UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Antônio Sérgio Barcala Jorge

Análise da expressão de interleucina-6 (IL6), fator de necrose tumoral alfa (TNF-alfa) e Sirtuina 1(SIRT 1) no tecido adiposo branco, tecido adiposo marrom e tecido hepático de indivíduos clinicamente saudáveis, caquéticos sarcopênicos e obesos mórbidos.

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Área de Concentração: Mecanismos e Aspectos Clínicos das Doenças

Orientador: Prof. Dr. Alfredo Maurício Batista de Paula

Coorientador: Prof. Dr. Sérgio Henrique Sousa Santos

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UNIVERSIDADE ESTADUAL DE MONTES CLAROS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Unimontes

CANDIDATO: ANTÔNIO SÉRGIO BARCALA JORGE

TÍTULO DO TRABALHO: "Análise comparativa da expressão de IL6 e TNF-alfa no tecido adiposo e hepático de pacientes obesos graves".

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Dedico este estudo:

Ao meu filho Antônio,

“porque se chamavam homens, também se chamavam sonhos,
e sonhos não envelhecem...” *(Milton Nascimento)*

E ao meu pai Antônio,

“as pessoas não morrem, ficam encantadas.” (*Guimarães Rosa)*

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Omnia possum in eo qui me confortat.
(Philippenses IV)

RESUMO

A obesidade leva a distúrbios das condições de saúde do organismo e essas alterações podem ser representadas por distúrbios psicológicos, sociais e o aumento de risco de doenças de grande morbi-mortalidade. Com o aumento no tecido adiposo e a infiltração de macrófagos neste tecido, numerosas citocinas pró - inflamatórias, incluindo o factor de necrose tumoral - (TNF - alfa) e a interleucina - 6 (IL - 6) estão concomitantemente aumentadas e este estado de stress inflamatório resulta em uma condição de resistência à insulina que, por sua vez, induz ao aumento subsequente das citocinas e ácidos graxos circulantes, acaretando um estado de lipotoxidade. No primeiro estudo fizemos uma comparação da expressão molecular de IL6 e TNF-alfa (RT-PCR) no tecido adiposo visceral de pacientes obesos graves, caquéticos e eutróficos destas citocinas. O segundo estudo trata de uma análise comparativa da expressão de IL6 (RT-PCR) no tecido adiposo branco e marron de pacientes obesos graves e suas interações com alguns marcadores do perfil metabólico, lipídico e inflamatório destes pacientes. O terceiro artigo avaliou a associação e correlação da expressão destas citocinas IL6 e TNF-alfa (RT-PCR) com a gravidade da doença hepática gordurosa não alcoólica (DHGNA) em pacientes obesos graves. Nossos resultados nos permitem demonstrar que os obesos graves apresentam maior expressão das citocinas TNF alfa e IL6 no tecido adiposo abdominal, em comparação com o grupo de adultos de peso normal e que os pacientes caquéticos apresentam uma expressão semelhante destas citocinas em relação a estes obesos. Em pacientes obesos a expressão de IL6 no tecido adiposo marron e branco tem níveis de expressão e repercursões diferentes no metabolismo humano. Também observamos que a expressão quantitativa destas citocinas estava associada ao IMC e que a maior expressão de TNF alfa se associou com a maior gravidade de DHGNA. Concluimos que pacientes obesos graves apresentaram uma expressão elevada destas citocinas que se associa direta e indiretamente com a modulação do processo inflamatório crônico descrito na fisiopatologia da obesidade e de suas comorbidades, sendo que o tipo histológico do tecido adiposo influencia diferentemente na expressão e ação de IL6 em pacientes obesos.

Palavras-chave: Obesidade mórbida. Doença hepática gordurosa não alcoólica. Tecido adiposo. Citocinas. Expressão de mRNA em tempo real-PCR.

ABSTRACT

Obesity leads to disturbances of the health conditions of the organism and these changes can be represented by psychological disorders, social and increased risk of morbidity and mortality from major diseases. With the increase in adipose tissue, and macrophage infiltration in this tissue, numerous pro - inflammatory cytokines, including tumor necrosis factor - alfa (TNF alfa) and interleukin - 6 (IL - 6) are concomitantly leading to an increased inflammatory state and a condition of chronic insulin resistance, which in turn induces a subsequent increase in circulating cytokines and fatty acids causing a state of lipotoxicity. In the first study is a comparison of the molecular expression of these cytokines in visceral adipose tissue of morbid obese patients, cachectic and normal. The second study is a comparative analysis of IL-6 expression in white adipose tissue and brown adipose tissue of morbid obese patients and their interactions with the metabolic profile, lipid and inflammatory these patients. The third article evaluated the association and corelação the expression of these cytokines with the severity of nonalcoholic fatty liver disease (NAFLD) in morbid obese patients. Our results establish that in morbidly obese patients have higher expression of cytokines TNF alpha and IL6 in abdominal fat compared to the group of adults of normal weight and obese patients IL6 expression in brown and white adipose tissue have different repercussions on human metabolism. We also observed that the quantitative expression of these cytokines was associated with BMI and that most TNF alpha expression was associated with greater severity of NAFLD. We conclude that morbid obese patients had an expression of these cytokines is associated directly and indirectly with the modulation of chronic inflammatory process described in the pathophysiology of obesity and its comorbidities, and the histological type of adipose tissue had different influences on the expression and IL-6 action in patients obese.

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Keywords: Morbid obesity. Nonalcoholic fatty liver disease. Tissue adipose. mRNA expression of cytokines tissue. real-time PCR.

SUMÁRIO

1 INTRODUÇÃO

A obesidade é um problema em todo o mundo sendo possivelmente o problema mais grave e dispendioso para saúde deste século, e representa uma ameaça crescente para a população mundial (1). A obesidade é caracterizada pelo acúmulo excessivo de gordura corporal com potencial prejuízo para a saúde e decorrente de vários fatores como padrões alimentares, genéticos, ambientais, sedentarismo além de fatores de susceptibilidade biológicos individuais (2,3). A obesidade e suas comorbidades relacionadas têm em comum uma condição inflamatória de baixa intensidade, desenvolvida pelo menos em parte por uma produção e ou ativação anormal de citocinas inflamatórias e de suas vias de sinalização que regulam o proliferação e ativação de células do sistema imunológico (4, 5).

O tecido adiposo branco constitui o tecido mais expansível e o maior órgão endócrino que secreta vários hormônios chamados coletivamente de adipocinas. Nos obesos este tecido adiposo branco é caracterizado pelo aumento da produção e secreção de um amplo painel de moléculas inflamatórias (citocinas) que tem efeitos locais sobre a fisiologia deste tecido, alterando a função de secreção dos adipócitos, além de efeitos potenciais sobre outros órgãos e tecidos resultando em um estado metabólico alterado de todo o organismo (6,7). Embora todos os mecanismos que controlam este estado de estresse oxidativo ainda não estejam claros, é provável que as citocinas, pequenas proteínas com funções na regulação do sistema imunitário e que constituem o maior grupo de substâncias biologicamente ativas produzidas no tecido adiposo estariam diretamente envolvidas neste processo (8). O excesso e a hipertrofia dos adipócitos do tecido adiposo visceral induzem os leucócitos mononucleares (linfócitos e monócitos) presentes neste tecido a secretarem várias citocinas, tais como a interleucina-6 (IL-6) e fator de necrose tumoral alfa (TNF-alfa), tipicamente pró-inflamatórias e que desempenham um papel direto na resistência à insulina induzida pela obesidade, bem como modulam também a expressão de várias outras citocinas relacionadas com a obesidade (9).

A primeira indicação do aumento da liberação de citoquinas na obesidade foi fornecida pela identificação da expressão e secreção de TNF-alfa, pelo tecido adiposo onde os

seus níveis estão diretamente associados com o grau de adipocidade e com o estado de resistência à insulina (10,11). A IL-6 por sua vez é mais conhecida como uma citoquina pró-inflamatória que regula a imunidade inata e a resposta de fase aguda na inflamação (12), no entanto ela também tem efeitos específicos nos tecidos e que podem ser diferentes, dependendo do contexto, e do tempo de estímulo. Do ponto de vista metabólico elas podem promover a resistência à insulina de forma autócrina e ou apócrina aumentando a secreção de leptina, a oxidação da gordura, reduzindo a expressão e a atividade da lípase lipoproteica e inibindo a atividade dos receptores de insulina da membrana plasmática (13).

Tem sido observado de forma consistente que as vias inflamatórias ativadas em indivíduos com diabetes Tipo 2 e obesidade estão intimamente associados com um número de diferentes manifestações clínicas, incluindo a hipertensão arterial sistêmica (HAS), hipertrigliceridemia, HDL baixo e disfunção endotelial (8, 14). Além disso, o acúmulo de gordura associado às perturbações metabólicas podem predispor os pacientes a desenvolverem um estado de inflamação e alterações vasculares de baixo grau que favorecem o processo de aterosclerose (15) e ao desenvolvimento de esteatose hepática, considerada hoje como componente hepático da síndrome metabólica, e tida como uma das doenças atuais mais prevalentes relacionadas com a obesidade (16). Por isso a importância de se procurar uma correlação entre as citocinas e os parâmetros clínicos da obesidade, com o objetivo de identificar situações de risco relacionadas com a obesidade, e assim tentar preveni-las e ou tratá-las precocemente.

Neste contexto tem sido amplamente reconhecido que a obesidade é um estado de inflamação de baixo grau, onde o tecido adiposo é responsável pela geração de quantidades substanciais de moléculas pró-inflamatórias, mas com mecanismos fisiopatológicos ainda pouco compreendidos. Demonstrar variantes no comportamento biológico destas citocinas, associadas com um aumento da susceptibilidade à obesidade e as suas comorbidades nos apresenta como um potencial marcador para identificar relações causais em futuras investigações na compreensão de qual é a contribuição dos genes inflamatórios na predisposição e evolução da obesidade, nos seus mecanismos moleculares e em futuras opções terapêuticas para a prevenção desta pandemia.

2 OBJETIVOS

2.1 Objetivo geral

Estudar a expressão de interleucina-6 (IL-6) e fator de necrose tumoral-alfa (TNF-alfa) em tecido adiposo e hepático em pacientes obesos graves e a sua associação com as medidas clínicas, bioquímicas, antropométricas e com algumas comorbidades associadas.

