UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Daniela Fernanda de Freitas

Avaliação do tecido adiposo branco na obesidade e sua influência no processo inflamatório: papel das armadilhas extracelulares dos neutrófilos (NETs) e modulação metabólica pela espécie *Acosmium dasycarpum*

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Área de Concentração: Mecanismos e Aspectos Clínicos das doenças

Orientador: Prof. Dr. Sérgio Henrique Sousa Santos

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[X] **APROVADO(A)** [ ] **REPROVADO(A)**

Ao meu amado irmão **Denarte Guilherme de Freitas** (*in memorian)* exemplo de generosidade e alegria. Aqueles que amamos nunca morrem, apenas partem antes de nós.

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“Deus é minha fortaleza e minha força e ele perfeitamente desembaraça o meu caminho”.

2 Samuel 22:33

RESUMO

A obesidade é definida como o aumento excessivo ou anormal da deposição de gordura corporal, resultado de um desequilíbrio entre a ingestão e o gasto de calorias. O crescimento alarmante da prevalência mundial dessa doença influencia significativamente os indicadores de morbimortalidade e os gastos com serviços de saúde. Assim, buscam-se novas alternativas que sejam mais efetivas, seguras, simples e de baixo custo para o tratamento da obesidade. Nesse sentido, o presente estudo teve como objetivo avaliar de maneira inédita duas condições no tecido adiposo branco de pessoas com obesidade: Papel dos *Neutrophil Extracellular Traps* (NETs), que constiui-se como uma recente função dos neutrófilos e consiste na formação de uma rede extracelular de material genético, sendo ativada em distintas situações, como na inflamação são armadilhas recentemente descobertas, liberadas pelos neutrófilos e parecem afetar essas condições inflamatórias e a modulação metabólica pela espécie nativa do cerrado *Acosmium dasycarpum.* Assim, o objetivo deste trabalho foi avaliar os efeitos da casca da raiz da *Acosmium dasycarpum* no perfil corporal e metabólico de camundongos com obesidade induzida por dieta, bem como a influência da produção e liberação de armadilhas extracelulares de neutrófilos (Neutrophil Extracellular Traps – NETs) no tecido adiposo branco de indivíduos com obesidade. Artigo 1: avaliou-se o efeito da casca da raiz da *Acosmium dasycarpum* (Vog.) Yakovlev, espécie presente no Cerrado norte-mineiro sobre a obesidade induzida pela dieta hipercalórica em camundongos. Os extratos foram caracterizados por meio da realização das análises fitoquímicas e cromatográficas (CG-EM), e foram avaliados os seus efeitos na tolerância à glicose e sensibilidade à insulina, perfil lipídico, redução de peso corporal e adiposidade. Investigou-se a histologia, imagens de ultrassom e PCR quantitativo em tempo real da proteína de ligação de estimulante (C/EBP α), translocase de ácido graxo (FAT) e estearoilcoenzima A desaturase-1 (SCD-1). Os resultados indicaram que a casca da raiz de *A. dasycarpum* possui efeito satisfatório na tolerância a glicose e sensibilidade à insulina, reduz os triglicerídeos, o LDL e aumenta o HDL, além de reduzir o peso corporal e a adiposidade quando comparados com o grupo controle obeso. Provavelmente isso ocorre pela interação dos efeitos da presença dos metabólitos secundários. Artigo 2: avaliou-se a produção de NETs em indivíduos com obesidade e verificaram-se os possíveis mecanismos associados à liberação de NETs no tecido adiposo branco. Tecido adiposo branco foram obtidos de humanos e camundongos magros e com os perfis lipídico, glicêmico e leucocitário foram avaliados, assim como os níveis de NET e seus marcadores relacionados também foram. Análises de bioinformática e proteômica foram realizadas e as

principais proteínas identificadas foram medidas. Os principais achados indicam que os marcadores inflamatórios interleucina 8 (IL-8), proteína de choque térmico 90 (HSP90) e família de proteínas de choque térmico E 1 (HSPE1) podem ser modulados pelos níveis de NETs na obesidade. Em conclusão, os achados experimentais deste estudo sugerem que a inibição das NETs pode ser uma alternativa terapêutica potencial no tratamento de comorbidades da obesidade e que a casca da raiz de *A. dasycarpum*, nativa possui efeitos benéficos à perda de peso e à redução da adiposidade, porém mais estudos devem ser realizados para esclarecer outros mecanismos envolvidos para o uso como agente no tratamento da obesidade.

Palavras-chave: Obesidade. Adiposidade. Neutrophil extracellular traps. Plantas do Cerrado. *Acosmium dasycarpum*.

ABSTRACT

Obesity is defined as the excessive or abnormal rise in body fat deposition resulting from an imbalance between intake and caloric expenditure. The alarming increase in the global prevalence of this disease has a significant influence on morbidity and mortality indicators and health care expenditures. Thus, new alternatives are sought that are more effective, safe, simple and of low cost for the treatment of the obesity. In this sense, the present study aimed to unprecedentedly evaluate two conditions in the white adipose tissue of people with obesity: the Role of Neutrophil Extracellular Traps (NETs), which is a recent function of neutrophils and consists of forming an extracellular network from material genetics, being activated in different situations, as in inflammation are newly discovered traps, released by neutrophils and appear to affect these inflammatory conditions and metabolic modulation by the native species of the cerrado Acosmium dasycarpum. The objective of this work was to evaluate the effects of Acosmium dasycarpum root bark on the body and the metabolic profile of mice with diet - induced obesity, as well as the influence of the production and release of extracellular neutrophil traps (NETs) on white adipose tissue. of individuals with obesity. Article 1: the effect of the root bark of the Acosmium dasycarpum (Vog.) Yakovlev, a species present in the northern Brazilian Cerrado on the obesity induced by the hypercaloric diet in mice, was evaluated. The extracts were characterized by phytochemical and chromatographic analyzes (CG-MS), and their effects on glucose tolerance and insulin sensitivity, lipid profile, body weight reduction and adiposity were evaluated. Histology, ultrasonography, and real-time quantitative PCR of the stimulator-binding protein (C / EBPα), fatty acid translocase (FAT) and stearoylcoenzyme A desaturase-1 (SCD-1) were investigated. The results indicated that the bark of A. dasycarpum has a satisfactory effect on glucose tolerance and insulin sensitivity, reduces triglycerides, LDL and increases HDL, besides reducing body weight and adiposity when compared to the obese control group. This is probably due to the interaction of the effects of secondary metabolites. Article 2: the production of NETs in individuals with obesity was evaluated and the possible mechanisms associated with the release of NETs in white adipose tissue were verified. White adipose tissue was obtained from human and lean mice and, with the lipid, glycemic and leukocyte profiles, were evaluated, as well as NET levels and their related markers. Bioinformatics and proteomics analyzes were performed and the main proteins identified were measured. The major findings indicate that inflammatory markers interleukin 8 (IL-8), heat shock protein 90 (HSP90) and E1 (HSPE1) heat shock protein family (HSPE1) can be modulated by TNF levels in obesity. In conclusion, the

experimental findings of this study suggest that inhibition of NETs may be a potential therapeutic alternative in the treatment of obesity comorbidities and that the native bark of A. dasycarpum has beneficial effects on weight loss and reduced adiposity, but additional studies should be performed to clarify other mechanisms involved for use as an agent in the treatment of obesity.

Keywords: Obesity. Adiposity, Neutrophil Extracellular Traps. Cerrado plants. *Acosmium dasycarpum*

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AMP/AMPK	Proteína quinase ativada
CCL2	Quimiocina ligante CCL2
C/EBPα	Proteína de ligação estimulante
FAT	Translocase de ácido graxo
IL-1β	Interleucina 1 Beta
IL-6	Interleucina 6
IL-10	Interleucina 10
IMC	Índice de massa corporal
IRS	Receptor de insulina
MPO	Mieloperoxidase
NE	Neutrófilo elastase
NETS	Neutrophil Extracellular Traps
OMS	Organização Mundial de Saúde
PAD4	Proteína arginina desaminase 4
PPGCS	Programa de pós graduação em Ciências da Sáude
RBP4	Proteína ligante de retinol
RNAm	RNA mensageiro
SCD-1	Estearoilcoenzima A desaturase-1
TA	Tecido adiposos
TAB	Tecido adiposo branco
TAM	Tecido adiposo marrom
TAS	Tecido adiposo subcutâneo
TAV	Tecido adiposo viceral
TNFα	Fator de Necrose Tumoral
Unimontes	Universidade Estadual de Montes Claros

SUMÁRIO

1 INTRODUÇÃO

A obesidade é uma doença crônica não transmissível, caracterizada pelo acúmulo excessivo ou anormal da deposição de gordura corporal, principal e gordura visceral. Resulta do desequilíbrio entre a ingestão e o gasto de energia e manifesta-se como um processo inflamatório de baixo grau, que é envolvido na fisiopatologia de várias doenças, como o diabetes mellitus, doença cardiovascular de etiologia aterosclerótica, dislipidemia etc. (1). É considerada uma epidemia mundial que acomete todas as faixas etárias (2). Estudos populacionais fortalecem a existência de uma epidemia de obesidade em todo o mundo. Segundo a OMS, em 2016, 39% dos adultos (com idade igual ou maior que 18 anos) apresentavam sobrepeso e 13% obesidade. Em números absolutos, mais de 1,5 bilhão de adultos apresenta sobrepeso e obesidade (World Health Organization. Obesity and overweight, 2018).

Ainda, vale a pena ressaltar que a obesidade é fator de risco para várias outras doenças, tais como o diabetes mellitus, a doença hepática gordurosa não alcoólica, as doenças cardiovasculares, a colecistolitíase, o câncer, a osteoartrite e a apneia obstrutiva do sono (3). As comorbidades associadas à obesidade comprometem a qualidade de vida dos indivíduos que as possuem (5), incrementam os gastos com serviços de saúde e aumentam as taxas de morbimortalidade em diversos grupos de doenças (4). Os gastos com a obesidade e suas doenças associadas equivalem a cerca de US$ 2 trilhões para a saúde no mundo (5). Dessa maneira, existe um grande interesse em identificar e delinear soluções para este problema de saúde pública mundial.

Vários são os desafios e os obstáculos relacionados à prevenção e ao tratamento da obesidade e apesar dos avanços no entendimento da sua fisiopatologia e de sua relação com outras doenças, muito ainda é requerido, principalmente a descoberta de novas opções preventivas e de tratamentos mais eficazes e mais baratos e a amplificação da compreensão sobre os processos fisiopatológico e imunológico. Nesse sentido, o nosso grupo de pesquisa da Pós-Graduação em Ciências da Saúde (PPGCS-Unimontes), orientado pelo Professor Doutor Sérgio Henrique Sousa Santos, possui como foco principal de trabalho o estudo dos mecanismos fisiopatológicos e a elucidação de opções preventivas e terapêuticas para a obesidade, tendo como foco de compreensão de processos moleculares em tecidos metabólicos.

Estudos sobre os mecanismos relacionados à obesidade têm sido foco de grande interesse científico, seja pela busca do entendimento de sua patogenia seja pela investigação

de novas abordagens para o tratamento, o que justifica a execução dos trabalhos apresentados nesta tese. Desse modo, os estudos deste trabalho têm como objetivo avaliar de maneira inédita o papel do tecido adiposo em duas situações distintas: papel dos Neutrophil Extracellular Traps (NETs) e a modulação metabólica pela espécie nativa do Cerrado *Acosmium dasycarpum*, na busca de maiores esclarecimentos sobre novos eventos moleculares envolvidos na patogenia da doença.

2 OBJETIVOS

- Avaliar a influência da produção e liberação de armadilhas extracelulares de neutrófilos (neutrophil extracellular traps – NETs) no tecido adiposo branco de indivíduos com obesidade, bem como os efeitos da casca da raiz da *Acosmium dasycarpum* no perfil corporal e metabólico de camundongos com obesidade induzida por dieta.

2.2 Objetivos específicos

Os objetivos específicos foram delineados com a finalidade de responder aos questionamentos principais, relacionados a seguir. Cada questionamento possibilitou a elaboração de um produto científico dessa tese.

A) Qual é o papel do tecido adiposo branco na relação entre obesidade, Neutrophil Extracellular Traps (NETs) e inflamação? Qual o papel das NETs no tecido adiposo branco de humanos e camundongos com obesidade?

• Delinear rede de interações *in silico* entre obesidade, NETs e inflamação;

• Investigar a expressão de marcadores inflamatórios correlacionados com obesidade e NETs de maneira sistêmica no tecido adiposo.

B) A espécie nativa do Cerrado norte-mineiro *Acosmium dasycarpum* pode modular metabolicamente o tecido adiposo branco de camundongos com obesidade?

• Caracterizar a espécie *Acosmium dasycarpum*, verificando os principais metabólitos secundários presentes;

• Investigar e comparar os efeitos da administração da raiz, da casca e do extrato diclorometano de *Acosmium dasycarpum*, além do princípio ativo lupeol e ainda avaliar a expressão dos genes associados à adipogênese, como o *CCAAT – enhancer binding protein α* (C/EBPα), translocase de ácido graxo (FAT) e estearoilcoenzima A desaturase-1 (SCD-1) em camundongos com obesidade.

3. REVISÃO DE LITERATURA

3.1 Obesidade

A obesidade é definida como o aumento excessivo ou anormal da deposição de gordura corporal, resultado de um desequilíbrio entre a ingestão e o gasto de calorias (1). O indivíduo é considerado com obesidade quando apresenta o Índice de Massa Corporal (IMC = peso em Kg/altura^2 em m) maior ou igual a 30 ou circunferência abdominal superior a 88 cm na mulher e 102 cm no homem (6, 7).

Os mecanismos envolvidos na etiologia da obesidade são multifatoriais e complexos (8), relacionados a fatores socioeconômicos, ambientais, comportamento pessoal e interações genótipo-fenótipo. Esses fatores parecem afetar o consumo de alimentos, absorção de nutrientes, a regulação de vias metabólicas e a mobilização de gordura corporal (9, 10).

Além disso, a obesidade está associada ao desenvolvimento de *diabetes mellitus* tipo II, doenças cardiovasculares, alguns tipos de câncer e outras condições patológicas adversas (11, 12). Algumas dessas comorbidades são consideradas características da síndrome metabólica, comumente considerada um fator de risco prevalente para doença cardiovascular e *diabetes mellitus* tipo 2 (2, 13). Estudos populacionais demonstram que indivíduos com obesidade possuem maior risco para morte, quando comparados aqueles com peso normal (14, 15). Assim, existe uma grande preocupação em prevenir e tratar a obesidade a qual tem sido considerada uma epidemia mundial do século XXI (1).

O aumento da prevalência de sobrepeso e de obesidade influencia expressivamente os indicadores de morbimortalidade, bem como os gastos com serviços de saúde (16). Segundo a Organização Mundial de Saúde (OMS), em 2014, a prevalência da obesidade em adultos foi maior que 2,1 bilhões em todo o mundo, sendo que, destes, 1,5 bilhões estavam acima do peso e 640 milhões eram obesos (Figura 1) (17). A estimativa padronizada por idade da prevalência da obesidade em 2014 foi de 10,8% entre os homens adultos e 14,9% entre mulheres adultas (18-20).

É necessário ressaltar que estudos semelhantes são relatados na literatura em todo o mundo (20, 21), bem como no Brasil. Segundo dados do Ministério da Saúde (2014), o total de obesos adultos tem crescido a cada ano, chegando a 17,9%. Esse aumento também foi observado nas crianças e adolescentes (2-19 anos de idade) do mundo inteiro, com uma estimativa de 110 milhões (20, 22). Entre 2006 e 2016, o índice de brasileiros com obesidade passou de 11,8% para 18,9%, um aumento de 60% em dez anos.

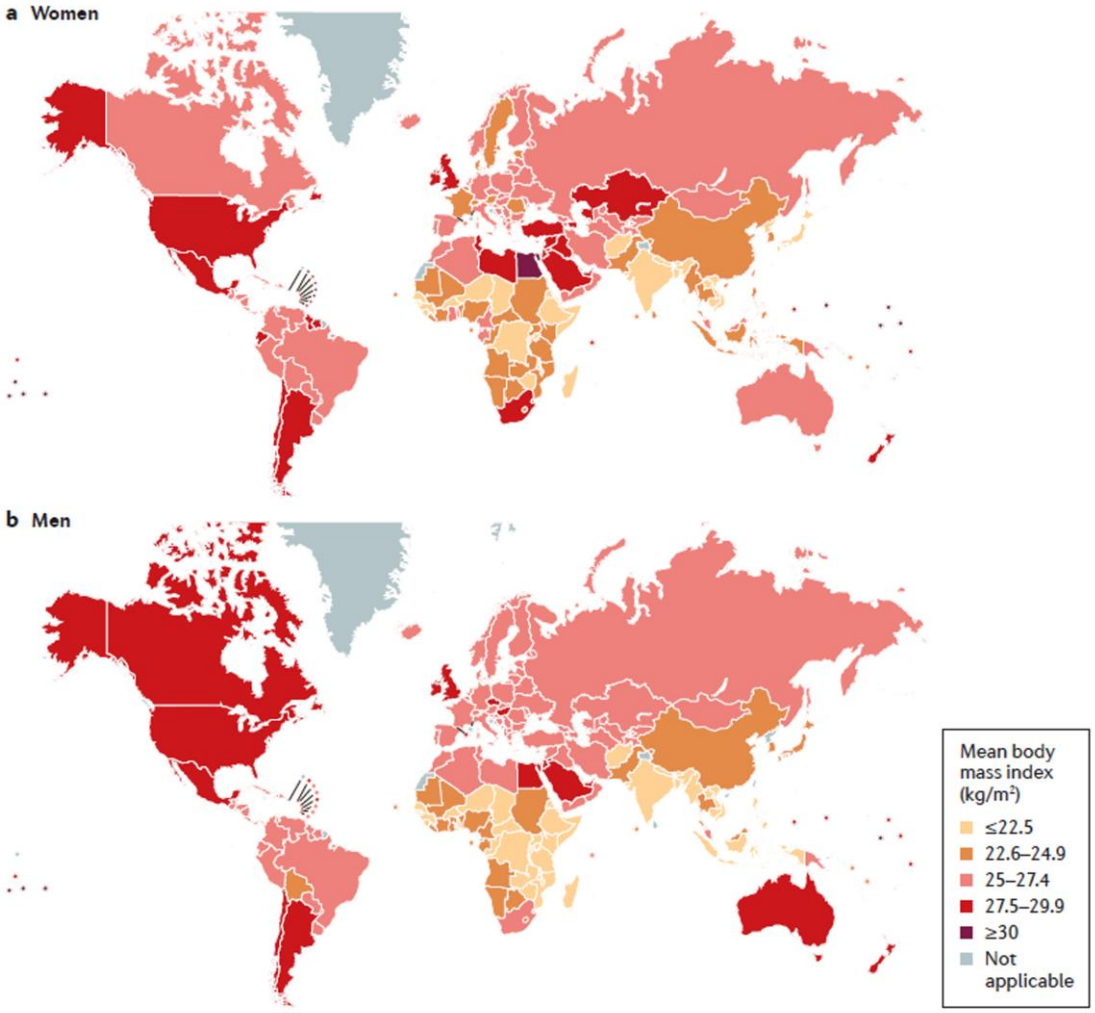

Figura 1. Índice de massa corporal global em homens e mulheres: Índice de massa corporal global padronizado por idade para mulheres (parte a) e homens (parte b). Fonte: González-Muniesa et al., 2017(1).

Esses dados expressam uma tendência do aumento da obesidade e suas comorbidades nos próximos anos, o que representa um importante desafio para a saúde pública em termos de formulação de novas políticas sobre os cuidados de saúde em torno dessa doença. Esta prevalência configura um custo equivalente a 2,8% do produto interno bruto mundial, ou aproximadamente US$ 2 trilhões para a saúde (5). Para impedir essa epidemia, estratégias individualizadas de tratamento, que modifiquem principalmente o estilo de vida, devem ser implementadas com abordagens mais amplas baseadas na população, incluindo a prevenção.

Nesse sentido, os Estados Unidos e o Reino Unido diminuíram a tributação de produtos com baixo teor de gordura e açúcar, elementos que, em excesso, resultam na disfunção do tecido adiposo (23, 24).

A disfunção do tecido adiposo e dos adipócitos reflete defeitos primários na obesidade associados às alterações metabólicas e às doenças cardiovasculares.

3.2 Modulações metabólica do tecido adiposo

O Tecido Adiposo Branco (TAB) é conhecido como o maior órgão do corpo humano e sua principal função é armazenar gordura em condições de excesso de calorias, podendo ser liberado durante o jejum e na privação prolongada de alimentos. O TAB é originário de células tronco mesodérmicas e se subdivide em subcutâneo (formando uma camada subdérmica) e o visceral (circundando órgãos internos) (25).

O TAB passou a ser considerado um órgão endócrino complexo e altamente ativo (26-28). Este secreta vários peptídeos bioativos que, além de influenciar a função adipocitária (função autócrina e parácrina), afetam diversas vias metabólicas por meio da circulação sanguínea. Os adipócitos produzem uma modesta quantidade de substâncias, contudo o *pool* dessas substâncias colabora para a disfunção do tecido adiposo (29, 30).

A disfunção do TAB faz parte das anormalidades precoces no desenvolvimento da obesidade e parece ser um mecanismo determinante para o risco individual do advento das comorbidades metabólicas e cardiovasculares (31, 32). Essa disfunção ocorre em condições de balanço energético positivo contínuo em pacientes com capacidade de expansão diminuída do TAS (33). O estudo de Gealekman et al. (2011) mostrou que o aumento do acúmulo de gordura diminui a capacidade de expansão do TAS com consequente diminuição da angiogênese que se correlaciona com a resistência à insulina e sugere que a vascularização prejudicada pode contribuir para o surgimento das doenças metabólicas (34). A incapacidade de armazenar o excesso de gordura no TAS representa o desenvolvimento da deposição de gordura ectópica (35, 36), o que inicia vários mecanismos, incluindo hipertrofia dos adipócitos, hipóxia, estresse, autofagia e inflamação, que são ativados como sequência ou paralelamente, levando à disfunção do tecido adiposo.

Assim, resumidamente, a disfunção do TA é caracterizada por acúmulo de gordura predominantemente visceral (ectópico) (31), alterações na matriz celular e intracelular na composição do TA (fibrose no tecido adiposo) (37, 38), aumento da infiltração de células do sistema imunológico no TA (39, 40), aumentado os adipócitos, aumento de autofagia (41) e

apoptose (41), bem como alterações no RNAm e na proteína AT padrões de expressão. Com o desenvolvimento da disfunção da TA, a secreção de adipocina é significativamente alterada em direção a um padrão pró-inflamatório, aterogênico e diabetogênico (42). A existência de um estado inflamatório envolvendo o tecido adiposo e seu potencial papel na obesidade foi descoberta pela demonstração da secreção de Fator de Necrose Tumoral (TNFα) pelo tecido adiposo (43).

3.3 Inflamação e Obesidade

A obesidade é uma doença cuja a patogênese revela um quadro inflamatório crônico de baixa intensidade (44, 45). Tem sido destacada a importância da inflamação no desenvolvimento das comorbidades associadas a essa doença, bem como sua mediação via citocinas (43). A primeira ligação entre obesidade e inflamação foi sugerida com o descobrimento do fator de necrose tumoral alfa (TNFα) e citosina com atividade pró-inflamatória, que é expressa de forma exacerbada no tecido adiposo de roedores obesos (46).

Devido ao fato de o TAB produzir uma série de citocinas ou adipocitocinas, que estão presentes nesse processo inflamatório, existem diversos mecanismos que podem esclarecer a atividade inflamatória relacionada com a adiposidade. Uma questão relevante é que o aumento de TAB tem relação com a resistência à insulina, a qual, por sua vez, tem relação com doenças cardiovasculares (43). Sabe-se que a relação da resistência à insulina e o processo inflamatório é bidirecional, ou seja, o processo inflamatório crônico relacionado à obesidade induz resistência à insulina, frequentemente associada à obesidade central, que por sua vez exacerba o processo inflamatório (47).

Em geral, as respostas inflamatórias são iniciadas com um propósito de defesa do organismo, reparo tecidual ou celular em função de condições de estresse. Contudo, quando a inflamação tecidual se torna crônica e permanece não resolvida (como é o caso da obesidade), ela avança para uma condição patológica e sistêmica, caracterizada por resistência à insulina, hiperglicemia e desenvolvimento do diabetes (48).

