes de gordura, Nuvilab CR-1, Nuvital Nutrientes SA, Colombo, PR, Brasil) enquanto os grupos ShamL, OvxL e OvxL+E2 receberam dieta hiperlipídica (3,60 kcal/g, 45% gordura) enriquecida com banha de porco (Cooperativa Central Aurora de Alimentos, Chapecó, Santa Catarina, Brasil). A dieta rica em gordura foi preparada adicionando-se à dieta padrão (p/p) 18% de banha, 2% de óleo de soja, 20% de caseína, 10% de sacarose e 0,02% de hidroxitolueno butilado (BHT) como anti-oxidante.

A massa corporal e a eficiência alimentar de 24 horas foram avaliadas semanalmente. A ingestão alimentar e calórica foi medida como o valor médio de consumo para cada rato. A eficiência alimentar foi calculada como: (ganho de massa corporal/consumo de energia) x 100 (Iwasa et al, 2018).

4.2 Testes comportamentais

4.2.2. Labirinto em cruz elevado

O teste do labirinto em cruz elevado foi realizado durante a décima semana de tratamento. A aparelhagem deste teste consiste em dois braços abertos e dois braços fechados conectados por uma plataforma central no alto do solo e iluminados por uma luz fraca. No dia do teste, as ratas foram transportadas individualmente para a sala de testes e colocadas na plataforma central de frente para um braço aberto. O teste foi realizado e gravado durante cinco minutos por uma câmera de vídeo para avaliação posterior. O número de entradas nos braços abertos e fechados foi medido por dois observadores de modo duplo-cego (Pellow et al., 1985). O número de entradas nos braços fechados e o número total de entradas foram usados para avaliação da atividade locomotora (Rodgers & Johnson, 1995; Degroot et al, 2001; Zarrindast et al, 2008; Ebrahimi-Ghiri et al, 2019).

4.2.3. Nado forçado modificado

Na semana seguinte ao teste do labirinto em cruz elevado, os animais foram transportados para a sala de teste do nado forçado modificado, e colocados individualmente em um cilindro de acrílico (30 cm de diâmetro 30cm e 50 cm de altura), preenchido com água até a altura de 35 cm, volume suficiente para impedir que o animal tocasse o fundo com a cauda. Foi realizada inicialmente uma sessão de treinamento de 15 minutos. Após 24 horas, as sessões de teste de 5 minutos foram realizadas e gravadas para análises subsequentes. A cada 5 segundos, o comportamento predominante foi caracterizado em: natação (movimentos de natação por todo o cilindro), escalada (movimentos direcionados para cima com as patas dianteiras tocando a parede do cilindro) e imobilidade (flutuação com movimentos mínimos e cabeça logo acima da água). A latência para imobilidade (tempo em segundos antes do primeiro comportamento de imobilidade) também foi avaliada (Porsolt et al, 1977).

4.3 Análises séricas e teciduais

No final da décima segunda semana, após 24 horas de jejum, as ratas foram anestesiadas com tiopental (50 mg/kg, intraperitoneal) e eutanasiadas. O sangue foi coletado e o plasma ou soro armazenado a -80°C até análise. Útero e depósitos de gordura gonadal, mesentérica e retroperitoneal foram dissecados e pesados.

Os níveis séricos de glicose, colesterol total, HDL-colesterol e triglicerídeos foram determinados por kits colorimétricos enzimáticos disponíveis no mercado (Labtest Diagnóstica, Lagoa Santa, MG, Brasil). Os kits ELISA (Millipore, Bedford, MA, EUA) foram utilizados para determinar os níveis séricos de leptina (sensibilidade - 0,08 ng/mL; precisão intra-ensaio - 2,49%; precisão inter-ensaio - 3,93%); insulina (sensibilidade - 0,1 ng/mL; precisão intra-ensaio - 1,33%; precisão inter-ensaio - 6,71%) e adiponectina (sensibilidade - 0,4 ng/mL; precisão intra-ensaio - 1,18%; precisão inter-ensaio - 7,34%). O teor tecidual (RET) de TNF- α (sensibilidade - 2,4 ng/mL; precisão intra-ensaio - 4,98%; precisão inter-ensaio - 9,44%) e IL-6 (sensibilidade - 0,7 ng/mL; precisão intra-ensaio - 3,96%; precisão inter-ensaio - 8,64%) também foi determinado através de ELISA.

O índice HOMA-IR foi calculado como: HOMA-IR = (insulina em jejum (μ M) x glicose em jejum (mM)) /22,5. O índice HOMA- β foi calculado como: HOMA- β = (insulina x 20) / (glicose-3,5) (Matthews et al, 1985).

4.4 Proteoma e fosfoproteoma do RET

4.4.1 Preparo das amostras

Alíquotas de 800mg de RET foram homogeneizadas em 1 mL de tampão contendo bicarbonato de amônio 50 mM, desoxicolato de sódio a 1% (m/v), água deionizada e coquetel inibidor de proteases/fosfatases (Thermo Scientific, Rockford, IL, EUA) (Pasing et al, 2017). As amostras foram centrifugadas (19.000 x g por 30 minutos a 4°C) e a concentração de proteínas nos sobrenadantes foi determinada usando o kit colorimétrico BCA (Pierce - Thermo

Scientific, Rockford, IL, EUA). Alíquotas de 200 µg de proteína foram diluídas em bicarbonato de amônio 50 mM até um volume final de 85 µL. As amostras foram então submetidas à redução com 2,5 µl de DTT 100 mM a 60°C por 30 min e alquilação com 2,5 µl de iodoacetamida 300 mM, a temperatura ambiente por 30 min. As proteínas foram digeridas durante a noite usando tripsina (Promega, Fitchburg, WI, EUA) na proporção de 1: 100 (p:p) enzima: proteína a 37°C. Após digestão, as amostras foram centrifugadas (19.000 x g por 15 minutos a 4°C), os sobrenadantes foram coletados e um volume igual de acetato de etila foi adicionado. Em seguida, ácido trifluoroacético foi adicionado para uma concentração final de 0,5%. As amostras foram homogeneizadas em vórtex e centrifugadas (19.000 x g por 5 minutos a 4°C), os sobrenadantes foram coletados, transferidos para filtros Millex-GV (Millipore, Burlington, MA, EUA), recuperados, secos em SpeedVac e armazenados a -80°C até análise por espectrometria de massas.

4.4.2 Espectrometria de massas acoplada à cromatografia líquida

Após a reconstituição das amostras em ácido fórmico 1% em água (v/v), o teor de proteína foi determinado fluorimetricamente (Qubit® 3.0 Fluorometer, Thermo Scientific, Rockford, IL, EUA) e a concentração final foi ajustada para 0,5 µg/µL. As análises proteômicas e fosfoproteômicas foram realizadas pelo método aquisições de dados independentes através de espectrometria de massa acoplada à cromatografia líquida (ACQUITY UPLC M-class acoplada ao espectrômetro de massa Synapt G2-Si) (Waters, Milford, MA, EUA). Uma coluna ACQUITY UPLC HSS T3 nanoACQUITY (100 Å. 1.8 µm. 75 µm × 150 mm., Waters) foi utilizada para a separação de peptídeos (1 µg) em gradiente de acetonitrila começando em 7% (v/v) por 54 minutos, e então aumentado para 80% de acetonitrila por 13 minutos, a fluxo de 500 nL/min). A aquisição de dados foi realizada utilizando-se ionização por nano-ESI em modo positivo, no modo HDMS^E. O espectômetro de massas foi calibrado utilizando-se o peptídeo humano Glu-fibrinopeptídeo e os espectros de massa foram identificados no intervalo de 50 a 2000 *m/z*.

4.4.3 Identificação de proteínas e fosfopeptídios

O processamento dos dados da espectrometria de massa e a busca no banco de dados relacionada às sequências de *Rattus norvegicus* (banco de dados UniProtKB/Swiss-Prot, www.uniprot.org, 8680 entradas) foram realizados através do software Progenesis for Proteomics (versão 4.0, Waters). A identificação de peptídeos seguiu os seguintes parâmetros: máximo de 1 local de clivagem perdida para digestão com tripsina; carbamidometilação da cisteína como modificação fixa e oxidação da metionina como modificação variável. Para identificação das fosforilações, o programa utilizou-se das modificações variáveis em serina, treonina e tirosina. A taxa de *false discovery rate* (FDR) usada foi de 1%. A identificação de proteínas seguiu os critérios: mínimo de 2 íons-fragmento por peptídeo, 5 íons-fragmento por proteína e 2 peptídeos por proteína. A quantificação relativa foi realizada pela abordagem Hi-N, utilizando-se os três peptídeos mais abundantes, priorizando-se os peptídeos únicos (Silva et al, 2005). Os dados normalizados foram exportados para arquivos de Excel.

4.4.4 Análise de vias

As proteínas diferencialmente expressas e / ou fosforiladas foram incluídas no teste para determinar vias enriquecidas, usando a plataforma online Panther (<http://www.pantherdb.org>, versão 14.1, lançada em 2019-07-11). O proteoma de *Rattus norvegicus* foi usado como lista de referência e a busca por vias foi feita através do banco de dados Reactome Pathways.

4.5 Determinação da composição de ácidos graxos no RET

Os lipídios totais do RET foram extraídos em hexano/isopropanol (3:2 v/v) contendo hidroxitolueno butilado a 0,01% (BHT). Após adição de clorofórmio / metanol / água (2:1:1 v/v/v), as amostras foram centrifugadas (10.000 x g por 10 minutos) e a fase orgânica foi separada e evaporada completamente com nitrogênio livre de oxigênio (NLO). Os lípidos foram

novamente ressuspensos em clorofórmio/metanol/água (8:4:3 v/v/v) e a camada de clorofórmio foi seca sob NLO.

A análise de ácidos graxos foi realizada conforme protocolos pré-estabelecidos (Bueno et al, 2012). Para isso, os ésteres metílicos de ácidos graxos (FAME) foram obtidos por aquecimento das amostras com cloreto de acila a 15% em metanol em tubo selado a 70°C durante 3 horas. Uma solução de NaCl a 5% foi utilizada para interromper a reação, a temperatura ambiente, e os FAMEs foram extraídos através de 3 lavagens com éter de petróleo contendo 0,01% de BHT. A cromatografia gasosa com detector de ionização de chama (GC2010 Plus, SHIMADZU, Kyoto, Japão) foi realizada com uma coluna capilar TRACE TR-FAME (Thermo Scientific, Rockford, IL, EUA). A porcentagem dos ácidos graxos foi quantificada usando o software Labsolutions (SHIMADZU, Kyoto, Japão).

4.6 Análise estatística

A análise univariada foi realizada com o Statistica 12 Software (StatSoft, Tulsa, OK, EUA). A normalidade e a homogeneidade das variáveis massa corporal, variáveis comportamentais, massa de depósitos de gordura e variáveis dos parâmetros séricos foram avaliadas pelo teste de Shapiro-Wilk e Levene, respectivamente. As variáveis paramétricas, incluindo os dados do perfil de ácidos graxos, foram expressas como média ± erro padrão e analisadas por análise de variância (ANOVA) de uma ou duas vias conforme necessário, seguidas do teste *post hoc* de Tukey. As variáveis não paramétricas foram expressas em mediana (intervalo interquartil) e analisadas por Kruskal-Wallis, seguida de comparações múltiplas bicaudais dos valores de p. A significância estatística foi estabelecida em p <0,05.

Os dados de proteoma e fosfoproteoma foram transformados em log, normalizados pela escala de pareto e submetidos à análise multivariada através da plataforma online metaboanalyst (<http://www.metaboanalyst.ca>). Os dados foram obtidos através da análise de componentes principais (PCA) e análise discriminante por quadrados mínimos parciais (PLSDA). Considerou-se a importância variável na projeção (VIP) acima de 1,5 nos cinco componentes principais, a fim de identificar as proteínas e fosfopeptídeos que mais contribuíram para a discriminação entre os grupos.

As vias significantemente enriquecidas foram determinadas pelo teste exato de Fisher, seguido por correção de Bonferroni com significância definida em $p < 0,05$.

5. RESULTADOS E DISCUSSÃO

Os resultados e discussão estão apresentados na forma de dois artigos científicos:

Artigo 1- High-fat intake induces depressive-like behavior in ovariectomized rats

Publicado em Scientific Reports, 2018

Artigo 2 – Ovariectomy modifies lipid metabolism of visceral white fat in rats: a proteomic approach

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High-fat diet intake induces depressive-like behavior in ovariectomized rats

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This study tested the effects of ovariectomy, allied or not to high-fat feeding and estradiol replacement, on hormonal, metabolic and behavioral parameters, to explore the connection of obesity and depression after menopause. Wistar rats were either ovariectomized or sham-operated and fed with either standard chow or lard-enriched diet for twelve weeks. Sub-groups of ovariectomized rats received estradiol replacement. Depressive-like behaviors were assessed by the forced swim test and locomotor activity was assessed by the elevated plus maze test. Ovariectomy alone increased body weight gain and feed efficiency and induced hyperleptinemia and glucose intolerance while it increased caloric intake and body adiposity only marginally. High-fat intake alone induced obesity and, in combination with ovariectomy, accentuated the ovariectomy-induced alterations. Estradiol replacement attenuated the hormonal alterations only in chow-fed rats. Ovariectomy combined with high-fat intake induced depressive-like behaviors, which were marginally attenuated by estradiol. Depressive-like behaviors were associated with metabolic and body composition parameters and with estrogen status. The data indicate that the vulnerability to develop depression after menopause is influenced by high-fat intake. It is suggested that weight management is a crucial issue in postmenopausal women, probably having a beneficial role in preventing the appearance of mental health problems.

Characterized by the loss of ovarian function, menopause is a period in women's life in which the risk of developing central obesity is higher than in any other^{1–3}. Obesity brings a series of comorbidities, such as type 2 diabetes, heart diseases, and psychopathologies including mild to moderate depression^{4,5}. Both epidemiological and experimental data seem to have established the existence of a bidirectional association between obesity and depression^{6,7}.

Estrogen (mostly in the form of estradiol) participates in the regulation of energy homeostasis, having been shown to affect, among other mechanisms, fat accumulation and distribution, leptin sensitivity and glucose tolerance^{8–10}. Moreover, acting at central nervous system sites, estradiol has been suggested to attenuate the mood and body weight alterations caused by ovariectomy in rats¹¹. These considerations indicate the relevance of a better understanding of the obesity/depression association after menopause.

We have previously demonstrated that the intake of a high-fat diet by ovariectomized rats, while potentiating the ovariectomy-induced body adiposity gain and metabolic alterations, failed to affect depressive-like behaviors consistently¹². Among middle-aged women, a group that frequently includes a high participation of post-menopausal individuals, a positive relation between depressive symptoms and obesity markers has been shown^{13–15}.

The existence of a direct connection between menopause and depression itself still lacks ascertainment, since studies in both rodents and human subjects have yielded conflicting results. In rats and mice, ovariectomy has been shown to either induce depressive-like behaviors^{11,16–18}, or to fail to do so^{12,19–21}. In humans, the menopausal transition has been associated with high risk to develop depression²² while either a direct or an inverse relation between the menopausal stages and depression has been described in middle-aged women^{23,24}.

All the above data indicate that menopause, obesity, and depression connect in a complex and still unascertained manner. The elucidation of these aspects are of great importance to both public health and therapeutic issues. Hence, the present study evaluated metabolic, hormonal and behavioral parameters in an ovariectomized

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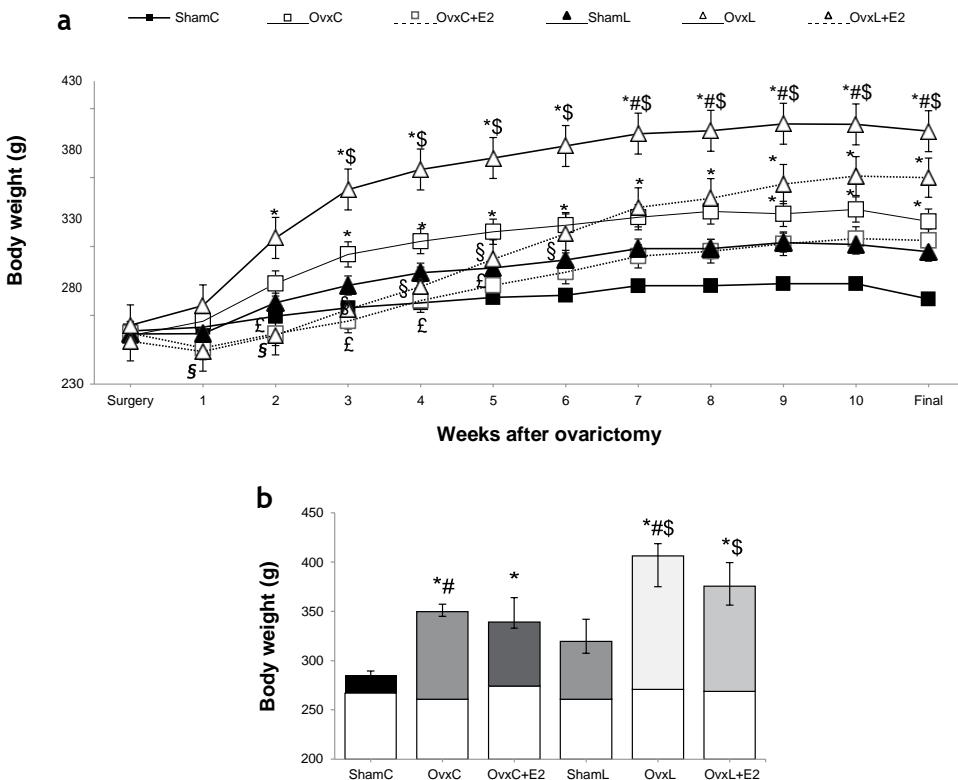


Figure 1. Weekly evolution of body weight (a) and 12-weeks cumulative body weight gain (b). The blank bars in Figure b represent the initial body weight. Groups were fed with either control diet - ShamC ($n = 18$), OvxC ($n = 18$), OvxC + E2 ($n = 18$) or high-lard diet - ShamL ($n = 17$), OvxL ($n = 18$), OvxL + E2 ($n = 17$), for twelve weeks. Data are expressed as medians and interquartile range (Q1–Q3) for both figures. * $p < 0.05$ vs. ShamC; # $p < 0.05$ OvxC vs. OvxL; \$ $p < 0.05$ vs. ShamL; ‡ $p < 0.05$ vs. OvxC + E2; ‡‡ $p < 0.05$ vs. OvxL + E2.

rat model, allied or not to high-fat feeding and estradiol replacement. The main purposes of these experiments were to address the questions of whether ovarian failure associates with depression and to what extent obesity is relevant to this connection.