2.2 Objetivos específicos

- Quantificar e comparar a expressão de IL-6 e TNF-alfa em pacientes obesos, caquéticos e eutróficos.
- Correlacionar a expressão de IL-6 e TNF-alfa com doenças sabidamente relacionadas à obesidade como deslipidemia e hiperglicemia.
- Comparar a expressão de IL-6 e TNF-alfa no tecido hepático com a gravidade da doença hepática gordurosa não alcoólica (NAFLD) em pacientes obesos graves.

3 REVISÃO DA LITERATURA

3.1 Obesidade

Segundo o conceito dado pela Organização Mundial da Saúde (OMS) baseada em padrões internacionais desenvolvidos para pessoas adultas descendentes de europeus, obesidade e obesidade grave se definem como um índice de massa corpórea (IMC) acima de 30 Kg/m^2 e um IMC acima de 40 Kg/m^2 ou acima de 35 Kg/m^2 (se houverem comorbidades associadas) respectivamente (17). De acordo com a Associação Brasileira para o Estudo da Obesidade e da Síndrome Metabólica, em suas diretrizes publicadas em 2010, em geral não é difícil reconhecer a obesidade ou até mesmo o sobrepeso, mas o diagnóstico correto requer que se identifiquem os níveis de risco, o que, frequentemente, necessita de algumas outras formas de quantificação, donde ser necessário entender-se que o IMC é apesar de ser muito utilizado, não está totalmente correlacionado com a gordura corporal (18). As principais limitações deste método são: não distinguir a massa gordurosa da massa magra; não refletir, necessariamente a distribuição da gordura corporal; não indicar, necessariamente, o mesmo grau de gordura em populações diversas, particularmente por causa das diferentes proporções corporais entre os indivíduos (18, 19).

A epidemia da obesidade tem crescido em todo o mundo, em países ricos ou pobres, entre todos os segmentos da sociedade, e as duas razões mais comumente alencadas para o aumento da prevalência da obesidade são algumas práticas de comercialização de alimentos e reduções na atividade física (20). O ganho de peso é um problema de saúde pública mundial e um fator de risco para várias doenças, sendo inegável a ligação entre as taxas crescentes de obesidade e aumento dos custos com assistência à saúde. A obesidade leva a distúrbios das condições de saúde do organismo e essas alterações podem ser representadas por distúrbios psicológicos, sociais, aumento do risco de morte prematura e o aumento de risco de doenças de grande morbi-mortalidade, como *diabetes mellitus* (DM), hipertensão arterial sistêmica (HAS), dislipidemias, doenças cardiovasculares (DCV), esteatose hepática não alcoólica (NAFLD) e câncer (21).

Dentre estas comorbidades ligadas à obesidade destaca-se uma das mais prevalentes doenças na sociedade moderna, o *diabetes mellitus*. O maior risco para o desenvolvimento desta doença se relaciona diretamente ao IMC, sendo que, quando este parâmetro está acima de 35, ocorre um aumento do risco do seu desenvolvimento em 93 vezes no sexo feminino e de 42 vezes no sexo masculino (22). Comparando-se ainda pessoas de peso normal, homens com 20% acima do peso desejável têm 20%, a mais de chance de vir a morrer do que a população eutrófica de mesma faixa etária; possui o risco duas vezes maior de falecer por diabetes, 40% a mais de chance de desenvolver disfunções na vesícula biliar e um aumento de 25% de chance de desenvolver doenças coronarianas. Já em homens com peso 40% acima do desejável a mortalidade é 55% maior que na população eutrófica na mesma faixa etária, sendo 70% maior a chance de desenvolverem doenças coronarianas, e com o risco de morte por diabetes quatro vezes maior (23).

Em estudo realizado por Oliveira, a estimativa de custos decorrentes da obesidade pelo sistema público de saúde no Brasil em 2001 foi de quase meio bilhão de reais, quando somamos os custos da obesidade aos custos das outras doenças associadas (comorbidades) que poderiam ter sido evitados se a obesidade tivesse sido prevenida. Como resultado, verificou-se que, em 2008 a 2009, nada menos do que 1,55 milhões de adultos brasileiros apresentavam obesidade grave, totalizando 0,81% da população brasileira, com uma maior prevalência na região sul, entre as mulheres e em pessoas melanodérmicas. Em 2011, os custos atribuíveis à obesidade no Sistema Único de Saúde (SUS) totalizaram R$487,98 milhões, correspondendo a 1,9% dos gastos com assistência à saúde de média e alta complexidade. Por outro lado, os custos da obesidade grave especificamente, perfizeram 23,8% dos custos com a obesidade, R$ 116,2 milhões, apesar de sua prevalência ser 18 vezes menor, ou seja, neste caso a obesidade grave apresentou custo proporcionalmente 4,3 vezes maior do que “obesidade não grave” (24).

3.2 Obesidade e inflamação

Originalmente o tecido adiposo era visto simplesmente como um órgão de armazenamento, mas nos últimos tempos o interesse no estudo do tecido adiposo tem aumentado substancialmente. Este tecido é visto agora como um órgão dinâmico

metabolicamente associado ao excesso de energia, que serve como um órgão endócrino capaz de sintetizar uma variedade de compostos biologicamente ativos que regulam a homeostase do organismo e que também podem causar em certas condições um estado de estresse oxidativo com um impacto significativo no aparecimento e progressão de várias doenças (25-27). Grande parte da fisiopatologia desta "síndrome", obesidade, encontra-se fundamentada na expressão de marcadores inflamatórios, que denotam um estado inflamatório crônico que se relaciona direta e ou indiretamente de forma apócrina, parócrina e endócrina com a secreção de citocinas pelo tecido adiposo.

Vários outos fatores contribuem para a obesidade e suas complicações, incluindo, mas não necessariamente se limitando, à dieta e ao estilo de vida (28). A estreita interação entre a função do sistema imunológico e o tecido adiposo contribui para uma plausível associação do processo inflamatório e obesidade. Adipócitos e macrófagos partilham muitas características funcionais semelhantes, e eles são de fato semelhantes visto que, por exemplo, os pré-adipócitos têm a capacidade, sob certos estímulos de se diferenciarem em macrófagos (29, 30). No entanto, evidências recentes sugerem que o aumento de citoquinas pró-inflamatórias não é apenas uma consequência da obesidade (31), mas um fator associado à resistência à ação da insulina e outras desordens associadas à obesidade, como hiperlipidemia e síndrome metabólica (definida por um conjunto de alterações metabólicas que incluem resistência à insulina, intolerância à glicose, hiperinsulinemia, aumento do VLDL colesterol, diminuição do HDL colesterol e HAS). A obesidade pode ser o resultado de uma doença inflamatória sistêmica mais complexa (32-35).

Na obesidade, a consequência do aumento no tamanho dos adipócitos nessa condição fisiopatológica é a necrose dessas células devido à hipóxia e a perda gradual de sua capacidade de neutralizar a produção dos radicais livres e manter a homeostase intracelular evitando assim a lise celular e o processo inflamatório subsequente. Esse perfil inflamatório do tecido adiposo disfuncional é caracterizado em parte pelo aumento da secreção das citocinas pró-inflamatórias, IL-6 e do TNF-alfa, da proteína quimiotática de monócitos 1 (MCP-1) e pela redução da expressão da adipocina anti-inflamatória como a adiponectina, dentre muitos outros. Além disso, a liberação de ácidos graxos insaturados pelo tecido adiposo hipertrófico proporciona também uma estimulação inflamatória adicional através de suas interações com receptores do tipo *toll*

like (TLRs) e subsequente ativação do fator nuclear κB (NF-κB) (36, 37). Na obesidade, existe uma sobrecarga da capacidade funcional do retículo endoplasmático (RE) e mitocondrial e este "estresse" leva à ativação de vias de sinalização inflamatórias agravando ainda mais a resistência insulínica e aumentando a produção mitocondrial de espécies reativas de oxigênio (EROS) (38). A ativação dos receptores Toll-like (TLRs) (receptores transmembrana que participam do reconhecimento de patógenos durante a resposta inflamatória) tanto por lipopolissacarídeos, quanto por ácidos graxos livres estão associados diretamente a estimulação da via de sinalização NFkB e indiretamente a expressão de genes de citocinas inflamatórias tais como TNF-alfa e IL-6 em adipócitos (39, 40).

Nesse sentido, o estresse oxidativo crônico é fonte de várias das alterações metabólicas decorrente do excesso de tecido adiposo no organismo, havendo muitas questões a serem respondidas sobre qual a origem e a função dos marcadores inflamatórios na obesidade. Existem algumas hipóteses levantadas:

- A primeira é aquela que remete a produção e liberação destas citocinas primariamente a partir de órgãos que não o tecido adiposo (o fígado e células do sistema imunológico principalmente).
- A segunda explicação é que o tecido adiposo secretaria fatores e citocinas que estimulariam a produção de marcadores inflamatórios por outros órgãos.
- A terceira possibilidade é que os próprios adipócitos são uma fonte imediata de alguns, ou de muitos desses marcadores inflamatórios.
- Existe também a possibilidade de haver uma combinação dessas três situações (41).

3.3 Tecido adiposo

A ocorrência de complicações da obesidade depende não apenas do excesso de peso, mas também da distribuição da gordura corporal, que pode estar localizada na região central ou abdominal. Consequências adversas para a saúde são relacionados com a obesidade, no entanto, estão menos relacionadas à deposição de gordura corporal total do que com a distribuição desta gordura no corpo, onde a presença de tecido adiposo intra-abdominal é um fator de risco importante para os distúrbios metabólicos (42).

O tecido adiposo pode ser classificado:

- Pela sua disposição em relação aos outros orgãos como no subcutâneo; periórgão e intersticial;
- De acordo com a sua função onde encontramos o tecido adiposo branco relaciona-se com o isolamento térmico e armazenamento de energia; o tecido adiposo marrom associa-se à termogênese; o tecido adiposo mamário é o responsável pelo crescimento do epitélio mamário e produção de leite e o tecido adiposo da medula óssea encarregado da hematopoiese e osteogênese (43).
- Quanto à função metabólica o tecido adiposo pode ser classificado como tecido adiposo subcutâneo superficial e profundo e tecido adiposo interno visceral e não visceral. Dentre os tecidos adiposos, o tecido subcutâneo é o mais abundante e detém a maior capacidade de expansão nos seres humanos enquanto o tecido adiposo interno compreende a gordura corporal total menos o subcutâneo sendo que o tecido adiposo interno não-visceral de maior interesse metabólico é relacionado aos músculos (44).