Esse evento provém da hipertrofia dos adipócitos, isto é, do aumento do volume da célula adiposa devido à acumulação excessiva de triacilgliceróis, sobretudo os viscerais, que são mais ativos e relacionados à resistência ao efeito antilipolítico da insulina e ao aumento da ação das catecolaminas, levando à elevação do fluxo dos ácidos graxos não esterificados para o fígado, via sistema porta, culminando em maior produção de glicose hepática, redução da degradação de apolipoproteína B e aumento da produção de triacilgliceróis (49).

O aspecto inflamatório do tecido adiposo branco leva à secreção de várias substâncias pelos adipócitos, sendo esse processo acompanhado por um aumento da liberação de ácidos graxos livres e desregulação da secreção de diversos produtos, dentre eles a leptina, a adiponectina, a resistina e a proteína ligante de retinol (RBP4) (50). Desse modo, estudos experimentais mostraram que ratos com obesidade aumentam não apenas os níveis de TNF-α, mas também de outras adipocinas pró-inflamatórias, incluindo a interleucina 6 (IL-6) e interleucina 1-beta (IL-1β), quimiocina ligante (CCL2), dentre outras (51). Em contrapartida, esses animais diminuem os níveis de adipocinas anti-inflamatórias, como a interleucina 10 (IL-10) (52).

3.4 Armadilhas extracelulares de neutrófilos – Neutrophil Extracellular Traps (NETs)

Neutrófilos são componentes importantes da imunidade inata, necessários para manter a homeostase do organismo. São granulócitos polimorfonucleares de curta duração e constituem a defesa primária contra infecções microbianas (52). Na inflamação aguda, os neutrófilos circulantes na corrente sanguínea são recrutados rapidamente para o sítio da infecção, em resposta a fatores quimiotáticos liberados por patógenos ou células hospedeiras. Após a ligação ao endotélio, os neutrófilos deixam os vasos sanguíneos e se movem em direção ao local da infecção, adquirem a capacidade de matar agentes patogênicos. Para cumprir essa tarefa, os neutrófilos usam estratégias, tais como a fagocitose, a degranulação e a formação recentemente descoberta das armadilhas extracelulares (53).

As armadilhas (redes) extracelulares dos neutrófilos, do inglês *neutrophil extracelular traps* (NETs), representam um mecanismo de defesa inata, as quais imobilizam e matam micro-organismos invasores para evitar a sua propagação e garantir uma elevada concentração de agentes antimicrobianos para degradar fatores de virulência e matar os patógenos (54, 55). O processo de morte celular para originar NETs é denominado netose (figura 2), que é um tipo de morte celular, com vias diferentes da necrose e apoptose, mas seu mecanismo não é totalmente esclarecido (53, 54). A netose é caracterizada pela presença das fibras de cromatina dos neutrófilos no espaço extracelular. As redes são produzidas por neutrófilos em contato com agentes patogênicos tais como bactérias, fungos, vírus e protozoários e outros (53). Assim, um pré-requisito para netoses é a modificação de resíduos de arginina de histonas para citrulina mediado por PAD4 (proteína arginina desaminase 4), que muda a carga das histonas, levando à enorme descondensação cromatina (56).

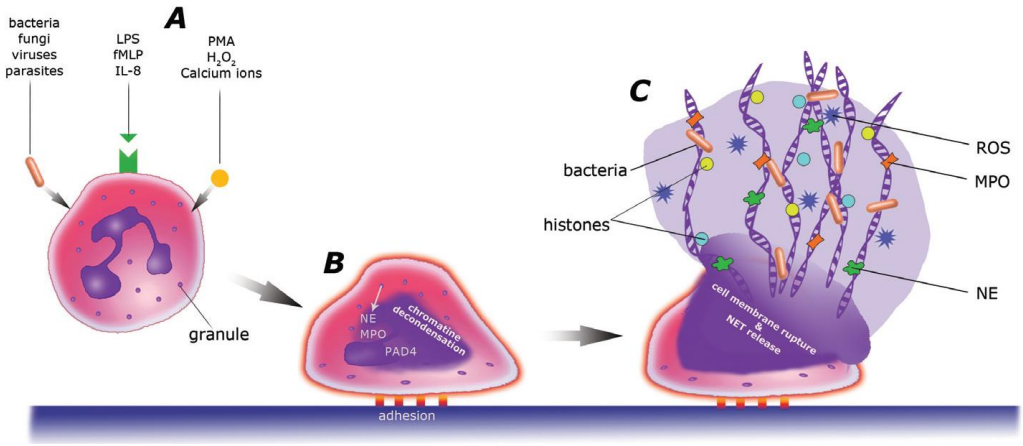

Figura 2. Mecanismo de liberação das NETs: Estimulação dos receptores (A) por disparadores (por exemplo: bactérias, fungos, vírus, parasitas, fatores químicos como a PMA ou LPS), que implica na adesão de neutrófilos ao endotélio, na qual ocorre descondensação da cromatina devido à clivagem de histona por hipercitrulinação NE e MPO e histona por PAD4 (B). Na fase final, as redes são liberadas em prender bactérias (C). Fonte: (53).

Apesar da função antibacteriana, a formação excessiva de NETs pode originar efeitos patológicos. Embora provisória, a nova estrutura interage com componentes do sangue ou tecido. Estudos de citotoxicidade das NETs em células endoteliais e epiteliais demonstraram histonas, mieloperoxidase (MPO), elastase (NE) e catepsina G, como principais componentes citotóxicos e de destruição tecidual (57).

A relação entre obesidade, inflamação e NETs ainda não é esclarecida e requer mais investigações. Essas informações poderão ajudar a explicar a fisiopatologia das doenças associadas à obesidade, bem como sugerir novos tratamentos para a obesidade. Atualmente, existem grandes esforços nesse sentido, visto que essa doença causa um profundo impacto na qualidade de vida do indivíduo obeso.

Diferentes abordagens e tratamentos individuais foram desenvolvidos e prescritos, tais como controle e educação alimentar, programas de atividade física, farmacoterapia e cirurgia bariátrica (58).

Em geral, os recursos terapêuticos convencionais das disfunções metabólicas causadas pela obesidade possuem efeitos colaterais indesejáveis e nocivos, além de induzir o uso abusivo de medicamentos e serem onerosos. Desse modo, é requerido um tratamento eficaz e com fácil adesão para o paciente (59, 60). O uso dos produtos naturais, incluindo extratos brutos e compostos isolados, pode ser efetivo e seguro na estratégia terapêutica, além de ser mais econômico. Os compostos polifenóis, saponinas triterpênicas e esteroides, alcaloides e

carotenoides, demonstraram reduzir o peso corporal e prevenir a obesidade e suas comorbidades (61, 62).

3.5 Plantas medicinais

As plantas são fontes importantes de medicamento para a maioria da população mundial. O elevado padrão tecnológico sustenta-se em larga extensão, na permanente introdução de novos produtos químicos. Nesse contexto, os vegetais têm sido importantes fornecedores desses novos produtos, tanto em nível de sustâncias químicas propriamente ditas como de modelos necessários para a sua produção industrial, por síntese ou cultivo de células (60, 63, 64).

Atualmente existe um grande interesse na utilização de medicamentos à base de plantas (fitoterápicos) e suplementos, por isso têm aumentado ao longo das últimas três décadas. Cerca de 80% das pessoas em todo o mundo usam esse tipo de produto como parte dos cuidados primários à saúde. Isso se deve principalmente ao fato de que as drogas sintéticas são importadas e possuem custos elevados e, portanto, são inacessíveis à maioria da população (65), além de estarem associadas a efeitos colaterais indesejáveis.

Nesse sentido, o Brasil possui a maior biodiversidade do mundo, com mais de 40.000 espécies diferentes de plantas, representando 20% da flora mundial. Em destaque, o bioma Cerrado (57% do território mineiro) abrange mais de 204 milhões de hectares, localizado na parte central do Brasil. É a savana tropical mais rica do mundo em termos de biodiversidade e o segundo bioma mais extenso do Sul da América (66).

Possui cerca de 4.400 espécies endêmicas de plantas (67) e cerca de 30% dessa biodiversidade são razoavelmente conhecidas. A flora do Cerrado engloba gramíneas, ervas e de 30% a 40% de plantas lenhosas; árvores e arbustos que exibem galhos contorcidos e troncos espessos com cascas resistentes ao fogo e folhas coriáceas brilhantes, um exemplo é a espécie *Acosmium dasycarpum*.

Acosmium dasycarpum (Vog.) Yakovlev é uma espécie ornamental brasileira, pertencente à família Fabáceas, sub-família Faboideae (68). É também conhecida como “chapada”, “chapada-do-campo”, “chapadinha”, “amargosa”, “amargozinho”, “pau-para-tudo” e “unha-d'anta” (69).

É uma planta característica e exclusiva dos cerrados e cerradões, restrita à região central e nordeste do Brasil, tendo ocorrência no Cerrado brasileiro entre os estados da Bahia, Minas Gerais, São Paulo, Mato Grosso e Goiás. Sua altura varia de 4 e 6m, dotada de copa

pequena, tronco tortuoso com casca suberosa, folhas alternadas, flores brancas e frutos do tipo legume (vagem achatada). São atribuídas às cascas da raiz propriedades terapêuticas, como tranquilizante, hipotensor, antineoplásico, antisifílico, antirreumático e no tratamento de afecções cutâneas, além da ação diurética atribuída às suas folhas (70-72). Em sua constituição química, é destacado na literatura o isolamento do lupeol, composto com grande potencial farmacológico. O lupeol é um fitoesterol e triterpeno amplamente encontrado em plantas, frutas comestíveis e legumes (72). Pesquisas têm demonstrado várias atividades farmacológicas em potencial do lupeol, tais como atividade contra artrite (73), doença renal (74), doença cardiovascular (75), diabetes (76), inflamação (77), antimicrobiana (77) e anticâncer (78). Ainda, estudos fornecem evidências de que o lupeol modula a expressão de alguns genes (79).

4 PRODUTOS

4.1 Produto 1: Effects of the bark of *Acosmium dasycarpum* (Vog.) Yakovlev on obesity induced by hypercaloric diet in mice, formatado segundo as normas para publicação do periódico British Journal of Nutrition.

4.2 Produto 2: Neutrophil extracellular traps (NETs) modulate inflammatory profiles in obese individuals formatado segundo as normas para publicação do periódico Inflammation.

4.1 PRODUTO 1

Effects of the of *Acosmium dasycarpum* (Vog.) Yakovlev root bark on obesity induced by hypercaloric diet in mice

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Running title: Bark of *Acosmium dasycarpum* reduce adiposity.

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Abstract

Obesity is a chronic disease that involves excess body fat and is considered a serious global public health problem. Side effects related to current pharmacological treatments have motivated a search for alternative approaches that are less harmful and also more accessible, so that the use of natural products has been investigated. Acosmium dasycarpum (Vog.) Yakovlev, a brazilian ornamental plant popularly known as "nata d'anta", exclusive of Cerrado region, has already demonstrated tranquilizer, hypotensive, antineoplastic, anti-septic, anti-rheumatic and diuretic properties, but its effect on obesity has not yet been evaluated. The extracts derived from root bark of *A. dasycarpum* were characterized (phytochemical screening and GC-MS analysis) and their effects (dose of 10mg/1000 mg/weight) on glucose tolerance and insulin sensitivity (tolerance and sensitivity test), lipid profile [histology, ultrasound imaging and adipogenic genes expression analysis of stimulator binding protein (C / EBP α), fatty acid translocase (FAT), and stearoyl coenzyme A desaturase-1 (SCD-1)]. Phytochemical screening of *A. dasycarpum* root bark allowed the identification of alkaloids, tannins and saponins in higher quantities and also phenolic and flavonoids compounds in moderate amounts. Results indicated that *A. dasycarpum* root bark has a satisfactory effect on glucose tolerance and insulin sensitivity, improves lipid profile by reducing triglycerides / LDL and increasing HDL, reduces body weight and adiposity and suppresses the expressions of adipogenic genes, when compared to the obese control group. Thus, we suggest the use of *A. dasycarpum* root bark as an accessible adjuvant treatment for obesity in local Brazilian Cerrado regions. In this sense, this is the first study to evaluate the effect of *Acosmium dasycarpum* (Vog.) Yakovlev root bark on obesity induced by hypercaloric diet in mice.

Key words: Obesity. Adiposity. Cerrado plant. *Acosmium dasycarpum.*

Introduction

Obesity is characterized by excessive fat accumulation in adipose tissues associated with a low-grade chronic inflammatory process, which may have a negative impact on health[1]. This is a growing public health problem that affects both developed and developing countries, being already described in the literature as a pandemic[2-4]. The comorbidities associated with obesity are numerous, and include higher risk of cardiovascular diseases, type 2 diabetes, metabolic syndrome, psychological problems, among others that significantly compromise life quality. However, this risk can be decreased with efforts to reduce adiposity, either by lifestyle changes or by drug treatment[5, 6].

The conventional therapeutic resources of metabolic dysfunctions caused by obesity have undesirable and harmful side effects, in addition to being costly and inducing abusive use of medication. Thus, a combined treatment that is both efficient and facilitates patient compliance is desirable[7, 8]. The use of natural products, including crude extracts and isolated compounds derived from plants have long been used for human populations in folk medicine, particularly in developing countries, being a safe and cost-effective strategy to treat numerous diseases, and also, in some cases, serving as a basis for the development of new drugs. Plant compounds, such as polyphenois, triterpenic saponins and steroids, alkaloids and carotenoids have been shown to reduce body weight and prevent obesity and its comorbidities[9, 10].

Brazil has the largest biodiversity in the world, with more than 40,000 different plant species representing 20% of the world's flora. Brazilian Savanna (57% of Minas Gerais) covers more than 204 million hectares. Besides being located in central Brazil, it is the richest tropical savanna in the world in terms of biodiversity and the second largest biome in South America[11]. Among these are the *Acosmium dasycarpum* (Vog.) Yakovlev, a Brazilian ornamental plant species belonging to the Fabaceae family, popularly known as "unha d'anta" (Tapir nail), "perobinha do campo" (meadow peroba) and endemic in the Brazilian Savanna. In this regard, the objective of this study is to evaluate the pharmacological potential of plants originating from the vicinity of the Pandeiros river basin in the treatment of metabolic alterations in animal models of obesity and metabolic syndrome. This is an area of environmental protection in the Pandeiros river basin, according to the technical report of the Institute of Applied Geosciences (IGA, 2006), encompassing the basin of São Francisco river located on the left bank of the middle course of the river, in the northern end of the State of Minas Gerais, Brazil, between the geographic coordinates 45°95'W, 15°88'S and 43°95'W, 14°40'S.

Therapeutic properties such as tranquilizer, hypotensive, antineoplastic, anti-sphiliac, antirheumatic and beneficial effects in the treatment of cutaneous conditions have already been attributed to the root bark of this species, where their leaves have diuretic property [12-14]. Several chemical compounds were isolated and identified in *A. dasycarpum* root bark, such as diaza-adamantane and quinolizidine alkaloids, 4-methoxy-6- (p-hydroxystyryl) -α-pyrone and lupeol[15]. Among them, lupeol is a phytosterol and triterpene widely found in plants, edible fruits and vegetables[16]. Research studies have demonstrated a number of potential pharmacological properties of lupeol, such as the activity against arthritis[17], kidney disease[18], cardiovascular disease[19], diabetes[20], inflammation[21], microorganisms[22] and cancer[23]. Furthermore, studies have provided evidence that lupeol modulates the expression

or activity of several molecules such as cytokines IL-2, IL4, IL5, ILβ, proteases, α-glucosidase, cFLIP, Bcl-2 and NFκB[16]. However, to our knowledge, none of these studies evaluated the effect of root bark extracts of *A. dasycarpum,* in obesity. Here, we evaluated the effect of oral administration of *A. dasycarpum* root bark extracts in obesity induced by hypercaloric diet in mice.

Methods

Plant Material

The root bark samples of *Acosmium dasycarpum* (Vog.) Yakovlev were havested in the county of Bonito de Minas city, Minas Gerais state, Brazil (15°13'31.4 "S 44°55'01.5" W) in April and May during the spring season of 2018, previously authorized by SISBIO (Biodiversity Authorization and Information System) under protocol number 58008-1, approved by the State Institute of Forestry (IEF) and registered with SisGen (National System for Management of Genetic Heritage and Traditional Knowledge Associated) under protocol A6B40FC. The samples were identified by D'Angelo-Neto, and one specimen was deposited at the BHCB-ICA herbarium, Federal University of Minas Gerais and identified as number (1,071).

Extract Preparation

The dichloromethane extract from the root bark of *A. dasycarpum* was prepared according to the Brazilian Pharmacopoeia (1988)[24]. The plant material was macerated in 80% dichloromethane solution, in the proportions of 1:10 (w/v) in amber glass, shaken for seven days. After extraction, the solution was filtered on filter paper, then reduced in an oven at 35 °C and stored under refrigeration 10° C [25]. The aqueous and methanolic extracts were prepared in the same manner and used in the chromatographic analyzes, whereas the lupeol standard was purchased from Sigma-Aldrich®, purity> 94%.

Phytochemical Characterization

The presence of secondary metabolites in the root bark was verified using Pb $(C_2H_3O_2)_2$ 10% (neutral lead acetate) and 2% FeCl3 (iron chloride) for tannins. For the detection of flavonoids, 2% FeCl3 and Shinoda reagent were used, while for Mayer alkaloids, Bouchadart, Betrand and Dragendorf assays were applied, and finally saponins were assessed by the resistant foam test [26-28].

Quantification of Lupeol in extracts by CG-MS

In an internally conical vial containing 0.0020 g of each extract, 100 µL BSTFA and 60 µL pyridine were added. The reaction volume was heated to 50 °C for 30 minutes in a glycerin bath. Subsequently, the entire volume was transferred to injection vial (2 mL) with insert and analyzed by GC-MS.

Chromatographic analyses were performed on Agilent Technologies (GC 7890A) Gas Chromatography Mass Spectrometry (GC-MS) and DB-5 capillary column (Agilent Technologies, 30m long x 0.25mm internal diameter x 0.25µm film thickness). Helium (99.9999% purity) was used as the entrainment gas at a rate of 1.0ml min^{-1}. Using autoinjector (CTC combiPaL), 1µL of the sample was injected into the chromatograph at the ratio of 1:5. The split/splitless injector was maintained at 290 °C. The chromatographic column initially at 100 °C, isotherm for 1 min, was heated at a rate of 6 °C min^{-1} to 220 °C, remaining 1.23 min, and then the temperature was raised to 290 °C at a rate of 10 °C min^{-1}, finally heated at 40 °C min^{-1} to 310 °C, remaining for 7.5 min[29]. The interface temperature was maintained at 280 °C and the ionization was performed with 70 eV impact. The scanning range of *m/z* was from 30 to 600 Da. The lupeol identification in the extracts was performed by comparing the mass spectra of the device database (NIST 2.0), with standard solution injection at 5 mg L^{-1}. Quantification was performed by comparing the peak area identified in the extracts with the peak area detected in the standard solution [29].

Animals

The experimental protocol was approved by the Committee on Ethics in Animal Experimentation of the State University of Montes Claros, according to the procedure No. 133/2017. Swiss mice (*Mus musculus*), 12 week old males with ± 30g body mass, were obtained from the Montes Claros - Unimontes State University Animal Hospital. The animals were kept in cages under the same environmental conditions, cycle of 12 hours (light/dark), temperature between 22 and 25 °C. Throughout this period, the animals received standard diet and water *ad libitum* and were kept according to the ethical guidelines of the Animal Use Ethics Committee (CEUA).

Animal Diet and Experimentation

The experiment was conducted with 40 mice, randomly divided into five groups (n = 8) and fed the following experimental diets for 60 days: a standard diet group (ST) (standard diet-Labina, Purina, St. Louis, MO , USA composed of: 50.3% carbohydrate, 22% protein and

7.8% fat with a total of 2.18 kcal/g diet) and the other groups with high fat diet (HFD) (High-fat diet components purchased from Rhoster LTDA, São Paulo, Brazil, diet consisting of 24% carbohydrate, 15% protein, and 61% fat, representing a total of 5.28 kcal per 1g of diet) for control and induction of obesity and metabolic dysfunction, respectively[30].

After the obesity induction period (60 days), mice were treated daily for 14 days[16, 31], with a dose of 10 mg per 1000 mg of mouse body weight[32-34] as follows: standard diet (ST) + vehicle; high fat diet (HFD) + vehicle; high fat diet + *A. dasycarpum* root bark powder; high-fat diet + *A. dasycarpum* extract containing lupeol; and high-fat diet + lupeol active principle.

Body mass determination and food consumption of the animals were measured weekly. At the end of the treatment, the animals were sacrificed using the guillotine decapitation technique[35] and samples of blood and visceral and subcutaneous white adipose tissue were collected. The removed tissues were weighed and then frozen in liquid nitrogen and immediately stored in an ultra-freezer (-80 ° C) for further analysis.

Glucose Tolerance and Insulin Sensitivity Tests

The glucose tolerance test was performed to evaluate the glycemic profile: mice were fasted overnight and in the morning received D-glucose (2 mg/kg body weight) injected intraperitoneally. Tail blood samples were collected at 0, 15, 30, 60 and 90 min after glucose administration, and blood glucose levels were measured using an Alere ™ G2 glycosimeter. The insulin sensitivity test was performed on mice fed after intraperitoneal injection of insulin (0.75 units/kg body weight, Sigma, St. Louis, MO). Tail blood samples were collected at 0, 15, 30 and 60 min after insulin injection in order to measure blood glucose levels[36].

Determination of serum parameters

After euthanasia, peripheral blood samples (1 mL) were collected in mice for analysis of plasma expression of apolipoproteins (total cholesterol, triglycerides, High Density Lipoprotein -HDL, Low Density Lipoproteins - LDL), glutamic-oxaloacetic transaminase-TGO, glutamine-*pyruvate transaminase* TGP and alkaline phosphatase liver transaminases using enzymatic kits (Wiener Laboratories, Rosario, Argentina). The measurements were performed on a Wiener BT-3000 plus Chemistry Analyzer (Wiener Laboratories, Rosario, Argentina).

Ultrasound images

Animals were studied in supine position, on the day before sacrifice, by the same trained radiologist, using a Medison® ultrasound equipment (Seoul, South Korea), with a multifrequency linear transducer (7.0 to 12 MHz). All imaging procedures were performed in fundamental brightness mode (B-mode), with optimization of the gain and the time gain compensation settings, which were kept constant throughout the experiment. The acoustic focus was placed in the center of the target organ (epididymal fat pad), with measurement of epididymal fat pad thickness[37, 38]. Epididymal thickness was measured in centimeters and then compared between groups.

Histology

The samples of adipose tissues were fixed in buffered formalin for later histopathological processing and preparation of slides for qualitative and quantitative analyses of adipose tissues. Subsequently, the tissues were stained with hematoxylin and eosin[39]. For each slide, 10 fields were photographed using at 20 × optical magnifications using an Olympus BX50 microscope 87.

To estimatives of the number, diameter (μm), and area (μm2) of the adipocytes from white adipose tissue (WAT) were counted by using ImageJ software and for each slide, 10 fields were photographed. Adipose tissue quantification was performed using ImageJ software (version 1.51p), which was used in the automatic mode to identify and measure the markings of the marker used in the present study.

Gene expression analysis of adiposity gene markers

In order to verify the effects of *A. dasycarpum* root bark on obesity, a real-time quantitative PCR of the stimulatory binding protein (C / EBPα), fatty acid translocase (FAT) and stearoylcoenzyme A desaturase-1 (SCD-1) specific genes to assess adiposity. Total RNA was extracted from white adipose tissue using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was treated with DNAse (Promega) and the cDNA was obtained via reverse transcription M-MLV (Invitrogen Corp.) using random hexamer primers.

Gene expression was normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA levels of the genes were determined by qRT-PCR and amplified using specific primers and reagent SYBR green (Appllied Biosystems, USA) on 384-well Quant Studio 6 flex equipment (Applied Biosystems®). The primer sequences used were:

GAPDH (NM_011146) Forward primer: 5’ AAGAAGGTGGTGAAGCAGGCATC3’ and Reverse primer: 5’ CGAAGGTGGAAGAGTGGGAGTTG3’;
C/EBPα (NM_007678) Forward primer: 5’ TTCAGCTCTGGGATGACCTT3’ and Reverse primer GCCGTTAGTGAAGAGTCTCAGTTTG3’;
FAT (NM_007643) Forward primer: 5’ TAGTAGAACCGGGCCACGTA3’ and Reverse primer 5’ CAGTTCCGATCACAGCCCAT3’;
SCD1 (NM_009127) Forward primer: 5’CATCGCCTGCTCTACCCTTT3’ and Reverse primer 5’GAACTGCGCTTGGAAACCTG3’[40]

To the relative quantification, the standard dietary group (control group) was employed as calibrator. The results were quantified as Ct values, where Ct was defined as the PCR threshold cycle in which the amplified product is first detected and defined as relative gene expression (the target/endogenous ratio). The qRT-PCR was analyzed by 2^-ΔΔCt method[41].