Results

High-fat intake accentuates the body weight gain and adiposity changes induced by ovariectomy. Figure 1a depicts the weekly evolution of body weight and Fig. 1b shows the cumulative body weight gain throughout the treatment. The animals presented similar body weight in the beginning of the treatment. Body weight gain differed significantly among the groups [$H(5) = 73.21$, $p = 0.001$]. All groups gained more weight than the ShamC group, although not significantly so in the ShamL group (157% higher, $p = 0.252$). Ovariectomy caused a significant increase in body weight gain, regardless the diet. Figure 1a shows that estradiol replacement reduced body weight gain during the first 5 weeks of treatment in the control-fed groups (OvxC vs. OvxC + E2) and during the first 6 weeks in the lard-fed groups (OvxL vs. OvxL + E2). However, by the end of the treatment, this effect was no longer significant.

The feed efficiency was significantly different among the groups [$H(5) = 69.21$, $p < 0.001$] (Fig. 2a). Ovariectomy induced significantly higher feed efficiencies than those of the Sham groups throughout the treatment, regardless of the diet. Estradiol replacement was effective in reducing feed efficiency up to the 5th week whereas in the remaining weeks this effect was lost, partially due to a non-significant but continuous decrease in the Ovx groups values.

The comparison of the cumulative caloric intake of the six groups [$H(5) = 86.84$, $p < 0.001$] is represented in Fig. 2b. It demonstrates that ovariectomy did not affect caloric intake significantly, independently of the diet consumed. In contrast, the intake of the high-fat diet *per se* increased caloric intake and estradiol replacement failed to alter this parameter.

Table 1 shows that the uterus weight differed among the groups [$H(5) = 84.98$, $p < 0.001$], and that ovariectomy caused a significant uterus hypotrophy. Estradiol replacement, although causing a small increase in uterus weight, did not eliminate the significant difference of the Ovx groups in comparison to the Sham groups, regardless the diet.

The sum of fat depots also showed to be significantly different among the groups [$H(5) = 66.01$, $p < 0.001$] in a diet-dependent manner, with the high-fat groups presenting increased fat depots compared to the respective control groups. Ovariectomy itself produced non-significant increases in fat depots in both diet groups. The intake of the high-fat diet by the Ovx rats further increased fat depots weight. Estradiol supplementation tended to decrease fat depots but the effect was not significant.

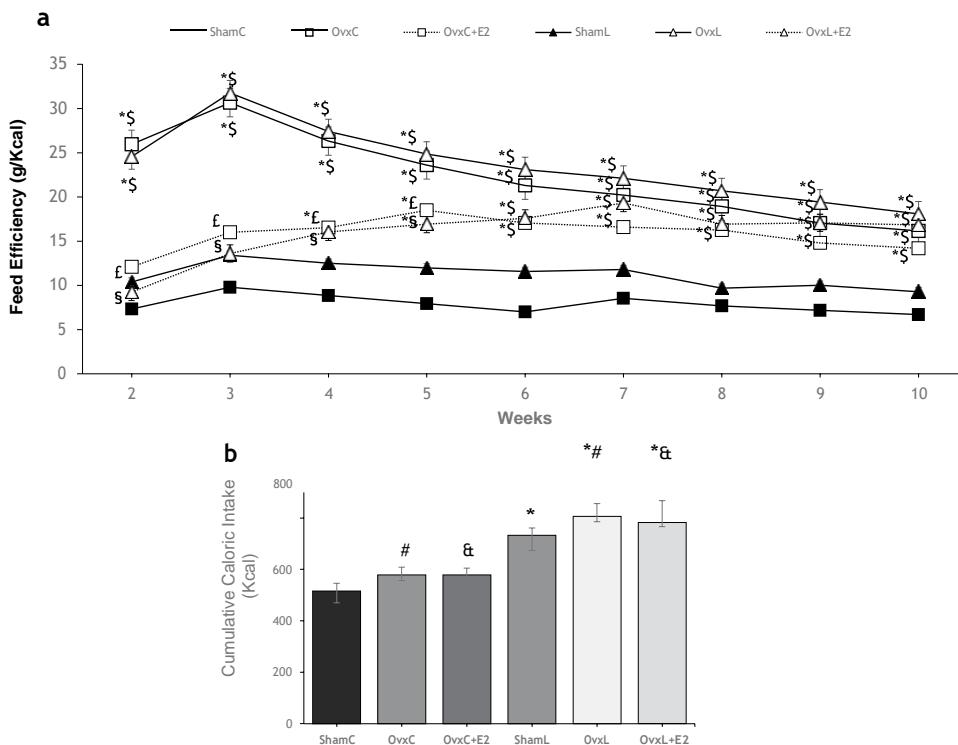


Figure 2. Feed efficiency (a) and cumulative caloric intake (b). Groups were fed with either control diet - ShamC ($n = 18$), OvxC ($n = 18$), OvxC + E2 ($n = 18$) or high-lard diet - ShamL ($n = 17$), OvxL ($n = 18$), OvxL + E2 ($n = 17$), for twelve weeks. Data are expressed as medians and interquartile range (Q1-Q3) for both figures. * $p < 0.05$ vs. ShamC; ** $p < 0.05$ OvxC vs. OvxL; *** $p < 0.05$ vs. ShamL; & $p < 0.05$ OvxC + E2 vs. OvxL + E2; ^ $p < 0.05$ vs. OvxC + E2; \$ $p < 0.05$ vs. OvxL + E2.

High-fat intake impairs the ability of estradiol replacement to attenuate the deleterious effects of ovariectomy on glucose homeostasis. Also highlighted by Table 1 are the serum parameters results. Glucose levels were different among the groups ($F_{[5,98]} = 34.12$, $p < 0.001$), being increased in the animals that received the high-fat diet.

Significant differences in insulin levels [$H(5) = 20.38$, $p = 0.001$], HOMA-IR [$H(5) = 22.43$, $p = 0.004$] and HOMA- β [$H(5) = 12.48$, $p = 0.028$] were also present among the groups. The results showed that insulin levels and HOMA -IR were affected by ovariectomy, being higher in the OvxC and OvxL groups than in the ShamC group. On the other hand, HOMA- β increased only in the OvxC group compared to the ShamC group. Estradiol replacement was able to normalize insulin levels only on the group fed with control diet.

Leptin levels varied significantly among the groups [$H(5) = 43.99$, $p < 0.001$], with increases in the Ovx groups on both diets, compared to the respective Sham groups, demonstrating an effect of ovariectomy. Estradiol replacement was able to normalize leptin levels only in the group fed with the control diet. Leptin/Adiponectin ratio also varied among the groups [$H(5) = 49.69$, $p < 0.001$]. Adiponectin, triglycerides, and total and HDL cholesterol levels did not differ significantly among the groups.

High-fat intake induces depressive-like behaviors in ovariectomized rats. The results of the EPM test showed that both the number of entries in the enclosed arms and the total number of entries were similar among the groups (Fig. 3a,b), indicating that the treatments did not affect locomotor activity.

According to the FST test, swimming frequency differed significantly among the groups ($F_{[5,98]} = 11.16$, $p = 0.001$). Figure 4a shows a decrease in the swimming frequency of both OvxL and OvxL + E2 groups compared to both Sham groups.

The immobility frequency (Fig. 4b) also differed among the groups [$H(5) = 27.89$, $p < 0.001$], increasing in OvxL and OvxL + E2 groups compared to both Sham groups.

The latency to immobility showed significant differences among the groups [$H(5) = 22.89$, $p = 0.002$], with the OvxL group showing the lowest latency. Estradiol replacement normalized this parameter (Fig. 4c).

The number of climbing events differed among the groups [$H(5) = 26.82$, $p = 0.001$], with significantly fewer events showed by the OvxL group when compared to the ShamC and ShamL groups. Estradiol replacement normalized this parameter (Fig. 4d).

Obesity and lack of estradiol associate with behavioral parameters. The following parameters were included in the correlation analysis: body weight gain, feed efficiency, sum of fat depots, all measured serum parameters and all measured parameters of the behavioral tests. Data from 60 animals were included in the analysis (10/group). Table 2 shows the variables with at least one significant correlation.

	ShamC	OvxC	OvxC + E2	ShamL	OvxL	OvxL + E2
Uterus (g)	0.57 (0.44–0.72) (n = 18)	0.11 (0.07–0.13) [§] (n = 18)	0.15 (0.14–0.18)* (n = 18)	0.65 (0.47–0.69) (n = 17)	0.09 (0.06–0.09)* ^s (n = 18)	0.15 (0.13–0.18)* ^s (n = 17)
Sum of fat depots (g/100 g)	3.17 (2.92–3.53) (n = 18)	5.69 (5.26–6.04) [#] (n = 18)	4.73 (4.17–5.64) ^{&} (n = 18)	6.37 (5.95–7.14)* (n = 17)	7.99 (7.32–8.27)* [#] (n = 18)	7.01 (6.76–7.65) ^{&} (n = 17)
Glucose (mg/dL)	89.1 ± 2.01 (n = 18)	95.8 ± 4.3# (n = 18)	101.4 ± 4.1& (n = 18)	108.1 ± 3.9* (n = 17)	112.2 ± 4.3*# (n = 18)	122.2 ± 4.7* ^{&} (n = 17)
Insulin (ng/mL)	0.6 (0.4–0.7) (n = 10)	2.1 (1.6–2.2)* (n = 10)	1.2 (1.1–1.4) (n = 10)	1.4 (1.1–1.9) (n = 10)	2.1 (1.4–2.9)* (n = 10)	2.2 (1.1–3.1)* (n = 10)
HOMA-IR	3.8 (2.4–4.5) (n = 10)	12.1 (9.2–15.4)* (n = 10)	7.9 (6.3–8.8) (n = 10)	9.6 (7.7–12.05) (n = 10)	14.5 (10.1–20.3)* (n = 10)	19.3 (7.7–24.9)* (n = 10)
HOMA- β	0.19 (0.15–0.26) (n = 10)	0.42 (0.37–0.49) [§] (n = 10)	0.24 (0.22–0.34) (n = 10)	0.29 (0.17–0.36) (n = 10)	0.40 (0.21–57) (n = 10)*	0.33 (0.21–0.43) (n = 10)
Leptin (ng/mL)	2.1 (1.85–2.44) (n = 13)	8.6 (6.1–10.1)* (n = 13)	6.9 (4.5–9.4) (n = 13)	5.8 (5.2–9.1) (n = 13)	12.1 (9.9–13.4)* ^s (n = 13)	14.1 (10.9–0.6)* ^s (n = 13)
Adiponectin (μg/mL)	5.06 (4.2–7.2) (n = 13)	7.39 (6.23–11.22) (n = 13)	5.57 (5.14–8.22) (n = 13)	5.92 (5.27–6.99) (n = 13)	6.97 (5.97–8.34) (n = 13)	5.02 (4.59–9.01) (n = 13)
Leptin/Adiponectin Ratio	0.34 (0.2–0.5) (n = 13)	0.94 (0.6–1.6)* [#] (n = 13)	0.79 (0.59–1.5)* ^{&} (n = 13)	0.87 (0.5–1.1) (n = 13)	1.85 (1.5–2.7)* ^s (n = 13)	2.43 (0.9–3.3)* ^{&s} (n = 13)
Triglycerides (mg/dL)	101.4 (93.6–106.7) (n = 18)	116.6 (104.1–26.6) (n = 18)	126.1 (97.4–131.9) (n = 18)	89.7 (81.8–115.3) (n = 17)	109.4 (88.1–117.3) (n = 18)	101 (93.1–119.8) (n = 17)
Total cholesterol (mg/dL)	106.8 (91.5–20.9) (n = 18)	113.1 (104.9–39.5) (n = 18)	120.8 (115.7–128.1) (n = 18)	103.2 (92.5–109.9) (n = 17)	98.6 (93.0–114.3) (n = 18)	112.2 (91.7–123.1) (n = 17)
HDL cholesterol (mg/dL)	39.3 (36.7–47.1) (n = 18)	34.2 (31.4–38.7) (n = 18)	38.5 (30.1–47.3) (n = 18)	35.8 (32.5–37.9) (n = 18)	32.4 (28.8–38.4) (n = 18)	38.6 (29.4–42.4) (n = 18)

Table 1. Uterus weight, sum of fat depots and serum parameters of the groups ShamC, OvxC, OvxC + E2, ShamL, OvxL and OvxL + E2. Data presented as mean ± SEM for variables with normal distribution and medians- interquartile range (Q1–Q3) for variables not normally distributed. *p < 0.05 vs. ShamC; #p < 0.05 OvxC vs. OvxL; §p < 0.05 OvxC + E2 vs. OvxL + E2; \$p < 0.05 vs. ShamL.

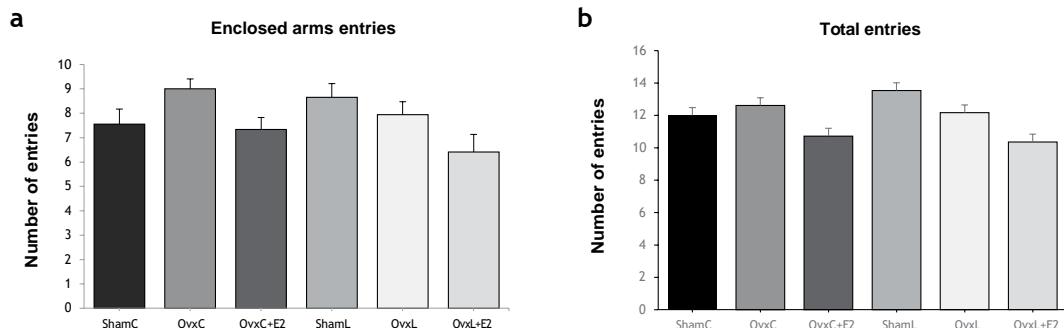


Figure 3. Elevated plus-maze test: Number of enclosed arms entries (a) and number of total entries (enclosed and opened arms). (b) Groups were fed with control diet-ShamC (n = 18), OvxC (n = 18), OvxC + E2 (n = 18), and groups fed with high-lard diet-ShamL (n = 17), OvxL (n = 18), OvxL + E2 (n = 17). Data are expressed as means ± SEM.

In the FST test, the swimming frequency was negatively correlated to body weight gain ($p < 0.001$), feed efficiency ($p = 0.003$), sum of fat depots ($p = 0.003$), insulin levels ($p = 0.025$), HOMA-IR ($p = 0.031$) and leptin levels ($p < 0.001$) and positively correlated with uterus weight ($p < 0.001$). The immobility frequency showed positive correlation to body weight gain ($p < 0.001$), feed efficiency ($p = 0.001$), sum of fat depots ($p = 0.001$), insulin levels ($p = 0.025$), HOMA-IR ($p = 0.034$) and leptin levels ($p < 0.001$) while it correlated negatively to uterus weight ($p < 0.001$).

The latency to immobility showed negative correlation to body weight gain ($p = 0.001$), feed efficiency ($p = 0.017$), sum of fat depots ($p = 0.002$), insulin levels ($p = 0.047$), HOMA-IR ($p = 0.047$) and leptin levels ($p = 0.003$), and positive correlation to uterus weight ($p = 0.007$).

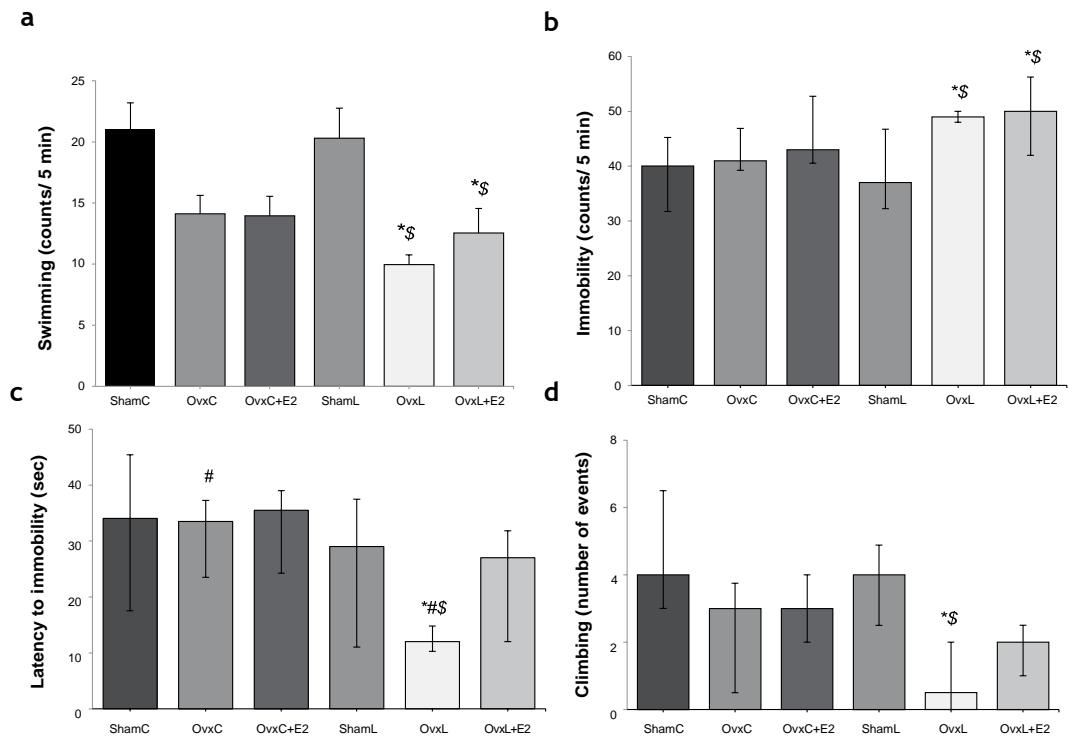


Figure 4. Modified forced swim test: Swimming frequency (**a**), Immobility frequency (**b**), latency to immobility (**c**) and climbing events (**d**) in groups fed with control diet-ShamC (n = 18), Ovx C (n = 18), Ovx C + E2 (n = 18), and groups fed with high-lard diet-ShamL (n = 17), Ovx L (n = 18), Ovx L + E2 (n = 17). Data are expressed as means \pm SEM for swimming frequency and as medians and interquartile range (Q1-Q3) for the other parameters. *p < 0.05 vs. ShamC; #p < 0.05 Ovx C vs. Ovx L; \$p < 0.05 vs. ShamL.