O tecido adiposo é constituído de, além dos adipócitos, também por uma matriz de tecido conjuntivo, nervoso, células do estroma vascular, nódulos linfáticos, células do sistema imune (linfócitos e macrófagos), fibroblastos e pré-adipócitos. A gotícula de gordura dos adipócitos é revestida por fosfoproteínas, designadas de perilipinas, as quais participam dos processos de armazenamento e liberação de ácidos graxos pela célula, elas previnem a lipólise em condições basais impedindo o acesso das lipases citosólicas à gotícula de ácidos graxos intracelulares (45). Esta célula adiposa acumula ácidos graxos durante seu ciclo de vida onde o aumento do tamanho do adipócito é acompanhado pelo aumento dos processos de captação de glicose, lipólise e lipogênese até um ponto de inflexão onde o limite de estocagem no adipócito é atingido e neste momento as vias lipogênicas são diminuídas, há diminuição da sensibilidade à insulina e direcionamento da glicose à lactatogênese, o que caracteriza um processo tardio e adaptativo que limita o acúmulo adicional de gordura neste tecido já saturado (46-48).

O significado patológico de acúmulo de gordura visceral é atribuído às características específicas destes adipócitos, especificamente, às diferenças nas características estruturais, fisiológicas e metabólicas em comparação com a gordura subcutânea branca e marron. Cerca de 85% da massa total de tecido adiposo, em seres humanos magros e ou obesos, é subcutânea, enquanto que os 15% restantes constituem a gordura intra-abdominal, incluindo a visceral e a retroperitoneal (49). A gordura visceral está mais

propensa à lipólise, pois expressa maior número de receptores de glicocorticóides, além de ser mais sensível a catecolaminas, e apresentar menor expressão do substrato do receptor de insulina da membrana citoplasmática (IRS-1) sendo, assim, menos sensível à ação deste hormônio (50). O excesso de gordura visceral apresenta um estado hiperlipolítico, liberando altas concentrações de ácidos graxos livres (AGLs) na circulação que podem resultar em deposição ectópica de gordura em vários órgãos (fígado, músculo esquelético, pâncreas, rins, coração e artérias), além de estresse oxidativo, estresse do retículo endotelial e apoptose (51). Este tipo de tecido apresenta também uma maior produção de adipocinas inflamatórias como o inibidor do ativador do plasminogênio-1 (PAI-1), IL-6 e o TNF-alfa que contribuem para a resistência à insulina, e para o estado próinflamatório, pró-trombótico e pró-hipertensivo da obesidade visceral, além disto, a proteína atrativa de monócitos-1 (MCP-1), também secretada pelo adipócito, atrai macrófagos, que se infiltram no tecido adiposo e produzem citocinas, fato este que contribui mais ainda para a perpetuação do quadro inflamatório crônico (52, 53). A gordura subcutânea, por outro lado, parece ter uma função metabolicamente protetora, já que apresenta alta atividade da lipase lipoprotéica (LPL), que hidrolisa os triglicerídeos (TAGs) circulantes, permitindo a entrada de ácidos graxos (AGLs) nos adipócitos, o que protegeria os órgãos de altas concentrações de AGLs pela sua maior capacidade de armazenamento de AGLs (50).

A disfunção desencadeada pelos lipídeos é chamada de lipotoxicidade e seu extremo é a morte celular programada (lipoapoptose) (54). Consequências adversas à saúde estão associadas a indivíduos com gordura corporal aumentada e, em particular, com excesso de gordura visceral (55). No fígado principalmente a exposição crônica a ácidos graxos livres liberados pelos adipócitos viscerais promove a gliconeogênese hepática, e uma redução da atividade das enzimas envolvidas na oxidação de AGLs, aumentando a lipogénese e a concentração de triglicéridos no fígado (55, 56). O acúmulo promovido pela obesidade no teor de triglicerideos intra-hepática assim como níveis aumentados de vários hormônios e ou citocinas derivadas de tecido adiposo são indicadores altamente correlacionados à disfunção metabólica, mas entretanto ainda a associação explícita e direta entre o aumento da massa adiposa visceral e desregulação metabólica não está completamente definida (57, 58).

3.4 Obesidade e as Citocinas TNF-alfa e IL6

As citocinas são hormônios protéicos tipicamente conhecidos como mediadores e reguladores de respostas imunes e inflamatórias, mas outros efeitos como, por exemplo sensores do balanço energético também têm sido atribuídos a algumas citocinas. A estrutura protéica, assim como a função fisiológica das adipocinas identificadas até o momento, é altamente variada e compreende proteínas relacionadas ao sistema imune, como as citocinas clássicas (TNF-alfa e IL-6), fatores de crescimento (fator transformador de crescimento TGF-b) e proteínas da via complemento alternativa (adipsina) dentre outras (49). Com a hipertrofia e hiperlasia do tecido adiposo e a infiltração de macrófagos neste tecido, numerosas citocinas pró-inflamatórias, incluindo o TNF-alfa e IL-6 são concomitantemente aumentadas tanto neste tecido como nos níveis séricos levando a um estado de estrese inflamatório sistêmico (60, 61).

A citocina TNF-alfa foi clonada em 1985 e está localizada no cromossomo (6p21), sendo atualmente conhecida como apenas um dos polipeptídeos da superfamília de 10 ligantes transmembrânicos glicoproteicos. Em seres humanos o TNF-alfa é sintetizado e secretado principalmente pelas células do estroma vascular e da matriz intersticial, incluindo os macrófagos, entretanto, sob condições não fisiológicas o tecido adiposo é capaz de produzir uma quantidade relativamente grande de TNF-alfa, fato este que levou esta citocina a ser considerada como uma adipocina (62,63). Ao que parece, existe uma hierarquia das citocinas dentro do tecido adiposo branco onde o TNF-alfa desenvolve um papel fundamental relacionado com a produção e regulação de várias outras citocinas e adipocinas (64, 65).

Esta citocina é um potente regulador interno do tecido adiposo que atuando de forma autócrina e parácrina influencia vários processos intracelulares, incluindo a apoptose celular. As ações do TNF-alfa são mediadas principalmente por dois receptores distintos e a ligação do TNF-alfa a esses receptores induz a transdução de sinais intracelulares que podem resultar na ativação de diferentes eventos como na regulação de apoptose, indução e ativação da transcrição de genes de resposta inflamatória inclusive de genes codificadores de outras citocinas como IL-1, IL-6 e IL-10 (66-68). Estudos em modelos animais indicaram que o TNF-alfa desempenha também um papel importante na mediação de resistência à insulina na obesidade através da sua super-expressão em tecido gorduroso (69, 70) e nos macrófagos que se encontram infiltrados no tecido

adiposo e que também são responsáveis pela secreção de altas concentrações de TNF-alfa, contribuindo para manutenção do estado de inflamação crônica acompanhado do metabolismo alterado de triglicérides e do aumento da lipólise (71, 72). No metabolismo lipídico age reprimindo a expressão de genes envolvidos na captação e armazenamento de ácidos graxos livres estimulando a lipólise por aumentar a atividade da lipase hormônio-sensível que retira os lipídeos estocados no tecido adiposo e libera na circulação (73,74). Quanto ao metabolismo glicídico o TNF-alfa contribui para a desregulação da via da sinalização insulínica através da fosforilação em serina dos substratos do receptor desse hormônio (IRS) além de alterar o metabolismo da glicose diminuindo a expressão do transportador de glicose GLUT4 (75-78).

A IL-6 é uma citocina com imuno-modulação, sendo que nos seres humanos é codificada pelo gene localizado no cromossomo 7 sendo secretada principalmente por células T e macrófagos para estimular a resposta imune e regular à homeostase celular (79, 80). É conhecida como uma citoquina pró-inflamatória que regula a imunidade inata e a resposta de fase aguda, no entanto também tem efeitos específicos nos tecidos que podem ser diferentes, dependendo do contexto e do tempo de estimulação. Uma das funções mais conhecidas e estudadas da IL-6 é estimular a produção Proteína C-reativa (CRP), que é a principal mediadora da resposta inflamatória de fase aguda, e possui funções imuno-reguladoras importantes como o recrutamento e ativação do sistema complemento, o aumento da reatividade leucocitária, o estímulo da liberação de citocinas (IL-1, IL-18, da própria IL-6 e o TNF-alfa) e também diversas outras ações implicadas na patogênese de doenças cardiovasculares, síndrome metabólica, intolerância à glicose e diabetes tipo 2 (81,82, 83). Acredita-se que o tecido adiposo seja responsável pela secreção de mais de 35% da IL-6 circulante em pessoas saudáveis (84- 86).

Produzida principalmente pelo tecido adiposo visceral de modo semelhante ao TNF-alfa, a IL-6 está associada também à obesidade e à resistência insulínica, suprimindo a expressão de adiponectina e dos receptores e sinalizadores de membrana da insulina (87, 88). Parece que a IL-6 pode agir de formas distintas, dependendo da sua concentração orgânica, tanto nos tecidos periféricos quanto no sistema nervoso central, influenciando o peso corporal, a homeostase energética, a sensibilidade insulínica e a fome sendo que dentre os fatores que podem induzir a sua expressão estão o TNF-alfa,

a IL-1, outros agentes ativadores de linfócitos e macrófagos e a própria insulina e catecolaminas via receptores beta-adrenérgicos (75,89, 90). Dados recentes sugerem que, enquanto o TNF-alfa age de forma parácrina no adipócito, a IL-6 circula no plasma em concentrações relativamente altas, sendo portanto, muito mais importante sistemicamente no quadro metabólico global, além disso a IL-6 derivada do tecido adiposo, tem efeitos autócrinos que aumentam a secreção de leptina e a oxidação da gordura pelo adipócito (91, 92).