Statistical analysis

For statistical analysis, GraphPad Prism software (version 5.0®, San Diego, California, USA) was used, with 95% reliability ($p < 0.05$). Data were expressed as mean ± SEM. The statistical significance of the mean values for the distinct groups were estimated by *one-way* ANOVA and *two-way* ANOVA (plasma glucose and body weight), with post-test Tukey for multiple comparisons.

Results

Phytochemical screening of *A. dasycarpum* bark

Phytochemical screening of the plant root bark allowed the identification of alkaloids, tannins and saponins in higher quantities and some phenolic compounds and flavonoids in moderate amounts (Table 1 and 2).

Chromatographic Analysis

Analysis of the aqueous, dichloromethane and methanol extracts of *A. dasycarpum* by GC-MS indicated the presence of lupeol. This finding was further confirmed by the commercial standard which showed a retention time of 35 min. The *m/z* scanning range was from 30 to 600 Da and the quantification was performed by comparing the area of the peak identifying the extracts with the area of the peak detected in the standard solution (Fig. 1 and Table 3).

Table 1. Qualitative test of secondary metabolites of *A. dasycarpum* bark.

Class	Test	Root bark
Alcaloids	Mayer	+++
	Dragendorff	+++
	Bertrand	+
	Bouchadart	-
Phenolic Compounds	Ferric Chloride	++
	Sodium hydroxide	++
	Green coloration	++
Flavonoids	Ferric chloride	+
	Sodium hydroxide	+
Taninos	Ferric chloride	+++
	Copper acetate	+++
	Neutral lead acetate	++

(-) Negative, (+) Weak positive, (++) Moderate positive, (+++) Strong positive.

Table 2. Qualitative test of saponins.

H_2O	Extract	Root bark
5	-	-
4	1	+
3	2	+
2	3	++
1	4	++
-	5	+++

(-) Negative, (+) Weak positive, (++) Moderate positive, (+++) Strong positive.

Obesity mice treated with bark and extract of *A. dasycarpum* exhibited weight and adiposity reduction

A decrease in the weight of the animals treated with the bark and the dichloromethane extract of *A. dasycarpum* compared to the HFD group (Fig. 2A) was observed when compared to the animals of the obese control group HFD (53.7±0.23), HFD+BAR (47.95±0.23) ($p < 0.05$), HFD+EXT (48.89±0.46) ($p < 0.05$). Food intake was higher in the control group of the standard diet, but in contrast, the energy intake was lower when compared to the other groups (Fig. 2B and C) ST (0.37 ± 0.01) HFD (0.61±0.01) HFD+BAR (0.54±0.03), HFD+EXT (0.52±0.04) and HFD+LUP (0.53±0.03) ($p < 0.01$) < 0.05).

Supporting data on weight loss, adiposity was significantly decreased in the groups treated with *A. dasycarpum* bark and dichloromethane extract compared to the obese control group HFD (Fig. 2D-G) (4.64±0.28). HFD+BAR (2.94±0.06) ($p < 0.05$), HFD+EXT (2.46±0.56) ($p < 0.01$).

The bark and extract of *A. dasycarpum* improves glucose homeostasis and serum lipid profile

The results of glucose tolerance and insulin sensitivity tests and lipid profile are summarized in fig 3. After 60 days submitted to HFD, the animals had a high glycemic response to glucose overload and a reduced insulin response compared to animals fed with the standard diet ST (6441±159.4), HF (8638±639.3) ($p < 0,05$) HFD+BAR (5554±155.6), ($p < 0,01$), HFD+EXT (5173±433), ($p < 0,01$). Treatments with the bark and dichloromethane extract of *A. dasycarpum* added to the hypercaloric diet to improved the glucose intolerance induced by HFD (Fig. 3A and B) ST (15100±2050), HF (24040±749) ($p < 0.01$) HFD+EXT (16660±2638), ($p < 0.05$).

The levels of triglycerides were reduce for the three treatments when associated with the hypercaloric diet but with statistical significance for the groups treated with dichloromethane extract of *A. dasycarpum* and lupeol when compared to the obese control group HF (219.3±33.8), HFD+EXT (109.5±7.41), ($p < 0.001$), HFD+LUP (73.3±12.0) ($p < 0.001$) (Fig. 3C).

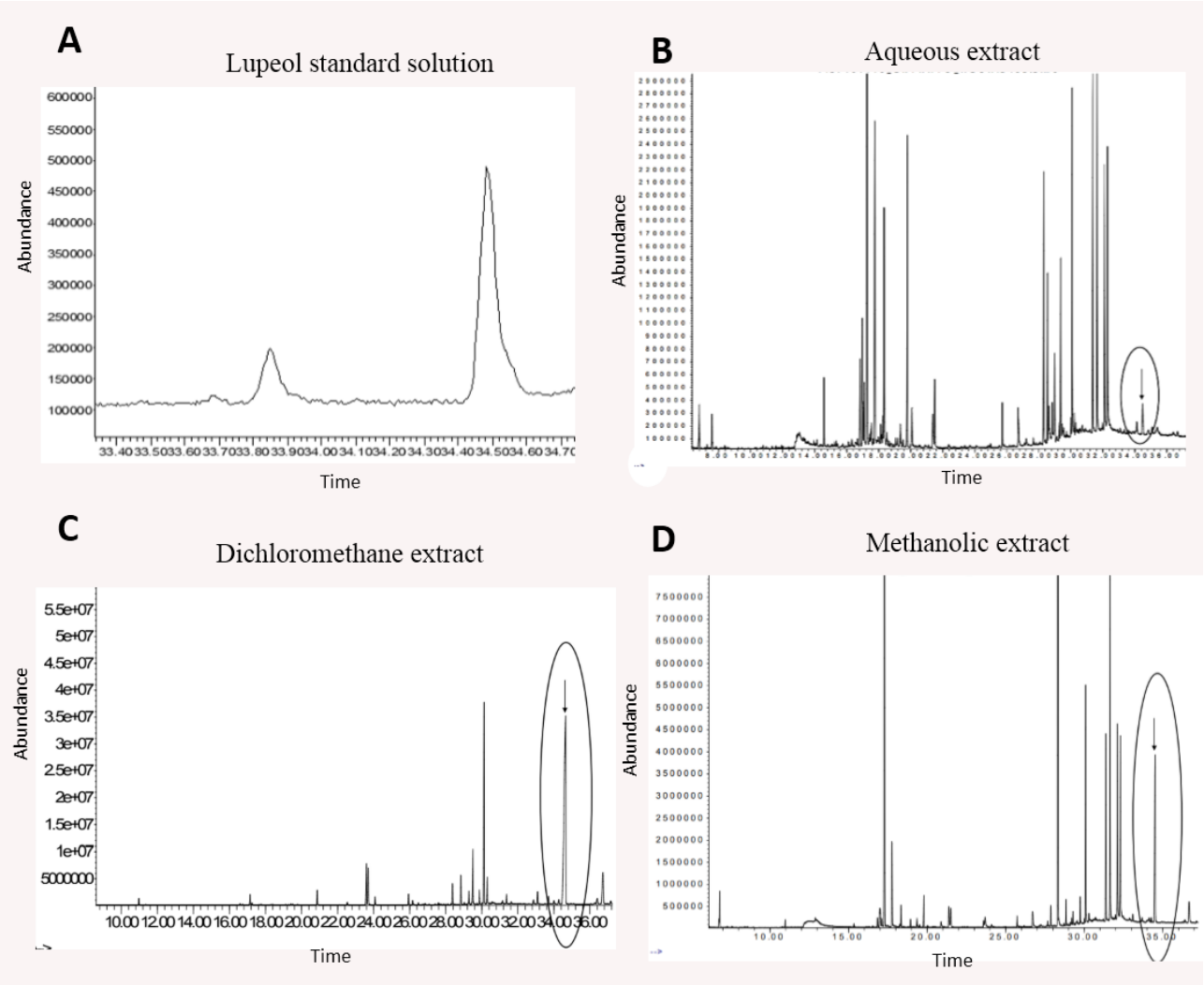


Figure 1. Total ion chromatogram of the lupeol 5 mg L^{-1} standard (A); total ion chromatogram of the aqueous *A. dasycarpum* bark extract (B), dichloromethane (C) and methanolic (D).

Table 3. Quantification of lupeol in each extract

Extract	**Concentration of lupeol in reaction volume (mg L^{-1})**	**Concentration of lupeol in dry extract (mg g^{-1})**
Water	3.080	0.250
Methanol	55.48	4.44
Dichloromethane	1045.41	83.6

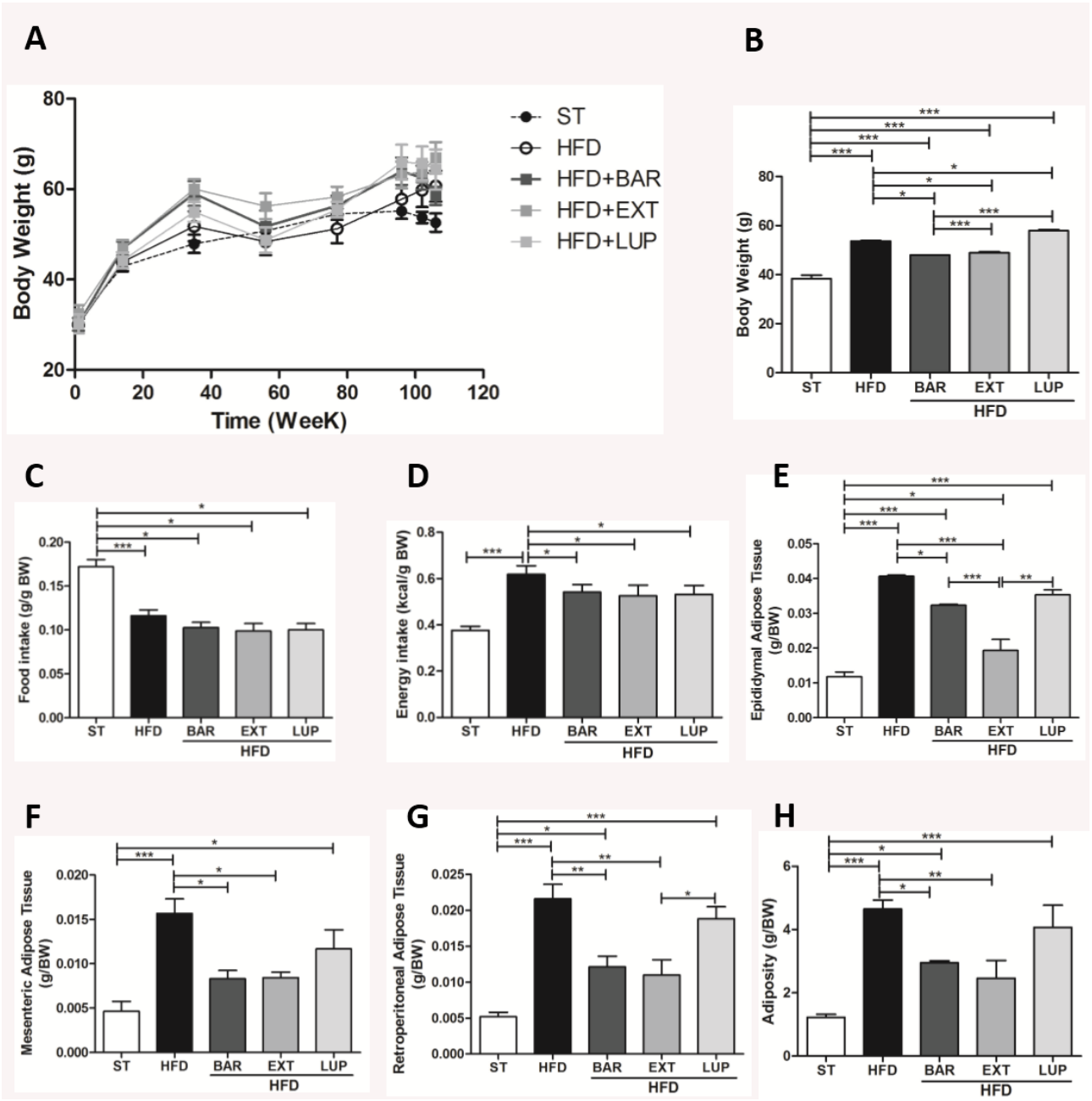


Figure 2. Energy consumption and body composition of mice fed with standard diets (ST), hypercaloric diet (HFD), hypercaloric diet supplemented with bark powder (HFD+BAR), hypercaloric diet supplemented with dichloromethane extract (HFD+EXT), hypercaloric diet supplemented with the active principle lupeol (HFD+LUP). A) Body weight gain (g) over time (days) and area under the curve; B) Final body weight of the treatment (g); C and D) Energy consumption (Kcal/body weight); E) Weight of adipose tissue of the epididymis (g/BW); F) Weight of the mesentic adipose tissue (g/BW); G) Weight of retroperitoneal adipose tissue (g/BW); H) Total adiposity (g/BW) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. BW: body weight.

The HDL levels were significantly increased for the three treatments evaluated in this work HF (66.93±5.54), HFD + BAR (105.5±6.70), (p <0.05), HFD+EXT (112 (P <0.05), HFD+LUP (106.0±10.17) (p <0.05). LDL levels were significantly reduced for the group of *A. dasycarpum* bark when compared to the obese HF group (8638±639,3) (p <0.05) HFD+BAR (5554±155.6), (p <0.01) (Fig. 3D and E).

GPT levels were lower among treatments when compared to the standard diet group and GOT levels were similar (Fig. 3F and G).

The *A. dasycarpum* decreased epididymal thickness and adipocyte surface area

In the evaluation of the thickness of epididymal adipose tissue using ultrasound imaging, a significant reduction was observed for the groups treated with the bark and the dichloromethane extract of *A. dasycarpum* when compared to the obese HF control group (0.760±0.01), HFD+BAR (0,570±0.01), (p <0.05), HFD+EXT (0.560±0.06) (p <0.05), (Fig. 4A). These data can be evidenced in the histology results with the reduction of the adipocyte area for the three treatments compared to the HF (76.55±4.66), HFD+BAR (45.11±5.49) (p <0,001), HFD+EXT (31.8±2.04) (p <0.001), HFD+LUP (21.94±1.98) (p <0.001) (Fig. 4B).

For this study, the dosing choice was based on based on previous studies that evaluated the toxicological effect[16, 31]. In the phytochemical analysis, the presence of alkaloids, phenolic compounds, flavonoids, tannins and saponins as secondary metabolites present in the *A. dasycarpum* root bark sample was detected, whereas the GC-MS analysis detected lupeol triterpene in a qualitatively and quantitatively manner (Fig. 1 and Table 2).

In this work it was interestingly observed that the presence of these substances and their synergistic effect probably contributed to the satisfactory result on glucose and insulin, lipid profile, as well as weight loss and decreased adiposity of the HFD group treated with the bark of *A. dasycarpum* (Fig. 2-5).

Commonly, extracts of medicinal plants have more pronounced biological activities than their isolated compounds[48]. This is often due to the synergistic interactions between these components, involving, in general, the action on several target motives that we suggest for the elucidation of our results.

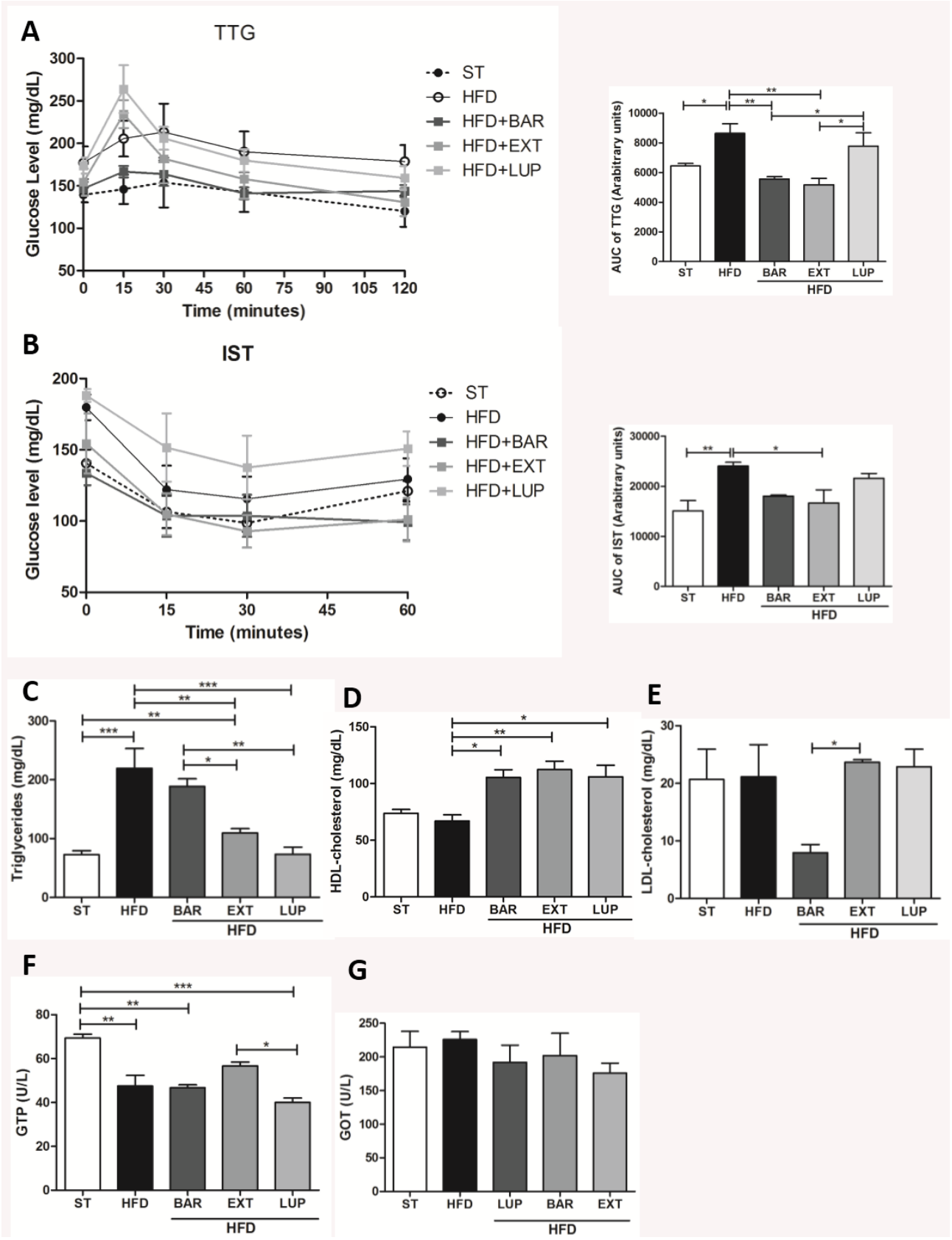


Figure 3. Biochemical profile of mice fed with standard diets (ST), hypercaloric diet (HFD), hypercaloric diet supplemented with bark powder (HFD+BAR), hypercaloric diet

supplemented with dichloromethane extract (HFD+EXT), hypercaloric diet supplemented with active ingredient lupeol (HFD+LUP). A) intraperitoneal glucose tolerance test (IPGTT), blood glucose (mg/dL) and area under the curve; B) intraperitoneal insulin sensitivity test (IPIST) glycemia (mg/dL) and area under the curve; C) serum triglycerides (mg/dL); D) serum HDL cholesterol (mg/dL); E) serum LDL cholesterol (mg/dL); F) GPT / AST levels (U/LI); G) GOT/ALT levels (U/L); GOT/GPT ratio (U/L). * p <0.05; ** p <0.01; *** p <0.001. HDL: high density lipoprotein; GOT/AST: aspartate aminotransferase; GPT/ALT: alanine aminotransferase.

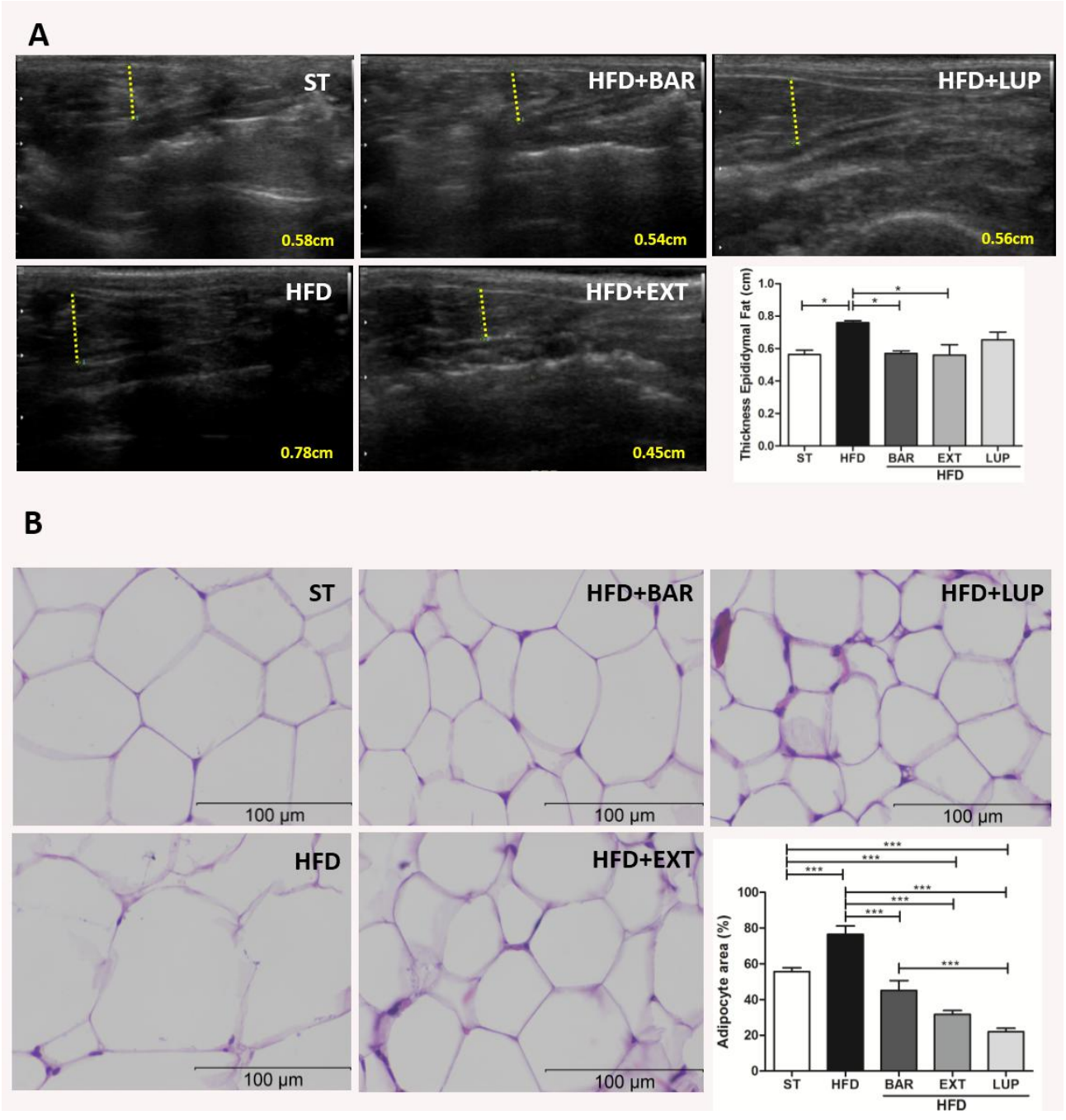


Figure 4. Thickness of epididymal adipose tissue and H & E staining of mice fed with standard diet (ST), high calorie diet (HFD), hypercaloric diet supplemented with bark powder (HFD+BAR), hypercaloric diet supplemented with dichloromethane extract (HFD+EXT), hypercaloric diet supplemented with active principle lupeol (HFD+LUP). A) Ultrasonography of epididymal adipose tissue; B) Epidydimal adipose tissue adipocyte area (μm^2). * p <0.05; ** p <0.01; *** p <0.001.