	Swimming frequency	Immobility frequency	Latency to immobility	Climbing
Body weight gain(g)	-0.5233*	0.5514*	-0.3986*	-0.4669*
Feed efficiency (g/kcal)	-0.5467*	0.5765*	-0.3808*	-0.4856*
Sum of fat depots(g)	-0.3680*	0.3739*	-0.3732*	-0.3012*
Uterus (g)	0.5094*	-0.5699*	0.3668*	0.5219*
Glucose (mg/dL)	-0.1734	0.1616	-0.1622	-0.1224
Insulin (ng/mL)	-0.2937*	0.2986*	-0.2650*	-0.2627*
Homa-IR	-0.2859*	0.2847*	-0.2585*	-0.2341
Leptin (ng/mL)	-0.4452*	0.4815*	-0.3691*	-0.4441*

Table 2. Spearman correlation between body/serum parameters and behavioral variables. n = 60; *p < 0.05 (two-tailed).

The climbing behavior showed negative correlation to the body weight gain (p < 0.001), feed efficiency (p = 0.006), sum of fat depots (p = 0.006), insulin (p < 0.001) and leptin levels (p < 0.001), while showing positive correlation to the uterus weight (p < 0.001).

Multiple linear regression models were constructed, using the same animals presented in Table 2, in order to identify predictors for the variations of behavioral parameters. All the variables which presented significant correlations with these behavioral parameters were tested in the models (Table 3).

The uterus weight was shown to significantly influence the swimming frequency as a positive predictor, and body weight gain as a negative one, explaining 32.4% of the variations.

The frequency of immobility was negatively influenced by uterus weight and positively influenced by body weight gain, and the equation explained 38.8% of the variations.

Climbing was positively influenced by uterus weight and negatively influenced by body weight gain. The equation predicted 29.7% of the climbing variations.

Behavior	Predictor	Beta coefficient	Standard error	P-value	Adjusted R ²
Swimming frequency	Uterus (g) Body weight gain(g)	13.500 -0.059	5.333 0.021	0.01414* 0.00758**	0.324
Immobilization frequency	Uterus (g) Body weight gain (g)	-20.048 0.072	6.282 0.025	0.00230** 0.00591**	0.388
Climbing	Uterus (g) Body weight gain (g)	6.121 -0.017	2.076 0.008	0.00462** 0.04704*	0.297

Table 3. Multiple linear regression for behavior dependent variables. n = 60; *p < 0.05, **p < 0.01.

Discussion

Ovariectomy alone increased body weight gain significantly and caused a 79% ($p > 0.05$) increase in body adiposity. This may be partly attributed to the observed increased feed efficiency, in agreement with our previous observations^{12,25}, since the cumulative caloric intake of the OvxC group showed only a non-significant 12% increase in relation to that of the ShamC rats. Ovariectomy has been shown to stimulate food intake in rats²⁶, although some studies have been shown that this effect is a transient one, disappearing after the first weeks after ovaries removal^{27,28}.

Ovariectomy induced hyperleptinemia and hyperinsulinemia, indicating resistance to these hormones, the latter confirmed by increased HOMA-IR and HOMA- β values. High leptin levels have been described both in ovariectomized rodents and in post-menopausal women, and suggested to depend on the estrogen deficiency^{8–10}. Our data are also in agreement with reports of hyperinsulinemia and insulin resistance in postmenopausal women²⁹.

Estradiol replacement for twelve weeks failed to significantly restore body weight gain and feed efficiency in the control-fed rats. Actually, estradiol was effective in reducing these parameters significantly only up to week five. This could be attributed to a low subcutaneous estradiol replacement dose used in the present study, of 2.8 $\mu\text{g}/\text{day}/90$ days, in comparison to that (4.2 $\mu\text{g}/\text{day}/60$ days) reportedly able to decrease body weight gain and food intake and to increase metabolic rate of ovariectomized rats³⁰. High doses of hormone replacement have also been shown to decrease body weight but not body adiposity in rats³¹.

In contrast, we presently observed that, by the end of the treatment, the hormonal replacement was sufficient to improve insulin and leptin levels, leptin/adiponectin ratio, and HOMA-IR and HOMA- β values. This agrees with the demonstrations that the anti-inflammatory properties of estrogen are related to its metabolic actions³² and that the chronic administration of an agonist of the estrogen receptor alpha improved the insulin sensitivity of diabetic mice³³.

The intake of the high-fat diet by the non-ovariectomized rats (ShamL group) increased caloric intake and body adiposity but not body weight or feed efficiency, in comparison to the ShamC group. The effect of high-fat intake was more pronounced in the ovariectomized group, as it accentuated feed efficiency, body mass and adiposity gain, in relation to ovariectomy alone. This corroborates a study showing that ovariectomized mice were more vulnerable to weight gain and fat mass increase when fed a high-fat diet, due to high degrees of adipose tissue inflammation and pro-inflammatory cytokines production³⁴.

The high-fat diet alone only tended to increase leptin and insulin levels while it did induce hyperglycemia, but no significant increase of HOMA-IR. Altogether, the results indicated that the consumption of this 45%-lard diet for 12 weeks had mild effects, perhaps due to the caloric and lipidic density of the diet as well as to the period of diet exposure. In agreement with this suggestion, other authors reported that the intake of a 45% lard-rich diet for 8 weeks by female rats failed to increase body fat percentage³⁵, while a 62%-lard diet for 50 weeks doubled the body weight gain of female rats³⁶. In male mice, the intake of a 60%-lard diet 16 weeks induced pronounced changes in body adiposity, glucose homeostasis, and leptinemia³⁷.

The combination of ovariectomy and high-fat diet increased leptinemia and insulinemia, and led to significantly increased HOMA-IR and HOMA- β values, showing a further deterioration of the parameters affected by either ovariectomy or high-fat intake alone. These findings are consistent with the demonstration that impaired glucose homeostasis influence adipose tissue inflammation during high-fat intake³⁸. The prominent increases in leptinemia induced by ovariectomy alone or combined with high-fat intake are likely to coexist with leptin resistance, probably due to multiple mechanisms including down-regulation and/or desensitization of hypothalamic leptin receptors and impairment of receptor signaling and leptin transport into brain³⁹.

Differently from the observations in the control-fed rats, in the lard-fed ones the estradiol replacement failed to improve insulin and leptin levels, leptin/adiponectin ratio, and HOMA-IR, although it did reduce the HOMA- β value. It is hypothesized that, due to the poorer metabolic status of the lard diet-fed ovariectomized rats, the present estradiol treatment, of 2.8 $\mu\text{g}/\text{day}$, was not sufficient. This suggestion agrees with a similar experiment in ovariectomized mice fed a high-lard diet, in which a dose of 1.7 $\mu\text{g}/\text{day}$, high in comparison to the present one in rats, protected from insulin resistance⁹.

Having demonstrated the high-fat intake potentiated the alterations induced by ovariectomy, we decided to evaluate behavioral aspects in these animals, due to the controversy around the incidence of mood disorders and its relation to obesity after menopause^{5,22}.

In the ovariectomized animals fed with the control diet, we found no significant effects on the depressive-like behaviors measured in the FST. While these results agree with previous reports in rodents utilizing the same behavioral paradigm^{12,19–21}, other studies have found ovariectomy to induce depressive-like behaviors^{11,17,18}. These contradictory findings may be attributed to methodological discrepancies among these studies. Indeed, depressive

behaviors have been observed when either ovariectomy was performed at an earlier age or the behavioral test was performed more acutely after the surgery, agreeing with review papers in humans concluding that the risk to develop depression was inversely associated with the age at menopausal onset² and that the menopausal transition is a period of vulnerability to the development of depression²².

The high-fat diet alone also failed to induce depressive-like behaviors in the FST, a result apparently inconsistent with the epidemiological data linking obesity and depression⁵. This may be attributed to the relatively mild degree of the alterations induced by the present high-fat regimen, as discussed above. Accordingly, a more potent obesogenic regimen in male mice induced depressive-like behaviors, which was attributed to both high-fat intake and impaired central leptin signaling³⁷.

In contrast, the ovariectomized group fed with the high-lard diet exhibited clear depressive-like behaviors in the FST. Importantly, no deficit in locomotor activity was observed during the EPM test, ruling out that impaired mobility due to obesity influenced the results, as previously suggested⁴⁰.

We have previously demonstrated that the combination of ovariectomy and high-lard intake decreased the latency to immobility in the FST but, unlike the present results, failed to affect the immobility time and the swimming frequency, indicating lack of a consistent depressive effect¹². This difference may be related to the present longer period of diet treatment after ovariectomy (12 weeks), leading to potent accentuation of body adiposity and metabolic impairment, in comparison to our previous study (8 weeks). These findings agree with epidemiological studies linking visceral fat and depressive symptoms in middle-aged women and appointing visceral adiposity as the factor responsible for increased risk of depression in postmenopausal women^{13,14}. Moreover, these findings corroborate the present correlation and regression data showing that the depressive-like behaviors were significantly influenced by the metabolic and body composition parameters and by the estrogen status, as indicated by uterus weight.

The present estradiol replacement regimen failed to consistently reverse the depressive-like behavior observed in the OvxL group, probably due to a low dosage, as discussed above. This suggestion agrees with the notion that estrus levels of estradiol are necessary to decrease depression behavior in rats¹⁶.

In conclusion, the present data indicate that the vulnerability to develop depression after menopause is positively influenced by obesity. It is suggested that weight management is a crucial issue in postmenopausal women, probably having a beneficial role in preventing the appearance of mental health problems.

Material and Methods

Animals and surgery conditions. All procedures were approved by and conducted according to the guidelines of the Committee in Research Ethics of the Universidade Federal de São Paulo (CEUA No.: 2172030315/2016), following the ethics procedures established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA). Twelve-week-old female Wistar rats were submitted to either bilateral ovariectomy (Ovx, n = 71) or sham operation (Sham, n = 35) under ketamine/xylazine anesthesia (66/33 mg/kg, ip). For estrogen replacement, 35 Ovx animals were implanted with a subcutaneous pellet of 17 β -estradiol (0.25 mg/pellet, 90-day release; Innovative Research of America, Sarasota, Florida, USA). Immediately after the surgery, all animals received penicillin (60.000U, i.m.) and ibuprofen (1 mg/kg body weight, v.o.).

Additional ibuprofen doses were administered once daily for 2 days. After the surgery all the animals were housed two to three per cage and maintained under controlled lighting (12 h light/dark cycle, lights on at 6am) and temperature conditions ($23 \pm 1^\circ\text{C}$), with free access to food and water. Upon housing, the above groups were randomly sub-divided according to the diet offered. ShamC, OvxC and OvxC + E2 received standard rat chow (2.87 kcal/g, 15% of energy from fat, Nuvilab CR-1, Nuvital Nutrientes SA, Colombo, PR, Brazil) while the ShamL, OvxL and OvxL + E2 groups received a high-fat diet (3.60 kcal/g, 45% energy from fat) enriched with lard (Cooperativa Central Aurora de Alimentos, Chapecó, Santa Catarina, Brazil) to the chow diet. The high-fat diet was prepared by adding, to the standard chow, 18% (w/w) lard, 2% soybean oil, 20% (w/w) casein, 10% sucrose and 0.02% (w/w) of butylhydroxytoluol. Body weight and feed efficiency were measured weekly. Food and caloric intake were measured as the mean consumption value for each housed rat, during the treatment. Feed efficiency was calculated as follows: (body weight gain/energy intake) × 100³⁶.

Behavioral assessment. *Elevated plus-maze (EPM).* The test apparatus consists of two open arms and two closed arms connected by a central platform high off the ground and lit by a dim light. On the day of the test, the rats were transported individually into the testing room and placed on the central platform facing an open arm. The test was performed and recorded during five minutes by an overhead video camera for later quantification. The number of entries in the arms was measured by two observers⁴¹. The number of entries in the enclosed arms and the total number of entries evaluate locomotor activity⁴²⁻⁴⁵.

Modified forced swim (FST). At the first day of the tenth week, the rats were transported to the FST test room and placed individually in a plexiglas cylinder filled with water (diameter 30 cm, height 50 cm) for the 15-minutes training session. The volume of water ($25 \pm 1^\circ\text{C}$) was enough to prevent the animal from touching the bottom with the tail. After 24 hours, the 5-minutes test session was performed while being recorded for subsequent analyses. Every 5 seconds, the predominant behavior was characterized as swimming (movements throughout the swim cylinder), climbing (upward-directed movements with the forepaws along the cylinder walls) and immobility (floating with minimal movements with head just above the water). Latency (total of seconds before the first immobility account) was also assessed⁴⁶.

Serum measurements. At the end of the twelfth week, the rats were euthanized after 24 hour fast (after thiopental anesthesia), trunk blood was collected and serum stored at -80°C until analyzed. Uterus, liver, retroperitoneal, gonadal and mesenteric fat pads were dissected and weighed.

Serum glucose, cholesterol, HDL-cholesterol and triglycerides levels were determined using commercially available enzymatic colorimetric kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil). Elisa kits (Millipore, Bedford, MA, USA) were used to determine the serum levels of leptin (sensitivity 0.08 ng/mL; intra assay precision- 2.49%; inter-assay precision- 3.93%), insulin (sensitivity 0.1 ng/mL; intra-assay precision- 1.33%; inter-assay precision- 6.71%) and adiponectin (sensitivity – 0.4 ng/mL; intra-assay precision- 1.18%; inter-assay precision- 7.34%). The leptin/adiponectin ratio was then calculated. The Homeostasis Model Assessment Insulin Resistance index (HOMA-IR) was calculated as follows: HOMA-IR = (fasting insulin (μ M/mL) x fasting glucose (mmol/L))/22.5. HOMA- β was calculated as follows: HOMA- β = (insulin x20)/(glucose-3.5)⁴⁷.

Data analysis. Statistical analysis was performed in Statistica 12 Software (StatSoft, Tulsa, OK, USA). The variables were tested for distribution and homogeneity (Shapiro-Wilks and Levene test respectively). Parametric variables were expressed as means \pm standard error, and analyzed by two-way analysis of variance (ANOVA) with Tukey post hoc test. The non-parametric variables were expressed as median (interquartile range) and analyzed by Kruskal-Wallis ANOVA followed by two tailed multiple comparisons of p-values.

The interaction between variables was tested by the Spearman's correlation coefficient. Multiple linear regressions were applied to determine the predictors of behavioral parameters among the variables presenting significant correlations. The level for statistical significance was set at $p < 0.05$.

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Author Contributions

V.T.B. and A.P.P. performed all experiments and wrote the manuscript; N.I.P.N. and A.P.S.D. participated in the *in vivo* experiments; C.M.O.N. and L.M.O. participated in results interpretation, E.B.R. supervised the whole project and participated in the manuscript writing.

Additional Information

Competing Interests: The authors declare no competing interests.

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ARTIGO 2: Ovariectomy modifies lipid metabolism of visceral white fat in rats: a proteomic approach

Abstract: Menopause is often accompanied by visceral obesity. With the aim of exploring the consequences of ovarian failure on visceral fat, we evaluated the effects of ovariectomy and estrogen replacement on the proteome/phosphoproteome and on the fatty acids profile of the retroperitoneal adipose depot (RET) of rats. Eighteen three months old female Wistar rats were either ovariectomized or sham-operated and fed with standard chow for three months. A subgroup of ovariectomized rats received estradiol replacement. RET samples were analyzed using data-independent acquisitions LC-MS and pathway analysis was performed with the differentially expressed/phosphorylated proteins. RET lipid profile was analyzed by gas chromatography.

Ovariectomy induced high adiposity and insulin resistance. Ovariectomy promoted down-regulation of 42 and up-regulation of 49 differentially expressed proteins compared to control group. Ninety-six proteins (106 peptides) were differentially phosphorylated, with decreased phosphorylation of 39 peptides and increased phosphorylation of 67 peptides. Pathway analysis showed that 5 pathways were significantly affected by ovariectomy, namely metabolism of lipids (included fatty acid metabolism and mitochondrial fatty acid β -oxidation), fatty acyl-CoA biosynthesis, innate immune system (included neutrophil degranulation), metabolism of vitamins and cofactors, and integration of energy metabolism (included ChREBP activates metabolic gene expression). Lipid profile analysis showed increased palmitic and palmitoleic acids content. The analysis of the data indicated that ovariectomy favored lipogenesis while it impaired fatty acids oxidation, and induced a pro-inflammatory state in the visceral adipose tissue. These effects are consistent with the findings of high adiposity, hyperleptinemia, and impaired insulin sensitivity. The observed alterations were partially attenuated by estradiol replacement. The data point to a role of disrupted lipid metabolism in adipose tissue in the genesis of obesity after menopause. It is suggested that weight management is crucial for postmenopausal women, having a beneficial role in preventing visceral fat accumulation and its consequences.

Keywords – ovariectomy; visceral adipose tissue; proteome/phosphoproteome; lipid profile; estrogen replacement

Introduction

The cessation of ovarian hormones production in menopause is often followed by increased body weight gain and adiposity, associated with obesity-related co-morbidities [1][2]. In both humans and rodents, a role of estrogens in metabolic, immune, and inflammatory processes has been established, although the complexity by which these effects occur is not fully understood [3][4]. The lack of estrogens after menopause has been associated with a shift of fat distribution from subcutaneous to visceral, with impairment of the sensitivity to insulin and leptin, predisposition to diabetes and to anxiety and depressive disorders [5][6][7].

A depot-specificity of functions has been described for the adipose tissue, which may result from differences intrinsic to pre-adipocytes and to factors secreted by neighboring organs [8]. During the development of obesity, the visceral adipose tissue suffers a higher degree of hypertrophy and macrophage infiltration than the subcutaneous depots, leading to a high production of inflammatory mediators that contribute substantially to the pathophysiology of obesity complications [9][10].