Com a hiperplasia e hipertrofia do tecido adiposo e com a infiltração de macrófagos neste mesmo tecido, o TNF -alfa e a IL-6 estão super expressos levando a um estado inflamatório crônico que favorece a liberação de proteínas quimioatraentes para macrófagos, promove um estado de hiperinsulinemia (resinstência a insulina) que por sua vez induz a um aumento subsequente da lipólise e de ácidos graxos circulantes levando o organismo a um estado de lipotoxidade (91) .

3.5 Doença gordurosa do fígado não alcoólica / DHGNA

A Doença hepática gordurosa não alcoólica (DHGNA) é a manifestação hepática da síndrome metabólica e frequentemente coexiste com obesidade, dislipidemia e resistência à insulina (RI) sendo a doença do fígado mais comum em países ocidentais, afetando 20% a 30% da população geral (94,95). É sabido que a prevalência de DHGNA tende a ser maior entre o sexo masculino, aumentando com a idade (a partir de 2,6% entre as crianças e até 26% entre as pessoas de 40-59 anos), apresentanto uma maior incidência entre as pessoas com diabetes (em torno de 50%) e nos pacientes obesos (76%), sendo que é quase universal entre as pessoas diabéticas portadoras de obesidade mórbida. A forma mais grave da doença NASH está presente em 18,5% dos obesos (contra 2,7% de indivíduos magros) e em 50% das pessoas com obesidade grave e diabetes (96, 97).

A definição e diagnóstico histológico da DHGNA consiste na presença de depósitos gordurosos (esteatose macrovesicular) em $\geq$ 5% dos hepatócitos, em indivíduos que consomem nenhum ou pouco álcool (ingestão diária inferior a 10 g para as mulheres, e inferior a 20 g para os homens), na ausência de evidência viral, auto-imune ou doença hepática induzida por drogas. Esta entidade compreende dois subtipos principais: o espectro histológico da DHGNA na sua forma benigna consiste em esteatose simples

denominada (NAFL), e a forma de esteato-hepatite não alcoólica (NASH), que é a forma progressiva da DHGNA que pode levar à cirrose, carcinoma hepatocelular (HCC) e ainda resultar em falência hepática e morte (94).

A DHGNA é uma doença poligênica e multifatorial, na qual a associação de genes relacionados é exuberante e a participação do ambiente, relacionando à dieta e ao sedentarismo, também tem sua importância fundamentada. Desta forma, sua fisiopatogênese é bastante complexa e envolve múltiplos fatores, dentre eles a deposição excessiva de gordura no fígado, o que pode decorrer do aumento da oferta de ácidos graxos (AG) do tecido adiposo, aumento da síntese de novos AG, aumento da ingestão de lipídeos dietéticos, diminuição da β-oxidação mitocondrial, diminuição da exportação de partículas de lipoproteína de muito baixa densidade (VLDL) ou destes fatores em combinação. A síndrome metabólica, "bypass" jejuno-ileal, desnutrição calórica-protéica, nutrição parenteral prolongada, uso de drogas e perda de peso superior a 1600g/semana também são também conhecidos como fatores desencadeantes da DHGNA (98).

Compreender a fisiopatologia da esteatose hepática é extremamente importante para desenvolver intervenções terapêuticas eficazes sendo que algumas hipóteses vêm sendo consideradas para explicar a patogênese da DHGNA e sua evolução para NASH, destacando-se dentre elas a teoria dos dois "hits" que apontam a resistência insulínica (RI) como condição inicial ("first hit") para acúmulo de ácidos graxos no hepatócito e o estresse oxidativo como o segundo estímulo para o desenvolvimento de inflamação e fibrose ("second hit"). Segundo essa hipótese, a hiperinsulinemia favorece a lipogênese e inibe a lipólise, inclusive no fígado, promovendo condições predisponentes para a infiltração gordurosa da glândula e se associando com a progressão para as formas mais graves de DHGNA, NASH e cirrose (97, 98). Associada à RI, a síndrome metabólica constitui um achado frequente em pacientes de DHGNA sendo observado que a presença de 3 critérios de síndrome metabólica em pacientes portadores de DHGNA eleva em 3,5 vezes o risco para desenvolver fibrose acentuada e ou doença hepática crônica (96, 99).

O estresse oxidativo ("second hit"), gerado neste contexto, por sua vez, seria importante na evolução de esteatose para esteatohepatite e fibrose. A lesão hepatocelular resultaria do estresse oxidativo, peroxidação lipídica e toxicidade celular

direta de ácidos graxos de cadeia curta (AGCCs). Foram propostos vários mecanismos sobre lesão do hepatócito dentre eles o aumento da expressão da isoforma do citocromo P450 CYP2E1 (mais expressiva em pacientes com NASH) e relacionada com aumento da produção de espécies reativas de oxigênio, que são capazes de peroxidar as membranas celulares. É sabido também que o aumento da insulina levaria à lipólise periférica, resultando em excesso de ácidos graxos que catalisam os peroxissomas lipídicos e que estariam envolvidos no processo de oxidação de ácidos graxos (97, 98).

Neste contexto onde o estresse oxidativo teria um papel importante na fisiopatologia e evolução da doença observa-se que os níveis de algumas citocinas pró-inflamatórias, tais como o TNF alfa e IL-6 estão elevados tanto no tecido hepático como no adiposo (100) . Os mecanismos moleculares que levam da esteatose hepática para para um quadro mais grave de esteatohepatite ainda permanecem obscuros. Há necessidade de melhor compreensão do papel das células hepáticas inflamatórias, e das citocinas inflamatórias como TNF-alfa ou IL-6, parece óbvia, no entanto, ainda é uma questão em debate. O TNF é conhecido principalmente pelo seu papel central na resistência à insulina e é criticamente envolvido na hepatite não alcoólica, uma vez que a expressão da IL-6 é também estimulada pela ativação de TNF (98,101) e que por sua vez a expressão aumentada de IL-6 no fígado de pacientes com NASH está associada a níveis elevados da mesma no sangue, fato este relacionado com a gravidade da doença esteatótica do fígado não alcoólica (102).

4 PRODUTOS

Os produtos foram três artigos científicos

4.1 Produto 1: *Comparative Expression of Visceral Adipose Tissue Inflammatory IL6, TNF-α e SIRT 1 -Related Genes of Normal Weight, Cachectic, and Morbidly Obese Human,* formatado segundo as normas para publicação do periódico Journal of Obesity enviado.

4.2 Produto 2: *Interleukine-6 (IL-6) expression in white and brown adipose tissue of obese patients and its association with metabolic and inflammatory profile* formatado segundo as normas para publicação do periódico Journal of Inflammation enviado.

4.3 Produto 3: *Body mass index and interleukin-6 and tumor necrosis factor-alpha adipose tissue expressions are associated to morphological severity of non-alcoholic fatty liver disease in morbidly obese patients* formatado segundo as normas para publicação do periódico Hepatology enviado.

4.1 Produto 1

Comparative study of gene expression of IL-6, TNF-α and SIRT 1 in visceral adipose tissue of humans of normal weight, morbidly obese and cachectic

Running title: Inflammatory markers in human white adipose tissue

Antônio Sérgio Barcala Jorge[a], João Marcus Oliveira Andrade[a], Alanna Fernandes Paraíso[a], Gislaine Cândida Batista Jorge[a], Thaísa Soares Crespo[a], Cássio André Vieira[a], André Luiz Sena Guimarães[a], Alfredo Maurício Batista de Paula[a#], Sérgio Henrique Sousa Santos[a,b#]

[a] Health Science Post-graduate Programme. Health Research Laboratory. Universidade Estadual de Montes Claros (Unimontes), Montes Claros, Minas Gerais, Brazil.
[b] Department of Pharmacology. Institute of Biological Sciences. Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.
# Equally contributed to this study
Corresponding authors:
Sérgio H S Santos
Pharmacology Department. Universidade Federal de Minas Gerais.
Av. Antonio Carlos 6627 – ICB 31270-901, Belo Horizonte, Minas Gerais, Brazil.
FAX/Phone: +55313409-2695
Email: sergiosousas@hotmail.com

Alfredo Maurício Batista De-Paula.
Health Science Post-graduate Programme. Health Research Laboratory.
Universidade Estadual de Montes Claros (Unimontes).
Hospital Universitário Clemente de Faria (HUCF).
Av. Cula Mangabeira, 562. Bairro Santo Expedito. Montes Claros, Minas Gerais, Brazil. CEP: 39401-001. Phone: +553832248327.
E-mail: ambpatologi@gmail.com

ABSTRACT

Background: White adipose tissue (WAT) constitutes our most expandable tissue and largest endocrine organ secreting several hormones collectively named adipokines. Changes in WAT mass induce alterations in adipocyte secretion function and inflammation, which are linked to disturbed whole-body metabolism. Although the mechanisms controlling this are not clear they are dependent on changes in gene expression, a complex process which is regulated at several levels.

Aim: Compare the expression of inflammatory markers and related genes: TNF-α, IL-6, and a member of sirtuin family - SIRT1 in human sample of visceral adipose tissue of eutrophic, obese and cachectic subjects.

Material and Methods: The subjects were divided in three groups: control group (lean patients; n=14); obese patients groups (n=20); and cachectic patients group (n=14). The samples were collected during surgeries following previous protocols. Expression of TNF-α, IL6, and SIRT1 were measured by quantitative real time polymerase chain reactions (qRT-PCR). Morphometric analyses were performed using total number and area of adipocytes by conventional microscopy.

Results: Our findings showed higher expression to TNF-α and IL-6 in obese and cachectic groups when compared with control group (lean subjects). Additionally, it was observed lower expression to SIRT1 in obese and cachectic groups compared with control group. These results were accompanied to lower area and higher number/field in adipocytes to cachectic group and higher area and lower number/field in adipocytes to obese group in relation lean group.

Conclusions: Obesity and cachexia conditions present similar expression to TNF-α and IL-6, with a significant increase in relation to lean patients.Obese patients had a higher expression and a higher correlation between TNF-alpha and IL-6 expression between the groups which can be explained, in part, to the greater volume of adipose tissue in these patients. Although the expression of SIRT1 be significantly lower in the group of patients with cachexia and obese group in this study was not possible to correlate the expression of this sirtuin with citocianas IL6 and TNF-alpha

Key words: Obesity. Cachexia. White Adipose Tissue. Inflammatory Markers.