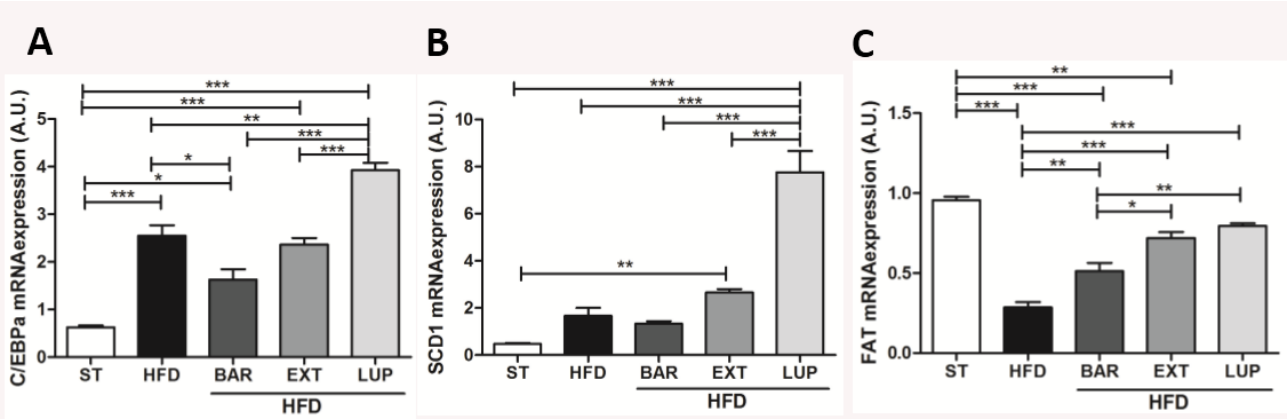


Figure 5. Expression analysis of adipocyte-specific genes A) C/EBPα mRNA expression (fold change)/GAPDH endogenous control; B) SCD-1 expression (fold change)/GAPDH endogenous control; C) FAT expression (fold change)/GAPDH endogenous control. * p <0.05; ** p <0.01; *** p <0.001. C / EBPα: stimulator binding protein, FAT: fatty acid translocase; SCD-1: stearoylcoenzyme A desaturase-1 and GAPDH: endogenous glyceraldehyde 3-phosphate dehydrogenase.

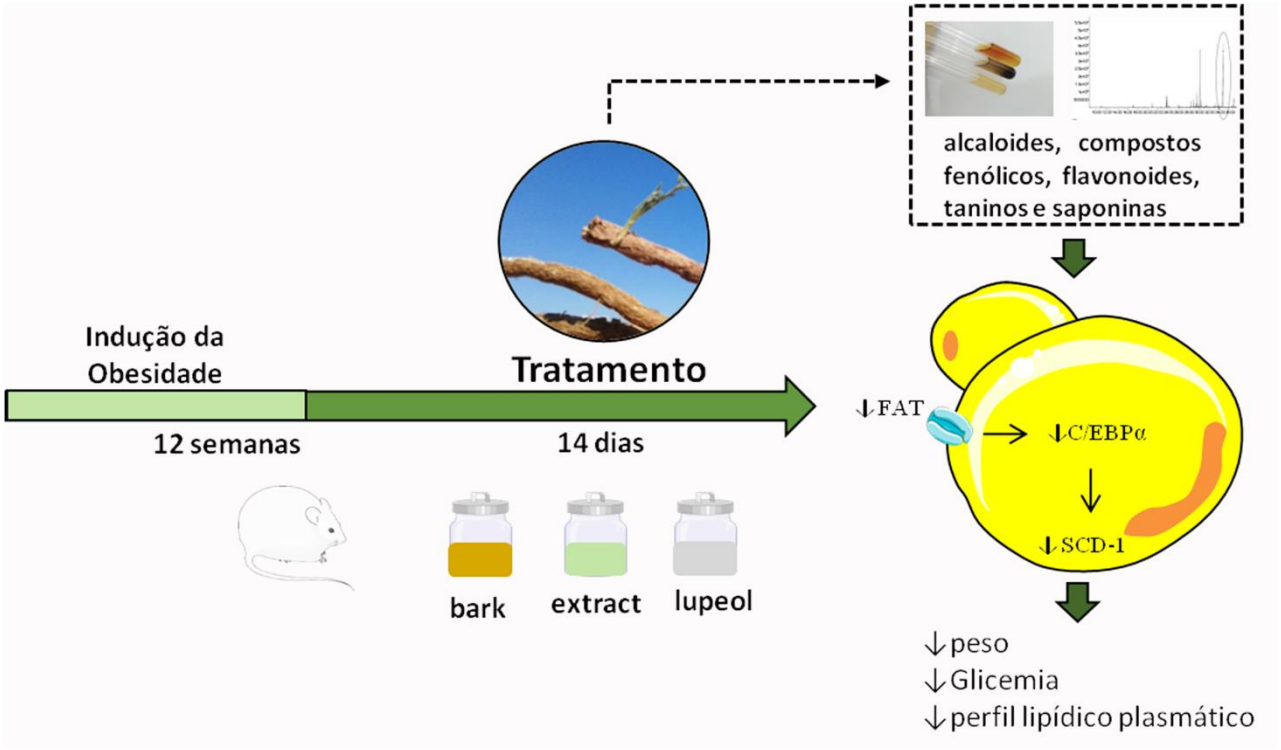
Regarding the effects of *A. dasycarpum* root bark on weight loss and adiposity, it is reported in the literature that adiposity is reduced by the inhibition of adipogenesis, which is associated with a reduction in the number and lipid content of adipocytes[49, 50].

Among the therapeutic strategies for obesity treatment are the balance in the consumption and energy expenditure, suppression in the differentiation and lipogenesis of the preadipocytes, as well as inductions of lipolysis and apoptosis of adipocytes[51].

In this study, we demonstrated that *A. dasycarpum* root bark significantly reduced the triglycerides and expressions of adipogenic C/EBPα transcription factors and SCD-1 and FAT

adipocyte-specific genes. SCD-1 regulates the expression of genes involved in lipogenesis and desaturation of fatty acids and FAT is a fatty acid binding protein[52].

In conclusion, our findings suggest that the root bark extract of *A. dasycarpum* has a beneficial effect on weight loss and on the reduction of adiposity. For this reason, may suggest its use as an adjuvant in the treatment of obesity. To the best of our knowledge, this is the first study that demonstrates the effect of *A. dasycarpum* bark on obesity, in that sense; further studies should be performed to clarify other mechanisms involved for its use as an agent for treating obesity.



Graphic abstract. The bark of *Acosmium dasycarpum* reduces body weight and adiposity

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Authors' contributions

D.F.F, S.H.S.S, A.L.S.G., A.M.B.P., designed the study, conducted research in the literature, wrote the manuscript.

D.F.F, carried out the biological assay and research in the literature.

L.M.A.B, V.M., reviewed the final version.

V.H.D.G, D.V.C, performed statistical analysis of the data**.**

Conflicts of interest

The authors declare no conflicts of interest.

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4.2 PRODUTO 2

Neutrophil extracellular traps (NETs) modulate inflammatory profiles in obesity individuals

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ABSTRACT

Neutrophil extracellular traps (NETs) are recently discovered traps released by neutrophils, and they appear to affect these inflammatory conditions. The present study aimed to evaluate NET production in obesity individuals and to verify the possible mechanisms associated with the release of NETs in adipose tissue. This is the first study to investigate NETs in human adipose tissue. Blood and white adipose tissues were obtained from lean and obesity individuals as well as from lean and obese mice. The lipid, glycemic and leukocyte profiles were assessed. NET levels and their related markers were also evaluated. Bioinformatics and proteomic analyses were performed, and the key proteins that were identified were measured. The main findings indicate that the inflammatory markers interleukin 8 (IL-8), heat shock protein 90 (HSP90) and heat shock protein family E 1 (HSPE1) might be modulated by NET levels in obesity. Obesity has been associated with increased cholesterol, glucose, ionic calcium and NET levels, as well as increased catalase and decreased superoxide dismutase

activities. The bioinformatics and proteomic analyses revealed that IL-8, HSP90 and HSPE1 were involved in obesity, inflammation and NET release. All markers were higher in obesity individuals than those in their lean. In conclusion, our study suggests that there is increased NET production during obesity. This result is confirmed by higher systemic NET levels in obesity individuals than those in eutrophic individuals, as well as the fact that NET production might be associated with important inflammatory markers such as IL-8, HSPE1 and HSP90.

Keywords: Neutrophil extracellular traps; Inflammation; Obesity; Proteomics.

INTRODUCTION

Obesity represents the most prevalent metabolic disorder and is characterized by a low-grade chronic inflammatory process. Thus, obesity is associated with the physiopathology of several inflammation-related diseases, including type 2 diabetes mellitus, non-alcoholic fatty liver disease, steatohepatitis, asthma, cancer, cardiovascular diseases and neurodegenerative diseases (1-3). However, the mechanisms involved in the stimulation of low-grade inflammation in obesity remain controversial in the literature (4).

An alternative strategy that has been used to evaluate the inflammation triggered by obesity includes both the total and differential leukocyte counts (5). The total white blood cell count is higher in obesity individuals than that in eutrophic individuals (6, 7) and has been related to unfavorable metabolic profiles (6) and cardiovascular events (8) after adjusting for body mass.

Among the leukocyte types, we are emphasizing neutrophils, which are phagocytic cells that play a key role in innate immune defense against different antigens (9). These cells have the capability to quickly infiltrate the infected tissue via several strategies, including degranulation, phagocytosis, and a neutrophil effector function that has been discovered more recently (10, 11), known as neutrophil extracellular traps (NETs) (11). The trap release mainly occurs via NETosis, a type of cell death process. NETs are primarily composed of cytosolic and granule proteins, and they act as a trap made of decondensed chromatin. This structure contains nuclei and mitochondrial deoxyribonucleic acid (DNA) and is able to protect the organism against the dissemination of microorganisms, including bacteria, fungi and viruses. However, growing evidence has demonstrated that NETs are also involved in the pathogenesis of immune-related diseases (11-13).

A possible relationship among obesity, inflammation and NETs requires further investigation, as it might help explain the physiopathology of obesity-associated diseases. Our hypothesis is that increased NET production modulates the inflammatory profile observed in obesity individuals. Thus, our study aimed to assess the NET levels and the expression of inflammatory markers in obesity individuals and mice. This represents the

first study to perform such an analysis in human adipose tissue and may facilitate new understandings of the molecular mechanisms involved in the etiology of obesity-associated diseases.

METHODS

Human samples

The biological materials and clinical data were derived from the Human Institutional Biological Bank (CONEP: B-013). All patients signed an informed consent agreement. Ethical approval for this study was obtained from the relevant Institutional Review Board (56905416.9.0000.5146, process number 1596711).

The samples consisted of a visceral white adipose tissue and blood from individuals submitted to bariatric surgeries (group as bore) and elective surgery (eutrophic group) who agreed to donate samples to the aforementioned biobank. Twenty samples, ten for each group (2 males and 8 females) and the clinical data from lean and obesity individuals of both sexes were retrieved, and the following information was registered: lean group (mean age 35.0±11.02 years old; BMI lower than 24.9 kg/m^2 (mean 22.32±1.4); with obesity group (mean age 37.3 ± 9.55, BMI higher than 29.9 kg/m^2 (mean: 41.95±6.0).

Animal experiments

The experiment was conducted with 14 Swiss male mice (aged eight weeks old) from the State University of Montes Claros (Montes Claros, Minas Gerais, Brazil). The groups were randomized and placed in an air-conditioned room (22 ± 2°C) with a 12 hour light-dark cycle. Following an adaptation period, the mice were separated into two groups (n=7) and were fed the following experimental diets for eight weeks: standard diet (ST) - standard diet-Labina, Purina, St. Louis, MO , USA composed of: 50.3% carbohydrate, 22% protein and 7.8% fat with a total of 2.18 kcal/g diet) and the other groups with high fat diet (HFD) - High-fat diet components purchased from Rhoster LTDA, São Paulo, Brazil, diet consisting of 24% carbohydrate, 15% protein, and 61% fat, representing a total of 5.28 kcal per 1g of diet) for control and induction of obesity and metabolic dysfunction, respectively (14). The mice had free access to food and water during the experimental period. At the end of the experiment, the animals were sacrificed by decapitation, and blood and white adipose tissue samples were collected, weighed and immediately frozen in dry ice and stored at -80°C for subsequent analysis. All procedures were performed in accordance with the principles of animal experimentation approved

by protocol number 134 from the Ethics Committee on the use of animals of the Universidade Estadual de Montes Claros (UNIMONTES), Brazil.

Plasmatic parameters

The biochemical data from obesity individuals were obtained from the preoperative laboratory exams (automated differential counting (XS-1000i-Sysmex) followed by the manual count described below) of the volunteer patients who agreed to participate in the study. The blood samples for the determination of the NETs were collected in a Vacuette tube (serum clot activator) before the t patients were anesthetized

The serum was obtained following centrifugation, and the levels of total serum cholesterol, high-density lipoprotein (HDL), triglycerides, low-density lipoprotein (LDL), glycated hemoglobin (Hbac) and ionic calcium were assayed using enzymatic kits (Wiener®, Argentina). IL-8 concentrations were measured by ELISA, as previously described by Alves-Filho et al. (2009) [15], using the Quant-iT PicoGreen Kit (Invitrogen) in a Flexstation 3 Microplate reader (Molecular Devices, California, USA).

Leukocyte count

A global leukocyte count was performed using the automatic process by the Hematology Analyzer Sysmex XS-1000i ™

Neutrophil extracellular trap quantification

The adipocytes from the white adipose tissue samples were isolated using the Rodbell method (Rodbell, 1964) with adjustments as described (15). Collagenase was used for digestion at 38°C with constant shaking for 30 minutes, followed by centrifugation at a low RPM (400 g) for 5 minutes. Inactivated fetal bovine serum was then added to inactivate the enzymes. The cells were filtered through a mesh cell strainer (40 μm nylon, BD® Falcon) and were washed three times with DMEM (Gibco®, New York, USA) plus 1% BSA. For the quantification of cfDNA/MPO (NET), the filtered tissue was used as well as the tissue that was retained at the time of filtration.

NET quantification was based on NET components (cell-free DNA/myeloperoxidase (cfDNA/MPO) as previously described (16). A 96-well flat-bottom plate coated with high-affinity anti-MPO antibody was used, and the amount of DNA bound to the enzyme was quantified using the Quant-iT™ PicoGreen® kit (Invitrogen)

(16). The fluorescence (emission at 485 nm wavelength) intensity relative to the DNA bound to MPO was quantified using a fluorescence reader (Fusion; PerkinElmer, Monza, Italy).

Oxidative stress

Tissue preparation

Adipose tissue samples were prepared according to Barreto (2012) (17). The tissue was homogenized with a mortar and pestle for 3 minutes in phosphate-buffered saline (pH 7.2). The homogenates were then centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was used to measure the antioxidant enzyme activities (superoxide dismutase and catalase).

Catalase activity

An aliquot of the previously prepared homogenate was added to 900 µL of catalase buffer and 150 µL of phosphate potassium buffer (50 mM, pH 7.8), and the reaction was initiated following the addition of hydrogen peroxide (300 mM). The absorbance was read using a spectrophotometer for 1 minute (at 15 second intervals) at 37°C in 340 nm.

Superoxide dismutase activity

An aliquot of homogenate was added to 780 µL of phosphate potassium buffer (50 mM, pH 7.8) and 20 µL of EDTA (ethylenediamine tetra-acetic acid), and the reaction was initiated after adding pyrogallol (2 mM). Absorbances were registered after 5 minutes at 37°C in the spectrophotometer.

Proteomic analysis

A proteomic analysis was performed as previously described (18). Human white adipose tissue samples were used. The proteins associated with obesity and NETs were submitted to a new bioinformatics analysis that revealed HSP90 and HSPE1, which were measured via immunohistochemistry and qPCR, respectively. The details of the methodology are available in the supplementary material.

Bioinformatics analysis

The bioinformatics analysis was undertaken following a previously described methodology (19) in two different phases. Initially, we performed a bioinformatics analysis of human genes related to “neutrophil extra cellular traps”, “inflammation” and “obesity” using the GeneCards database. The leader gene approach identified IL-8. Bioinformatics analysis validation was conducted via IL-8 measurements in the human blood samples. Following the proteomic analysis, a second bioinformatics analysis was performed based on the proteins retrieved from the proteomic analysis, where the leader gene was HSPE1 and STITCH was HSP90 (20-22). Further information is provided in the supplementary material.

Immunohistochemical reactions

Each resected tissue specimen was fixed in formalin, embedded in paraffin and cut into 3-mm serial sections. The HSP 90α/β antibody (SC-1065 - goat polyclonal IgG - Santa Cruz Biotechnology)was used and was incubated at 4°C for 18 hours. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide and Protein Blocker (EP-12-20532, Kit Easy Link One, Easy Path, Immunobiosciense, Corp, EUA). For antigen retrieval, the sections were heated in a steam cooker for 10 minutes at 121°C in Trilogy (cat. No. 920P-06; Cell Marque Corporation, Rocklin, CA). The primary antibody was detected using the Easy Link One Kit (Easy Path, Immunobiosciense, Corp, EUA). Signals were retrieved with 3’3-diaminobenzidine-tetrahydrochloride (Cat. No. 32750, Sigma-Aldrich, USA) for 3 minutes, and the slides were counterstained with Mayer’s hematoxylin (Cat. no. 109249, Merck, USA) for 60 seconds. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS).

For each microscope slide, 10 fields were photographed using the “hot spot” method, and the photos were obtained with the aid of a microscope (FSX100, Olympus, Center Valley, PA, USA) with a 40x objective. Adipose tissue quantification was performed using ImageJ software (version 1.51p), which was used in automatic mode to identify and measure the markers used in the present study.

Gene expression by real-time polymerase chain reaction (PCR)

Total ribonucleic acid (RNA) from white adipose tissue was extracted using TRIzol (Invitrogen Corp.®, San Diego, California, USA) and then treated with DNase and reverse-transcribed with M-MLV Reverse Transcriptase (Invitrogen Corp.®) using random hexamer primers. cDNA samples were amplified using specific primers and SYBR Green reagent (Applied Biosystems®, USA) in a PlusOne platform (Applied

Biosystems®). Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (internal control) and HSPE1-specific primers were used: GAPDH sequence forward, 5'-GAAGGTGAAGGTCGGAGTCAAC-3', and reverse, 5'-CAGAGTTAAAAGCAGCCCTGGT-3', (23); and HSPE1 sequence forward, 5'-AGTAATGGCAGGACAAGCGTT -3', and reverse, 5'- CTGGTTGAATCTCTCCACCCT -3'. Samples were analyzed in duplicate, and the method 2-delta-delta Ct was applied (24).

Statistical analysis

Bioinformatics analyses were carried out in Statistical Package for the Social Sciences (SPSS) (Version 18.0, IBM, New York, NY, USA). Kolmogorov-Smirnov and Shapiro-Wilk tests were conducted to evaluate data distribution. Samples presented normal distribution. Further analyses were performed in GraphPad Prism 5.0® (San Diego, USA), with t-tests applied to verify the statistical differences between the groups. Statistical significance was accepted at $p < 0.05$.

RESULTS

Human analysis

Obesity individuals presented greater significant differences in their mean body weight (113.4±4.5 p=0.0001) and their mean *body mass index* (BMI) (41.95±1.9, p=0.0001) relative to lean individuals [(61.60±2.1) and (22.32±41.95), respectively] (Figure 1a and b).

Furthermore, total plasma cholesterol levels (211.1±11.4 vs. 138.9±10.5, p=0.0005), low-density lipoprotein levels (112.6±10.3 vs. 82.3±9.8, p=0.04), high-density lipoprotein levels (57.6±3.7 vs. 44.02±2.4 p=0.01), triglyceride levels (192.5±36.0 vs. 68.0±11.5, p=<0.01), fasting glucose levels (102.4±7.7 vs. 82.7±3.3, p=0.03) and glycated hemoglobin levels (5.6±0.14 vs. 4.6±0.12, p=0.0002) were higher in the with obesity group than those in the lean group, respectively (Figure 1c-h). Moreover, the adipocyte area in the white adipose tissue was higher in obesity individuals than that in lean individuals (1408000 ± 130100 vs. 2339000 ± 246300 μm^2, respectively, p<0.003) (Figure 1i and j).

The release of NETs is mediated by the enzyme peptidyl arginine deiminase 4 (PAD 4) which is dependent on calcium, so ionic calcium levels were significantly higher in obesity individuals than those in lean individuals (4.3 ± 0.1 vs. 1.2 ± 0.2 mmol/L, respectively, p=0.0004) (Figure 2a). To evaluate tissue oxidative

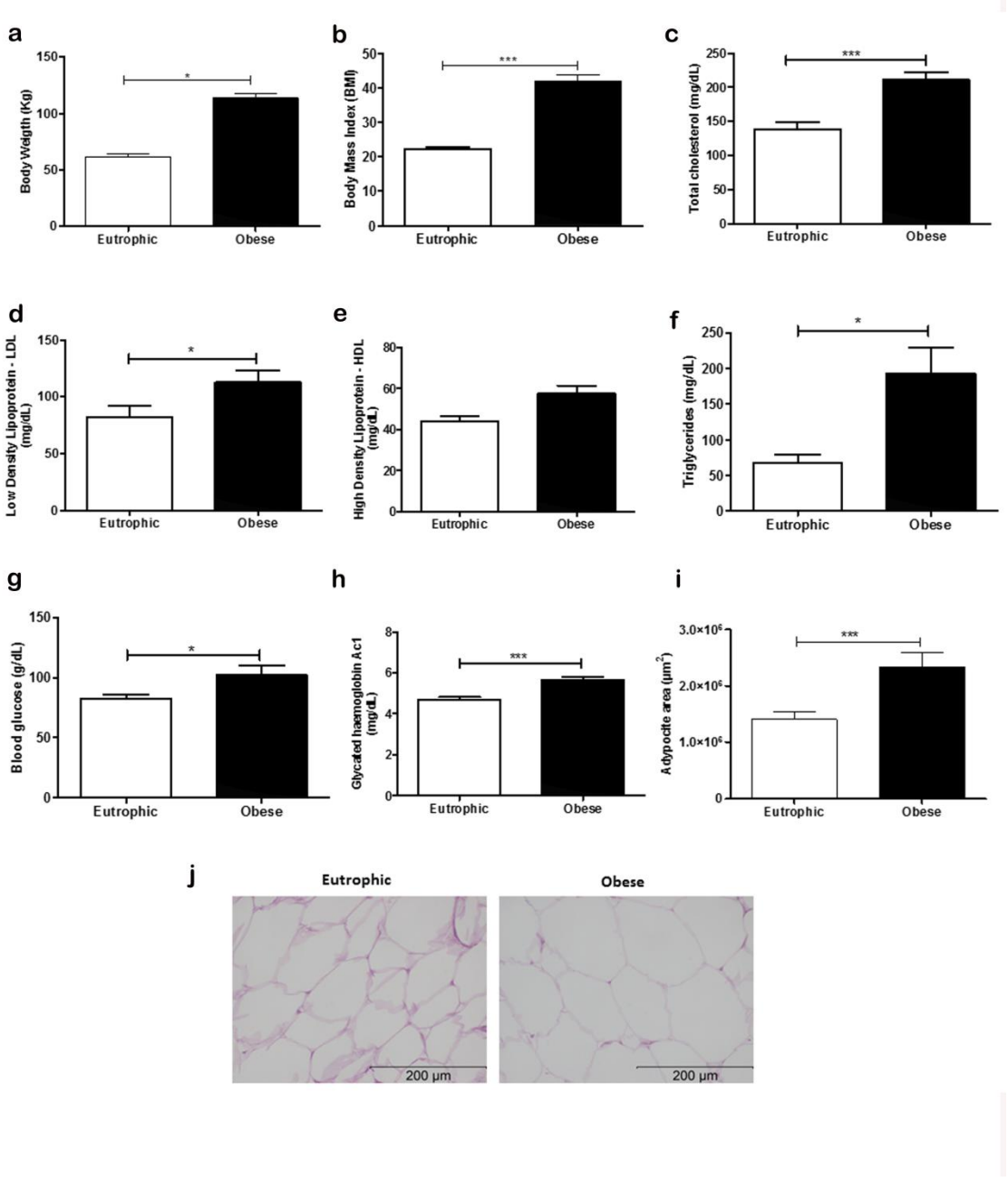


Fig. 1 Human samples characterization, lipid and glycemic profiles. a) Body weight (Kg); b) Body Mass Index (BMI); c) Total cholesterol (mg/dL); d) Low density lipoprotein (mg/dL); e) High density lipoprotein (mg/dL); f) Triglycerides (mg/dL); g) Blood glucose levels (g/dL); h) Glycated haemoglobin Ac1 (mg/dL). i) Adipocyte area (um2); j) H&E White adipose tissue staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

stress, we assessed catalase activity in visceral white adipose tissue adipose tissue, which was higher in obesity individuals than that in lean individuals (p=0.003) (Figure 2b). Conversely, lower superoxide dismutase activity was observed in obesity individuals than that in lean individuals (p=0.001) (Figure 2c). Serum levels in obesity individuals exhibited an increase in leukocytes and neutrophils compared to serum levels in lean individuals (Figure 2d and e). Furthermore, in investigating NET production, we observed greater serum cfDNA/MPO levels in obesity individuals than those in lean individuals (5.2±0.3 vs. 4.00±0.1, respectively, p=0.007) (Figure 2f).