Several disruptions of the metabolism of visceral adipose tissue have been reported in ovariectomized rodent models. Increased conversion of cortisone to cortisol, due to increased expression of type 1 11 β -hydroxysteroid dehydrogenase, has been found in visceral but not in subcutaneous adipose depots, contributing to fat redistribution [11]. Decreased glutathione peroxidase activity and superoxide dismutase protein levels, indicating impairment of anti-oxidant status, as well as low mRNA levels of the anti-inflammatory hormone adiponectin, have also been observed [12].

Using a gel electrophoresis-based proteomic approach, a recent study described that ovariectomy affected proteins involved in intermediate metabolism, energy transduction, cell structure, and immune system, in the periovarian adipose tissue of rats [13]. The use of a more sensitive and accurate proteomic technique could help unravel a more expressive number of proteins and pathways affected by ovarian failure in the visceral fat. Moreover, the identification of changes in protein phosphorylation is of high relevance, as it plays a pivotal role in a multitude of cellular functions.

Additionally, perturbations of visceral adipose tissue functions have been associated with its fatty acids composition in several conditions, such as diet-induced obesity and diabetes [14] [15]. However, data on the effects of obesity induced by the loss of ovarian hormones in visceral adipose tissue fatty acid composition are scarce. One study reported increased levels of short-chain saturated fatty acids levels in the visceral fat of postmenopausal women [16]. Further studies on the subject are necessary.

Aiming at expanding our knowledge on the consequences of ovarian failure on visceral fat, the present study examined the effects of ovariectomy and of estrogen replacement on the proteome/phosphoproteome as well as on the fatty acid profile of the retroperitoneal fat of rats.

Materials and Methods

Experimental procedures

All procedures were approved by and conducted according to the guidelines of the Committee in Research Ethics of the Universidade Federal de São Paulo (CEUA nº: 2172030315/ 2016), following the ethics procedures established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and performed according to previously reported data [7]. In summary, twelve-week-old female Wistar rats were submitted to either bilateral ovariectomy (Ovx, n=12) or sham operation (Sham, n=6) under ketamine/xylazine anesthesia (66/33mg/kg, ip). For estrogen replacement (Ovx+E2), six Ovx animals were implanted with a subcutaneous pellet of 17 β -estradiol (0.25 mg/pellet, 90-day release; Innovative Research of America, Sarasota, Florida, USA). Immediately after the surgery, all animals received penicillin (60.000U. i.m.) and ibuprofen (1 mg/kg body weight, v.o.). Additional ibuprofen doses were administered once daily for 2 days. After the surgery, all the animals were housed two to three per cage and maintained under controlled lighting (12h light/dark cycle, lights on at 6am) and temperature conditions ($23 \pm 1^{\circ}\text{C}$), with free access to food and water.

All 3 groups received standard rat chow (2.87 kcal/g, 15% of energy from fat, Nuvilab CR-1, Nuvital Nutrientes SA, Colombo, PR, Brazil) for 12 weeks. Body weight and 24-h food mass

intake were measured once a week. Feed efficiency was calculated as: (body weight gain / energy intake) x 100.

At the end of the 12 weeks, the animals were fasted overnight, anesthetized with thiopental (80mg/kg, ip) and euthanized. Trunk blood was collected and serum stored at -80°C until analyzed. White fat pads (retroperitoneal, gonadal and mesenteric) were dissected, weighed, frozen in liquid nitrogen and stored at -80°C.

Serum and tissue cytokines measurements

Serum glucose, total and HDL-cholesterol and triglycerides levels were measured by commercially available enzymatic colorimetric kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil). Elisa kits (Millipore, Bedford, MA, USA) were used to determine the serum levels of leptin (sensitivity 0.08 ng/mL; intra assay precision- 2.49%; inter-assay precision- 3.93%), insulin (sensitivity 0.1 ng/mL; intra-assay precision- 1.33%; inter-assay precision- 6.71%) and adiponectin (sensitivity – 0.4 ng/mL; intra-assay precision- 1.18%; inter-assay precision- 7.34%). RET content of TNF- α (sensitivity – 2.4 ng/mL; intra-assay precision- 4.98%; inter-assay precision- 9.44%) and IL-6 (sensitivity – 0.7 ng/mL; intra-assay precision- 3.96%; inter-assay precision- 8.64%) were also determined by Elisa (R&D Systems). HOMA-IR was calculated as (fasting insulin (μ mol/mL) x fasting glucose (mmol/L)/22.5) and HOMA- β was calculated as (insulin (μ mol/mL x 20)/ (fasting glucose (mmol/L) - 3.5).

Retroperitoneal fat proteome and phosphoproteome analyses

Aliquots (800 mg) of RET fat pads were homogenized in 1mL of buffer containing 50 mM ammonium bicarbonate, 1% sodium deoxycholate (m/v), and deionized water [17], with the addition of a protease/phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), and centrifuged at 19,000 x g for 30 minutes at 4°C. Protein concentration in the supernatants was determined using a Pierce BCA assay (Thermo Scientific, Rockford, IL, USA). Aliquots of 200 μ g of protein were diluted in 50 mM ammonium bicarbonate to a final volume of 85 μ L. Samples were then subjected to reduction with 2.5 μ L of 100 mM DTT at 60°C for 30 min and alkylation with 2.5 μ L of 300 mM iodoacetamide, at room temperature for

30 min. Proteins were digested overnight using trypsin (Promega, Fitchburg, WI, USA) at a 1:100 (wt:wt) enzyme:protein ratio at 37°C. After digestion, samples were centrifuged at 19,000 x g for 15 minutes at 4°C, the supernatants were collected and an equal volume of ethyl acetate was added. Then, trifluoroacetic acid was added to a final concentration of 0.5%. Samples were homogenized in vortex and centrifuged at 19,000 x g for 5 minutes at 4°C. Supernatants were collected, transferred to Millex-GV filters (Millipore, Burlington, MA, USA), recovered, dried in SpeedVac, and stored at -80°C until mass spectrometry analysis.

Liquid-chromatography mass spectrometry

After reconstitution of the samples in 1 % (v/v) formic acid, protein content was determined fluorimetrically (Qubit® 3.0 Fluorometer, Thermo Scientific, Rockford, IL, USA) and the final concentration was adjusted to 0.5 µg/µL. The proteomic and phosphoproteomic analyses were performed by data-independent acquisitions liquid-chromatography mass spectrometry (ACQUITY UPLC M-Class coupled to Synapt G2-Si mass spectrometer) (Waters, Milford, MA, USA). An ACQUITY UPLC HSS T3 nanoACQUITY Column (100 Å. 1.8 µm. 75 µm × 150 mm., Waters) was used for peptides separation (1µg) in acetonitrile gradient from 7% (v/v) for 54 min, and then increased to 80% of acetonitrile for 13 min, at a flow rate of 500 nL/min). Data were acquired using nanoESI ionization on positive mode, in HDMS^E mode using Transfer MS Collision Energy Low (eV) 19.0 and Transfer MS Collision Energy High (eV) 53.0. Human Glu-fibrinopeptide B was used for mass spectrometer calibration. MS identification was made between 50 and 2000 *m/z*.

Database search

Mass spectrometry data processing and database search against *Rattus norvegicus* sequences (UniProtKB/Swiss-Prot database, www.uniprot.org, 8680 entries) were performed with the Progenesis for Proteomics software (version 4.0, Waters). Peptides identification followed the parameters: maximum 1 missed cleavage site allowed for trypsin digestion; cysteine carbamidomethylation as fixed modification and methionine oxidation as variable modification. The search for phosphopeptides was made through variable modification of

serine, threonine and tyrosine phosphorylation; false discovery rate (FDR) less than 1%. Protein identification followed the criteria: minimum of 2 fragment ions per peptide, 5 fragment ions per protein, and 2 peptides per protein. Relative protein quantification was performed by the Hi-N approach, using the three most abundant peptides, with priority of the unique peptides' ones [18]. Normalized data were exported to Excel files.

Pathway analysis

The proteins differentially expressed and/or differentially phosphorylated among the groups were included in the test to determine enriched pathways, using the online Panther platform (<http://www.pantherdb.org>, version 14.1, released 2019-07-11). The whole *Rattus norvegicus* proteome was used as the reference list and the Reactome Pathways was used as the annotation data set. Significantly enriched pathways were based on Fisher's exact test followed by Bonferroni correction with significance set to $p < 0.05$.

RET fatty acid composition

RET total lipids were extracted in hexane/isopropanol (3:2 v/v) containing 0.01% butylated hydroxytoluene (BHT). After addition of chloroform/methanol/water (2:1:1 v/v/v), the samples were centrifuged (10,000 x g for 10 minutes) and the organic layers were separated and evaporated to complete dryness with oxygen-free nitrogen (OFN). The lipids were partitioned again in chloroform/methanol/water (8:4:3 v/v/v) and the chloroform layer was dried under OFN.

Fatty acid analysis was performed as previously described [19]. Briefly, fatty acid methyl esters (FAME) were obtained by heating the samples with 15% acyl chloride in dry methanol in a sealed tube at 70°C during 3 hours under OFN. A solution of 5% NaCl was used to stop the reaction, at room temperature, and FAMEs were extracted by 3 washings with petroleum ether containing 0.01% BHT. Gas chromatography with flame ionization detector (GC2010 Plus, SHIMADZU, Kyoto, Japan) was performed with a TRACE TR-FAME capillary column (Thermo Scientific, Rockford, IL, USA). The intensity of peaks was quantified using the software Labsolutions (SHIMADZU, Kyoto, Japan).

Statistical analysis

Univariate analysis was performed with Statistica 12 Software (StatSoft, Tulsa, OK, USA). Normality and homogeneity of variances of body weight, white fat depots mass and serum parameters variables were evaluated by Shapiro-Wilk test and Levene's test, respectively. Parametric variables, including the fatty acid profile data, were expressed as means \pm standard error and analyzed by one-way analysis of variance (ANOVA) with Tukey post hoc test. Non-parametric variables were expressed as median (interquartile range) and analyzed by Kruskal-Wallis ANOVA followed by two tailed multiple comparisons of p-values. Statistical significance was set at $p < 0.05$.

Proteome and phosphoproteome data were log transformed, normalized by pareto scaling, and submitted to multivariate analysis by the online platform MetaboAnalyst (<http://www.metaboanalyst.ca>). Data were submitted to principal component analysis (PCA) and partial least-squares discriminant analysis (PLSDA). Variable importance for the projection (VIP) values higher than 1.5 in all five principal components were considered in order to identify the proteins and phosphopeptides that most contributed to discrimination among the groups.

Results

Ovariectomy induced high adiposity and insulin resistance

As shown in table 1, the success of ovariectomy was confirmed by the low uterus weight of the ovariectomized groups. Initial body weight was similar among the 3 groups while both ovariectomized groups had higher body weights, feed efficiency and fat depots mass at the end of the 12 weeks. The elevations induced by ovariectomy in leptin and insulin levels, leptin/adiponectin ratio, HOMA-IR and HOMA- β were significantly attenuated by estradiol replacement. Total cholesterol, HDL-cholesterol and triglycerides levels did not differ significantly among the groups. RET levels of TNF- α and IL-6 were similar among the groups,

although IL-6 mean levels showed a 59% increment in the Ovx group, in relation to the Sham group ($p=0.21$).

Table 1: Body and serum parameters

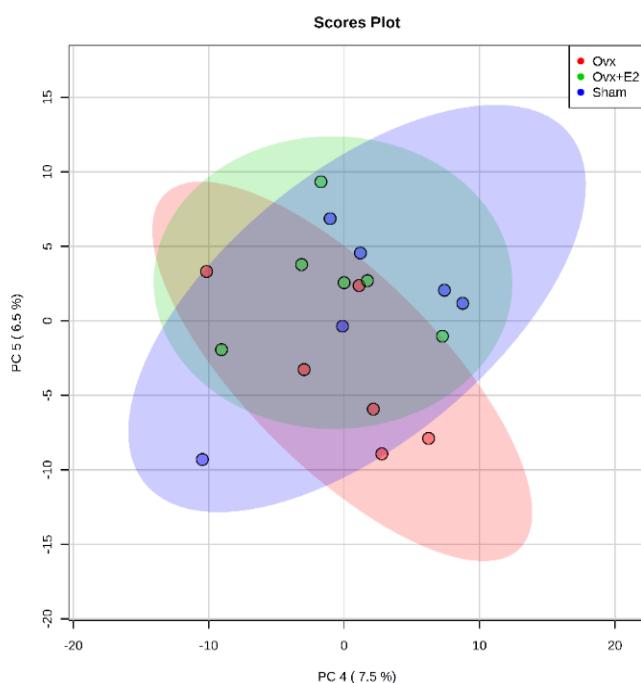
	Sham	Ovx	Ovx+E2
Initial body weight (g)	264.79 ± 5.22	263.00 ± 3.79	263.51 ± 5.02
Final body weight (g)	280.84 (272.38 - 285.75)	338.00 (336.00 - 339.50)*	335.71 (325.89 - 337.00)*
Cumulative food intake (Kcal)	526.36 ± 14.87	570.90 ± 12.48	613.63 ± 13.01*
Feed efficiency (g/Kcal)	2.72 ± 0.54	13.52 ± 0.66*	11.98 ± 0.81*
Uterus (g)	0.43 ± 0.03	0.11 ± 0.02*	0.15 ± 0.02*
Sum of fat depots (g/100g)	3.18 ± 0.31	5.21 ± 0.47*	4.79 ± 0.30*
RET mass (g/100g)	1.19 ± 0.19	2.06 ± 0.22*	1.78 ± 0.18*
Leptin (ng/mL)	2.17 (1.84 - 2.61)	10.06 (7.80-012.52)*#	5.97 (4.45 - 9.89)
Adiponectin (μg/mL)	5.65 ± 0.59	8.06 ± 1.04	7.67 ± 0.91
Leptin/Adiponectin	0.41 ± 0.06	1.42 ± 0.29*#	0.89 ± 0.18
TNF-α (pg/mg of protein)	7.04 ± 0.72	7.45 ± 0.78	6.96 ± 2.34
IL-6 (pg/mg of protein)	32.2 ± 5.28	54.6 ± 17.3	26.8 ± 2.62
Glucose (mg/dL)	92.80 ± 2.98	105.72 ± 6.38	103.88 ± 6.29
Insulin (ng/mL)	0.56 ± 0.06	2.32 ± 0.20*#	1.31 ± 0.12*
HOMA-IR	3.18 (2.32 - 4.25)	14.37 (12.08 - 19.07)*#	7.93 (6.26 - 8.28)
HOMA-β	0.18 (0.14 - 0.23)	0.52 (0.38 - 0.72)*#	0.29 (0.24 - 0.45)
Total cholesterol (mg/dL)	115.46 (104.96 - 121.76)	140.84 (119.08 - 193.13)	126.34 (119.85 - 133.97)
HDL cholesterol (mg/dL)	205.73 (179.01 - 251.91)	163.17 (146.18 - 174.05)	142.37 (135.50 - 157.63)
Triglycerides (mg/dL)	99.18 (94.67 - 108.20)	111.27 (105.33 - 118.85)	122.54 (107.79 - 220.49)

Data presented as mean ± SEM for variables with normal distribution or median-interquartile range (Q1-Q3) for variables not normally distributed. n=6 animals per group. * $p < 0.05$ vs Sham; # $p < 0.05$ vs Ovx+E2.

Ovariectomy induced changes in protein expression and phosphorylation

Among the 18 biological samples, 13.722 peptides, corresponding to 1.246 proteins, were identified, of which 994 proteins met the inclusion criteria and were included in the statistical analysis. The multivariate analysis found 91 RET proteins significantly modulated among the groups. The unsupervised PCA analysis followed by the supervised PLSDA analysis showed a separation between the Sham and the Ovx groups while the Ovx+E2 group appeared in an intermediate position (Figures 1A and 1B).

1A



1B

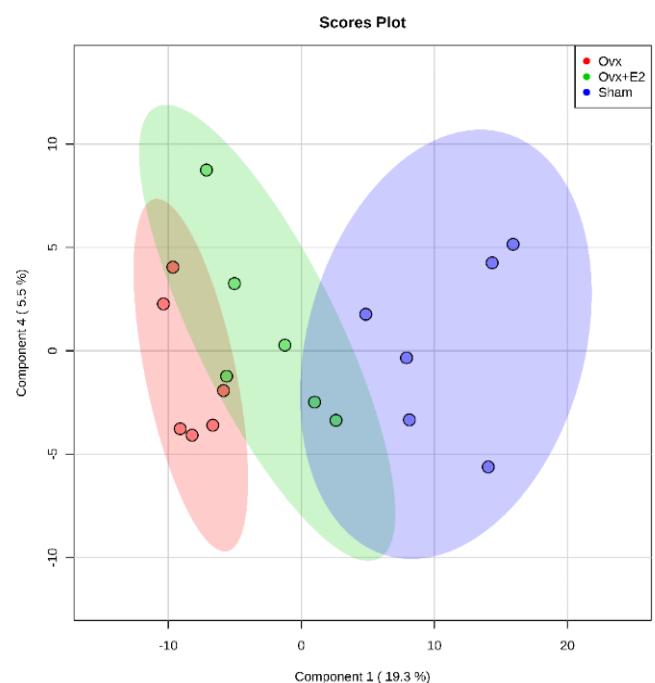


FIGURE 1: Separation of the groups using multivariate analyses of proteome data. Unsupervised PCA (A) and supervised PLSDA (B) analyses based on 994 protein intensities from shotgun analysis. The principal components explaining the separation are 4 and 5 for PCA and 1 and 4 for PLSDA.

Table 2 shows the proteins that discriminate the groups. Forty-two proteins were downregulated and 49 proteins were upregulated by ovariectomy, in relation to the Sham group. Of those, 72 proteins had a fold-change of 50% or more. The E2-replacement group presented 43 downregulated proteins and 48 upregulated proteins, in comparison to the Sham group, with 41 proteins showing a fold-change of 50% or more.