1. INTRODUCTION

White adipose tissue (WAT) is a dynamic organ, which is involved in body homeostasis and exerts important functions, such as storage and release of energy, thermally insulate and body mechanical protection (1). In addition, in the last two decades was clearly demonstrated that WAT is an active endocrine organ, which secretes a vast number of factors with local and/or systemic effects on several processes including lipid and glucose metabolism (2-3).

Obesity is characterized by excessive accumulation of body fat with potential injury to health. Arising from these various factors are genetic or environmental, dietary patterns, physical activity or individual biological susceptibility factors (4, 5). It is considered a chronic low-grade inflammatory disease (4, 6, 7). The white adipose tissue of obese subjects is characterized by increased production and secretion of a wide panel of inflammatory molecules. These inflammatory molecules may have local effects on WAT physiology in addition to their potential effects on other organs and tissues (2, 8, 9).

It seems clear that weight loss in obese patients improve the inflammatory state by decreasing the number of circulating inflammatory molecules (10,11), however, a significant loss in body weight can induce an inverse status in function of WAT. Cachexia is a complex metabolic disorder characterized by involuntary weight loss, adipose tissue and skeletal muscle depletion, anorexia and fatigue. Cachexia is often associated with systemic inflammation indicated by elevated plasma C reactive protein (CRP) and reduced albumin levels (12-13). The mechanism involved in inflammatory status in obese and cachectic patients still poor known, being necessary to clarify the pathways and genes involved in inflammatory status on obesity and cachexia but it is accepted that increased levels of cytokines such as interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) and other factors may be involved (14-15).

On the other hand have been studied the function of a protein family, called sirtuins (SIRT) in many cellular processes and mechanisms such as metabolism and aging (16-17). In humans, the sirtuin family is composed by the homolog of yeast silent

information-regulator 2 (Sir2), is comprised of a highly conserved family of proteins, with one or more sirtuins present in virtually all species from bacteria to mammals (18-19). The sirtuin family of proteins possesses NAD+-dependent deacetylase activity and/or ADP ribosyltransferase activity. The seven mammalian sirtuins (SIRT1–7) are localized differentially within the cell and have a variety of functions (20). SIRT1 is the most extensively studied member of the family and regulates several biological processes ranging from cell proliferation, differentiation, apoptosis, and metabolism (21).

In this context, the present study aimed to analyze the expression of inflammatory markers and related gene: TNF-α, IL-6, and a member of sirtuin family - SIRT1, in human sample of visceral adipose tissue of eutrophic, obese and cachectic subjects.

1. MATERIALS AND METHODS

2.1 Ethical aspects

Ethical approval for present study was obtained from a relevant local ethic committee (Plataforma Brasil – 85742/13-07-2012). All subjects of the study consent to signing the consent form to participate in this research.

2.2 Subjects

The study was divided in three groups: G1 – control group; G2 – obese patients groups; and G3 – cachectic patients group.

The control group consisted of 14 adult patients (mean age 50.01 ± 16.42), with a BMI within the normal range, clinically stable who underwent abdominal surgeries for performing ostomy. All control subjects showed mean weight of 61.30 Kg ± 9.59 Kg and mean body mass index (BMI) of 21.76 ± 2.01, range from 18.5 to 25 kg/m2.

Obese patients group (n = 20; mean age: 41.55 ± 12.44, all obese showed BMI above 38 Kg/m^2). The biopsies of white adipose tissue were obtained during the bariatric surgery. Bariatric procedures consisted of laparoscopic adjustable gastric banding or a

laparoscopic Roux-en-Y gastric bypass depending on the degree of obesity and other patient characteristics. A team of two surgeons executed all operations.
Cachectic patients composed the third group (n = 14; mean age: 72.14 ± 8.03). All obese and cachectic patients were categorized using previous criteria (Evans et al which took into account the weight loss in the last 12 months, decreased muscle strength, weakness, decreased mass index of body fat, anemia and hypoalbuminemia) and were not carriers of malignancies. The biopsies were obtained during gastrointestinal surgery. (Table1)

2.3Samples

The samples were collected from surgeries performed at Hospital Universitário Clemente Faria, Universidade Estadual de Montes Claros - Brazil. During the surgeries sample of visceral white adipose tissue were collected and immediately frozen, and stored at -80 °C.

2.4 RNA isolation and quantitative real-time PCR analysis

Total cellular RNA was extracted by using Trizol reagent according to the manufacturer's instructions (Invitrogen Corp.®, San Diego, California, USA). cDNAs were synthesized from 1 µg of total RNA by using the moloney murine leukemia virus reverse transcriptase (M-MLV RT) system (Invitrogen™, Life Technologies Corp., USA). The cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) with specific gene primers (Applied Biosystems®, USA) as follows (forward and reverse): Beta actin (5'- CGG CGA CGA CCC ATT CGA AC - 3' and 5'- GAA TCG AAC CCT GAT TCC CCG TC - 3)'; TNF-α (5'- GAG CTG AAC AAT AGG CTG TTC CCA - 3' and 5'- AGA GGC TCA GCA ATG AGT GAC AGT - 3'); IL-6 (5'- AAA TTC GGT ACA TCC TCG ACG GCA - 3'; 5'- AGT GCC TCT TTG CTG CTT TCA CAC - 3'), and SIRT1 (5'- ACT GCA ACA TTC CGG GAC TCT ACT -3'; 5'- AGA GAA CGG CCT TGT CCT TCT TGA - 3'). All qRT-PCR analyses were performed by using the Applied Biosystems StepOne™, Real-Time PCR Systems (Foster City, CA, USA). For each condition, mRNA expression of biomarkers was

quantified in duplicate. Beta actin was used as the endogenous control in the comparative cycle threshold (C_T) method.

2.5 Morphometric analysis of visceral white fat cells

Samples of visceral white adipose tissue samples were fixed in 10% neutral-buffered formalin at 40 C° overnight, dehydrated through a graded alcohol series, xylene and paraffin, and then embedded in paraffin. Sections of 5 μm were prepared for hematoxylin and eosin stain (H&E). Images (x40 objective lenses) were captured using an optical OlympusBH2 microscope (model CX31; RTSF, Miami, USA), with 10 ocular and 40 objective lenses and an ocular lattice. A total area of 1.84 mm2, containing at least 100 fat cells for each sample, was measured using the Image-Pro Plus - Media Cybernetics Software (Rockville, MD, USA).

2.6 Statistical analysis

All data were transferred and statistical tests performed with Graphic Pad Prism 5.0 software (San Diego, California, USA) and analyzed with 95% confidence. The association between TNF-α, IL-6, and SIRT1 expression in control, obese and cachectic groups were evaluated using Mann–Whitney U tests and Perarson's correlation . Data are expressed as the mean ± SD.

2. RESULTS

The mRNA expression to TNF-α, IL6, and SIRT1 were evaluated. According to our findings, TNF-α expression was higher in cachectic (0.957 ± 0.468 – p= 0.0453) and obese (0.973 ± 0.332 – p= 0.0055) groups compared with control group (0.645 ± 0.375) (Figure 1A). Similarly, IL6 mRNA expression was higher in cachectic (0.921 ± 0.423 – p= 0.0417) and obese (1.078 ± 0.482 – p= 0.0017) groups when compared with control group (0.641 ± 0.382).In relation to SIRT1 mRNA expression, our findings showed low in the expression for both groups (obese: 0.659 ± 0.271 – p=0.0026; cachectic: 0.926 ± 0.512 - p=0.0383) in relation to control group (1.484 ± 0.877) (Figure 1)

There was a significant correlation between the three groups of TNF-alpha and IL-6 expression. Performing linear regression observed in the obese group the β was higher

than in cachectic group and the control group individuals This study was not observed correlation between the expression of SIRT1 and the cytokine IL-6. The SIRT1 expression did not correlate with expression levels of IL6 and TNF-alpha (table2).

Obese patients had a cell number average of 10.6 cells/field and an area average of 251.79 μm^2, in cachectic group, the number of cells average was 45.4 cells/field and an area average of 60.4 μm^2. The control group had a cell number average 20.5 cells/field and an area average of 163.2 μm^2. (Figure 2).

3. DISCUSSION

The increase in TNF-α and IL-6 in samples of obese and cachectic patients in relation to eutrophic patients was expected, as indicated by previous reports (23-24). Interleukin-6 is a cytokine associated with immuno-modulation in obesity and cachexia in humans (25). IL-6 is commonly described as a pleiotropic cytokine, which is produced by a variety of cell types, and has the capacity to induce several different intracellular signaling pathways (26). Although many cytokines are involved in inflammatory processes and have the potential to be involved in cachexia, circulating IL-6 is well described as either elevated or a predictor of weight loss in human cachexia (27-28). IL-6 has been found in larger quantities in weight-losing patients with cancer than in patients with the same disease, but without weight loss (28), Iwase *et al*. (2004) reported that IL-6 was the only cytokine measured that was elevated in all 28 cachectic patients included in their study, and IL-6 levels increased in patients as they approached death (27).

IL-6 expression is also increased in obese adipose tissue; IL-6 expression in adipose tissue from obese individuals is 10-fold that in adipose tissue from eutrophic individuals (29). Also, IL-6 expression varies between adipose tissue sites: expression is higher in visceral than in peripheral adipocytes, and >90% of IL-6 expressed in adipose tissue is produced by cells other than adipocytes (29).

Understanding how IL-6 regulates the nutrients homeostasis by central and peripheral is complicated due to the conflicting and multi-systemic effects that this cytokine has under various physiological states. IL-6 is better known as a pro-inflammatory cytokine that regulates innate and acute phase response immunity (30-31). However IL-6 also has tissue-specific effects which may be different depending on the context and time of stimulation. They may promote systemic insulin resistance and this is possible because the expression of IL-6 can be modulated by the composition of fatty free acids (FFA) in the diet, and that the skeletal muscle cells might be target cells for IL-6 in some situation. Furthermore, production of cytokine IL-6 by adipocytes also presents autocrine effects that increase the secretion of leptin, and fat oxidation and may also reduce the expression and activity of lipoprotein lipase in human adipose tissue. (32).