Similarly, filtrated (2.676 ± 0.219 vs. 7.965 ± 1.377, p=0.019) and retained (1.219 ± 0.389 vs. 6.729 ± 0.839, p=0.004) adipose tissue cfDNA/MPO levels were higher in obesity individuals than those in lean individuals, respectively (Figure 2g and h).

High-fat-diet-fed mice displayed greater weight gain during treatment (56.16±1.0) than standard diet-fed mice (48.10±0.4, p=<0.0001) (Figure 3a). The food intake between groups did not differ (p=0.5217) (Figure 3b).

Similar to the human model, the plasma analysis revealed significant differences between the mouse groups in the following parameters: total cholesterol (81.67±2.3 vs. 62.67±4.0, p=0.01), high-density lipoprotein (36.4±0.4 vs. 49.2±2.2 p=0.0051), triglycerides (86.33±8.3 vs. 47.33±7.6, p=<0.02) and glucose levels (99.0±12.5 vs. 57.0±5.5 p=<0.037) (Figure 3c-f). The adipocyte area in the white adipose tissue was higher in HFD-fed animals than that in standard diet-fed animals (1344000 ± 113800 vs. 2339000 ± 246300, respectively, p<0.003) (Figure 3g and h).

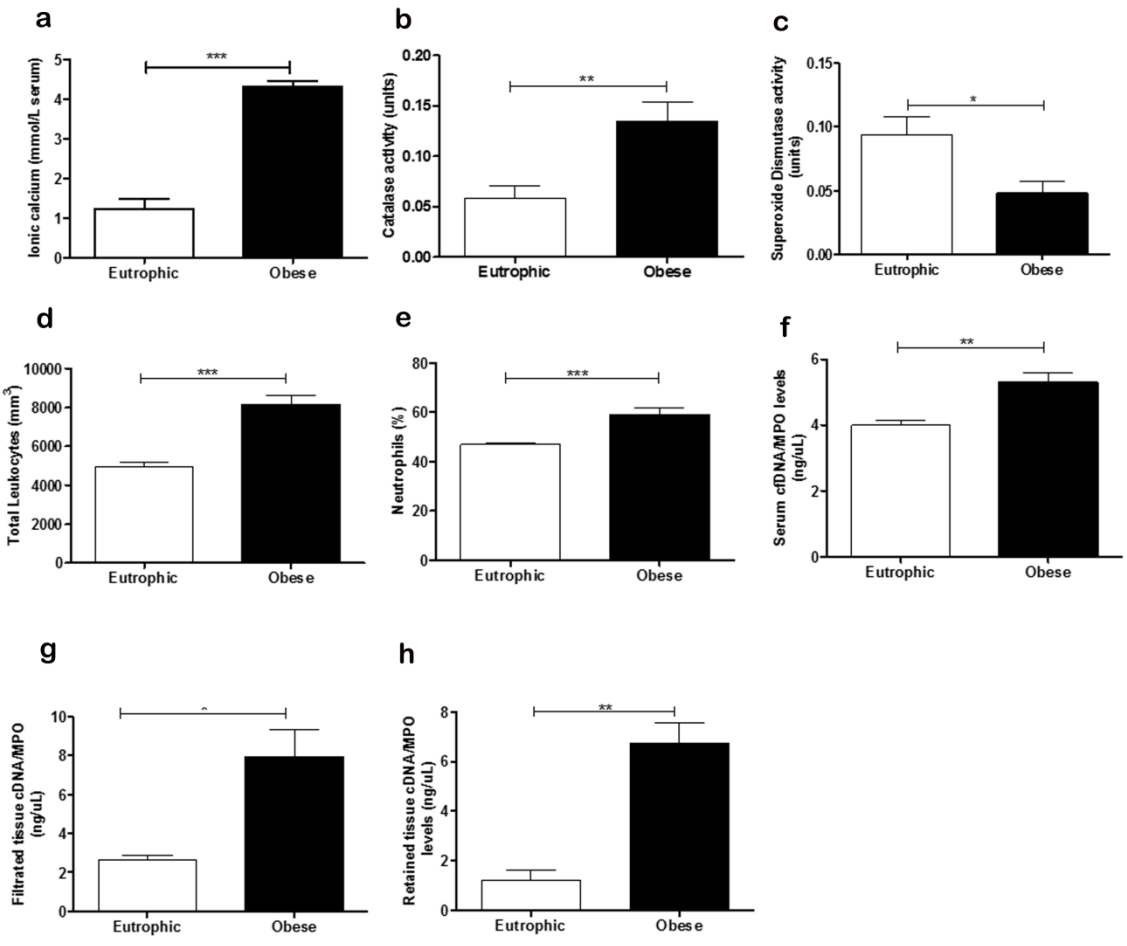


Fig. 2 Measurement of NETs in human samples, oxidative stress and inflammatory profiles. a) Serum ionic calcium (mmol/L); b) Catalase activity (units); c) Superoxide dismutase activity (units) (units); d) Total leucocytes (mm^3); e) Neutrophils (%); f) Serum cfDNA/MPO (NETs) levels (ng/uL); g) Filtrated tissue cfDNA/MPO (NETs) levels (ng/uL); h) Retained tissue cfDNA/MPO (NETs) levels (ng/uL). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

Mouse analysis

The results of the oxidative stress analysis in animals were similar to the results in humans, as catalase (0.018±001 vs. 0.007±0.0008, p=<0.01) and superoxide dismutase (0.002±0.0003 vs. 0.005±0.0003, p=<0.003) differed between obesity and lean individuals, respectively (Figure 4a and b). Calcium ion levels were higher in obese mice than those in lean mice (0.85±0.014 vs. 0.67±0.05, p=<0.0003), respectively (Figure 4c). Furthermore, although no differences in serum cfDNA/MPO concentrations were found between the groups, higher concentrations were observed in the retained (78.75 ± 4.197 vs. 176.2 ± 13.25) and filtrated (10.81 ± 1.889 vs. 42.71 ± 7.608) white adipose tissue of obese mice than those in the lean mice, respectively (Figure 4d-f).

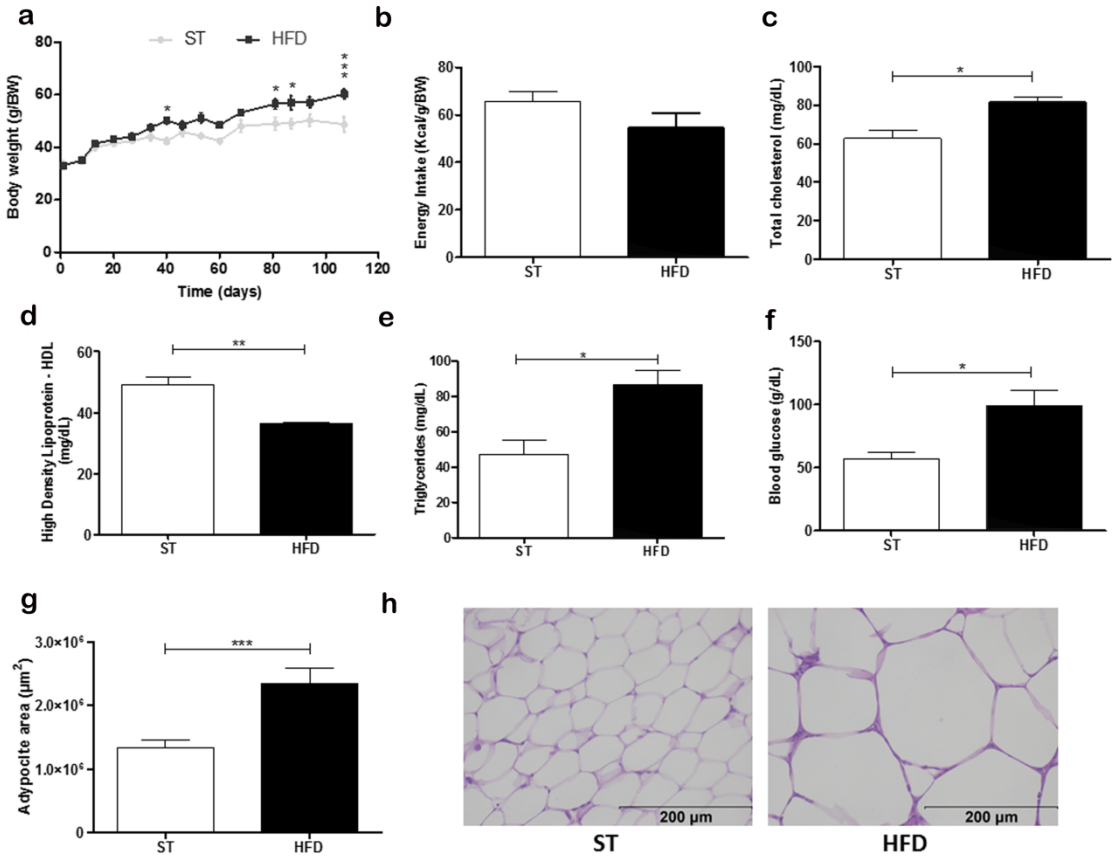


Fig.3 Mice samples characterization, lipid and glycemic profiles. a) Body weight (g/BW); b) Energy intake (Kcal/g/BW); c) Total cholesterol (mg/dL); d) High-density lipoproteins (mg/dL); e) Triglycerides (mg/dL); f) Blood glucose (g/dL); g) Adipocyte area (um2); H) H&E White adipose tissue staining * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

Bioinformatics analysis

The bioinformatics analysis aimed to identify the interaction of the genes involved in NET release and the activation process in obesity. The results indicated that interleukin-8 (IL-8) was the major gene, most likely connecting both pathological mechanisms. Further results are available as supplementary data (Figure 5a).

To confirm the bioinformatics findings, IL-8 serum levels were measured and were higher in obesity individuals than those in lean individuals (41.34±9.9 vs. 6.9±1.2, respectively, p=<0.002) (Figure 5b).

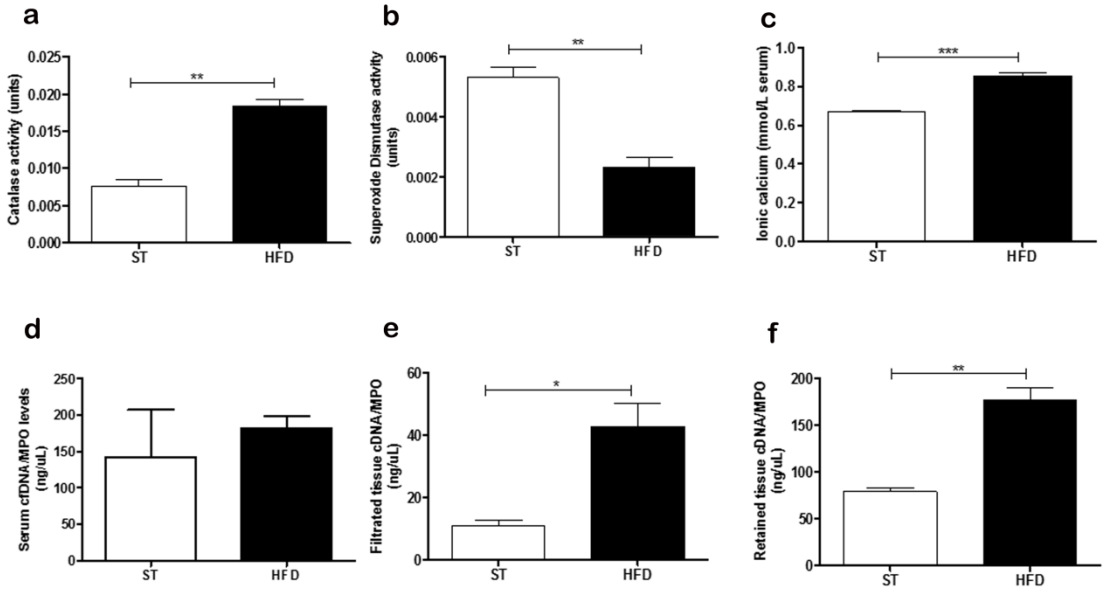


Fig. 4 Measurement of NETs in mice samples and oxidative stress profiles. A) Catalase activity (units); B) Superoxide dismutase activity (units); C) Ionic calcium (mmol/L); D) Serum cfDNA/MPO (NETs) levels (ng/uL); E) Filtrated tissue cfDNA/MPO (NETs) levels (ng/uL); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

Proteomic analysis

Proteomic analysis using the white adipose tissue of obesity and lean human individuals was performed to establish a profile of proteins that are more abundant in these two groups (Figures 6a). Bioinformatics analysis performed on the group of proteins described in the individuals with obesity group revealed the chaperones HSP90 and HSPE1 as targets (Figures 6b and c). Further details are provided in the supplementary data.

Thus, immunohistochemistry and qPCR analysis were performed on white adipose tissue and revealed that these markers were greater in the with obesity phenotype than those in the lean phenotype (Figure 6d-f).

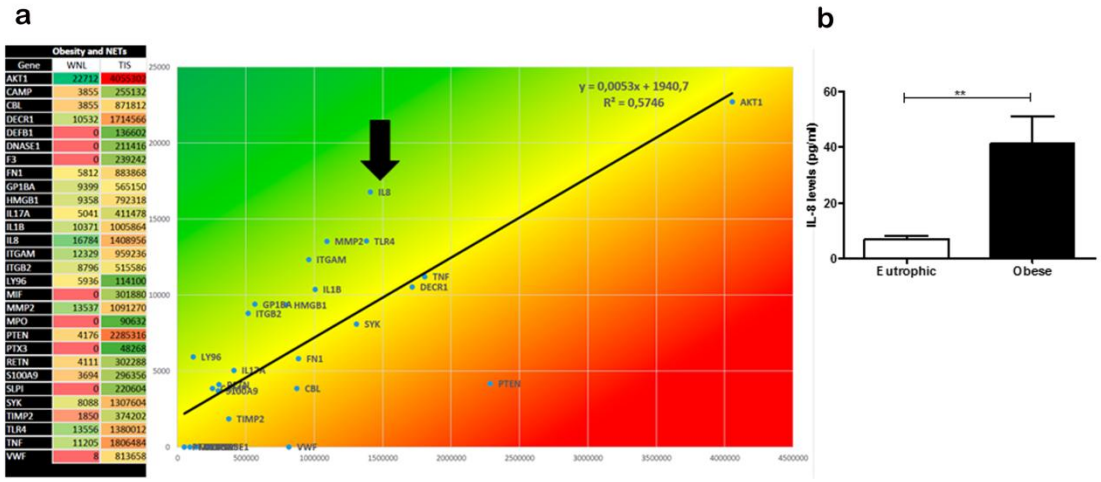


Fig.5 Bioinformatic analysis. a) Leader genes obtained from the bioinformatics analysis based on WNL (Weighted number of links) and TIS (Total interactions score). b) IL-8 serum levels (pg/ml). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

DISCUSSION

The main findings of the present study suggest that NET production is higher in obesity individuals than that in lean individuals and that NETs seem to modulate the inflammatory profile observed in the obesity state. To our knowledge, this study is the first to evaluate the role of adipose tissue in NET formation in with obesity humans. Obesity is associated with increased leukocytes and neutrophils in the peripheral blood. The importance of neutrophils in the pathogenesis of obesity-associated diseases has been the focus of recent studies. This suggests that neutrophils and chronic inflammation represent a possible link between chronic hypertension and obesity and represent some of major risk factors of obesity (25). In the present study, increased leukocytes were observed in the serum of obesity individuals, which reinforces the relationship among inflammation, leukocyte activation and obesity. Our analyses also suggest that obesity is related to increased NET levels in the serum and that the IL-8 gene may constitute a link among obesity, inflammation and NETs.

In contrast, a study conducted by Braster and colleagues tested the effects of peptidyl arginine deiminase 4 inhibitor Cl-amidine, a compound that prevents histone citrullination and subsequent NET release,

in a mouse model of adipose tissue inflammation. The authors observed that although high-fat-diet-fed mice usually developed insulin resistance, no significant effects were observed between groups. Furthermore, no effects were observed regarding leukocyte infiltration and activation in the adipose tissue or liver, suggesting

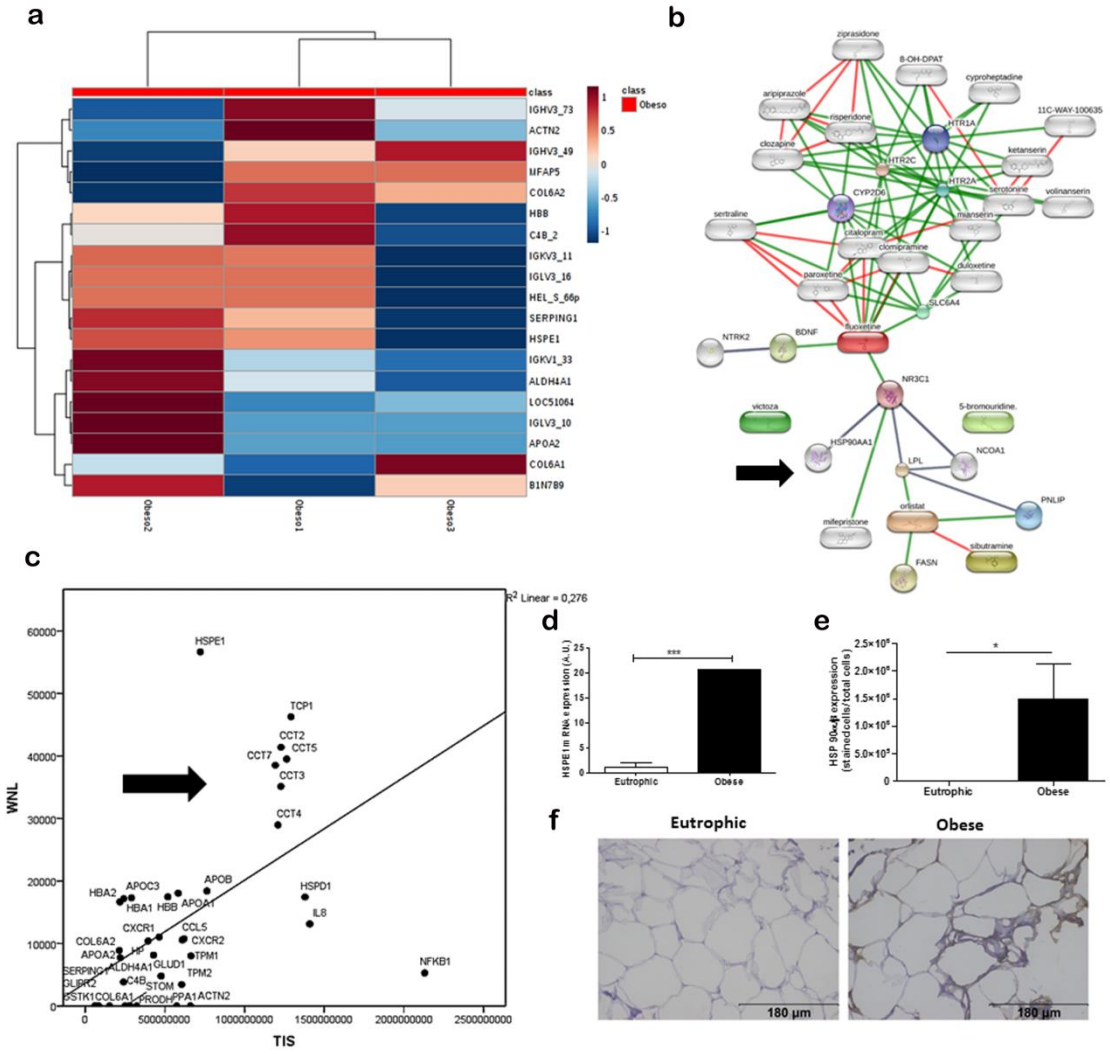


Fig. 6 Proteomic analysis and inflammatory markers expression. a) Heat map from with obesity human samples; b) Leader genes obtained from the bioinformatics analysis based on WNL (Weighted number of links) and TIS (Total interactions score); c) STITCH bioinformatics approach; d) HSPE1 mRNA expression (fold change)/ GAPDH endogenous control; e) HSP90 protein expression by immunohistochemistry. f) Immunohistochemistry HSP90 staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus indicated groups by the bars

that the inhibition of NET release might not have clinical relevance in the early pathogenesis mediated by obesity in the adipose tissue and liver (26). In contrast, some studies have shown that mice fed a high-fat diet are more prone to spontaneous neutrophil (NET) trapping (27, 28). Other studies have shown that preventing the formation or degradation of NETs improves obesity-related comorbidities (29, 30); thus, we believe in the direct benefit in this condition.

Given that adipose tissue promotes a potentially neutrophilic inflammatory environment (25), it was necessary to evaluate the role of adipose tissue in NET formation. Adipocytes are capable of secreting adipokines such as *tumor necrosis factor-alpha* (TNF-α), interleukin-6 (IL-6), and IL-8, which has been

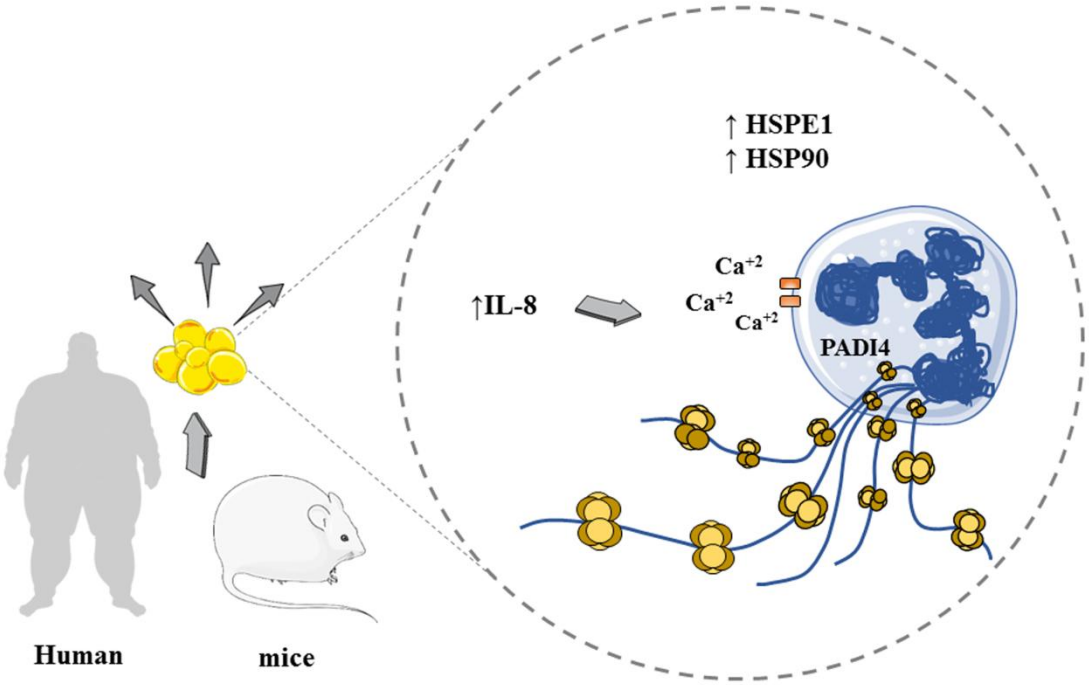
associated with increased peripheric neutrophils, superoxide radicals and NET formation due to its proinflammatory properties. However, the inflammation effects in adiposity and their relationship with NETosis remain unclear (10, 31, 32).