Table 2: Retroperitoneal fat proteins with significantly altered expression

Protein (UniProt ID)	Gene symbol	Unique Peptides	Confidence score	Ovx/Sham	Fold-Change	
					Ovx+E2/Sham	Ovx+E2/Ovx
Proteins down-regulated by ovariectomy						
Alpha-1B-glycoprotein (Q9EPH1)	A1bg	17	157.79	0.10	0.27	0.08
Contactin-associated protein like 5-4 (Q0V8T3)	Cntnap5d	1	14.11	0.10	0.09	0.97
Ras-related protein Rab-3C (P62824)	Rab3c	1	34.66	0.11	0.13	0.95
Mitochondrial import inner membrane translocase subunit Tim13 (P62076)	Timm13	2	12.49	0.16	0.23	0.83
Aminoacylase-1B (Q6PTT0)	Acy1b	1	16.34	0.17	0.56	0.84
<i>Synaptic vesicle membrane protein VAT-1 homolog (Q3MIE4)</i>	Vat1	15	180.30	0.20	0.27	0.81
Mitochondrial ribonuclease P catalytic subunit (B5DF07)	Mrpp3	1	12.43	0.23	0.89	1.06
T-kininogen 1 (P01048)	Map1	3	173.49	0.23	0.25	0.72
Thioredoxin (P11232)	Txn	5	41.21	0.25	0.23	0.76
Contactin-associated protein like 5-3 (Q0V8T4)	Cntnap5c	1	13.43	0.26	0.35	0.93
Myosin light chain 4 (P17209)	Myl4	1	22.76	0.33	0.48	1.02
Calmodulin-like protein 3 (Q5U206)	Calml3	3	28.90	0.37	0.44	0.11
Zinc finger protein 518A (Q499R0)	Znf518a	3	56.53	0.37	0.51	0.88
Myosin-4 (Q29RW1)	Myh4	14	238.83	0.39	0.54	1.07
<i>NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial (Q66HF1)</i>	Ndufs1	14	195.45	0.40	0.54	1.05
Protein AF1q (Q5M971)	Mllt11	1	16.22	0.40	0.94	0.91
Cytochrome b5 (P00173)	Cyb5a	2	41.30	0.41	0.47	0.80
<i>Integrin beta-1 (P49134)</i>	Itgb1	17	182.16	0.42	0.57	0.82
Aldehyde dehydrogenase, dimeric NADP-preferring (P11883)	Aldh3a1	1	23.14	0.43	0.40	0.80
Hsp90 co-chaperone Cdc37 (Q63692)	Cdc37	4	27.96	0.44	0.45	0.64
Keratin, type II cytoskeletal 75 (Q6IG05)	Krt75	5	88.84	0.44	0.86	0.78

Ig gamma-1 chain C region (P20759)	ENSRNOG00000030332	11	151.63	0.47	0.60	0.26
Kelch-like protein 3 (F1LZ52)	Klhl3	3	30.75	0.48	0.37	0.39
60S ribosomal protein L9 (P17077)	Rpl9	5	41.56	0.49	0.71	0.95
Tyrosine-protein kinase Lyn (Q07014)	Lyn	5	57.61	0.49	0.61	0.92
T-kininogen 2 (P08932)	Kng1	7	208.51	0.51	0.56	0.54
<i>C-reactive protein</i> (<i>P48199</i>)	Crp	3	36.76	0.54	0.58	0.49
Enoyl-CoA delta isomerase 1, mitochondrial (P23965)	Eci1	7	110.57	0.54	0.56	0.16
Ras-related protein Rab-11B (O35509)	Rab11b	3	99.71	0.54	0.58	0.79
Alpha-amino adipic semialdehyde dehydrogenase (Q64057)	Aldh7a1	14	136.18	0.55	0.73	0.82
Ceruloplasmin (P13635)	Cp	25	245.46	0.55	0.59	0.23
Pyruvate carboxylase, mitochondrial (P52873)	Pc	47	512.13	0.56	0.68	0.59
Endoplasmic reticulum resident protein 29 (P52555)	Erp29	11	114.00	0.59	0.66	0.40
Short-chain specific acyl-CoA dehydrogenase, mitochondrial (P15651)	Acads	10	165.91	0.59	0.63	0.51
cAMP-dependent protein kinase catalytic subunit alpha (P27791)	Prkaca	6	79.52	0.60	0.77	0.83
AP-2 complex subunit beta (P62944)	Ap2b1	5	39.13	0.61	0.59	0.48
Long-chain-fatty-acid-CoA ligase 5 (O88813)	Acsl5	10	138.83	0.61	0.72	0.73
40S ribosomal protein S16 (P62250)	Rps16	2	41.57	0.62	0.69	0.51
Acyl-coenzyme A thioesterase 2, mitochondrial (O55171)	Acot2	7	106.48	0.62	0.70	0.37
Cytoplasmic aconitase hydratase (Q63270)	Aco1	25	362.22	0.64	0.69	0.67
<i>Elongation factor 1-delta</i> (<i>Q68FR9</i>)	Eef1d	11	94.35	0.65	0.76	0.43
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (P49432)	Pdhb	15	170.94	0.66	0.76	0.68
DNA polymerase subunit gamma-1 (Q9QYV8)	Polg	1	13.92	0.81	0.78	1.10

Proteins up-regulated by ovariectomy

GTPase KRas (P08644)	Kras	1	18.15	1.16	1.04	1.12
Peroxiredoxin-4 (Q9Z0V5)	Prdx4	6	86.83	1.40	1.47	1.03
Amphoterin-induced protein 1 (Q80ZD7)	Amigo1	2	17.39	1.46	1.22	0.57
Aldose reductase-related protein 1 (Q5RJP0)	Akr1b7	1	27.39	1.51	1.37	0.57

cAMP-dependent protein kinase type II-beta regulatory subunit (P12369)	Prkar2b	10	188.75	1.51	1.42	0.38
Vacuolar protein sorting-associated protein 45 (O08700)	Vps45	6	31.18	1.51	1.25	0.99
Platelet glycoprotein 4 (Q07969)	Cd36	15	207.10	1.53	1.33	0.55
Transmembrane emp24 domain-containing protein 7 (D3ZTX0)	Tmed7	4	29.87	1.53	1.18	0.78
Gamma-synuclein (Q63544)	Sncg	17	117.13	1.59	1.33	0.99
Myosin-9 (Q62812)	Myh9	19	164.92	1.60	1.21	0.87
Acyl-coenzyme A thioesterase 1 (O88267)	Acot1	2	59.78	1.61	1.30	0.86
Golgin subfamily A member 4 (Q5U4E6)	Golga4	8	63.93	1.61	1.28	0.17
Transmembrane emp24 domain-containing protein 10 (Q63584)	Tmed10	3	50.32	1.64	1.63	0.58
Patatin-like phospholipase domain-containing protein 2 (P0C548)	Pnpla2	15	145.53	1.67	1.42	0.84
<i>All-trans-retinol 13,14-reductase (Q8VHE9)</i>	Retsat	24	204.05	1.75	1.52	0.49
Unconventional myosin-X (D3ZJP6)	Myo10	1	23.52	1.75	1.42	0.93
Coatomer subunit beta (O35142)	Copb2	3	33.09	1.77	1.29	0.94
Monoglyceride lipase (Q8R431)	Mgll	13	228.13	1.77	1.59	0.75
NAD(P)H dehydrogenase [quinone] 1 (P05982)	Nqo1	2	28.46	1.80	1.51	0.60
Coronin-1A (Q91ZN1)	Coro1a	5	63.58	1.82	0.86	0.54
Acyl-CoA-binding protein (P11030)	Dbi	6	84.83	1.84	1.78	0.79
Enoyl-CoA delta isomerase 2, mitochondrial (Q5XIC0)	Eci2	5	51.13	1.86	1.67	0.64
ATP synthase subunit d, mitochondrial (P31399)	Atp5pd	5	54.50	1.88	1.84	0.25
Nucleosome assembly protein 1-like 1 (Q9Z2G8)	Nap1l1	7	54.35	1.91	1.73	0.79
60S ribosomal protein L23 (P62832)	Rpl23	2	48.81	1.92	1.37	0.78
<i>Serine protease inhibitor A3M (Fragment) (Q63556)</i>	Serpina3m	9	121.48	1.95	1.75	0.93
Bifunctional purine biosynthesis protein PURH (O35567)	Atic	22	232.03	1.98	1.57	0.03
Fructose-bisphosphate aldolase B (P00884)	Aldob	4	37.22	2.00	1.49	0.67
2-oxoglutarate dehydrogenase, mitochondrial (Q5XI78)	Ogdh	30	292.24	2.05	1.34	0.50
Cytochrome b5 type B (P04166)	Cyb5b	2	12.37	2.05	1.55	0.74
Ras-related protein Rab-13 (P35286)	Rab13	1	25.88	2.10	1.36	1.01

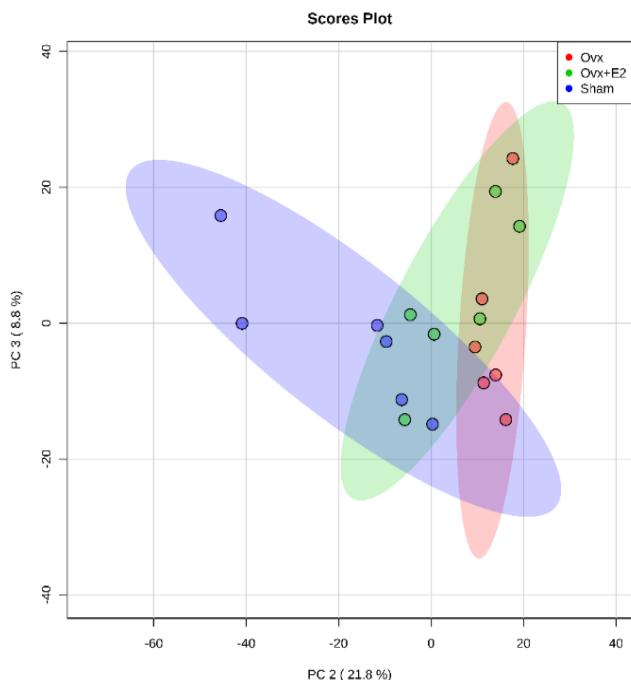
<i>Adenylyl cyclase-associated protein 1 (Q08163)</i>	Cap1	15	184.31	2.12	1.01	0.56
Glutathione S-transferase alpha-1 (P00502)	Gsta1	1	36.67	2.12	3.17	0.82
<i>Lipoprotein lipase (Q06000)</i>	Lpl	9	88.27	2.15	2.03	0.10
40S ribosomal protein S21 (P05765)	Rps21	4	18.59	2.16	1.18	0.61
Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (P25235)	Rpn2	18	151.69	2.19	1.95	0.23
Polypyrimidine tract-binding protein 1 (Q00438)	Ptbp1	3	23.06	2.38	1.76	0.80
2'-3'-cyclic-nucleotide 3'-phosphodiesterase (P13233)	Cnp	1	17.48	2.40	1.39	0.91
Transmembrane emp24 domain-containing protein 9 (Q5I0E7)	Tmed9	4	40.11	2.41	1.76	0.54
Eukaryotic initiation factor 4A-II (Q5RKI1)	Eif4a2	15	170.85	2.49	2.31	0.81
Coiled-coil domain-containing protein 184 (Q4V8F1)	Ccdc184	1	11.68	2.52	2.21	0.72
E3 ubiquitin-protein ligase NEDD4 (Q62940)	Nedd4	21	191.96	2.60	2.62	0.10
Guanine nucleotide-binding protein subunit alpha-11 (Q9JID2)	Gna11	3	33.88	2.66	2.13	0.78
Acid ceramidase (Q6P7S1)	Asah1	3	21.57	2.69	1.77	0.73
Ras-related protein Rab-3A (P63012)	Rab3a	1	42.94	2.90	3.47	0.96
NADH-cytochrome b5 reductase 1 (Q5EB81)	Cyb5r1	1	24.84	2.93	4.17	0.91
Retinol-binding protein 4 (P04916)	Rbp4	4	55.17	3.04	2.34	0.73
Protein S100-A10 (P05943)	S100a10	2	43.19	4.45	1.52	0.31
<i>Class I histocompatibility antigen, Non-RT1.A alpha-1 chain (P15978)</i>	RT1-Aw2	2	34.65	12.80	2.61	0.41

Abbreviation: UniProt: Universal Protein Resource.

Differently expressed and phosphorylated proteins

Considering the phosphoproteome results shown in table 3, 1074 peptides were identified with at least one phosphorylation site, corresponding to 468 proteins. The multivariate analysis found 106 peptides with a characteristic phosphorylation pattern for each group, corresponding to 96 proteins. The analysis based on principal components showed a separation of Sham and Ovx groups, while the replacement group showed to be in an intermediate position (Figures 2A and 2B).

2A



2B

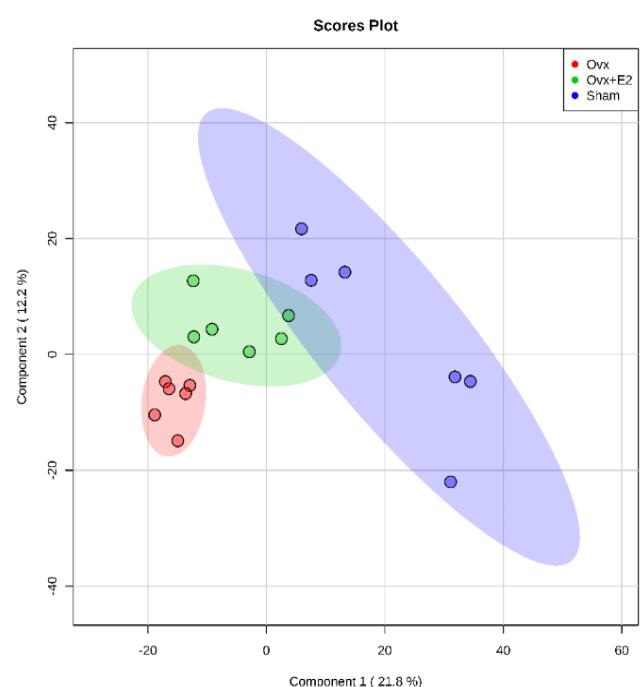


FIGURE 2: Separation of the groups using multivariate analyses of phosphoproteome data. Unsupervised PCA (A) and supervised PLSDA (B) analyses based on 1074 peptide intensities. The principal components explaining the separation are 2 and 3 for PCA and 1 and 2 for PLSDA.

In comparison to the Sham group, the Ovx group showed decreased phosphorylation of 39 peptides, of which 10 peptides showed simultaneous differences in two or more phosphorylation sites. Among the 67 peptides with increased phosphorylation, 14 showed simultaneous differences in two or more phosphorylation sites. The Ovx+E2 group showed downregulation of 41 peptides, of which 12 showed simultaneous differences in two or more phosphorylation sites. Among the 65 peptides with increased phosphorylation, 12 showed simultaneous differences in two or more phosphorylation sites.

Additionally, 10 proteins showed differences in both the expression and phosphorylation intensities, and are depicted in Tables 2 and 3.

Table 3: Retroperitoneal fat proteins differentially phosphorylated

Protein (UniProt ID)	Gene symbol	Unique Peptides	Confidence score	Phosphorylation site	Peptide Sequence	Ovx/ Sham	Fold-Change Ovx+E2/S ham	Fold-Change Ovx+E2/ Ovx
Proteins down-regulated by ovariectomy								
Proteasome subunit alpha type-4 (P21670)	Psma4	9	65.17	Thr ⁹ /Ser ¹³	TTIFSPEGR	0.01	0.01	1.24
Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial (P26284)	Pdha1	10	115.31	Ser ²⁷⁵	SGKGPILMELQTYR	0.01	0.01	0.86
Heat shock-related 70 kDa protein 2 (P14659)	Hspa2	15	241.77	Ser ¹⁴²	VQS AVITVPAYFNDNSQR	0.02	0.53	29.52
Heterogeneous nuclear ribonucleoprotein A3 (Q6URK4)	Hnrnpa3	5	62.74	Thr ¹⁵⁴	IET IEVMEDRQSGK	0.02	0.03	1.58
Alpha-actinin-4 (Q9QXQ0)	Actn4	28	470.02	Ser ⁶⁵⁶	QFAS QANMVGPWIQTKMEEIGR	0.04	0.07	1.49
Peroxisomal acyl-coenzyme A oxidase 3 (Q63448)	Acox3	6	73.24	Ser ⁵⁷⁸	FHE HTHSSSVPPSLR	0.04	0.24	5.67
Extended synaptotagmin-1 (Q9Z1X1)	Esyt1	36	369.36	Ser ¹⁰⁵¹	SNSSFMS RER	0.05	0.11	2.10
Phosphoglucomutase-1 (P38652)	Pgm1	24	232.48	Thr ⁷¹²	TGGSEVVV TNK	0.05	1.24	22.82
Elongation factor 1-gamma (Q68FR6)	Eef1g	15	152.72	Thr ⁵	AAGT LYTYPENWR	0.06	0.29	4.96
Isoaspartyl peptidase/L-asparaginase (Q8VI04)	Asrgl1	8	125.93	Thr ¹⁵⁷	GAEKFAADMGIPQT PAEK	0.07	0.33	4.46
Maleylacetoacetate isomerase (P57113)	Gstz1	10	106.96	Ser ⁴⁷ /Thr ⁵²	GIDYEIVPINLIKDGQQF SEEFQTLNPMK	0.09	0.17	1.92
Caveolin-1 (P41350)	Cav1	5	82.31	Thr ⁹³ /Thr ⁹⁵	ASFTTF TV TK	0.11	0.18	1.60
Acetyl-CoA carboxylase 1(P11497)	Acc1	55	527.66	Thr ²²⁷⁸	QL TEEDGVRSVIEENIK	0.12	0.84	7.21
Protocadherin Fat 3 (Q8R508)	Fat3	8	73.13	Thr ³³¹	DGGTPALSTAATVSIDL TDVNDNPPR	0.12	0.04	0.31
Aflatoxin B1 aldehyde reductase member 2 (Q8CG45)	Akr7a2	6	63.51	Ser ³³	SPAPRAVSGAPLRPGTVLGTMEMGR	0.13	0.20	1.55
Serine protease inhibitor A3M (Fragment) (Q63556)	Serpina3M	9	121.48	Ser ¹²⁵	L SQPGDQVK	0.13	0.26	2.06