The first indication for increased cytokine release in obesity was provided by the identification of increased expression of TNF-α, a proinflammatory cytokine, in the adipose tissue of obese mice in the early 1990s (33-34). TNF-α is expressed in and secreted by adipose tissue, its levels correlating with the degree of adiposity and the associated insulin resistance (34). Targeting TNF-α and/or its receptors has been suggested as a promising treatment for insulin resistance and type 2 diabetes (35-36). TNF-α levels are associated com cachexia conditions, being a major cytokines involves this process (24). Beutler and Cerami were the first to describe a linking between TNF-α, initially named cachectin, and a cachectic phenotype characterized by anorexia, anemia, and weakness (37). To date, a causal relationship between TNF-α-induced inflammatory signaling and muscle wasting has been firmly established in experimental models (38), and associations with acute and advanced disease have been found (39).

Cachexia syndrome appears to be multifactorial, often associated with the underlying disease process, and related to both peripheral and central neurohormonal signals regulating both appetite and energy expenditure. Inflammatory cytokines, such as TNF-α and IL-6 have been postulated to play a key pathogenic role in the decreased food intake and increased energy expenditure seen in most chronic conditions associated with the anorexia and cachexia syndrome (40).

Leptin levels are significantly lower in patients with inflammatory states such as cancer (41-42), despite correction for body fat. These low levels of leptin, however, are not associated with greater appetite or lower energy expenditure, as might be expected. Disturbances in the feedback mechanism in the hypothalamus and/or release of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, are thought to be responsible for cachexia in this setting. These circulating cytokines result in insulin resistance, lipolysis, and loss of skeletal muscle mass (43). Cytokines also suppress gastric production of the orexigenic peptide ghrelin that decreases production of inflammatory cytokines TNF-α, IL-6, and IL-1β.

Pathophysiological concentrations of cytokines, such as IL-6 and TNF-α centrally in cerebrospinal fluid may additively or synergistically act by decreasing the feed, and this effect may be present in cachexia (44). This chronic subclinical inflammation may be a marker of functional limitations in the elderly through various diseases. Inflammatory cytokines produced by adipose tissue, especially visceral fat, accelerated muscle catabolism and thus contribute to a vicious cycle that begins and sustains the sarcopenia framework. Either in obesity or sarcopenia, the muscle resistence is weak, it is a common characteristic and is associated with elevated levels of circulating pro-inflammatory cytokines (45).

Our results showing a significantly lower statistical expression in obese and cachectic. The role of sirtuin 1 in obesity and cachexia conditions are poor known. However, studies indicate increased levels of SIRT1 in caloric restriction and reduction of their levels in cases of overfeeding . Gillum, et al. found that SIRT1 depletion causes anorexia, stimulating production of inflammatory factors in the white adipose tissue (46). Additonal, SIRT1 has been shown to increase muscle precursor cell proliferation. These findings could have clinical significance since muscle precursor cell proliferation has important implications in regulating skeletal muscle growth, maintenance, and repair, and the aging-related loss of skeletal muscle mass (47).

In conclusion, in obesity and cachexia TNF-α expression and IL-6 showed a significant increase compared to healthy patients. Obese patients had a higher expression and a higher correlation between TNF-alpha and IL-6 expression between the groups which can be explained, in part, to the greater volume of adipose tissue in these patients, which is where most of the IL-6 is produced in the body. Although the expression of SIRT1 be

significantly lower in the group of patients with cachexia and obese group in this study was not possible to correlate the expression of this sirtuin with citocianas IL6 and TNF-alpha, which may indicating that other pathways may be involved in the inflammatory modulation by sirt 1.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

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FIGURE LEGENDS:

Table 1

Analysis of sex distribution in relation to the nutritional diagnosis.

Table 2

Correlation between expression of IL-6, TNF-alpha and Sirt 1 in adipose tissue of patients of three groups.

Figure 1

Figures A-C: TNF-α, IL-6 and SIRT1 mRNA expression by qRT-PCR.

Figure 2

Figures A-C: Morphometric analysis of white fat cells of obese, cachectic and control patients.

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Table 1. Analysis of sex, age and BMI: distribution in relation to the nutritional diagnosis.

Nutritional diagnosis	**Male (n)**	**Female (n)**	**Age (median)**	**BMI**
Control	*7*	7	50.01(± 16.42)	21.76(± 2.01)
Cachetic	*10*	4	72.14(± 8.03)	*
Obeses	*3*	17	41.55 (±12.44)	42.61 (± 4.00)

- Based on the criteria of Evans et al 2008

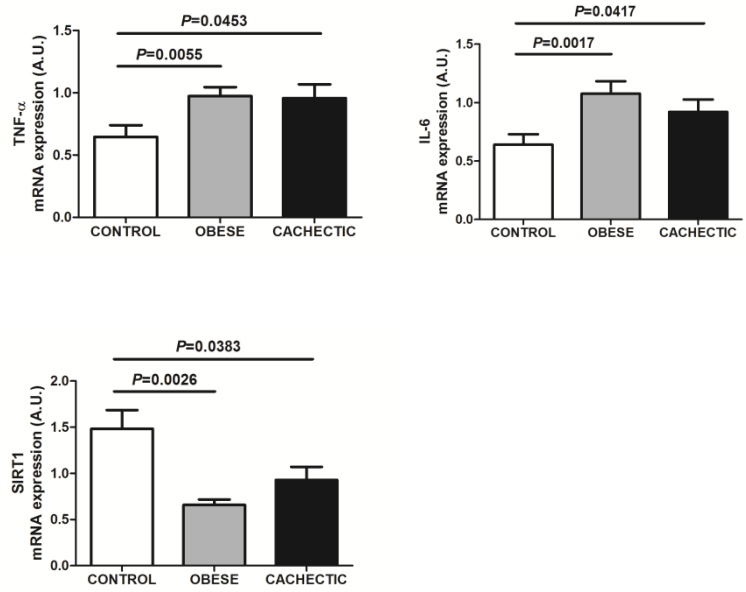

Figure 1: TNF-α, IL-6 and SIRT1 mRNA expression by qRT-PCR. Tests were performed by using Mann-Whitney U test * p values significant ($p<0.05$). We can observe a significant relationship of according to our findings, TNF-α expression was higher in cachectic (0.957 ± 0.468 – p= 0.0453) and obese (0.973 ± 0.332 – p= 0.0055) groups compared with control group (0.645 ± 0.375)). IL6 mRNA expression was higher in cachectic (0.921 ± 0.423 – p= 0.0417) and obese (1.078 ± 0.482 – p= 0.0017) groups when compared with control group (0.641 ± 0.382). SIRT1 mRNA expression, our findings showed low in the expression for both groups (obese: 0.659 ± 0.271 – p=0.0026; cachectic: 0.926 ± 0.512 - p=0.0383) in relation to control group (1.484 ± 0.877)

Table 2: Correlation between expression of IL-6, TNF-alpha and Sirt 1 in adipose tissue of patients of three groups.

	Control (n = 14)		**Cachectic (n = 14)**		**Obese (n = 21)**		**All (n = 49)**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
TNF-α vs. IL6	**0,80**	**0,00***	**0,68**	**0,01***	**0,70**	**0,00***	**0,80**	**0,00***
TNF-α *vs.* SIRT1	**0,01**	**0,95**	**0,07**	**0,80**	**0,.11**	**0,62**	**0,01**	**0,95**
IL6 *vs.* SIRT1	**0,20**	**0,39**	**0,04**	**0,87**	**0,13**	**0,56**	**0,20**	**0,39**

Table 2: Tests were performed by using Pearson's correlation * p values significant ($p<0.05$). There was a significant correlation between the three groups of TNF-alpha and IL-6 expression. Performing linear regression observed in the obese group the β (1.25) was higher than in cachectic β (0.56) group and the control group individuals β (0.46).

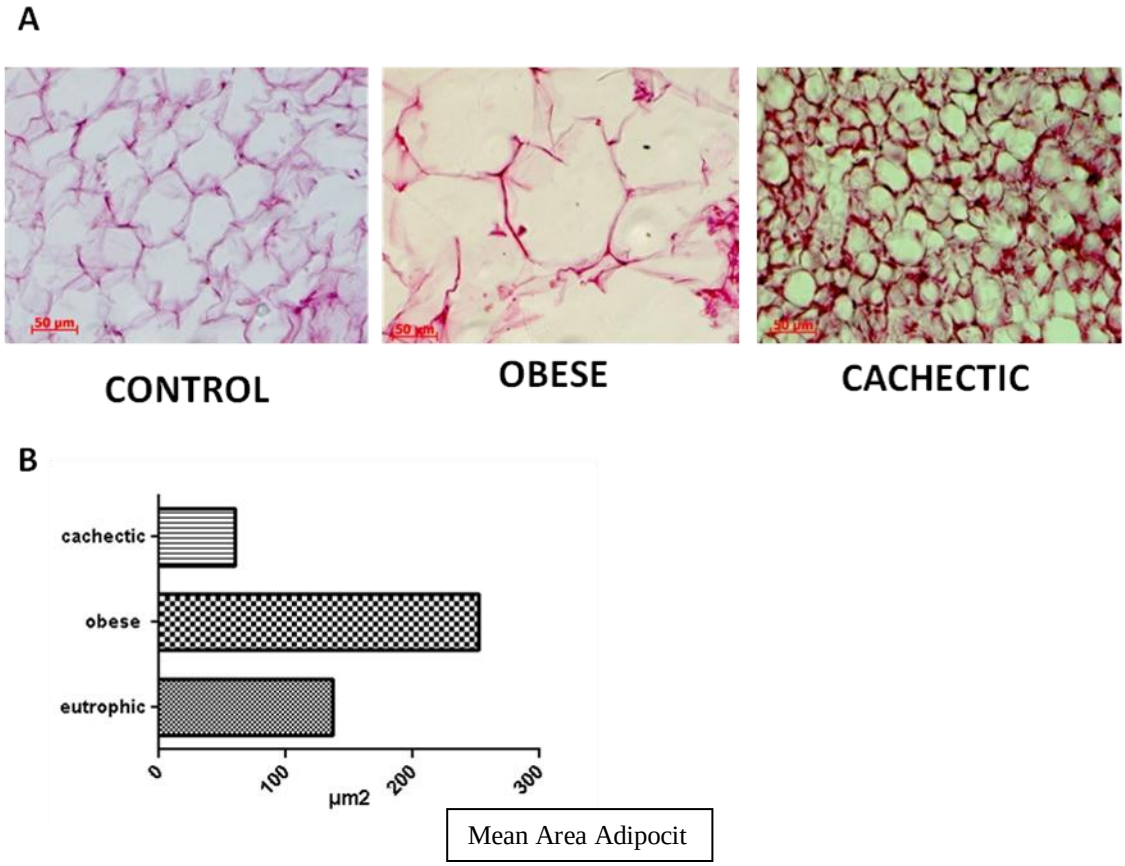

Figure 2

Figures A-B: Morphometric analysis of white fat cells of obese, cachectic and control patients.