Our findings suggest that NET serum levels in obesity might regulate inflammatory markers such as IL-8, HSP90 and HSPE1. The heat shock response (HSR) is an essential defense mechanism against several pathological, physiological and environmental stress agents. The HSR involves the immediate activation of several highly conserved heat shock proteins (HSPs), known as molecular chaperones, and their transcription factors (HSFs) (33, 34). The HSR is also one of the principally impaired pathways observed in insulin resistance induced by obesity. HSPs may additionally be released in the circulation; however, unlike the case of their cellular functions, they exert an immunostimulatory effect via interactions with pattern recognition receptors such as toll-like receptors (TLRs), thus activating the host inflammatory response (35). Another important molecular marker that might be involved in NET release in adipose tissue is HSPE1, whose role in inflammation and NET formation has received little discussion. HSPE1 is the cochaperone for HSP 60 inside the mitochondria, but it also resides outside the organelle. HSPE1 plays a critical role in inhibiting the inflammatory responses to a number of other stressors (35-37). Several studies have demonstrated that HSPE1 therapies reduce the clinical signs of experimental autoimmune diseases. In a study performed by Fonseca (2017), human neutrophils activated by methyl-leucyl-phenylalanine (FMLP) were evaluated by proteomics, and HSPE1 was identified as a regulated protein in the analysis of total extracts of unenhanced, upregulated neutrophils.

Our findings revealed "*in situ*" the most-used drugs in obesity treatment that act on HSP90 and validated these drugs "*in vivo*" by immunohistochemistry. These findings might be explained by the fact that HSP90 is a molecular chaperone that participates in several processes, including cellular motility and wound healing, in addition to being an essential factor in the maintenance of cellular homeostasis (38-40). The HSP90A isoform was found outside the cell, and its secretion is stimulated by oxygen-reactive species, heat, hypoxia, irradiation and cytokines released by injured tissue. These conditions are also observed in obesity and promote the activation of inflammatory and NET signaling pathways. Gupta et al. (2014) demonstrated that treatment with ascomycin and cyclosporine A (calcineurin pathway antagonists) reduces the release of NETs via IL-8 (41). Further evidence among NETs and HSP90 involves the release and activation of reactive oxygen species (ROS). ROS may contribute to the etiology and physiology of several inflammation-associated diseases, such as cancer and cardiovascular diseases. The use of HSP90 inhibitors is suggested to be beneficial in the treatment of these diseases. However, it remains unknown whether ROS modulation is a mechanism by which these compounds are

effective. In our study, we have shown that increased ROS in human and mouse white adipose tissues was correlated with increased HSP90, corroborating the results of previous studies. Chen et al. (2011) revealed that HSP90 stabilizes the production of superoxide via the interaction between this protein and the C-terminal domain (42). Furthermore, Hattori et al. (2010) identified NADPH oxidase-dependent ROS as key regulators of neutrophil chemotactic migration.

In conclusion, our findings suggest that NET serum levels in obesity might modulate inflammatory markers such as IL-8, HSP90 and HSPE1. We suggest that NET inhibition might be a potential therapeutic alternative in the treatment of obesity comorbidities, although findings in animal models remain controversial. In this sense, further studies should be performed to clarify whether NET inhibition is an effective approach as an obesity treatment agent.



Graphic abstract legends

NETs serum and tissue levels are increased in obesity and might be regulated by inflammatory markers such as IL-8, HSP90 and HSPE1.

Acknowledgements

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COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of interest

The authors declare no conflicts of interest.

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Neutrophil extracellular traps (NETs) modulate inflammatory profiles in obese individuals

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Supplementary data
Additional methods
Bioinformatics Analysis

We initially performed a bioinformatics analysis of human genes related to "neutrophil extra cellular traps", "inflammation" and "obesity" using the GeneCards database (1) and NCBI (https: //www.ncbi. Nlm.nih.gov/mesh/) (2). Owing to the probability of gene interactions, we also performed a more specific bioinformatics analysis designed to reveal the interaction network between genes expressed in the following processes: "neutrophil extra cellular traps," "inflammation" and "obesity". STRING software version 10.0 (3, 4) was used to construct the interaction map between the genes identified. The network obtained was expanded only once, revealing new possible genes associated with the pathological mechanisms searched. The combined score for each gene was adjusted by multiplying by 1,000 to obtain a single score, called the weighted number of links (WNL) (5). The total interaction score (TIS) represents all gene interactions in the entire STRING database. All interactions of a gene in the total STRING database were summed and adjusted by multiplying by 1,000 to obtain the TIS value (5-7). Genes with no interactions were defined as orphan genes (6, 7). Genes were ranked according to this parameter in clusters by the clustering method K-means (8-10). The differences between

the various classes in WNL terms were assessed using Kruskal-Wallis tests ($p < 0.001$). WNL and TIS values are used for different purposes (7-11). The TIS is associated with great interactions while WNL is related to specific network interactio The TIS is associated with greater interactions whereas WNL is related to specific network interactions (9). The categories with both higher WNL and TIS were chosen to identify the genes that have more interactions.

Topological and ontological analysis

Cytoscape was used for ontological analysis, and BinGO was applied (12). All three structures' (biological processes, molecular functions, and cellular components) controlled vocabularies were used to describe gene products. The results suggest the molecular pathways involved in the process.

Proteomic analysis

White adipose tissue from eutrophic and obese patients was used for proteomic analysis as described in the main manuscript.

In brief, the supernatants were dried in a speed vacuum (Eppendorf, Hamburg, Germany). All peptides obtained were suspended in 80 µL of a solution containing 20 mM of ammonium formiate and 150 fmol/µL of Enolase (Waters Corporation, Manchester, UK) (MassPREPTM protein).

Nanoscale LC separation of tryptic peptides was performed using an ACQUITY UPLC® M-Class system (Waters Corporation, USA) equipped with a XBridge® Peptide 5 µm BEH130 C18 300 µm x 50 mm precolumn; Trap, 2D Symmetry® 5 µm BEH100 C18, 180 µm x 20mm column and Peptide CSH™ BEH130 C18 1.7 µm, 100 µm x 100 mm analytical reversed-phase column (Waters Corporation, USA). The peptides were separated using a gradient of 3% at 45% of acetonitrile, with a flow rate of 2.000 µL/min. The lock mass was used for calibration of the apparatus, using a constant flow rate of 0.2 µL/min at concentrations of 200 fmol protein GFP ([Glu1]-Fibrinopeptide B human (Sigma-Aldrich, St. Louis, MO, USA). Mass spectrometry analysis was performed on a Synapt G1 MSTM (Waters, USA) equipped with a nanoelectronspray source and two mass analysers: a quadrupole and a time-of-flight (TOF) operating in TOF V-mode. Data were obtained using the instrument in the MSE mode, which switches the low energy (6 V) and elevated energy (40 V) acquisition modes every 0.4 s. Samples were analysed from three replicates.

Data processing and protein identification

The mass spectrometer data obtained from the LC-MSE analysis were processed and searched using the ProteinLynx Global Server version 3.0.2 (Waters, Manchester, UK).

The data were subjected to automatic background subtraction, deisotoping and charge state deconvolution. After processing, each ion comprised an exact mass-retention time (EMRT) that contained the retention time, intensity-weighted average charge, inferred molecular weight based on charge and m/z, and the deconvoluted intensity. The processed spectra were then searched against Homo sapiens (Human) protein sequences (http://www.uniprot.proteomes/query=homo+sapiens&sort=score) alongside reverse sequences. The protein identification criteria also included the detection of at least 2 fragment ions per peptide, 5 fragments per protein and the determination of at least 1 peptide per protein. The identification of the protein was permitted with a maximum 4% false positive discovery rate in at least three technical replicate injections. The searches were performed with fixed modification of carbamidomethyl-C, and variable modifications were

phosphorylation of serine, threonine and tyrosine. One missed cleavage site was allowed. Protein tables were generated by ProteinLynx Global Server.

The proteins obtained from obese individuals in the proteomic analysis were then used to perform a new bioinformatics analysis (as described below):

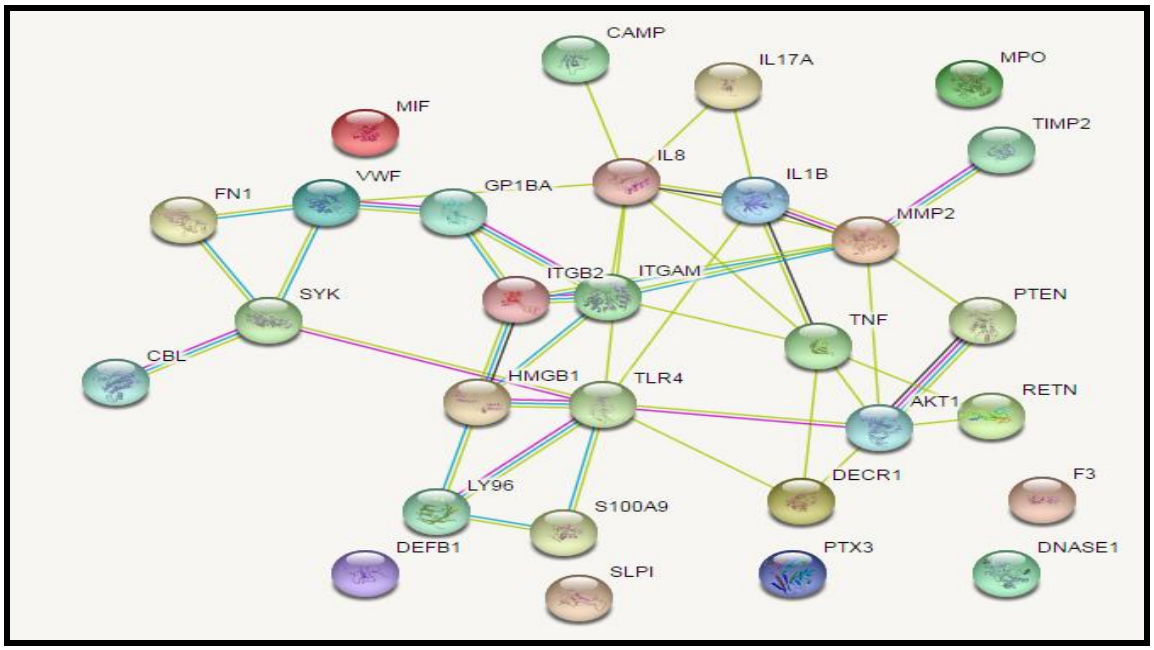
STOM	HBB
SERPING1	GLUD1
MFAP5	COL6A2
LOC51064	COL6A1
IGLV3-16	cDNA FLJ54370
IGLV3-10	C9orf19
IGKV3-11	C4B_2
IGKV1-33	B1N7B9
IGHV3-73	APOA2
HSPE1	ALDH4A1
HEL-S-66p	ACTN2
hCG_40889	IGHV3-49

The bioinformatics results pointed to HSPE1 as the leader gene. In addition, another bioinformatics tool – STITCH – was used, evidencing HSP90 modulation by the drugs commonly used for obesity treatment.

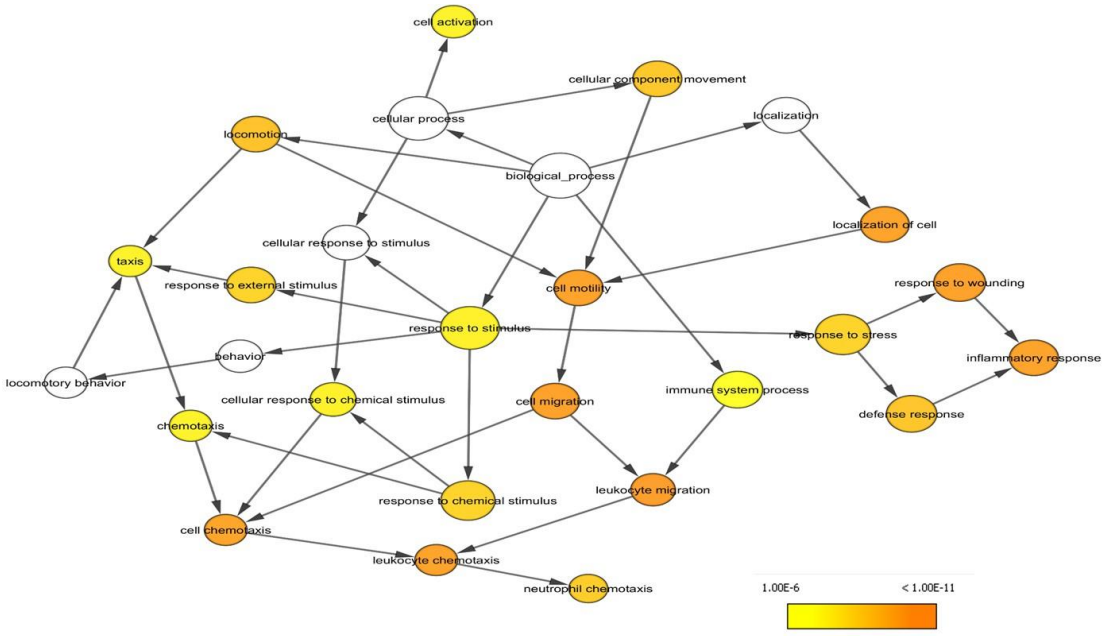
The validation of these markers was tested using human tissue samples via qRT-PCR and immunohistochemistry (data presented in the main manuscript).

Additional results

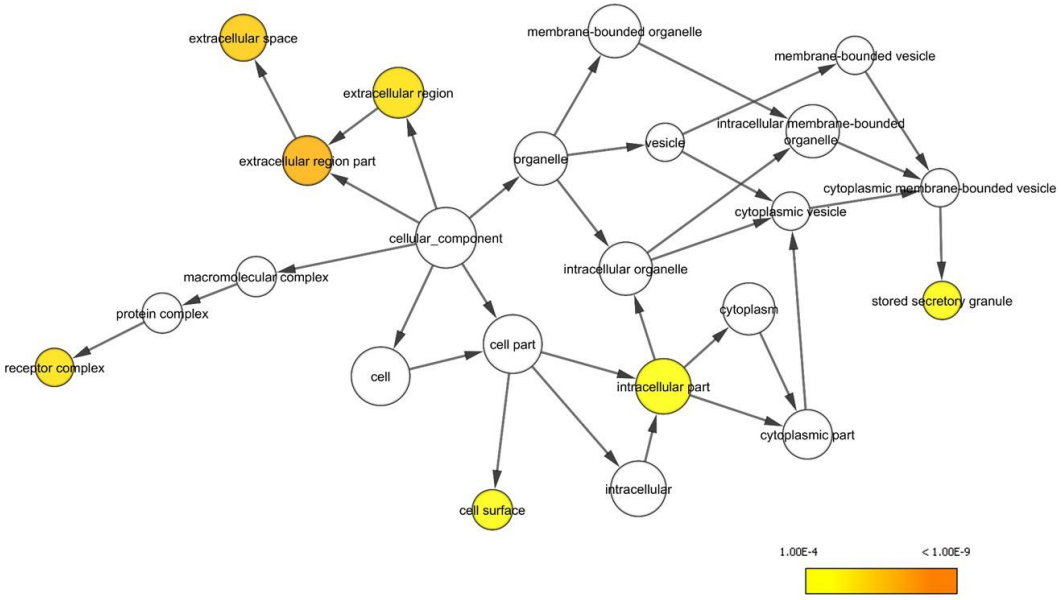
Network obtained from the first bioinformatics analysis with the keywords “neutrophil extra cellular traps”, “inflammation” and “obesity”.



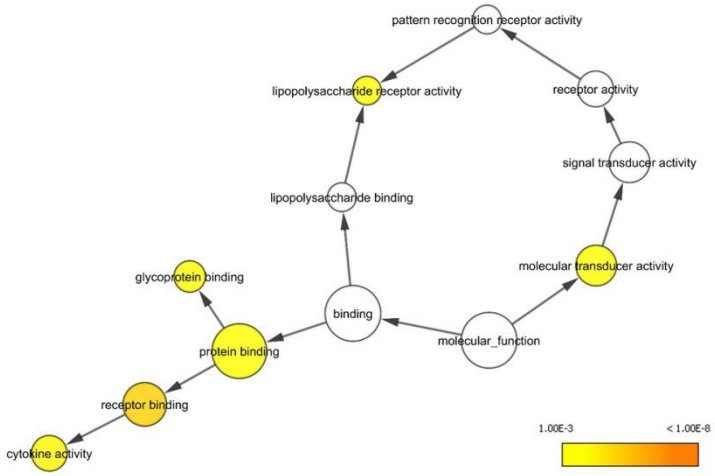
Biological network process obtained with ontological analysis



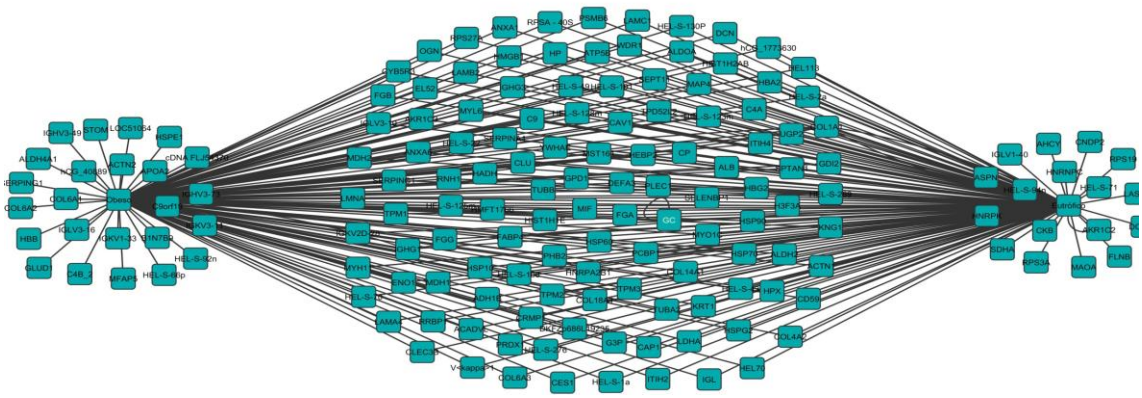
Cellular process network obtained with ontological analysis.



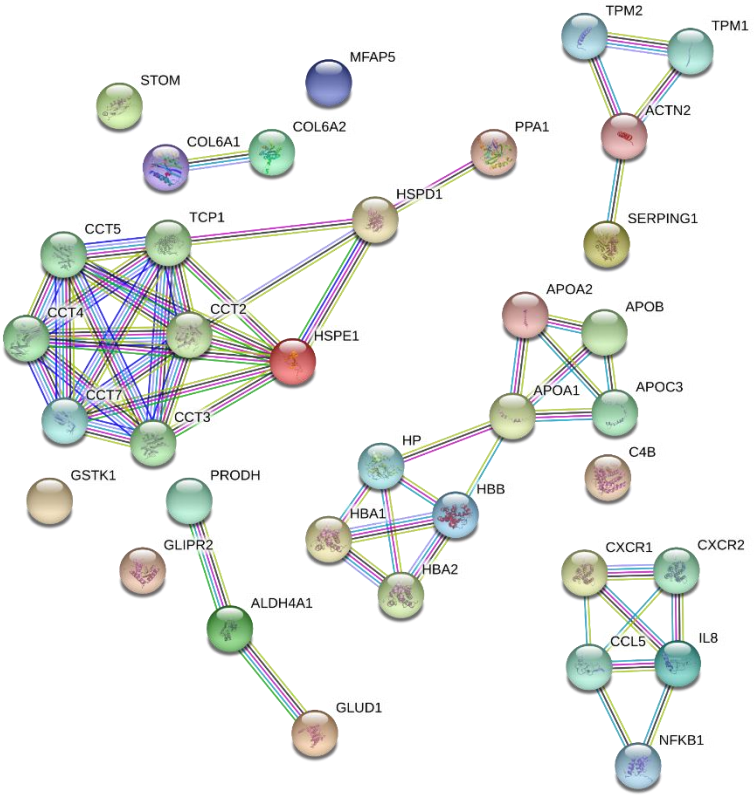
Molecular process network obtained with ontological analysis.



Proteins identified and stratified by groups (eutrophic and obese).



Network obtained after bioinformatics analysis performed with the proteins obtained by the proteomic analysis.



Biological network process obtained with ontological analysis.

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5 CONCLUSÃO

A busca de novas abordagens para o tratamento da obesidade é necessária, devido ao aumento das implicações clínicas, ao impacto financeiro e ao social na vida dos indivíduos. Assim, verificamos a influência do tecido adiposo no processo inflamatório. Nossos dados foram os primeiros a avaliar essa influência relacionando NETs, obesidade e inflamação, bem como os efeitos da casca da raiz da *Acosmium dasycarpum.* Com base nas investigações experimentais realizadas, concluímos que os níveis séricos de NETs na obesidade podem modular marcadores inflamatórios como IL-8, HSP90 e HSPE1 e que o uso da casca da espécie *Acosmium dasycarpum* parece reduzir o peso corporal e a adiposidade, além da expressão dos genes e C/EBP α, FAT, SCD-1, demonstrando, assim, seu efeito antiadipogênico em camundongos obesos.

Em conclusão, coletivamente, os achados experimentais deste estudo em modelo humano e animal sugerem que a inibição das NETs pode ser uma alternativa terapêutica potencial no tratamento de comorbidades da obesidade, embora os achados em modelos animais permaneçam controversos. Acresça-se a isso que a casca da raiz de *A. dasycarpum*, nativa da região do rio Pandeiros possui efeitos benéficos à perda de peso e à redução da adiposidade, porém mais estudos devem ser realizados para esclarecer outros mecanismos envolvidos para o uso, como agente no tratamento da obesidade.

Ademais, futuras investigações são necessárias para esclarecer outros mecanismos envolvidos para o uso como agente no tratamento da obesidade. Além disso, estudos translacionais, que visem a aplicar os conhecimentos adquiridos em pesquisas clínicas, devem ser fortemente encorajados.

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ANEXOS

ANEXO A – Parecer do Comitê de Ética e Pesquisa

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO
E BEM-ESTAR ANIMAL

Unimontes

PARECER CONSUBSTANCIADO

Montes Claros, 07 de Julho de 2017.

Processo N.º 133
Título do Projeto: Avaliação do efeito da *Acosmium dasycarpum* (Vog.) Yakovlev (Unha-d'anta) e *Hancornia speciosa* Gomes var. gardneri (Mangaba) em modelos murinos de síndrome metabólica: Papel do Lupeol.
Orientador: **Prof. Sérgio Henrique Sousa Santos**

Histórico
A síndrome metabólica (SM) é um estado de resistência à insulina, estresse oxidativo e inflamação crônica. Recentemente é considerada como uma epidemia mundial e caracteriza-se pela coexistência variável do excesso de gordura corporal, hiperinsulinemia (resistência à insulina e glicose intolerância), dislipidemia (altos níveis de triglicerídeos e níveis totais de colesterol de plasma), e hipertensão.
As plantas são fontes importantes de medicamento para a maioria da população mundial. O bioma Cerrado (57% do território mineiro) abrange mais de 204 milhões de hectares, localizado na parte central do Brasil, é a savana tropical mais rica do mundo em termos de biodiversidade e o segundo bioma mais extenso do Sul da América. Bioma pouco explorado, como por exemplo, o efeito da *Acosmium dasycarpum* (Unha-d'anta) planta do cerrado, sobre a síndrome metabólica não está elucidado na literatura. Em geral poucos estudos descrevem os efeitos do fruto da *Hancornia speciosa* (Mangaba) principalmente sobre a obesidade um dos critérios brasileiros para a síndrome metabólica, o que justifica a execução deste trabalho.

Mérito
O objetivo geral do estudo é avaliar os efeitos terapêuticos do princípio ativo Lupeol advindos do cerrado, da planta *Acosmium dasycarpum* (Vog.) Yakovlev (Unha-d'anta) e do fruto *Hancornia speciosa* Gomes var. gardneri (Mangaba) em modelos murinos de Síndrome Metabólica.

Parecer
A Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes analisou o processo 133 e entende que o protocolo de procedimentos preenche todos os requisitos éticos do CEEBEA/Unimontes enquadrando na categoria de **Aprovado.**

Prof°. Orlando Raphael Lopasso Júnior
Presidente da Comissão de Ética em Experimentação
e Bem-Estar Animal da UNIMONTES

ANEXO B

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A6B40FC

A atividade de acesso ao Patrimônio Genético/CTA, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A6B40FC**
Usuário:	**Universidade Federal de Minas Gerais**
CPF/CNPJ:	**17.217.985/0001-04**
Objeto do Acesso:	**Patrimônio Genético/CTA**
Finalidade do Acesso:	**Pesquisa**

Espécie

Davilla elliptica

Acosmium dasycarpum

Lafoensia pacari

Davilla elliptica, Lafoensia pacari, Acosmium dasycarpum.