Cytochrome b-c1 complex subunit 2, mitochondrial (P32551)	Uqcrc2	10	102.22	Ser ²²⁵ /Ser ²²⁷	MALVGLGV <u>SHSILK</u>	0.14	0.35	2.53
<i>NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial (Q66HF1)</i>	Ndufs1	14	195.45	Thr ¹⁷¹ /Thr ¹⁷⁴	NIGPLVK <u>TIMTR</u>	0.15	0.80	5.31
Transketolase (P50137)	Tkt	35	403.36	Ser ⁴⁴⁹	SVPMSTVFYPS <u>SDGVATEK</u>	0.15	0.59	3.82
Transketolase (P50137)	Tkt	35	403.36	Ser ²⁹⁵	ILATPPQEDAP <u>SVDIANIR</u>	0.15	0.31	2.15
Nucleoside diphosphate kinase A (Q05982)	Nme1	4	119.78	Tyr ¹⁴²	EISLWFQPEELVD <u>YK</u>	0.16	0.34	2.19
<i>C-reactive protein (P48199)</i>	Crp	3	36.76	Thr ³⁶ /Ser ⁶³	QAFVFPGVSA <u>TAYVSLEAESKKPLEAFTV</u> CLYAHA <u>DVS</u> R	0.17	0.17	1.00
Murinoglobulin-1 (Q03626)	Mug1	23	849.52	Thr ¹²⁸⁸	<u>TPLVTIQSSGSFSQK</u>	0.17	0.67	3.95
Peroxiredoxin-6 (O35244)	Prdx6	13	209.66	Ser ³⁸	FHDFLGDSWGILF <u>SHPR</u>	0.18	0.49	2.75
NADPH-cytochrome P450 reductase (P00388)	Por	23	174.48	Thr ²⁸⁷	SYENQKPPFDAKNPFLAAV <u>TANR</u>	0.19	0.37	1.99
Contactin-6 (P97528)	Cntn6	4	52.79	Thr ⁵⁷⁸	NIQLGHSGKYLCT <u>VQTTLER</u>	0.22	0.58	2.68
Myosin light polypeptide 6 (Q64119)	Myl6	9	100.30	Thr ⁴⁴	ILYSQCGDVMRALGQNPT <u>NAEVLK</u>	0.22	0.30	1.38
<i>Elongation factor 1-delta (Q68FR9)</i>	Eef1d	11	94.35	Ser ⁴⁴⁷	ENIQKSLAGSSGPASSGP <u>GGDHSDLIVR</u>	0.23	0.34	1.52
Gelsolin (Q68FP1)	Gsn	34	400.93	Ser ⁵⁷⁰ /Ser ⁵⁷¹	<u>ASSSGATRAVEVMPK</u>	0.24	0.31	1.28
Aldo-keto reductase family 1 member C21 (Q6AYQ2)	Akr1c21	8	64.82	Thr ²⁷⁰	GIVVNLNT <u>SLKEER</u>	0.25	0.42	1.66
Complement C3 (P01026)	C3	74	894.22	Ser ¹³²¹	LLWESGSLLR <u>SEETK</u>	0.28	0.27	0.95
14-3-3 protein theta (P68255)	Ywhaq	6	188.28	Thr ¹⁴¹	KQT <u>IENSQGAYQEAFDISK</u>	0.34	0.09	0.26
<i>Synaptic vesicle membrane protein VAT-1 homolog (Q3MIE4)</i>	Vat1	15	180.30	Thr ²⁹⁵	VV <u>TYGMANLLTGPKR</u>	0.37	0.35	0.97
Glutathione peroxidase 1 (P04041)	Gpx1	13	131.27	Ser ⁷ /Ser ¹⁸ /Ser ³²	L <u>SAVAQSTVYAFSARPLAGGEPVSLGSLR</u> GK	0.41	1.32	3.22
Myosin-3 (P12847)	Myh3	30	340.89	Ser ¹⁶⁰⁸	TVETMQGALDAEVR <u>SR</u>	0.45	3.01	6.70
Unconventional myosin-Ic (Q633550)	Myo1c	40	420.21	Thr ⁵⁵⁴	<u>ETMCSS</u> TNPIMAQCFDK	0.58	0.50	0.86

<i>All-trans-retinol 13,14-reductase (Q8VHE9)</i>	Retsat	24	204.05	Ser ⁴⁰⁴ /Tyr ⁴⁰⁹	EELKLQSTNYYVYFDTDMDK	0.96	2.95	3.08
Heat shock protein 75 kDa, mitochondrial (Q5XHZ0)	Trap1	30	254.79	Ser ¹²⁰ /Ser ¹²³	SLYSEKEVFIRELISNASDALEK	0.96	0.28	0.29
Protein AMBP (Q64240)	Ampb	10	151.27	Ser ¹⁷⁰	DSLLQE FREVALS VGIPENSIVFMADR	0.97	1.20	1.24
Proteins up-regulated by ovariectomy								
Protein S100-A11 (Q6B345)	S100a11	4	48.22	Ser ¹¹	MPTETERCIES LIAVFQK	1.01	0.93	0.93
Fructose-bisphosphate aldolase A (P05065)	Aldoa	24	290.12	Thr ⁵²	LQSIGTENT EE ENR	1.08	0.76	0.71
ES1 protein homolog, mitochondrial (P56571)	RGD1303003	5	58.47	Thr ¹¹⁶	IT NLAQLSAANHDA IFPGGF GAAK	1.20	1.41	1.17
Vesicle-trafficking protein SEC22b (Q4KM74)	Sec22b	4	37.81	Ser ¹⁶⁴ /Ser ¹⁶⁸	GEAL SALD KANNLSSL SK	1.26	1.57	1.24
Phosphatidylethanolamine-binding protein 1 (P31044)	Pebp1	11	94.97	Ser ¹⁰⁴	GNDI SSGTVL SEYVGSGPPK	1.27	1.27	1.00
Keratin, type II cytoskeletal 5 (Q6P6Q2)	Krt5	6	116.47	Ser ⁴⁷⁸ /Ser ⁴⁹⁵	LLEGEECRL S GEGVGPVNISVVTNSL SSGY GGR	1.37	0.78	0.57
Fibrinogen gamma chain (P02680)	Fgg	11	129.52	Tyr ³⁰⁶	LTYAY Y	1.38	1.18	0.86
Complement C3 (P01026)	C3	74	894.22	Ser ⁵²⁷	EPGQDLVVLSLPITPEFIPSFR	1.39	1.39	1.00
Reticulon-4 (Q9JK11)	Rtn4	20	262.51	Ser ³⁰⁰	GSPKG E SAILVENTK	1.39	1.39	0.99
Glyceraldehyde-3-phosphate dehydrogenase (P04797)	Gapdh	19	249.04	Se ⁹⁶	DPANIKWGDAGAEYVVESTGVFTTMEK	1.42	0.97	0.68
4-trimethylaminobutyraldehyde dehydrogenase (Q9JLJ3)	Aldh9a1	20	205.19	Ser ⁹	MSTGTFVV S QPLNYRG GAR	1.60	1.29	0.81
Sideroflexin-1 (Q63965)	Sfxn1	7	62.38	Tyr ⁵⁴	VVHD Y RQGIVPAGL T ENELWR	1.72	1.56	0.91
Isocitrate dehydrogenase [NADP] cytoplasmic (P41562)	Idh1	23	281.54	Thr ³⁵⁰	AKLDNN T ELSF F ANALEEV C IETIEAGFMTK	1.73	1.23	0.71
Desmin (P48675)	Des	8	156.78	Ser ⁵⁷ /Ser ⁶⁸	VYQVSRTSGGAGGLG S LR	1.74	1.77	1.01
Guanine nucleotide-binding protein G(s) subunit alpha isoforms short (P63095)	Gnas	7	234.25	Thr ²⁴²	KWIQCFNDVT A IIFVVASSSYNM VIR	1.74	1.64	0.95
<i>Lipoprotein lipase (Q06000)</i>	Lpl	9	88.27	Tyr ¹⁹¹	ITGLDPAGPNFE Y AEAPSR	1.78	1.04	0.58

Elongation factor 2 (P05197)	Eef2	34	374.40	Ser ⁵⁴¹	LAKSDPMVQCIIEE <u>S</u> GEHIIAGAGELHLEIC LK	1.80	0.47	0.26
Cell surface glycoprotein MUC18 (Q9EPF2)	Mcam	19	248.85	Ser ¹⁹⁴	VHIQ <u>S</u> SQTVESSGLYTLK	1.83	2.63	1.43
Perilipin-1 (P43884)	Plin1	37	377.03	Ser ^{258/Ser²⁵⁹}	GHSLAMWIPGVAPL <u>S</u> SLAQWGASAAMQV VSR	1.88	1.88	1.00
Guanine nucleotide-binding protein G(t) subunit alpha-3 (P29348)	Gnat3	11	91.69	Thr ^{163/Ser¹⁷⁷}	LT <u>A</u> PGYVPNEQDV <u>LH</u> SRVK	1.93	0.97	0.50
Neurofilament medium polypeptide (P12839)	Nefm	21	331.79	Ser ^{2/Tyr¹⁴}	MS <u>T</u> LDSLGNPSA <u>Y</u> RR	1.93	1.03	0.54
Tubulin alpha-1B chain (Q6P9V9)	Tuba1b	6	313.28	Thr ⁷³	AVFVDLEPT <u>T</u> VIDEV <u>R</u>	1.96	2.86	1.46
Dihydropyrimidinase-related protein 2 (P47942)	Dpysl2	21	337.64	Ser ⁵¹⁷	TVTP <u>A</u> SSAK	1.97	1.93	0.98
Biglycan (P47853)	Bgn	11	101.78	Tyr ³³²	RAY <u>Y</u> NGISLFNNPVPYWEVQPATFR	2.05	1.17	0.57
Endoplasmin (Q66HD0)	Hsp90b1	30	329.65	Ser ²⁸³	YSQFIN <u>P</u> IYVV <u>W</u> SSK	2.11	2.66	1.26
Fatty acid-binding protein 5 (P55053)	Fabp5	9	126.16	Thr ¹⁰⁶	WEGKE <u>S</u> TIR	2.12	1.47	0.69
Integrin beta-1 (P49134)	Itgb1	17	182.16	Ser ²⁶³	ISGNLDSPEGGFDAIMQVA <u>VCG</u> <u>S</u> LIGWRN VTR	2.23	2.27	1.02
Guanine deaminase (Q9WTT6)	Gda	33	383.90	Ser ^{116/Tyr¹²⁴}	RFQ <u>S</u> TDVAEEV <u>Y</u> TR	2.27	0.92	0.40
Guanine nucleotide-binding protein G(i) subunit alpha-2 (P04897)	Gnai2	9	213.36	Tyr ¹⁵⁵	EYQLND <u>SAA</u> <u>Y</u> YLN <u>D</u> LER	2.41	2.43	1.01
Gamma-enolase (P07323)	Eno2	2	133.70	Ser ²²¹	YGKDATNVGDEGGFAPNILE <u>N</u> SEALELVK	2.42	2.85	1.18
Ras-related protein Rab-7a (P09527)	Rab7a	15	154.96	Thr ⁹¹	GADCCVLVF <u>D</u> V <u>T</u> APNTFK	2.43	3.25	1.34
Complement C3 (P01026)	C3	74	894.22	Ser ^{1142/Thr¹¹⁴⁴}	NTKEAD <u>V</u> <u>S</u> LA <u>F</u> V <u>L</u> IALQE <u>A</u> R	2.51	1.49	0.59
Endoplasmic reticulum chaperone BiP (P06761)	Hspa5	37	446.42	Ser ⁵⁶⁷	NELES <u>Y</u> AYSLKNQIGDK	2.54	2.50	0.99
Fatty acid synthase (P12785)	Fasn	120	1368.34	Ser ⁷²⁵	SARWL <u>S</u> TIPEAQW <u>Q</u> <u>SSL</u> AR	2.82	1.95	0.69
PDZ and LIM domain protein 3 (Q66HS7)	Pdlim3	3	18.50	Thr ⁸⁵	AE <u>TR</u> LCPAV <u>S</u> EDGK	2.82	1.52	0.54
Cell surface glycoprotein MUC18 (Q9EPF2)	Mcam	19	248.85	Tyr ²⁴⁹	ESKEVTVP <u>V</u> L <u>Y</u> PAEK	2.83	2.95	1.04

Guanine nucleotide-binding protein G(s) subunit alpha isoforms short (P63095)	Gnas	7	234.25	Thr ²⁰⁴ /Ser ²⁰⁵	CRVL <u>T</u> S GIFETK	2.86	2.44	0.85
Cell division control protein 42 homolog (Q8CFN2)	Cdc42	3	69.43	Thr ¹⁷	CVVVGDGAVGK <u>T</u> CLLISYTTNK	2.87	2.14	0.74
Pyruvate kinase PKM (P11980)	Pkm	39	455.37	Ser ⁷⁷	SGMNVARLNFS <u>H</u> GTHEYHAETIK	3.18	2.51	0.79
Alpha-1-inhibitor 3 (P14046)	A1i3	24	877.18	Tyr ¹⁶⁶	NLHPLNELFPLA <u>Y</u> IEDPKMNR	3.30	2.64	0.80
Ras-related protein Rab-14 (P61107)	Rab1	11	117.21	Ser ⁷⁹	<u>S</u> YYRGAAGALMVYDITR	3.39	2.48	0.73
Ras-related protein Rab-26 (P51156)	Rab26	10	79.12	Thr ⁹⁷	DGAFLAGTF <u>I</u> STVGIDFRNK	3.43	2.31	0.67
Xanthine dehydrogenase/oxidase (P22985)	Xdh	25	220.59	Thr ⁴³⁴	V <u>T</u> SGMRVLFKP GTIEVQELSLCFGGMADR	3.43	3.04	0.89
Ig gamma-2A chain C region (P20760)	Igg-2a	30	212.31	Thr ¹²²	<u>T</u> KDVLTTILTPK	3.58	3.78	1.05
EH domain-containing protein 2 (Q4V8H8)	Ehd2	37	543.72	Ser ¹⁵⁹	FMCAQLPNQVLESISIIDTPGIL <u>S</u> GAKQR	3.61	2.35	0.65
Endoplasmin (Q66HD0)	Hsp90b1	30	329.65	Ser ⁷³	<u>S</u> EKF AFQAEVNR	3.66	1.34	0.37
EH domain-containing protein 1 (Q641Z6)	Ehd1	22	356.36	Ser ¹⁵⁹	FMCAQLPNPVLD SISIIDTPGIL <u>S</u> GEKQR	3.74	3.19	0.85
Long-chain-fatty-acid-CoA ligase 1 (P18163)	Acsl1	67	720.21	Thr ²⁷ /Thr ⁴⁰	QYVRTLP <u>T</u> NTLMGFGAFAAL <u>T</u> TFWYATR PK	3.79	2.26	0.60
Unconventional myosin-Ic (Q633550)	Myo1c	40	420.21	Thr ⁸⁸⁰	<u>L</u> FIST <u>TR</u> LGTEEISPR	3.85	3.12	0.81
ATP-citrate synthase (P16638)	Acly	48	537.26	Ser ³⁶⁷	AIRDYQ <u>G</u> SLKEHEVTIFVR	3.90	2.73	0.70
Angiotensinogen (P01015)	Agt	11	113.28	Thr ¹¹	<u>A</u> TIFCILTWVSLTAGDR	3.97	1.64	0.41
Very long-chain specific acyl-CoA dehydrogenase_mitochondrial (P45953)	Acadvl	15	179.91	Ser ⁴¹⁸	DFQIEAA <u>I</u> SK	4.49	1.75	0.39
6-phosphogluconate dehydrogenase, decarboxylating (P85968)	Pgd	16	214.30	Tyr ¹³⁷	GILFVGSGVSGGEEGAR <u>Y</u> GPSLMPGGNK	4.53	2.66	0.59
Extended synaptotagmin-1 (Q9Z1X1)	Esyt1	36	369.36	Tyr ⁴⁷⁵	GITSRPEPPSAA <u>I</u> LVV <u>Y</u> LDR	4.59	2.10	0.46

Endoplasmic reticulum protein (Q66HD0)	Hsp90b1	30	329.65	Ser ⁶⁴	TDDEVVQREEEAIQLDGLNA <u>S</u> QIR	4.75	3.50	0.74
Complement C4 (P08649)	C4	46	501.39	Thr ¹⁷⁵	VFALDQKMRPSTD T TVENS H G L R	4.83	5.20	1.08
Complement component C9 (Q62930)	C9	20	167.24	Thr ⁴⁹²	LSPIYNLIPL <u>T</u> MK	4.97	4.75	0.96
N(G),N(G)-dimethylarginine dimethylaminohydrolase 2 (Q6MG60)	Ddah2	11	115.40	Ser ²⁵	CSHALIRGVPE <u>S</u> LA <u>S</u> GEGAGAGLPALDLA <u>K</u>	5.92	2.37	0.40
Angiotensinogen (P01015)	Agt	11	113.28	Thr ³⁷¹	AIRL <u>T</u> LPQLEIR	8.01	4.01	0.50
Citrate synthase, mitochondrial (Q8VHF5)	Cs	12	91.97	Tyr ⁸⁰ /Ser ⁸³	GLV <u>Y</u> ETSVLD P DEGIR	8.36	1.78	0.21
Rab GDP dissociation inhibitor beta (P50399)	Gdi2	21	317.96	Tyr ⁵³	KVLHMDQNPYYGGESASITPLEDL <u>Y</u> K	9.23	8.95	0.97
Annexin A2 (Q07936)	Anxa2	34	407.55	Thr ⁶¹ /Ser ⁶⁴	GVDEVTIVNIL <u>T</u> NRSNAQR	11.84	5.29	0.45
Peripherin (P21807)	Plin1	16	184.82	Thr ⁴⁵⁰	VV <u>T</u> ESQKEQHSELDK	11.88	6.20	0.52
<i>Class I histocompatibility antigen, Non-RT1.A alpha-1 chain (P15978)</i>	RT1-Aw2	2	34.65	Ser ²⁹⁵	EQNYTCRVEHEGLPE <u>P</u> L <u>S</u> QR	12.79	2.35	0.18
Zinc finger protein 518A (Q499R0)	Znf518a	3	56.53	Thr ¹²²	MCAKILNF <u>T</u> CSK	15.27	7.77	0.51
Adenylyl cyclase-associated protein 1 (Q08163)	Cap1	15	184.31	Ser ³⁰⁰ /Thr ³⁰⁶ /Ser ³⁰⁷	SGPKPF <u>S</u> APKPQT <u>S</u> PSPKPATK	18.49	1.20	0.07
Long-chain specific acyl-CoA dehydrogenase, mitochondrial (P15650)	Acadl	20	243.85	Ser ²¹⁰	VFITNGWL <u>S</u> DLVIVVAVTNREAR	27.25	7.84	0.29