4.2 Produto 2

Interleukine-6 (IL-6) expression in white and brown adipose tissue of obese patients and its association with metabolic and inflammatory profile.

Antônio Sérgio Barcala Jorge[1]; Gislaine Candida Batista Jorge[1], Alanna Fernandes Paraíso[1], João Marcus Oliveira Andrade[1]; Raíssa Mendonça Porto Franco[2]; Lara Jhullian Tolentino Vieira[2]; Aline Mourão Hilzenderger[4]; André Luiz Sena Guimarães[1]; Alfredo Maurício Batista de Paula[1]; Sérgio Henrique Sousa Santos[1,3].

[1] *Nucleus of Epidemiological and Molecular Research Catrumano.Health Research Laboratory.Health Science Post-graduate Programme. Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil.*
[2]*Medical School. Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil.*
[3] *Department of Pharmacology and Biochemistry. Universidade Federal de Minas Gerais, 31270901 Belo Horizonte, MG, Brazil.*
[4]*Department of Pharmacodynamics, University of Florida, PO Box 100487, Gainesville, FL, 32610, USA.*

Address correspondence to:
Prof. Dr. Sérgio Henrique Sousa Santos
Programa de Pós-graduação em Ciências da Saúde. Sala 7.
Hospital Universitário Clemente de Faria, Universidade Estadual de Montes Claros. Avenida Cula Mangabeira, 562. Bairro Santo Expedito. Montes Claros, Minas Gerais, Brazil. CEP: 39401-001. Phone: 55-21-38 32248327
e-mail: sergiosousas@hotmail.com

Running-title: IL6 expression in adipose tissue of obese patients.

ABSTRACT

Background: The incidence of obesity,a major health problem throughout the world, is increasing continuously together with associated morbidity and mortality. This persistent state of low-grade chronic inflammation induced by obesity is characterized by abnormal cytokine production, altered adipokine profile and activation of inflammatory pathways.

Aim: Study the distribution of IL 6 expression in white and brown adipose tissue in obese individuals and their correlations with some anthropometric, inflammatory and metabolic data associated with obesity and its comorbidities**.**

Methods: Samples of brown and white adipose tissue from obese patients (n = 27) were utilized for the analysis of IL-6 expression by quantitative real time polymerase chain reactions. Plasma glucose levels, hepatic transaminases and cholesterol were assessed.

Results: Our results demonstrated that IL-6 expression was increased in brown adipose tissue of females. In both white and brown adipose tissue, the expression levels of IL-6 is associated with hyperglycemia. Elevated levels of IL-6 in brown adipose tissue is associated with elevated serum levels of fibrinogen and leptin, and reduction of LDH levels and IL6 expression in brown fat is positively associated with abdominal fat accumulation. **Conclusions** Our results showed that the expression of IL-6 in white and brown adipose tissue is associated with alterations in the metabolism of carbohydrates. Brown adipose tissue was also associated with elevated serum levels of inflammatory markers (fibrinogen and leptin)

Key words: IL6. White adipose tissue. Brown adipose tissue. Inflammation.

1. INTRODUCTION

Obesity is a major health problem throughout the world, possibly the most serious and costly health problem of this century, and represents a growing threat to the population [1]. Obesity and related comorbidities have in common an inflammatory condition of low intensity, developed by an abnormal activation or production of inflammatory cytokines and signaling pathways [2, 3, 4, 5, 6] that regulate growth and activation of immune cells and mediate the inflammatory response [7].

Adipose tissue is one of the most important reservoirs of energy in the body. It consists of adipocytes, connective tissue matrix (collagen and reticular fibers), nervous tissue, vascular stroma cells, lymph nodes, immune cells (leukocytes and macrophages), fibroblasts, pre-adipocytes (adipose stem cells) [8]. Research related to the physiology of adipose tissue demonstrated that adipocytes are more than just the energy stockcell; it is an important endocrine organ that secrete hormones for the regulation of insulin action, inflammation and energy homeostasis. In mammals there are two main types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). The BAT is specialized in the thermogenesis and, therefore, actively participates in the regulation of body temperature [9]. BAT is localized in subscapular, interscapular, axillary, intercostal regions and also along the major blood vessels of the abdomen and thorax. The WAT, in contrast, has broader functions with depots located in various regions of the body, also infiltrate organs and internal structures provides mechanical protection and energy storage [8].

It has been consistently observed that the activated inflammatory pathways in individuals with type 2 diabetes and obesity are closely associated with a number of different clinical manifestations, including hypertension, hypertriglyceridemia, low HDL cholesterol, and endothelial dysfunction, which were grouped into a designation of the metabolic syndrome [10,11]. This inflammatory condition is also associated with thromboembolic profile, characterized by an imbalance between pro-coagulation factors and fibrinolytic agents, which is described primarily by an increase in fibrinogen and, possibly, by activated coagulation pathways [12]. In addition, the excessive

accumulation of visceral fat in the liver induces this organ to secrete fibrinogen and serum amyloid A protein potentializing further thromboembolic effect [13, 14, 15].Due to accumulation of fat and metabolic disorders of the syndrome, those patients develop a state of low-grade inflammation and vascular changes that favor the atherosclerotic process [16] and hepatic steatosis process [17].

Cytokines, small proteins with predefined roles in the regulation of the immune system, constitute the largest group of biologically active substances produced in adipose tissue. [10]. The excess of visceral fat induces mononuclear leukocytes (lymphocytes and monocytes) to secrete various cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α) modulating growth factor beta (TGF-β) [13]. TNF-alpha, a proinflammatory cytokine typically produced by cells of the immune system, plays a direct role in obesity-induced insulin resistance [18], as well as modulates the expression of several other cytokines related to obesity, such as IL-6, leptin, IL-8, interleukin-18 (IL-18), monocyte chemotactic protein-1 (MCP-1), adiponectin and interleukin-10 (IL-10) [3]. Leptin is one of the most potent cytokines in metabolic regulation. Leptin regulates body weight and nutritional status signaling to other organs, particularly the hypothalamus, which produces neuropeptides and neurotransmitters that modulate food intake and energy expenditure [19]. The interconnection of leptin receptors in various areas of the nervous system, particularly the hypothalamus, induces an increase in sympathetic nervous system activity, which induces peripheral stimulation and release of noradrenaline from the sympathetic nervous system terminals located in adipose tissue [18].

IL-6 is a pleiotropic cytokine with many pathophysiologic roles in humans. IL-6 gene is located on chromosome 7 and it is secreted by monocytes, macrophages, osteoblasts, adipocytes, endothelial cells, pancreatic cells andmyocytes β. IL-6 is a cytokine hormonally regulated. Its production is suppressed by glucocorticoids and estrogens, and is stimulated by catecholamines [20, 21]. IL-6 has many endocrine functions, and is one of the major cytokines that stimulate the hypothalamus-pituitary-adrenal axis during the inflammatory stress. Visceral adipose tissue produces three times more IL-6 than the subcutaneous tissue. Approximately one third of IL-6 is produced in adipose tissue is

drained through the portal venous system blood passing through the liver and therefore give the importance of the impact of this cytokine in liver physiology [21,22, 23].

This study aims to correlate and compare the expression of the cytokine IL-6 in white and brown adipose tissue in obese individuals with some anthropometric, inflammatory and metabolic data associated with obesity and its comorbidities.

2. METHOD

Ethical aspects

Ethical approval for present study was obtained from an ethic committee (CONEPE - 85742/2012). All subjects of the study signed the consent form to participate in this study.

Inclusion and exclusion criteria

These patients were invited to participate after annual survey of the annual program worker health in clinical nutrition ambulatory of University Hospital Clemente de Faria - UNIMONTES / Brazil. This study included individuals with age between 18 and 65,with a BMI> 35 kg / m², unsuccessfully treated with long-term clinical follow-up. The exclusion criteria included patients with uncontrolled psychiatric disorder, and severe decompensated cardiopulmonary disease.

Patients

The study involved 27 obese subjects (male: female ratio: 3.8: 1) with a mean age of 40.48 years (± 5.74 years, range 27-51 years), included on an anti-obesity program. Evaluation of patients included detailed medical history, physical, nutritional, metabolic, cardiopulmonary, and psychological evaluations. For the comparative study we divided the patients into four groups according to the average expression of IL-6 in white and brown adipose tissues: LowIL6W, LowIL6B, HighIL61W and HighIL6B. The LowIL6W and LowIL6B groups represent those individuals with lower expression of IL-6 than the average in WAT and BAT, respectively. The HighIL61W and HighIL6B groups represent those individuals with higher than average interleukin in WAT and BAT expression, respectively.

Samples

Blood samples were collected after overnight fast period in the same laboratory. Clinical biochemistry laboratory blood assays were performed for evaluation of oxidative stress inflammatory, intolerance of glucose and deslipydemic in obesity. Very-low-density lipoprotein [VLDL], high-density lipoprotein-colesterol [HDL-C], total cholesterol [TC], plasma levels of glucose, insulin, ferritin, fibrinogen , leptin, LDH and glycosylated hemoglobin [GH] were measured by using enzymatic methods and chromatography. Biopsy between the shoulder blades and abdominal subcutaneous adipose tissue were obtained through liposuction with thick needle on an out patient basis under local anesthesia. Samples were rinsed in RNA holding and criopreserved at -80 °C.

Anthropometric data

Anthropometric data such as weight, height, body mass index (BMI), waist circumference, lean weight, fat weight and fat percentage were measured and calculated during the initial nutritional assessment.