Fonte do CTA

CTA de origem não identificável

Título da Atividade:	**Potencial Terapêutico e Farmacológico de Espécies Vegetais Nativas da Bacia do Rio Pandeiros no Tratamento de Doenças Metabólicas: Incentivo à Preservação da Flora.**

Equipe

Bruna Mara Aparecida de Carvalho	**UFMG**
Diego Vicente da Costa	**UFMG**

Junio Cota Silva	UFMG
Igor Viana Brandi	UFMG
João Marcus Oliveira Andrade	Unimontes
Janaina Ribeiro Oliveira	Unimontes
Amanda Souto Machado	Unimontes
Deborah de Farias Leite	Unimontes
Daniela Fernanda de Freitas	Unimontes
Daniel Silva Moraes	Unimontes
Luis Paulo Oliveira	Unimontes
Natália Gonçalves Ribeiro	Unimontes
Jaciara Neves Sousa	Unimontes
Victor Hugo Dantas Guimarães	Unimontes
Fábio Ribeiro do Santos	UFMG
Alfredo Mauricio Batista de Paula	Unimontes
André Luiz Sena Guimarães	Unimontes
Daniele Cristina Moreira	Unimontes

Parceiras Nacionais

22.675.359/0001-00 / Universidade Estadual de Montes Claros

Data do Cadastro: 06/11/2018 16:17:33

Situação do Cadastro: Concluído

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em 15:35 de 26/12/2018.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

ANEXO C

GOVERNO DO ESTADO DE MINAS GERAIS
SECRETARIA DE ESTADO DE MEIO AMBIENTE E DESENVOLVIMENTO SUSTENTÁVEL
INSTITUTO ESTADUAL DE FLORESTAS
DIRETORIA DE PROTEÇÃO À FAUNA
GERÊNCIA DE PROJETOS E PESQUISAS

AUTORIZAÇÃO PARA PESQUISA CIENTÍFICA NO ESTADO DE MINAS GERAIS

Número da Autorização	Data da Emissão	Prazo de Validade
107/2017	29/12/2017	29/12/2018

INFORMAÇÕES DO RESPONSÁVEL E DO PROJETO

Título do Projeto	"Potencial terapêutico e farmacológico de espécies vegetais nativas da Bacia do Rio pandeiros no tratamento de doenças metabolicas: incentivo à preservação da flora".
Instituição	Universidade Federal de Minas Gerais
Responsável	Sergio Henrique Sousa Santos — CPF: 055.482.156-71
Logradouro	Rua Cosme e Damião
Nº/KM	260 — Complemento: — Bairro/Localidade: Santo Expedito
Município	Montes Claros — UF: MG — CEP: 39401-302 — Cx. Postal:
Telefone	(38) 2101-7710 — Celular: (38) 99912-1033
E-mail	sergiosousas@hotmail.com

INTEGRANTES DA EQUIPE

Nome	Instituição	CPF/RG	Função
Alfredo Maurício Batista de Paula	UFMG	860.835.899-49	Pesquisador Colaborador
Bruna Mara Aparecida de Carvalho	UFMG	060.680.356-46	Pesquisadora Colaboradora
Diego Vicente da Costa	UFMG	354.789.668-19	Pesquisador Colaborador
Júnio Cota Silva	UFMG	067.236.616-88	Pesquisador Colaborador
Igor viana Brandi	UFMG	814.462.926-49	Pesquisador Colaborador
João Marcus Oliveira Andrade	UNIMONTES	081.794.126-60	Pesquisador Colaborador
Janaína Ribeiro Oliveira	UNIMONTES	068.789.696-70	Colaboradora
Amanda Souto Machado	UNIMONTES	105.743.826-46	Colaborador
Deborah de Faria Lelis	UNIMONTES	108.815.546-42	Colaborador
Daniela Fernanda de Freitas	UNIMONTES	055.384.946-20	Colaboradora
Daniel Silva Moraes	UNIMONTES	098.038.796-50	Colabordor
Luis Paulo Oliveira	UNIMONTES	080.724.386-89	Colaborador
Natália Gonçalves Ribeiro	UNIMONTES	100.329.536-31	Colaboradora
Jaciara Neves Sousa	UNIMONTES	047.078.015-01	Colaboradora
Victor Hugo Dantas Guimarães	UNIMONTES	116.318.186-27	Colaborador
Fernando Lopes Ferreira	UFMG	121.465.316-22	Colaborador
Fábio Ribeiro dos Santos	UFMG	108.799.876-00	Colaborador

INFORMAÇÕES DAS ATIVIDADES

Tipo de Atividade: ( ) Captura; (X) Coleta; (X) Transporte; ( ) Sem Coleta/Captura; ( )Abiótica; ( ) Microrganismos; ( ) Fungo; (X) Botânica; ( ) Invertebrados; ( )Anfíbios; ( ) Répteis; ( ) Aves; ( ) Mamíferos; ( ) Ictiofauna

OBSERVAÇÕES

Esta autorização permite coleta/transporte botânico de sementes, folhas e ramos das espécies citadas na tabela de estimativa de coleta.
Esta autorização não permite coleta de espécies ameaçadas.
Esta autorização permite até cinco (05) integrantes da equipe a cada campanha.

LOCAL DA ATIVIDADE – EM UNIDADE DE CONSERVAÇÃO ESTADUAL

Unidade de Conservação	Responsável pela UC	Contato (Telefone e e-mail)	Endereço da UC	Assinatura do Responsável pela UC

Assinatura do responsável pela Autorização	Número de Processo SIGED/SIPRO – IEF/DFAU/GPROP
Janaína A. B. Aguiar Gerente de Projetos e Pesquisas do IEF Masp: 1159560-0	SIGED 00000241 2101 2018

GOVERNO DO ESTADO DE MINAS GERAIS
SECRETARIA DE ESTADO DE MEIO AMBIENTE E DESENVOLVIMENTO SUSTENTÁVEL
INSTITUTO ESTADUAL DE FLORESTAS
DIRETORIA DE PROTEÇÃO À FAUNA
GERÊNCIA DE PROJETOS E PESQUISAS

Refúgio de Vida Silvestre Estadual do Rio Pandeiros	Neilton Viana Neves	(38) 3621-0100 neilton.viana@meioambiente.mg.gov.br	BR 479, Vila da CEMIG, S/nº Distrito de Pandeiros. CEP: 39.480-000	
APA Estadual do Rio Pandeiros	Alexsandro Martins da Fonseca	(38) 3625-6222 (38) 3625-6208 alexsandro.fonseca@meioambiente.mg.gov.br	Rua Joaquim Borges Monteiro nº 180 – Bonito de Minas CEP: 39.480-000	

Esta autorização será válida apenas com a autenticação do responsável pela(s) Unidade(s) de Conservação.

LOCAL DA ATIVIDADE – FORA DE UNIDADE DE CONSERVAÇÃO ESTADUAL (apenas para material botânico)	
Município(s)	Não se aplica

TRANSPORTE – DESTINO DO MATERIAL COLETADO	
Instituição(ões)	ICA - UFMG
Endereço(s)	Montes Claros - MG

Outras Observações e Ressalvas:

1. Esta autorização não exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena, da unidade de conservação federal, distrital ou municipal, ou do proprietário, arrendatário, possuidor ou morador de área dentro dos limites de unidade de conservação estadual, cujo processo de regularização fundiária encontra-se em curso;
2. O pesquisador titular deverá contatar a administração desta unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade de conservação, quando for o caso;
3. O Instituto Estadual de Florestas não se responsabiliza por qualquer dano a equipamentos, acidentes ou lesões físicas ou pessoais, estando ainda, o pesquisador responsável e sua equipe ciente da vulnerabilidade da área de realização da pesquisa;
4. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior;
5. O titular da autorização e os membros de sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos e empregar esforços de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condições in situ, quando for o caso;
6. Esta autorização não permite captura/coleta/transporte:
   - para fins comerciais, industriais ou esportivos;
   - para realização de atividades integrantes do processo de licenciamento ambiental de empreendimentos, conforme resolução do CONAMA de nº 237 de 19/ 12/97, salvo quando especificado;
   - de espécies ameaçadas de extinção em lista oficial federal, salvo quando constante de projeto específico autorizado pelo SISBIO;
   - de espécies ameaçadas de extinção em lista oficial estadual, salvo quando constante de projeto específico autorizado pelo IEF;
   - de fauna e flora em áreas de domínio privado, sem o consentimento expresso ou tácito do proprietário nos termos do Código Civil;
7. Esta autorização não permite transporte internacional e internacional de material biológico;
8. Esta autorização não dispensa o cumprimento da legislação que dispõe sobre o acesso ao patrimônio genético, sobre a proteção e o acesso ao conhecimento tradicional associado e sobre a repartição de benefícios para conservação e uso sustentável da biodiversidade. Veja maiores informações em www.mma.gov.br;
9. O titular desta autorização, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição da mesma, poderá, mediante decisão motivada, ter a autorização suspensa ou revogada pelo IEF e o material biológico coletado apreendido nos termos da legislação em vigor;
10. O responsável poderá, durante o validade desta autorização e conforme Termo de Compromisso firmado, solicitar à Gerência de Projeto e Pesquisas do IEF Renovação, Cancelamento ou Conclusão, conforme instruções no site do IEF: (http://www.ief.mg.gov.br/biodiversidade/pesquisa-cientifica);
11. Esta autorização é válida somente sem emendas ou rasuras e exclusivamente no estado de Minas Gerais;
12. O pesquisador deverá estar sempre acompanhado desta autorização para apresentá-la às autoridades, quando solicitado.

ANEXO D – Parecer do Comitê de Ética e Pesquisa

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da produção de NETs pelo tecido adiposo de humanos obesos.
Pesquisador: Daniela Fernanda de Freitas Souza
Área Temática:
Versão: 1
CAAE: 56905416.9.0000.5146
Instituição Proponente: Universidade Estadual de Montes Claros - UNIMONTES
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.596.711

Apresentação do Projeto:
Estudo será realizando utilizando amostras do Biobanco Institucional - UNIMONTES/ Registro CONEP: B-013. Serão estudados casos de indivíduos obesos, totalizando 100 indivíduos participantes, com idade superior a 18 anos. Visando a conservação das amostras nos arquivos do Biobanco, salienta-se que uma quantidade mínima das amostras em blocos de parafina (10 cortes de 10 µm) será utilizada, as quais serão devolvidas ao Biobanco logo após seu uso, sem prejuízo para novas avaliações diagnósticas, caso sejam necessárias, além da amostra de sangue e/ou plasma. Será avaliada a produção de NETs no tecido adiposo de indivíduos obesos e eutróficos, além de verificar os níveis de colesterol total, HDL, triglicérides, glicose, ácidos graxos livres, e transaminases e os níveis séricos de adipocinas (adiponectina, leptina e resistina), e insulina serão avaliados utilizando kits de Espectrofotometria específicos. Ainda serão quantificadas as expressão proteicas e mRNA de biomarcadores no tecido adiposo e dosadas as NETs por Elisa/Fluorescência e Imunofluorescência.

Objetivo da Pesquisa:
Avaliar a produção das NETs pelo tecido adiposo de humanos obesos.

Avaliação dos Riscos e Benefícios:
Os riscos quanto à cessão do material estão relacionados ao tipo de procedimento realizado pela

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

equipe médica, necessário para diagnóstico e tratamento ao paciente na época da coleta de dados. Sendo que a coleta de material para o Biobanco envolve apenas o excedente material biológico proveniente desse procedimento médico.
Quanto aos benefícios, a cessão do material poderá favorecer a realização de pesquisas que buscam um melhor entendimento e possibilidade de controle e tratamento das doenças.

Comentários e Considerações sobre a Pesquisa:
Pesquisa importante que caracteriza-se por uma pesquisa experimental laboratorial. No modelo humano obeso as amostras de sangue e tecido adiposos serão provenientes do Banco de Materiais Biológicos Humano do Norte do Estado de Minas Gerais. Esse estudo será realizado com o auxílio da metodologia de Real-time PCR, western Blot, Imuno-histoquímica, Imunofluorescência e dosagem de NETS no sangue técnicas que permitem avaliar a expressão dos genes e proteínas envolvidas no processo obesidade, e ainda detectam a expressão de proteínas localizadas nas células dos tecidos utilizando o princípio antígeno/anticorpo servindo como base para a continuação da pesquisa em busca de alvos moleculares.

Considerações sobre os Termos de apresentação obrigatória:
Uso de material do Biobanco Unimontes, devidamente registrado no CONEP.

Recomendações:
Apresentação de relatório final por meio da plataforma Brasil, em "enviar notificação".

Conclusões ou Pendências e Lista de Inadequações:
Aprovado.

Considerações Finais a critério do CEP:
O projeto respeita os preceitos éticos da pesquisa em seres humanos, sendo assim somos favoráveis à aprovação do mesmo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações	PB_INFORMAÇÕES_BÁSICAS_DO_P	08/06/2016		Aceito

Endereço: Av.Dr Rui Braga s/n-Camp Univers Prof° Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

Continuação do Parecer: 1.586.711

Básicas do Projeto	ETO_730734.pdf	13:53:52		Aceito
Outros	Apendice.pdf	08/06/2016 13:52:59	Daniela Fernanda de Freitas Souza	Aceito
Outros	Declaracao.pdf	08/06/2016 13:48:29	Daniela Fernanda de Freitas Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	08/06/2016 13:46:28	Daniela Fernanda de Freitas Souza	Aceito
Outros	Parecer_Biobanco.pdf	08/06/2016 13:43:51	Daniela Fernanda de Freitas Souza	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	08/06/2016 13:40:13	Daniela Fernanda de Freitas Souza	Aceito
Folha de Rosto	Folha_de_rosto_Projeto_Nets.pdf	08/06/2016 12:51:32	Daniela Fernanda de Freitas Souza	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

MONTES CLAROS, 16 de Junho de 2016

Assinado por:
SIMONE DE MELO COSTA
(Coordenador)

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO
E BEM-ESTAR ANIMAL

Unimontes

PARECER CONSUBSTANCIADO

Montes Claros, 07 de julho de 2017.

Processo N.º **134**
Título do Projeto: **Avaliação da produção de NETs pelo tecido adiposo de camundongos obesos**
Pesquisador responsável: **Dr. Sérgio Henrique Sousa Santos**

Histórico
A obesidade é caracterizada pelo acúmulo excessivo de gordura corporal no indivíduo. Esta tem sido vista como uma condição inflamatória local e/ou sistêmica de baixa intensidade associada à deposição de gordura no tecido e com a produção de muitas citocinas. Estudos recentes tem demonstrado um novo mecanismo dos neutrófilos e sua relação com disfunções orgânicas onde ocorre a formação de armadilhas extracelulares denominadas NETs, onde o neutrófilo também libera uma rede de fibras de cromatina associadas com grânulos antimicrobianos e enzimas, tais como a MPO, elastase e catepsina G. A formação excessiva das redes também tem sido observada em muitos estados patológicos, além disso, há evidencias crescentes de que os mediadores inflamatórios agem sinergicamente com os produtos finais dos microorganismos para induzir a ativação de neutrófilos sistêmica, o que contribui para a sua acumulação em órgãos distantes e impede sua migração adequada ao foco da infecção.

Mérito
O projeto visa responder as seguintes questões: a produção de NETs esta aumentada nos obesos? Existe associação entre a produção das NETs pelo tecido adiposo que está inflamado? Objetivando avaliar a associação entre a expressão das NETs e os marcadores associados à obesidade no tecido adiposos e hepático de humanos e camundongos. O estudo caracteriza-se por uma pesquisa experimental laboratorial, realizado com protocolos aprovados pelo NIH, conforme o *Guide for the Care and Use of Laboratory Animals*. No modelo animal será induzida a obesidade e avaliada a produção de NETs, já no modelo humano obeso serão coletadas amostras de sangue e tecido adiposo após o consentimento do mesmo para analises posteriores. Para o experimento *in vivo* serão utilizados 30 camundongos machos da linhagem *Swiss*, 15 receberão dieta padrão e 15 dieta hiperlipídica, mantidos em gaiola em ambiente com ciclos de luminosidade de 12 horas com temperatura entre 22 e 25ºC e acesso à alimentação e água ad libitum. Coletas de sangue serão realizadas para avaliação do perfil glicêmico. Após o sacrifício dos camundongos será realizada a coleta de sangue e de amostras teciduais.

Parecer
A Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes analisou o processo **134** e entende que o protocolo de procedimentos preenche todos os requisitos éticos do CEEBEA/Unimontes enquadrando na categoria de **Aprovado.**

Prof°. Orlando Raphael Lopasso Júnior
Presidente da Comissão de Ética em Experimentação
e Bem-Estar Animal da UNIMONTES

ANEXO E – Normas para publicação no periódico British Journal of Nutrition

Instructions for contributors

Submission | Scope | Review Process | Publishing Ethics | Article Types | Detailed Manuscript Preparation Instructions| Copyright | Open Access | Green Open Access Policy | AuthorAID | Author Language Services | Accepted Manuscript | Proofs | Offprints | Digital Preservation Policy | Contact

British Journal of Nutrition (BJN) is an international peer-reviewed journal that publishes original papers and review articles in all branches of nutritional science. The journal welcomes submission of manuscripts that in which the primary aim is to develop nutritional concepts.

SUBMISSION

This journal uses ScholarOne Manuscripts for online submission and peer review.

Complete guidelines for preparing and submitting your manuscript to this journal are provided below.

SCOPE

BJN encompasses the full spectrum of nutritional science and submission of manuscripts that report studies in the following areas is strongly encouraged: Epidemiology, dietary surveys, nutritional requirements and behaviour, metabolic studies, body composition, energetics, appetite, obesity, ageing, endocrinology, immunology, neuroscience, microbiology, genetics, and molecular and cell biology. The focus of all manuscripts submitted to the journal should be to increase knowledge in nutritional science.

The articles published in BJN are expected to be directly relevant to human or animal nutrition. Please ensure that studies which involve the following experimental designs should meet the following criteria:

In vivo and in vitro models

Studies involving animal models of human nutrition and health or disease **will only be considered for publication** if the amount of a nutrient or combination of nutrients used could reasonably be expected to be achieved in the human population.

Studies involving in vitro models **will be considered for publication** if the amount of a nutrient or combination of nutrients is demonstrated to be within the range that could reasonably be expected to be encountered in vivo, and that the molecular form of the nutrient or nutrients is the same as that to which the cell type used in the model would encounter in vivo.

Extracts

Studies involving extracts **will be considered for publication** if the source of starting material is readily accessible to other researchers and that there are appropriate measures for quality control of the starting material and extract. The method of extraction must be described in sufficient detail for other researchers to replicate the experiment. Please ensure that the nutrient composition of the extract is characterised fully and that appropriate measures are used to control the composition of the extract between preparations. The amount of extract used should reasonably be expected to be achievable in a human population (or in animals if they are the specific target of an intervention).

Studies involving extracts in in vitro models **will only be considered for publication** if the above guidelines for studies involving extracts are followed, and that the amount and molecular form of the extract is the same as that which would be encountered by the cell type used in the model in vivo.

Probiotics

We encourage submission of experimental studies and reviews that focus only on the effects of probiotics on nutrient absorption and/or metabolism. However, manuscripts that report the effects of probiotics on any other outcomes will not be accepted for publication.

Coffee and caffeine

Studies of the effect of coffee consumption will be considered by the journal. Please ensure that the amount of coffee is within the range consumed habitually and that the findings show that any health or metabolic outcomes are due to nutritional effects. Studies on caffeine alone or that involve intakes of coffee above those consumed habitually are discouraged.

Dietary Inflammatory Index and Dietary Acid Load

Manuscripts reporting outcomes related to the Dietary Inflammatory Index will only be considered for publication if there is evidence from the study that the index is related to two or more biomarkers of inflammation.

Manuscripts reporting outcomes related to the Dietary Acid Load will only be considered for publication if there is evidence from the study of a causal association between the diet or dietary pattern and appropriate markers of acid–base balance.

Manuscripts reporting studies on the following topics are discouraged: Pilot studies; case studies; papers on food technology, food science or food chemistry; studies of primarily local interest; studies on herbs, spices or other flavouring agents, pharmaceutical agents or that compare the effects of nutrients to those of medicines, traditional medicines, complementary medicines or other substances that are considered to be primarily medicinal agents; studies in which a nutrient or extract is not administered by the oral route (unless the specific aim of the study is to investigate parenteral nutrition); studies using non-physiological amounts of nutrients (unless the specific aim of the study is to investigate toxic effects); caffeine, food contaminants.

REVIEW PROCESS

BJN uses a single blind review process.

As part of the online submission process, authors are asked to affirm that the submission represents original work that has not been published previously, and that it is not currently being considered by another journal. Authors must also confirm that each author has seen and approved the contents of the submitted manuscript. Finally, authors should confirm that permission for all appropriate uses has been obtained from the copyright holder for any figures or other material not in his/her copyright, and that the appropriate acknowledgement has been made to the original source.

At submission, authors are asked to nominate at least four potential referees who may then be asked by the Editorial Board to help review the work. Manuscripts are normally reviewed by two external peer reviewers and a member of the Editorial Board.

When substantial revisions are required to manuscripts after review, authors are normally given the opportunity to do this once only; the need for any further changes should at most reflect only minor issues. If a paper requiring revision is not resubmitted within 2 months, it may, on resubmission, be deemed a new paper and the date of receipt altered accordingly.

PUBLISHING ETHICS

BJN considers all manuscripts on the strict condition that:

- The manuscript is your own original work, and does not duplicate any other previously published work;
- The manuscript has been submitted only to the journal - it is not under consideration or peer review or accepted for publication or in press or published elsewhere;
- All listed authors know of and agree to the manuscript being submitted to the journal; and
- The manuscript contains nothing that is abusive, defamatory, fraudulent, illegal, libellous, or obscene.

The Journal adheres to the Committee on Publication Ethics (COPE) guidelines on research and publications ethics.

Text taken directly or closely paraphrased from earlier published work that has not been acknowledged or referenced will be considered plagiarism. Submitted manuscripts in which such text is identified will be withdrawn from the editorial process. If a concern is raised about possible plagiarism in an article submitted to or published in BJN, this will be investigated fully and dealt with in accordance with the COPE guidelines.

ARTICLE TYPES

BJN publishes the following: Research Articles, Review Articles, Systematic Reviews, Horizons in Nutritional Science, Workshop Reports, Invited Commentaries, Letters to the Editor, Obituaries, and Editorials.

Research Articles, Reviews, Systematic Reviews, Horizons Articles, Letters to the Editor and Workshop Reports should be submitted to http://mc.manuscriptcentral.com/bjn. Please contact the Editorial Office on bjn.edoffice@cambridge.orgregarding any other types of article.

Review Articles

BJN is willing to accept critical reviews that are designed to advance knowledge, policy and practice in nutritional science. Current knowledge should be appropriately contextualised and presented such that knowledge gaps and research needs can be characterised and prioritised, or so that changes in policy and practice can be proposed along with suggestions as to how any changes can be monitored. The purpose or objective of a review should be clearly expressed, perhaps as question in the Introduction, and the review's conclusions should be congruent with the initial objective or question. Reviews will be handled by specialist Reviews Editors. Please contact the Editorial Office with any queries regarding the submission of potential review articles. All reviews, including systematic reviews and meta-analyses, should present the uncertainties and variabilities associated with the papers and data being reviewed; in particular BJN cautions against uncritical acceptance of definitions and non-specific global terminology, the advice of advisory bodies, and reference ranges for example.

- **Reviews**: These articles are written in a narrative style, and aim to critically evaluate a specific topic in nutritional science.
- **Horizons in Nutritional Science**: These are shorter than Review articles and aim to critically evaluate recent novel developments that are likely to produce substantial advances in nutritional science. These articles should be thought-provoking and possibly controversial.
- **Systematic Reviews and meta-analyses**: A systematic review or meta-analysis of randomised trials and other evaluation studies must be accompanied by a completed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement checklist, a guideline to help authors report a systematic review and meta-analysis (see British Medical Journal (2009) 339, b2535). Meta-analysis of observational studies must be accompanied by a completed Meta-analysis of Observational Studies in Epidemiology (MOOSE) reporting checklist, indicating the page where each item is included (see JAMA (2000) 283, 2008-2012). Manuscripts in these areas of review will not be sent for peer review unless accompanied by the relevant completed checklist.