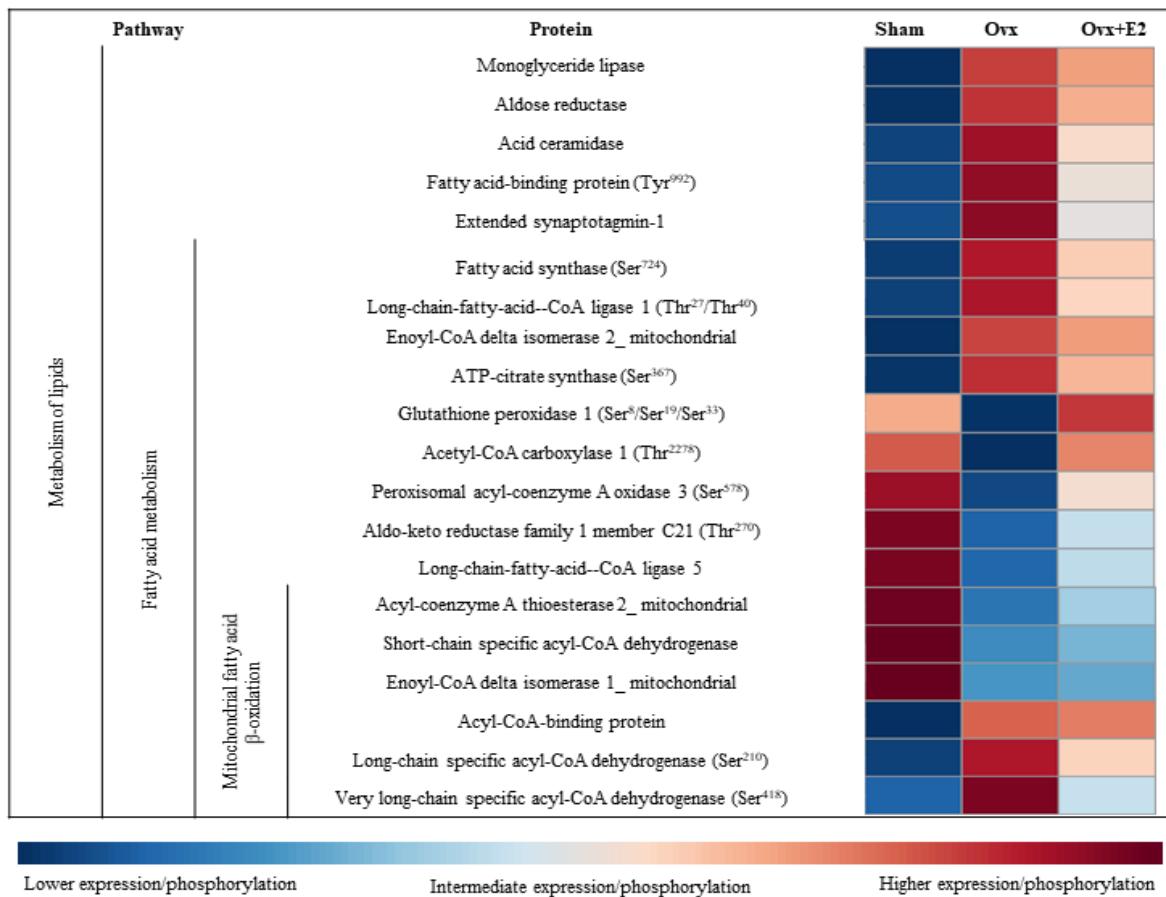
Abbreviation: UniProt: Universal Protein Resource
Differently expressed and phosphorylated proteins

Ovariectomy modified metabolic pathways in retroperitoneal fat

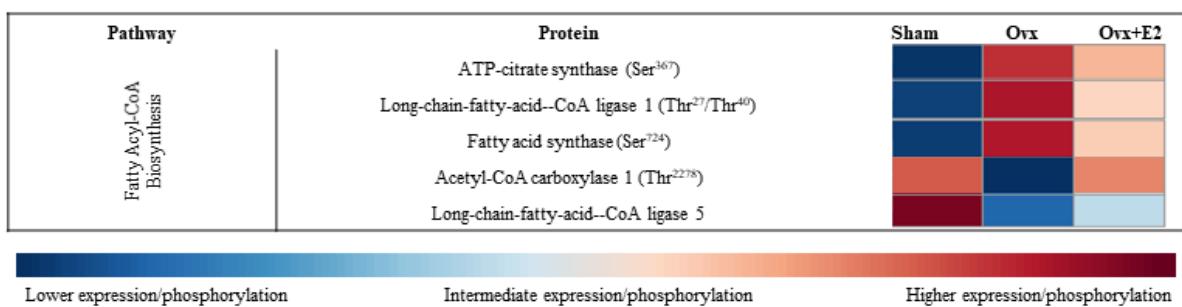
The pathway overrepresentation test showed significantly enrichment of 5 main pathways: metabolism of lipids pathway (9 proteins/11 phosphopeptides; $p = 5.30E-5$) included fatty acid metabolism (6 proteins/9 phosphopeptides; $p = 7.35E-8$) and mitochondrial fatty acid β -oxidation (4 proteins/2 phosphopeptides; $p = 1.65E-3$) (Figure 3A); fatty acyl-CoA biosynthesis pathway (1 protein/4 phosphopeptides; $p = 2.67E-2$) (Figure 3B); innate immune system pathway (10 proteins/16 phosphopeptides; $p = 1.82E-7$) included neutrophil degranulation (5 proteins/13 phosphopeptides; $p = 2.07E-10$) (Figure 3C); metabolism of vitamins and cofactors pathway (5 proteins/4 phosphopeptides; $p = 4.29E-2$) (Figure 3D); integration of energy metabolism pathway (3 proteins/6 phosphopeptides; $p = 5.74E-4$) included ChREBP activates metabolic gene expression (3 phosphopeptides; $p = 4.42E-2$) (Figure 3E).

It is worth noting that, when related to the Sham condition, the effects of Ovx on both protein expression and phosphorylation intensities were, in general, more pronounced than those of the Ovx+E2 condition (Figures 3A-E).

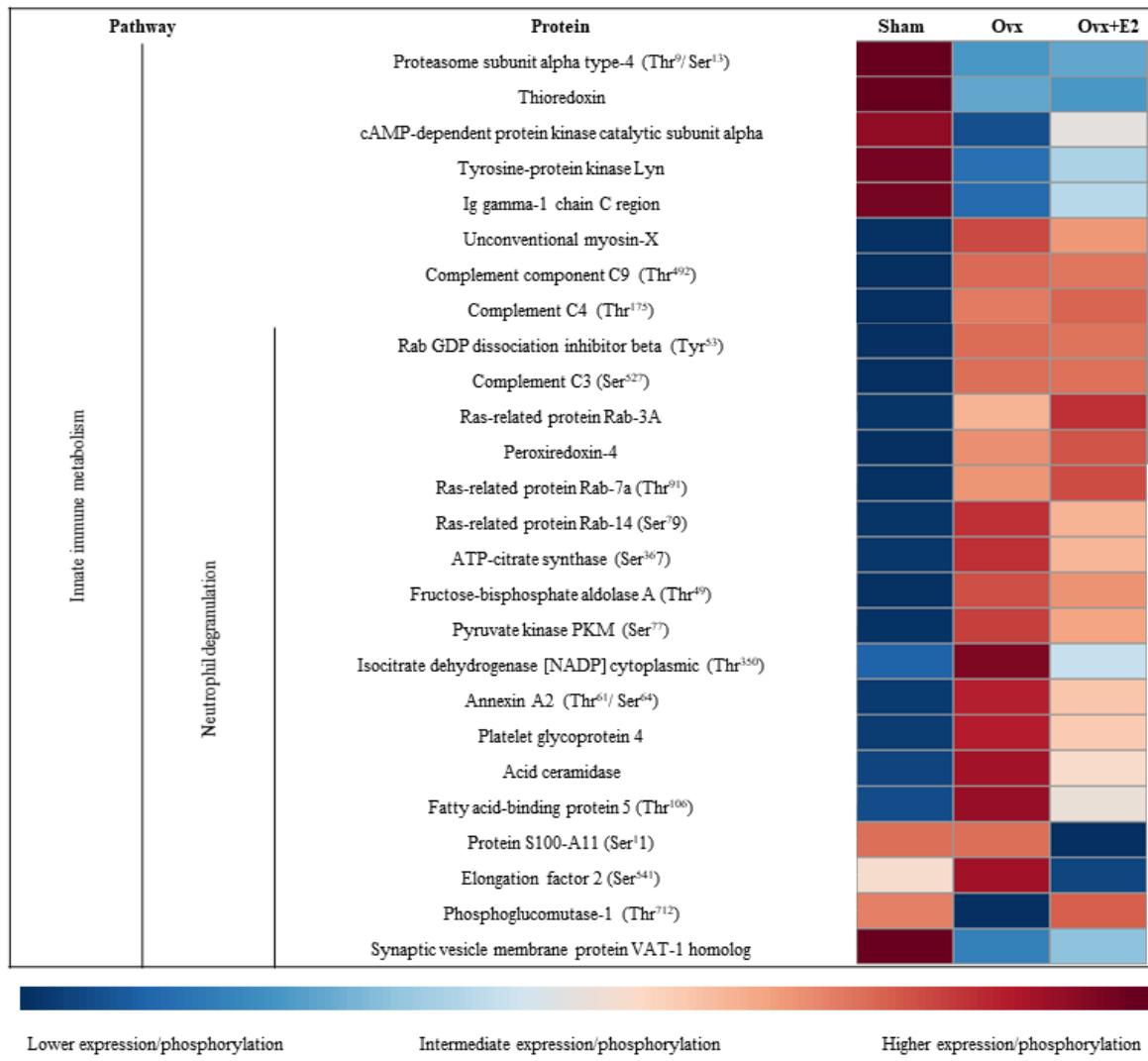
3A



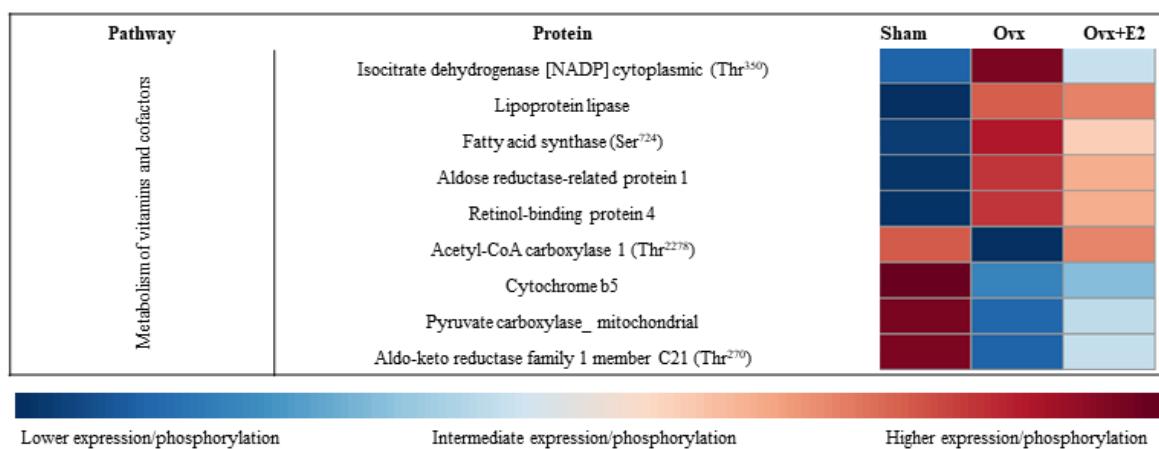
3B



3C



3D



3E

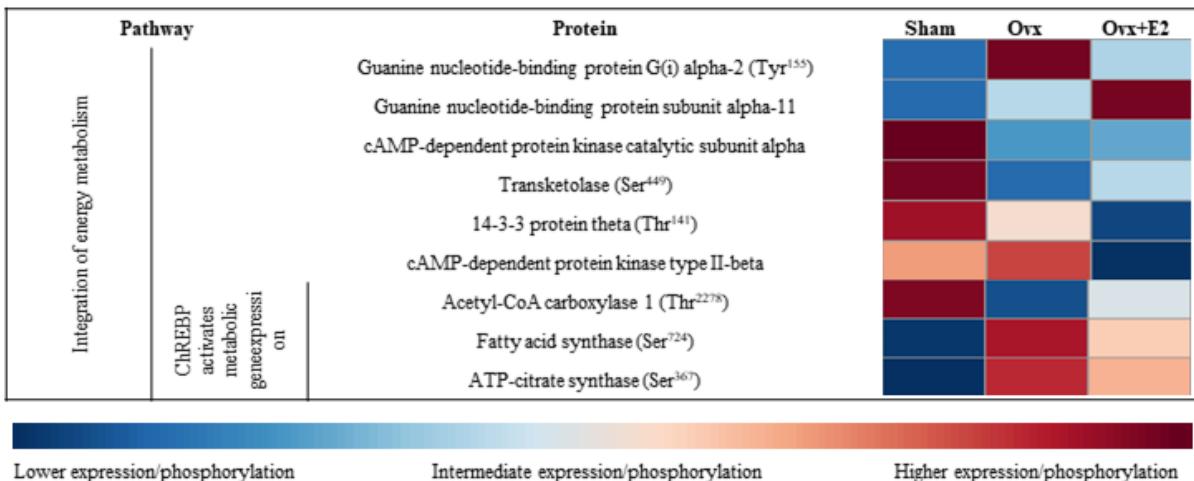


FIGURE 3: Pathways significantly enriched by ovariectomy and their associated proteins and phosphopeptides

Pathway enrichment analysis: Fisher's exact test followed by Bonferroni correction. The colored bars indicate the proteins expression/phosphorylation peak intensities.

Ovariectomy increased saturated fatty acid content and disturbed the polyunsaturated fatty acid ratios in RET

The Ovx group showed increased content of lauric, myristic and palmitic acids in relation to Sham. Increased total saturated fatty acids (SFA), alongside increased palmitic/stearic ratio, was also found in Ovx group. Estrogen replacement brought the content of lauric and myristic acids, as well as total SFA, back to levels comparable to Sham.

The monounsaturated fatty acids (MUFA) myristoleic and palmitoleic acids levels were higher in Ovx group as compared to Sham, but comparable to Sham when estrogen replacement was introduced. Eicosenoic acid was lower in both Ovx and Ovx+E2 groups as compared to Sham. The palmitic/palmitoleic ratio was significantly decreased in Ovx, but normalized in Ovx+E2 group.

Ovariectomy led to a significant disturbance in the proportions of various polyunsaturated fatty acids (PUFAs). The essential fatty acids alpha-linoleic and linoleic acids were respectively increased and decreased in Ovx group. Estrogen replacement was successful in returning

linoleic acid back to a level comparable to Sham, but the same was not found for alpha-linoleic acid. A significant increase in eicosapentaenoic acid (EPA) was found in Ovx and Ovx+E2 groups. An increase in arachidonic acid (AA) was also found in both groups, but such difference did not reach statistically significant levels.

Ovariectomy increased 0.33% of the total n-3 while decreasing 3% of the total n-6. Such differences reached statistically significant levels, and whilst they remained for total n-3 in Ovx+E2 group, they were no longer statistically different for n-6 in Ovx+E2 group. The total n-6/n-3 ratio was lower in Ovx and Ovx+E2 groups; however, the total SFA/PUFA ratio was higher in Ovx, and similar in Ovx+E2, as compared to Sham (table 4).

Table 4. Fatty acid composition of RET total lipid extract

Fatty acid		% of total fatty acids		
		Sham	Ovx	Ovx+E2
C12:0	Lauric acid	0.04 ± 0.01	0.05 ± 0.01*#	0.04 ± 0.01
C14:0	Myristic acid	0.62 ± 0.06	0.75 ± 0.07*#	0.67 ± 0.11
C16:0	Pamitic acid	18.7 ± 1.4	21.2 ± 1.4*	20.4 ± 1.2*
C18:0	Stearic acid	3.35 ± 0.21	3.21 ± 0.37	3.2 ± 0.15
C20:0	Arachidinic acid	0.06 ± 0.01	0.04 ± 0.01*	0.05 ± 0.01*
C22:0	Behenic acid	0.018 ± 0.001	0.009 ± 0.001*#	0.012 ± 0.001*
C16:0/C18:0		5.75 ± 0.39	6.67 ± 0.88*	6.3 ± 0.57
Σ SFA		22.8 ± 1.5	25.3 ± 1.5*#	24.4 ± 1.3
C14:1n-7	Myristoleic acid	0.02 ± 0.01	0.03 ± 0.01*#	0.02 ± 0.01
C16:1n-7	Palmitoleic acid	1.55 ± 0.3	2.36 ± 0.59*#	1.8 ± 0.52
C18:1n-9	Oleic acid	27.7 ± 1.2	27.5 ± 1.7	27.2 ± 0.9
C18:1n7	cis-vaccenic acid	2.32 ± 0.18	2.23 ± 0.12	2.31 ± 0.17
C20:1n9	Eicosenoic acid	0.18 ± 0.02	0.15 ± 0.01*	0.16 ± 0.01*
C18/C18:1		0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.01
C16:0/C16:1n-7		12.2 ± 1.7	9.38 ± 1.8*#	12.3 ± 2.9
Σ MUFA		31.8 ± 1.3	32.4 ± 2.3	31.4 ± 1.4
C18:3n-3	Alpha-linolenic acid	1.50 ± 0.18	1.81 ± 0.14*	1.84 ± 0.05*
C20:5n-3	Eicosapentaenoic acid (EPA)	0.02 ± 0.01	0.03 ± 0.001*	0.03 ± 0.01*
C22:5n-3	Docosapentaenoic acid (DPA)	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02
C22:6n-3	Docosahexaenoic acid (DHA)	0.16 ± 0.05	0.15 ± 0.06	0.15 ± 0.03
Σ n-3		1.75 ± 0.28	2.08 ± 0.17*	2.11 ± 0.08*
C18:2n-6	Linoleic acid	40.14 ± 2.0	37.1 ± 2.8*	38.5 ± 2.3
C18:3n-6	Gamma-linoleic acid	0.07 ± 0.02	0.1 ± 0.01*	0.09 ± 0.02*
C20:2n-6	Eicosadienoic acid (EDA)	0.19 ± 0.02	0.16 ± 0.03*	0.17 ± 0.2*
C20:3n-6	Dihomo-gamma linoleic acid	0.14 ± 0.02	0.14 ± 0.02	0.15 ± 0.01
C20:4n-6	Arachidonic acid (AA)	0.63 ± 0.17	0.69 ± 0.18	0.74 ± 0.14
C22:4n-6	Docosatetraenoic acid	0.23 ± 0.08	0.19 ± 0.06	0.20 ± 0.04
Σ n-6		41.4 ± 2.2	38.4 ± 3.0*#	39.9 ± 2.4
Σ n-6/ Σ n-3		24.1 ± 3.9	18.5 ± 0.7*	18.9 ± 0.9*
Σ PUFA		43.1 ± 2.4	40.5 ± 3.2	42.1 ± 2.5
Σ SFA/ Σ PUFA		0.53 ± 0.07	0.63 ± 0.08*	0.58 ± 0.06

Data presented as means ± SEM of the % of total FAs. n=6 for each group. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids *p < 0.05 vs Sham; #p < 0.05 Ovx vs Ovx+E2

Discussion

Menopause has been considered as an important factor leading to obesity, mainly due to the shift in fat distribution from subcutaneous to visceral [5]. We thus hypothesized that the loss of ovarian hormones could lead to impairment of protein and lipid regulation in the visceral adipose tissue. In order to test this hypothesis, we used shotgun proteome and phosphoproteome, along with fatty acid profiling, to determine proteins and lipids affected and to evaluate to what extent estrogen replacement would modify the ovariectomy-induced alterations.

Ovariectomy increased feed efficiency, body weight gain and adiposity, and insulin and leptin levels. These alterations were attenuated by estradiol replacement, in agreement with our previous observations [6][7].

RET protein expression and phosphorylation profiles that showed the most distinctive modulation by ovariectomy are discussed below.

Elongation factor 1- δ (EEF1D) is involved in the protein elongation steps during the synthesis of a variety of proteins, acting in the regulation of translation and transcription [20]. Insulin stimulates the activity of this enzyme, mainly through phosphorylation processes in adipose tissue, as observed after *in vitro* experiments using adipose-derived cells 3T3-L1 [21]. The fact that this protein showed downregulated expression and decreased phosphorylation is compatible with our suggestion of insulin resistance after ovariectomy.

The protein Integrin β -1 (ITB1) showed decreased expression and increased phosphorylation induced by ovariectomy. The protein is localized in the cell membrane and participates in the signaling communication between extracellular matrix and the intracellular environment, and dysfunctional integrins have been related to insulin resistance [22]. In visceral fat pads isolated from chow-fed rats, ITB1 potentiated the ability of insulin to enhance tyrosine phosphorylation of insulin receptor substrate 1 [23]. Increased phosphorylation at Ser²⁶³ described in the present study has been previously found in breast cancer, but the consequences of this cancer-induced alteration on ITB1 function have not yet been explored [24]. Since this modification appeared in the present study in an obese pathological condition, it may be not inferred whether it yielded a deleterious effect or, alternatively, whether it represented a counter-regulatory mechanism to insulin resistance.

Ovariectomy also modulated proteins involved in the adipogenesis process. The protein all-trans-retinol 13,14-reductase (RETSAT) showed increased expression and decreased phosphorylation. This protein favors adipocyte differentiation under normal metabolic conditions and its blockade has been shown to inhibit adipogenesis [25]. Its expression has been found to be reduced in diet-induced obese mice and in highly obese postmenopausal women, probably as a function of a high degree of macrophage infiltration in these well established and severe obese states [26]. Because the present Ovx animals showed mild increases of body fat and adipose tissue cytokine levels, it is suggested that the upregulated RETSAT indicates a state of active fat accumulation. Moreover, further studies are necessary to elucidate the consequences of reduced phosphorylation of the sites found in the present study (Ser⁴⁰⁴/Tyr⁴⁰⁹).

The protein lipoprotein lipase (LPL) showed increased expression and phosphorylation in the Ovx groups, indicating a high capacity of the retroperitoneal adipose tissue for uptake of lipoprotein-derived free fatty acids. Additionally, to its role in fat uptake, this enzyme has also been implicated in adipocyte differentiation and it has shown increased expression in adipose-derived mesenchymal stem cells from ovariectomized mice [27]. Moreover, estrogen has been shown to decrease its expression in subcutaneous fat of premenopausal women [28]. Although we were not able to find previous records of the phosphorylation site found in the present study (Tyr¹⁹¹), the phosphorylation at a tyrosine site could have a stimulatory effect, since the phosphorylation at a serine site (Ser³⁰⁴) was reported to increase with increasing insulin resistance in 3T3-L1 adipocytes [29].

The protein NADH-ubiquinone oxidoreductase (NDUS1), a crucial complex in the oxidative phosphorylation pathway, showed downregulated expression and decreased phosphorylation. Its expression was found to be downregulated in the epididymal white adipose tissue and muscle of insulin-resistant diet-induced obese mice [30][31]. The phosphorylation sites found in the present study (Thr¹⁷¹/Thr¹⁷⁴) have not been described yet, although decreased phosphorylation of Ser⁴⁶¹ has been described in liver mitochondria from obese mice, and associated with increased ketogenesis [32].

The adenylyl cyclase-associated protein-1 (CAP1) showed upregulated expression and increased phosphorylation. In monocytes, this protein was described to function as a receptor for adipocyte-derived resistin, which in turn regulates inflammation signaling, leading to the release of pro-inflammatory cytokines. Its expression was found to be increased in cells

extracted from white adipose tissue of obese humans [33]. The phosphorylations at Thr³⁰⁶/Ser³⁰⁷ identified in the present study were found to be increased in response to lipopolysaccharide-induced inflammation in macrophages [34], which reinforces the idea of an overall inflammatory status of our experimental model.

Class I histocompatibility antigen, Non-RT1.A alpha-1 chain (RT1-AW2) also showed upregulated expression and increased phosphorylation. This protein is involved in immune response and was shown to be upregulated in the secretome of hepatocytes of rats treated with hepato-carcinogenic substances, a condition also associated with a pro-inflammatory environment [35]. The phosphorylation site described in the present study (Ser²⁹⁵) has not been described yet but the Ser³⁵⁴ phosphorylation was found to be increased in liver mitochondria from obese mice [32].

Platelet glycoprotein 4 (CD36), a protein produced in macrophages, plays a role in the inflammatory response of the innate immune system, being considered a pro-inflammatory marker. This protein was found up regulated both in the visceral adipose tissue of postmenopausal women and in the perivascular adipose tissue of diabetic rats [36][37]. In the present study, the protein expression was found to be upregulated by ovariectomy.

Estrogen replacement attenuated the ovariectomy-induced changes regarding the proteins CAP1, RT1-AW2, and CD36. These results are compatible with the reduction of IL-6 tissue content and suggest an overall effect of estrogen replacement in reducing the inflammation status.

The pathway analyses indicated that ovariectomy had a large impact on the metabolism of lipids, affecting fatty acid metabolism/mitochondrial fatty-acids β-oxidation pathway and the fatty acyl-CoA biosynthesis pathway. Figure 4 depicts the main effects of ovariectomy in these pathways, which will be addressed below.

The activation of free fatty acids by their association with coenzyme-A, yielding fatty-acyl CoA, is promoted by the enzymes ligases and represents a crucial step both for β-oxidation and synthesis of triacylglycerols and other lipids. The fatty acid metabolism/mitochondrial fatty-acids β-oxidation pathways showed down regulation of the protein long-chain-fatty-acid-CoA ligase 5 (ACSL5), a feature that has been associated with low β-oxidation rates [38], and increased phosphorylation of the protein long-chain-fatty-acid-CoA ligase 1 (ACSL1). The present result on ACSL5 agrees with a previous report of its reduced gene expression in diabetic

mice [39]. The phosphorylation sites of the ACSL1) found in the present study (Thr²⁷ and Thr⁴⁰) have not been reported previously while a threonine phosphorylation site (Thr⁸⁵) was described in liver mitochondria of normal rats under physiological conditions [40].

The acyl-CoA dehydrogenases, responsible for fatty acyl-CoA desaturation in the β -oxidation pathway [41], also showed an effect of ovariectomy. The short-chain specific acyl-CoA dehydrogenase (ACADS) showed down regulated expression. This is in accordance with a report in the white adipose tissue from obese subjects [42]. The long- and very long- chain specific Acyl-CoA dehydrogenases (ACADL; ACADVL) presented increased phosphorylation (Ser²¹⁰ and Ser⁴¹⁸, respectively). While ACADVL has no phosphorylation sites previously reported, ACADL presents a previous description of decreased phosphorylation at serine sites (Ser²⁸/Ser³⁰) after ischemia in ovarian tumor grafts [43] and a demonstration that its decreased phosphorylation (Ser⁵⁸⁶) in fibrotic lung cells was associated with higher lipid peroxidation in comparison to normal lung cells [44].

Two other enzymes of the mitochondrial fatty-acids β -oxidation pathway, acyl-coenzyme A thioesterase type 2 (ACOT2) and enoyl-CoA delta isomerase-1 (ECI1), showed downregulated expression in response to ovariectomy. Located in the mitochondrial matrix, ACOT2 targets mainly long-chain fatty acyl-CoAs and catalyzes their hydrolysis to the free fatty acids and CoA. This mechanism has been proposed to enhance hepatic long-chain fatty acids oxidation by preventing accumulation of fatty acyl-CoA during high rates of hepatic β -oxidation [45][46]. ECI1 is involved in the process of oxidation of unsaturated fatty acids and was reported to be downregulated in white adipose tissue from Zucker diabetic-fatty female rats [47].

Ovariectomy also affected proteins involved in fatty acids synthesis. acetyl-CoA carboxylase-1 (ACC1) showed decreased phosphorylation at Thr²²⁷⁸, also attenuated by estradiol replacement. ACC1 converts acetyl-CoA into malonyl-CoA during *de novo* lipogenesis. Although the phosphorylation site found in the present study has not been previously described, the phosphorylation of at least other five sites was demonstrated to inhibit the protein activity, resulting in less malonyl-CoA content [48][49]. In addition, fatty acid synthase (FAS) showed increased phosphorylation at Ser⁷²⁵. FAS is a key enzyme in the lipogenesis process, catalyzing the conversion of malonyl-CoA to palmitic acid [50]. FAS phosphorylation at Ser⁷²⁵ was increased in the liver of mice after re-feeding, a situation favoring

lipogenesis [51]. Ovariectomy has been shown to increase FAS protein expression in the retroperitoneal adipose tissue of rats [52].

Those results from previous reports indicate that our present findings may represent a deleterious response, favoring disruption of fatty acids metabolism in the ovariectomized animals. Accordingly, estradiol replacement attenuated the observed ovariectomy-induced alterations.

Since ovariectomy caused obesity and affected many proteins involved in lipid metabolism, we hypothesized that fatty acid composition of RET could be affected. Data about the effects of ovariectomy-induced obesity on fatty acid profile are scarce, although the pro-inflammatory state of common obesity has been associated to SFA in the adipose tissue [53]. Our results showed that ovariectomy increased palmitic acid and total SFA contents of RET. Estrogen replacement was able to attenuate these effects. In postmenopausal obese women, visceral adipose tissue inflammation has been related to accumulation of SFA, especially palmitic acid [54]. Although palmitic acid has been shown to impact fatty acids metabolism by upregulating the expression of ACSLs [55][56], high levels have been found to lower fatty acid oxidation due to inhibition of carnitine-palmitoyltransferase 1A [57].

Ovariectomy also increased the percentage of the monounsaturated palmitoleic fatty acid. Endogenous non-dietary palmitoleic acid originates mainly from *de novo* lipogenesis in white adipose tissue, and has been recently considered as a lipokine, since it is released from the tissue and acts on distant cells. However, its metabolic effects have not been elucidated, as both deleterious and beneficial effects on adiposity, insulin sensitivity and lipid profile have been described [58]. In the present study, its higher levels after ovariectomy are consistent with the proteomic results indicating a high lipogenesis rate, attenuated by estradiol replacement.

Regarding polyunsaturated fatty acids, our results showed a decrease in linoleic acid (n-6) and an increase in alpha-linolenic acid (n-3). Linoleic acid is a precursor of pro-inflammatory lipid mediators such as prostaglandins and leukotrienes [59]. Although we did not measure these factors, it is fair to speculate that the decrease in linoleic acid in the ovariectomized animals was a consequence of its recruitment for the production of pro-inflammatory factors, since we had indications of an inflamed status induced by ovariectomy, as discussed above. Given the fact that the sum of PUFAs did not differ among the groups, the increase in n-3 was a direct consequence of the n-6 decrease.

In conclusion, the present results indicated that ovariectomy favored lipogenesis while it impaired fatty acids oxidation, and induced a pro-inflammatory state in the visceral adipose tissue. These effects are consistent with the findings of high adiposity, hyperleptinemia, and impaired insulin sensitivity. The observed alterations were partially attenuated by estradiol replacement. The data point to a role of disrupted lipid metabolism in adipose tissue in the genesis of obesity after menopause. It is suggested that weight management is crucial for postmenopausal women, having a beneficial role in preventing visceral fat accumulation and its consequences.

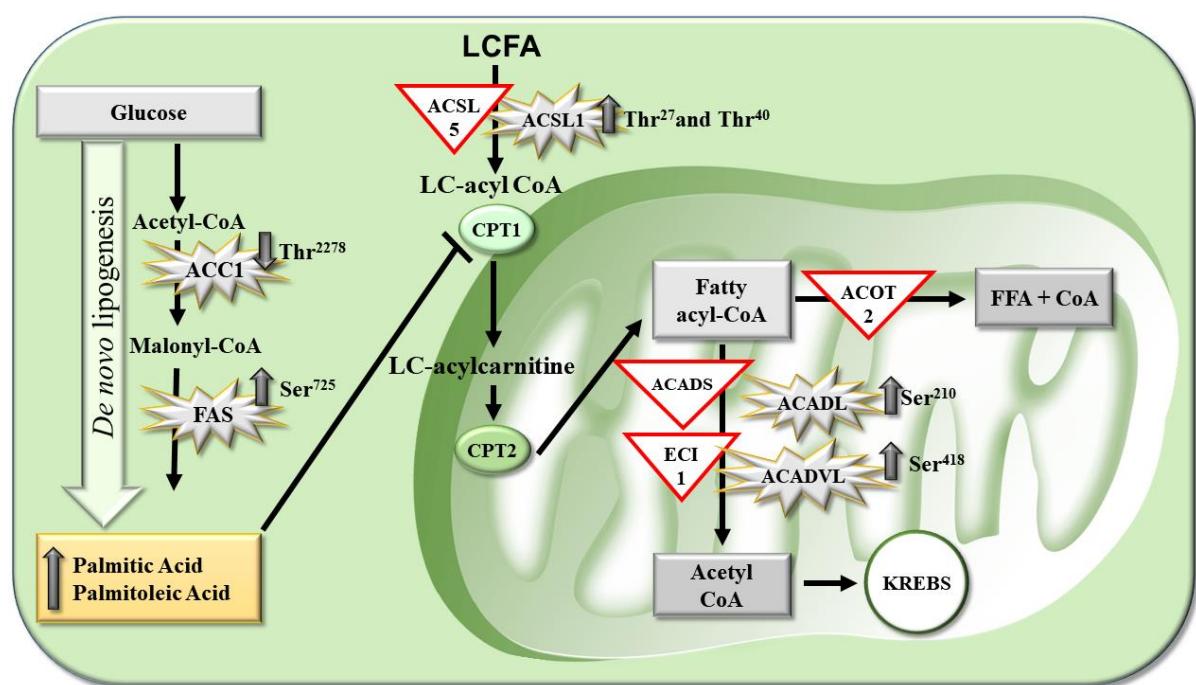


FIGURE 4: Summary of the alterations in lipid metabolism induced by ovariectomy, as indicated by the proteome/phosphoproteome analysis.

Red triangles indicate reduced protein expression; Arrows followed by amino acid symbol indicate reduced (\downarrow) or increased (\uparrow) phosphorylation of the residue in the protein shown. ACSL5: Long-chain fatty acid-CoA ligase 5; ACSL1: Long-chain fatty acid-CoA ligase 1; ACADS: Short-chain specific acyl-CoA dehydrogenase; ACADL: Long-chain specific acyl-CoA dehydrogenase; ACADVL: Very long-chain specific acyl-CoA dehydrogenase; ACOT2: Acyl-Coenzyme A thioesterase typ2; ECI1: Enoyl-CoA delta isomerase-1; ACC1: Acetyl-CoA carboxylase-1; FAS: Fatty acid synthase.

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6. CONCLUSÕES

Os dois estudos em ratas apresentados permitem concluir que:

- A vulnerabilidade para o desenvolvimento de depressão após a menopausa foi influenciada de forma direta pela obesidade.
- A falta de hormônios ovarianos afetou o metabolismo do tecido adiposo visceral, favorecendo a lipogênese e prejudicando a oxidação de ácidos graxos, além de induzir um estado inflamado no tecido. Estas alterações corroboram os dados de alta adiposidade, hiperleptinemia e resistência insulínica. A reposição de estradiol reverteu parcialmente os efeitos da ovariectomia.
- Em conjunto, os dados indicam que o controle de peso é muito importante em mulheres na pós-menopausa, podendo prevenir o surgimento de desordens de humor bem como a obesidade visceral e suas consequências.

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