Letters to the Editor

Letters are invited that discuss, criticise or develop themes put forward in papers published in BJN. They should not, however, be used as a means of publishing new work. Acceptance will be at the discretion of the Editorial Board, and editorial changes may be required. Wherever possible, letters from responding authors will be included in the same issue as the original article.

DETAILED MANUSCRIPT PREPARATION INSTRUCTIONS

Language

Papers submitted for publication must be written in English and should be as concise as possible. We recommend that authors for whom English is not their first language have their manuscript checked by someone whose first language is English before submission, to ensure that submissions are judged at peer review exclusively on academic merit. Please see the Author Language Services section below for more information.

Spelling should generally be that of the *Concise Oxford Dictionary* (1995), 9th ed. Oxford: Clarendon Press. Authors are advised to consult a current issue in order to make themselves familiar with BJN as to typographical and other conventions, layout of tables etc. Sufficient information should be given to permit repetition of the published work by any competent reader of BJN.

Published examples of BJN article types can be found below:

- Research Article
- Review Article
- Horizons Article
- Letter to the Editor

Authorship

The Journal conforms to the International Committee of Medical Journal Editors (ICMJE) definition of authorship, as described by P.C. Calder (Br J Nutr (2009) 101, 775). Authorship credit should be based on:

1. Substantial contributions to conception and design, data acquisition, analysis and/or interpretation;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

The contribution of individuals who were involved in the study but do not meet these criteria should be described in the Acknowledgments section.

Ethical standards

The required standards for reporting studies involving humans and experimental animals are detailed in an Editorial by G.C. Burdge (*Br J Nutr* (2014) **112**).

Experiments involving human subjects

The notice of contributors is drawn to the guidelines in the World Medical Association (2000) Declaration of Helsinki: ethical principles for medical research involving human subjects, with notes of clarification of 2002 and 2004 (http://www.wma.net/en/30publications/10policies/b3/), the *Guidelines on the Practice of Ethics Committees Involved in Medical Research Involving Human Subjects* (3rd ed., 1996; London: The Royal College of Physicians) and the Guidelines for the ethical conduct of medical research involving children, revised in 2000 by the Royal College of Paediatrics and Child Health: Ethics Advisory Committee (*Arch Dis Child* (2000) **82**, 177–182). Articles reporting randomised trials must conform to the standards set by the Consolidated Standards of Reporting Trials (CONSORT) consortium. A completed CONSORT Checklist (Consolidated Standards of Reporting Trials (CONSORT) consortium) must accompany manuscripts reporting randomised controlled trials. Submissions that do not include this information will not be considered for review until a completed CONSORT Checklist has been submitted and approved.

Required disclosures: A paper describing any experimental work on human subjects must include the following statement in the Experimental Methods section: "This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the [insert name of the ethics committee; a specific ethics number MUST be inserted]. Written [or Verbal] informed consent was obtained from all subjects/patients. [Where verbal consent was obtained this must be followed by a statement such as: Verbal consent was witnessed and formally recorded]." For clinical trials, the trial registry name, registration identification number, and the URL for the registry should be included.

PLEASE NOTE: As a condition for publication, all randomised controlled trials that involve human subjects submitted to BJN for review must be registered in a public trials registry. A clinical trial is defined by the ICMJE (in accordance with the definition of the World Health Organisation) as any research project that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes. Registration information must be provided at the time of submission, including the trial registry name, registration identification number, and the URL for the registry.

Experiments involving the use of other vertebrate animals

Papers that report studies involving vertebrate animals must conform to the 'ARRIVE Guidelines for Reporting Animal Research' detailed in Kilkenny et al. (*J Pharmacol Pharmacother* (2010) **1**, 94-99) and summarised at www.nc3rs.org.uk. Authors MUST ensure that their manuscript conforms to the checklist that is available from the nc3Rs website (the completed check list should be uploaded as a separate document during submission of the manuscript). The attention of authors is drawn particularly to the ARRIVE guidelines point 3b ('Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology', point 9c ('Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment') and point 17a ('Give details of all important adverse events in each experimental group'). The Editors will not accept papers reporting work carried out involving procedures that cause or are considered likely to cause distress or suffering which would confound the outcomes of the experiments, or experiments that have not been reviewed and approved by an animal experimentation ethics committee or regulatory organisation.

Required disclosures: Where a paper reports studies involving vertebrate animals, authors must state in the Experimental Methods section the institutional and national guidelines for the care and use of animals that were followed and that all experimental procedures involving animals were approved by the [insert name of the ethics committee or other approving body; wherever possible authors should also insert a specific ethics/approval number].

Manuscript Format

The requirements of BJN are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the ICMJE.

Typescripts should be prepared with 1.5 line spacing and wide margins (2 cm), the preferred font being Times New Roman size 12. At the ends of lines, words should not be hyphenated unless hyphens are to be printed. **Line numbering and page numbering are required.**

MANUSCRIPTS SHOULD BE ORGANISED AS FOLLOWS:

Cover letter

Papers should be accompanied by a cover letter including a brief summary of the work and a short explanation of the novelty of the study and

how it advances nutritional science. The text for the cover letter should be entered in the appropriate box as part of the online submission process.

Title Page

The title page should include:

1. The title of the article;
2. Authors' names;
3. Name and address of department(s) and institution(s) to which the work should be attributed for each author;
4. Name, mailing address, email address, telephone and fax numbers of the author responsible for correspondence about the manuscript;
5. A shortened version of the title, not exceeding 45 characters (including letters and spaces) in length;
6. At least four keywords or phrases (each containing up to three words).

Authors' names should be given without titles or degrees and one forename may be given in full. Identify each author's institution by a superscript number (e.g. A.B. Smith[1]) and list the institutions underneath and after the final author.

Abstract

Each paper must open with an unstructured abstract of **not more than 250 words**. The abstract should be a single paragraph of continuous text without subheadings outlining the aims of the work, the experimental approach taken, the principal results (including effect size and the results of statistical analysis) and the conclusions and their relevance to nutritional science.

Introduction

It is not necessary to introduce a paper with a full account of the relevant literature, but the introduction should indicate briefly the nature of the question asked and the reasons for asking it. It should be **no longer than two manuscript pages**.

Experimental methods

The methods section must include a subsection that describes the methods used for statistical analysis (see the section on statistical analysis in the Appendix) and the sample size must be justified by the results of appropriate calculations and related to the study outcomes.

Justification of sample size: All manuscripts that report primary research must contain a statistical justification of sample size that is stated explicitly in the Statistics sub-section of the Methods. Manuscripts that do not contain this information will be returned to the authors for correction before peer review. The amended versions will be treated as new submissions. The information required must include, but not be restricted to, the following:-

- Hypothesised effect size with appropriate justification.
- A statement regarding statistical power (typically 80%) and the two-sided significance level (typically 0.05).
- An explanation of how the statistical power was calculated.
- If sample size is determined by the feasibility of recruitment minimally detectable effect sizes should be provided instead of power analysis.

The only exceptions are:-

- Meta-analyses.
- Exploratory or secondary analysis of observational studies based on large sample sizes

For studies involving humans subjects or experimental animals, the Methods section must include a subsection that reports the appropriate ethical approvals for the study (see Ethical Standards above).

All analytical procedures must be accompanied by a statement of within and between assay precision.

Diets: The nutrient composition of diets used in studies published in BJN must be described in detail, preferably in a table(s). Experimentally relevant differences in composition between diets are essential. For instance, studies of fat nutrition should always include fatty acid compositions of all diets.

PCR analysis: Where experiments involve measurement of mRNA including microarray analysis, for analysis of individual genes, mRNA should be measured by quantitative RTPCR. A statement about the quality and integrity of the RNA must be provided together with the results of eletrophoretic analysis of the purity of the PCR products. Unless published elsewhere, full details of the oligonuceoltide primers and of the PCR protocol must be stated either in the text or in Supplementary Material. The stability of reference genes used for normalisation of PCR data must be reported for the experimental conditions described. Where possible, analysis of mRNA levels should be accompanied by assessment of either protein levels or activities.

Microarray analysis: Studies involving microarray analysis of mRNA must conform to the "Minimum Information about a Microarray Experiment" (MIAME) guidelines including deposition of the raw data in an appropriate repository (the Access Code must be state din the Methods). All microarray experiments must be accompanied by appropriate validation by quantitative RTPCR.

Results

These should be given as concisely as possible, using figures or tables as appropriate. Data must not be duplicated in tables and figures.

Discussion

While it is generally desirable that the presentation of the results and the discussion of their significance should be presented separately, there may be occasions when combining these sections may be beneficial. Authors may also find that additional or alternative sections such as 'conclusions' may be useful. The discussion should be **no longer than five manuscript pages**.

Acknowledgments

Here you may acknowledge individuals or organizations that provided advice and/or support (non-financial). Formal financial support and funding should be listed in the following section.

Financial Support

Please provide details of the sources of financial support for all authors, including grant numbers. For example, "This work was supported by the Medical research Council (grant number XXXXXXX)". Multiple grant numbers should be separated by a comma and space, and where research was funded by more than one agency the different agencies should be separated by a semi-colon, with "and" before the final funder. Grants held by different authors should be identified as belonging to individual authors by the authors' initials. For example, "This work was supported by the Wellcome Trust (A.B., grant numbers XXXX, YYYY), (C.D., grant number ZZZZ); the Natural Environment Research Council (E.F., grant number FFFF); and the National Institutes of Health (A.B., grant number GGGG), (E.F., grant number HHHH)".

This disclosure is particularly important in the case of research that is supported by industry. Support from industry not only includes direct financial support for the study but also support in kind such as provision of medications, equipment, kits or reagents without charge or at reduced cost and provision of services such as statistical analysis; all such support must be disclosed here and if no such support was received this must be stated. Where no specific funding has been provided for research, please provide the following statement: "This research received no specific grant from any funding agency, commercial or not-for-profit sectors."

In addition to the source of financial support, please state whether the funder contributed to the study design, conduct of the study, analysis of samples or data, interpretation of findings or the preparation of the manuscript. If the funder made no such contribution, please provide the following statement: "[Funder's name] had no role in the design, analysis or writing of this article."

Conflict of Interest

Please provide details of all known financial, professional and personal relationships with the potential to bias the work. Where no known conflicts of interest exist, please include the following statement: "None."

For more information on what constitutes a conflict of interest, please see the International Committee of Medical Journal Editors (ICMJE) guidelines.

Authorship

Please provide a very brief description of the contribution of each author to the research. Their roles in formulating the research question(s), designing the study, carrying it out, analysing the data and writing the article should be made plain.

References

References should be numbered consecutively in the order in which they first appear in the text using superscript Arabic numerals in parentheses, e.g. 'The conceptual difficulty of this approach has recently been highlighted[1,2]'. If a reference is cited more than once, the same number should be used each time. References cited only in tables and figure legends should be numbered in sequence from the last number used in the text and in the order of mention of the individual tables and figures in the text.

Names and initials of authors of unpublished work should be given in the text as 'unpublished results' and not included in the References. References that have been published online only but not yet in an issue should include the online publication date and the Digital Object Identifier (doi) reference, as per the example below.

At the end of the paper, on a page(s) separate from the text, references should be listed in numerical order using the Vancouver system. When an article has more than three authors only the names of the first three authors should be given followed by '*et al.*' The issue number should be omitted if there is continuous pagination throughout a volume. Titles of journals should appear in their abbreviated form using the NCBI LinkOut page. References to books and monographs should include the town of publication and the number of the edition to which reference is made. References to material available on websites should follow a similar style, with the full URL included at the end of the reference, as well as the date of the version cited and the date of access.

Examples of correct forms of references are given below.

Journal articles

1. Rebello SA, Koh H, Chen C *et al.* (2014) Amount, type, and sources of carbohydrates in relation to ischemic heart disease mortality in a Chinese population: a prospective cohort study. *Am J Clin Nutr* **100**, 53-64.
2. Villar J, Ismail LC, Victora CG *et al.* (2014) International standards for newborn weight, length, and head circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project.*Lancet* **384**, 857-868.
3. Alonso VR & Guarner F (2013) Linking the gut microbiota to human health. *Br J Nutr* **109**, Suppl. 2, S21–S26.
4. Bauserman M, Lokangaka A, Gado J et al. A cluster-randomized trial determining the efficacy of caterpillar cereal as a locally available and sustainable complementary food to prevent stunting and anaemia. *Public Health Nutr*. Published online: 29 January 2015. doi: 10.1017/S1368980014003334.

Books and monographs

1. Bradbury J (2002) Dietary intervention in edentulous patients. PhD Thesis, University of Newcastle.
2. Ailhaud G & Hauner H (2004) Development of white adipose tissue. In *Handbook of Obesity. Etiology and Pathophysiology*, 2nd ed., pp. 481–514 [GA Bray and C Bouchard, editors]. New York: Marcel Dekker.
3. Bruinsma J (editor) (2003) *World Agriculture towards 2015/2030: An FAO Perspective*. London: Earthscan Publications.
4. World Health Organization (2003) *Diet, Nutrition and the Prevention of Chronic Diseases*. Joint WHO/FAO Expert Consultation. WHO Technical Report Series no. 916. Geneva: WHO.
5. Keiding L (1997) *Astma, Allergi og Anden Overfølsomhed i Danmark – Og Udviklingen 1987–199I (Asthma, Allergy and Other Hypersensitivities in Denmark, 1987–1991)*. Copenhagen, Denmark: Dansk Institut for Klinisk Epidemiologi.

Sources from the internet

1. Nationmaster (2005) HIV AIDS – Adult prevalence rate. http://www.nationmaster.com/graph-T/hea_hiv_aid_ad... (accessed June 2013).

For authors that use Endnote, you can find the style guide for BJN here.

Figures

Figures should be supplied as separate electronic files. Figure legends should be grouped in a section at the end of the manuscript text. Each figure should be clearly marked with its number and separate panels within figures should be clearly marked (a), (b), (c) etc. so that they are easily identifiable when the article and figure files are merged for review. Each figure, with its legend, should be comprehensible without reference to the text and should include definitions of abbreviations. The nature of the information displayed in the figures (e.g. mean (SEM)) and the statistical test used must be stated.

We recommend that only TIFF, EPS or PDF formats are used for electronic artwork. Other non-preferred but usable formats are JPG, PPT and GIF files and images created in Microsoft Word. Note that these non-preferred formats are generally NOT suitable for conversion to print reproduction. For further information about how to prepare your figures, including sizing and resolution requirements, please see our artwork guide.

In curves presenting experimental results the determined points should be clearly shown, the symbols used being, in order of preference, ○, ●, Δ, ▲, □, ■, ×, +. Curves and symbols should not extend beyond the experimental points. Scale-marks on the axes should be on the inner side of each axis and should extend beyond the last experimental point. Ensure that lines and symbols used in graphs and shading used in histograms are large enough to be easily identified when the figure size is reduced to fit the printed page. Statistically significant effects should be indicated with symbols or letters.

Colour figures will be published online free of charge, and there is a fee of £350 per figure for colour figures in the printed version. If you request colour figures in the printed version, you will be contacted by CCC-Rightslink who are acting on our behalf to collect colour charges. Please follow their instructions in order to avoid any delay in the publication of your article.

Images submitted with a manuscript should be minimally processed; some image processing is acceptable (and may be unavoidable), but the final image must accurately represent the original data. Grouping or cropping of images must be identified in the legend and indicated by clear demarcation. Please refer to the Office of Research Integrity guidelines on image processing in scientific publication. Authors should provide sufficient detail of image-gathering procedures and process manipulation in the Methods sections to enable the accuracy of image presentation to be assessed. Authors should retain their original data, as Editors may request them for comparison during manuscript review.

Tables

Tables should be placed in the main manuscript file at the end of the document, not within the main text. Please do**not** supply tables as images (e.g. in TIFF or JPG format). Be sure that each table is cited in the text. Tables should carry headings describing their content and should be comprehensible without reference to the text.

The dimensions of the values, e.g. mg/kg, should be given at the top of each column. Separate columns should be used for measures of variance (SD, SE etc.), the ± sign should not be used. The number of decimal places used should be standardized; for whole numbers 1.0, 2.0 etc. should be used. Shortened forms of the words weight (wt) height (ht) and experiment (Expt) may be used to save space in tables, but only Expt (when referring to a specified experiment, e.g. Expt 1) is acceptable in the heading.

Footnotes are given in the following order: (1) abbreviations, (2) superscript letters, (3) symbols. Abbreviations are given in the format: RS, resistant starch. Abbreviations in tables must be defined in footnotes in the order that they appear in the table (reading from left to right across the table, then down each column). Symbols for footnotes should be used in the sequence: *†‡§‖¶, then ** etc. (omit * or †, or both, from the sequence if they are used to indicate levels of significance).

For indicating statistical significance, superscript letters or symbols may be used. Superscript letters are useful where comparisons are within a row or column and the level of significance is uniform, e.g. 'a,b,cMean values within a column with unlike superscript letters were significantly different (*P*<0•05)'. Symbols are useful for indicating significant differences between rows or columns, especially where different levels of significance are found, e.g. 'Mean values were significantly different from those of the control group: **P*<0•05, ***P*<0•01, ****P*<0•001'. The symbols used for *P* values in the tables must be consistent.

Supplementary material

Additional data (e.g. data sets, large tables) relevant to the paper can be submitted for publication online only, where they are made available via a link from the paper. The paper should stand alone without these data. Supplementary Material must be cited in a relevant place in the text of the paper.

Although Supplementary Material is peer reviewed, it is not checked, copyedited or typeset after acceptance and it is loaded onto the journal's website exactly as supplied. You should check your Supplementary Material carefully to ensure that it adheres to journal styles. Corrections cannot be made to the Supplementary Material after acceptance of the manuscript. Please bear this in mind when deciding what content to include as Supplementary Material.

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The British Journal of Nutrition has generous options to enable sharing of published articles through the Nutrition Society's Green Open Access policy (Burdge *et al.* Br J Nutr. 2016 116(4):571-572): All material is freely available one year after publication.

	Personal webpage	**Departmental/ Institutional Repository**	**Non-commercial subject repository**	**Commercial repository/Social media sites**
Accepted Manuscript*	On acceptance for publication	On acceptance for publication	On acceptance for publication	Abstract only in PDF or HTML format no sooner than the first publication of the full article
Version of record**	On publication	12 Months after first publication	12 Months after first publication	Abstract only in PDF or HTML format no sooner than the first publication of the full article

*The version that was accepted by the journal which has not been subjected to typesetting or other modification by the publisher

**The fully typeset version that appears in the printed and online issues of the journal.

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AUTHOR LANGUAGE SERVICES

BJN recommends that authors have their manuscripts checked by an English language native speaker before submission; this will ensure that submissions are judged at peer review exclusively on academic merit. We list a number of third-party servicesspecialising in language editing and/or translation, and suggest that authors contact as appropriate. Use of any of these services is voluntary, and at the author's own expense.

ACCEPTED MANUSCRIPT

Accepted manuscripts are published online as is (before copy-editing or typesetting) within approximately a week of final acceptance, provided we have received all final files and a completed license to publish form. At this point, the article will have a DOI and be considered published and citable. You will subsequently receive a proof of your typeset, edited article, which will eventually replace the accepted manuscript online and be considered the final version of record. For more information, please click here.

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PDF proofs are sent to authors in order that they make sure that the paper has been correctly set up in type. Only changes to errors induced by typesetting/copy-editing or typographical errors will be accepted.

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OFFPRINTS

A PDF file of the paper will be supplied free of charge to the corresponding author of each paper, and offprints may be ordered on the order form sent with the proofs.

ANEXO F – Normas para publicação no periódico Inflammation

GENERAL

The Council of Biology Editors Style Manual should be used as the style guide for the preparation of manuscripts, particularly with respect to such matters as the use of abbreviations, numbers, and symbols.

MANUSCRIPT SUBMISSION

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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AUTHORSHIP POLICY

Authorship should incorporate and should be restricted to those who have contributed substantially to the work in one or more of the following categories:
• Conceived of or designed study
• Performed research
• Analyzed data
• Contributed new methods or models
• Wrote the paper

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The title page should include:

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- A concise and informative title
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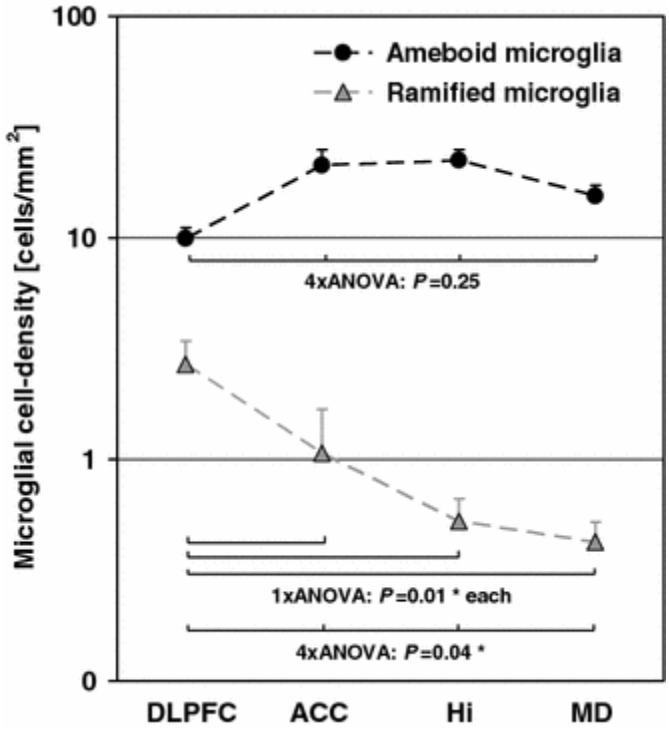

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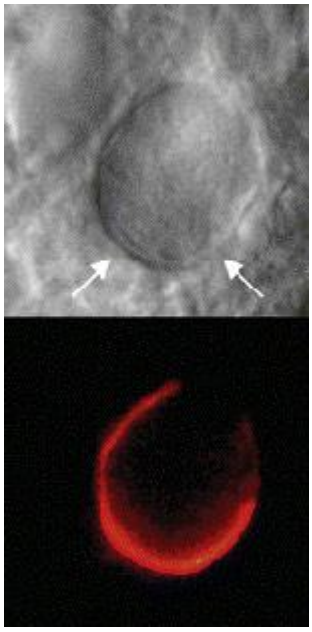

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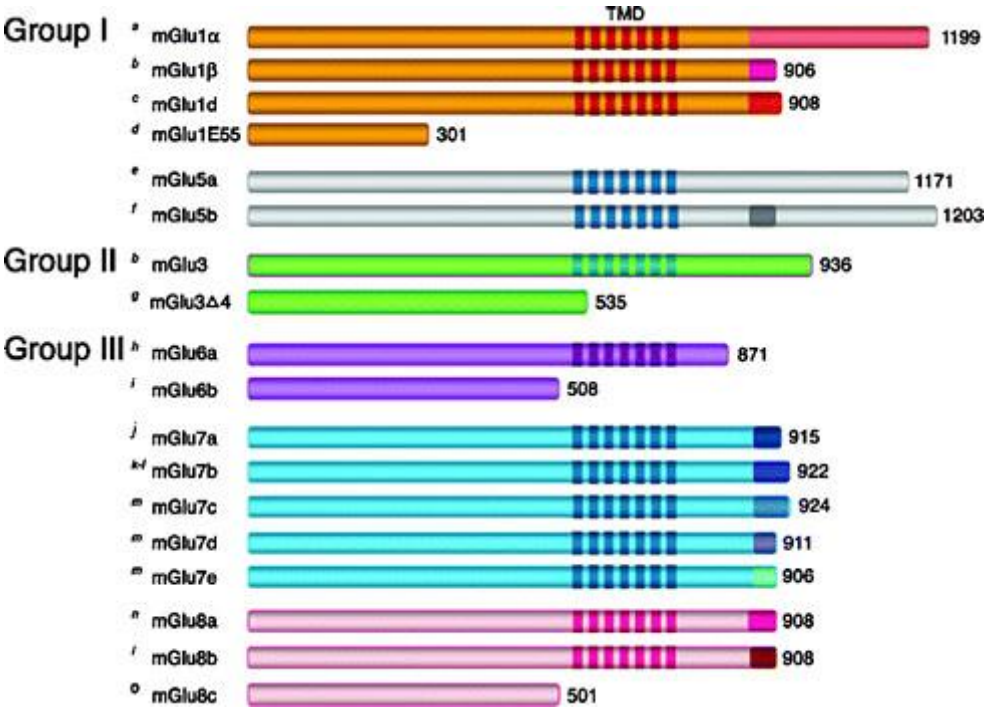

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