RNA isolation and real-time reverse transcriptase PCR analysis

Total cellular RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen Corp, San Diego, California, USA). cDNAs were synthesized from 1 µg of total RNA by using the moloney murine leukemia virus reverse transcriptase (M-MLV RT) system (Invitrogen™, Life Technologies Corp., USA). The cDNAwas used for quantitative real-time polymerase chain reaction (qRT-PCR) with specific gene primers (Applied Biosystems®, USA) as follows (forward and reverse): Beta actin (5'- CGG CGA CGA CCC ATT CGA AC - 3' and 5'- GAA TCG AAC CCT GAT TCC CCG TC - 3)' and IL-6 (5'- AAA TTC GGT ACA TCC TCG ACG GCA - 3'; 5'- AGT GCC TCT TTG CTG CTT TCA CAC - 3'). All qRT-PCR analyses were performedusing the Applied BiosystemsStepOne™, Real-Time PCR Systems (Foster City, CA, USA). For each condition, mRNA expression of the genes were quantified in duplicate. Beta actin was used as the endogenous control in the comparative cycle threshold (C_T) method.

Statistical analysis

All data were analyzed and statistical tests were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The association between the expression of IL-6 in white adipose

tissue and brown adipose tissue with anthropometric data, associated diseases, biochemical and inflammatory markers was performed by means of univariate statistical tests (exact tests of Pearson Chi-square and Fisher). Comparisons between groups were performed by means of univariate statistical tests and t Student and Mann-Whitney test. The significance level was set at 5%.

3. RESULTS

The sample consisted of 27 patients (21 men and 6 women),average age of 40.48 years, maximum of 51 and minimum of 27 years, an average height of 1,71m (± 0.60 m) and an average weight of these patients of 93.72 kilograms (± 16.68 kg) with a mean BMI of 38.18 kg / m² (± 5.98 kg / m^2). The fat percentage ranged from 25.12% to 42.87% (mean percentage 30.60%), the average fat weight was 31.24 kg (± 6.38 kg) and lean weight of the subjects was 70, 57 kg (± 10.79 kg). Moreover, there was a significant difference in the expression of IL-6 in two types of adipose tissue associating gender of patients, in this study that the BAT IL-6 was expressed more in females independent of the patient age (p 0.03). (Figure 1)

Pacients expressing high IL6 in BAT had higher percentage of fat (p 0.01) and fat weight (p 0.02) compared to patients expressing low IL6 in BAT. Although there was no a significant difference between the groups in BAT was also possible to observe a relationship between waist circumference and IL6 expression in brown adipose tissue (p 0.01) (Table 1).

The presence of diabetes, dyslipidemia, hypertension were studied in this group of subjects was not observed any relationship between these diseases and the expression of IL-6, both in white adipose tissue and brown adipose tissue. It was also observed a significant association between the presence of hyperglycemia and IL-6 expression in BAT (p 0.04) and WAT (p 0.01) (Figure 2). It was also possible to observe a relationship between glicated hemoglobin levels and the expression of IL-6 in the BAT without however be a significant difference between the groups in this tissue (p 0.01) (Table S 1)

Inflammatory condition was evaluated by RT-PCR based on the expression of IL-6 in WAT and BAT. When comparing the expression of IL6 between the white adipose tissue and brown observe a higher level of IL-6 expression in BAT, but not statistically significant when compared to TAW (p 0.80) (Figure 3). In brown adipose tissue in the minimum expression of IL-6 was associated with a lower serum level of leptin and fibrinogen and increased serum LDH. When comparing the groups with the highest expression of this cytokine in white adipose tissue and brown adipose tissue the lactate dehydrogenase levels (0.04) was associated inversely and fibrinogen levels (p 0.01) and leptin (p 0.02) were directly associated with the expression of IL6. (Table 2) (Figure 4)

4. DISCUSSION

The persistent state of low-grade chronic inflammation induced by obesity is characterized by abnormal cytokine production, altered adipokine profile and activation of inflammatory pathways [25]. High IL-6 concentrations were associated with obesity, which justifies the focus of the present study evaluating the distribution and correlation with white and brown adipose tissue expression, since it is a critical cytokine for the acute and chronic inflammatory processes control. [26, 27]

The metabolic syndrome consists of a set of clinical and biochemical characteristics, such as glucose intolerance, central obesity, hypertension and dyslipidemia. Central obesity in this context may be an important risk factor for this syndrome, even in patients with normal BMI [28], where visceral fat is an independent predictor of the state of oxidative stress [28, 29]. Waist circumference is recognized as a simple and variable parameter to estimate the accumulation of visceral fat. It is a predictable method to estimate the volume and the proportion of intra-abdominal fat, which has a strong impact on health and risk [30, 31], especially among patients with moderate BMI [32]. This study showed an association between IL-6 expression in brown adipose tissue with body fat. Recent studies have shown that healthy adult humans have significant deposits of brown adipose tissue metabolically active [33, 34, 35], which are positively induced by cold [34, 35] and inversely correlated with BMI and age [33, 34]. This adipose tissue is important in the control of obesity. BAT action is dependent on the mediator beta-adrenergic activation of lipolysis and fatty acid degradation by

subsequent uncoupling protein 1 (UCP1), which dissipates large amounts of chemical energy in the form heat [36]. The present data indicate that the activity of BAT is strongly correlated with the concentrations of catecholamines, and is negatively correlated with body fat, especially abdominal fat. In Shanghai, 14 patients with pheochromocytoma and 14 normal subjects were evaluated using tomographic measurements of positron emission tomography and plasma metanephrines, revealing that the metabolic activity of BAT was significantly higher in patients with high levels of metanephrines [37].The metabolic activity of BAT tissue was also inversely related to BMI, and the areas of visceral fat and waist circumference [38]. In this study, the inflammatory status (IL6) seems to be positively modulated by the amount of abdominal adipose tissue and not by BMI. The highest IL-6 expression in brown adipose tissue is positively correlated with a higher percentage of body fat and abdominal tissue.

The expression of IL-6 in WAT and BAT may contribute to the development of metabolic and cardiovascular disease. This is of great clinical importance and it is partially addressed by this paper, which aims to clarify a role of IL-6 cytokine in the chronic inflammatory state. Oxidative stress is a key element in both atherogenesis and in the metabolic syndrome, because the latter reactive oxygen species (ROS) are involved in all stages of the disease since endothelial dysfunction up to the formation of atherosclerotic plaques [39]

The development of type 2 diabetes is due, in part, to excess adipose tissue that produces a state of insulin resistance. The main causes of insulin resistance are: (1) the excess of free fatty acids in circulation; (2) cytokine release; and (3) the accumulation of adipose tissue in other fatty tissues such as muscle, liver and pancreas [40]. Obesity and insulin resistance are often present for many years before the appearance of other disorders such as hypertension, dyslipidemia, type 2 diabetesand cardiovascular diseases. [41]. The free fatty acids linked to albumin and derived mainly from triglycerides stored in fatty tissue deposits that are released by the action of the enzyme lipoprotein lipase [42]. The increase in free fatty acid concentrations are related to the expansion of adipose tissue and adipocyte hypertrophy, commonly found in type 2 diabetes and obese patients or patients. The mechanisms by which fatty acids alter the

metabolism of glucose are associated with changes in the activity of different proteins and (glucose transport), hexokinase (glucose phosphorylation), glycogen phosphorylase (glycogenolysis), phosphofructokinase (glycolysis) GLUT-4, pyruvate dehydrogenase [43]. Chronic exposure to free fatty acids causes the inhibition of proteins involved in insulin signaling, effects involved in the development of insulin resistance [44, 45]. This study showed that both IL-6 expression in WAT (p 0.01), as in BAT (p 0.04) were associated with elevated levels of glucose in serum. Cytokine expression in WAT showed that 83% of patients who had blood glucose levels below 100 mg / dL were LowIL6W group. The analysis of blood glucose compared to IL-6 expression in BAT, 75% of patients with blood glucose levels below 100 mg / dL were also below the mean group expressions (LowIL6B). Also in this study, we found that IL-6 expression in brown adipose tissue showed an association of this cytokine and glycosylated hemoglobin (p 0.01).

Understanding how IL-6 regulates nutrient homeostasis is complicated, because its effects are contradictory and multi-systemic, depending on the physiological state of the organism. In obesity associated with chronic inflammation there are secretion of proinflammatory cytokines such as resistin, TNF and IL-6 by adipocytes[45]. Plasma leptin levels are positively associated with WAT mass signaling to the central nervous system (CNS) to control caloric intake and modulate weight gain. A combination of high concentrations of leptin and accumulation of fat mass induces the mechanism of leptin resistance in the CNS of obese patients [46]. The secretion of leptin is increased by inflammatory stimuli and promote humoral and cellular immune response and is therefore stimulates the secretion of TNF-alpha and IL-6 from mononuclear cells and orchestrates the network inflammatory cytokines [46, 47, 48]. Adipocytes, pyruvate plays a versatile role in energy production, mainly from glycolysis. When the pyruvate is converted to alanine their metabolic fate is determined largely by the three reactions: efflux from the cell through the reduction of lactate lactate dehydrogenase (LDH); complete oxidation via decarboxylation to acetyl-CoA by pyruvate dehydrogenase complex (PDC); and oxaloacetate by the carboxylation (CEA) for pyruvate carboxylase (PC). Lactate production derived from glucose metabolism in adipocytes of obese and diabetic subjects was 35% higher in comparison to lean controls (5 -15%) [49]. These results suggest that the differentiation of adipocytes and subsequent hypertrophy, which

is characterized mainly by the accumulation of intracellular triglyceride (TG), may require adjustments in pyruvate metabolism and, consequently, changes in LDH levels [52] . In brown adipose tissue in the minimum expression of IL-6 was associated with a lower serum level of leptin and fibrinogen and increased serum LDH. When comparing the groups with the highest expression of this cytokine in white adipose tissue and brown adipose tissue the lactate dehydrogenase levels (0.04) was associated inversely and fibrinogen levels (p 0.01) and leptin (p 0.02) were directly associated with the expression of IL6. In white adipose tissue the dosage of lepitina and fibrinogen were also lower in the group with lower expression of IL6. Ferritin despite not maintain relationship with any group had their highest levels related to white adipose tissue. This association between these markers and IL-6 expression in brown adipose tissue can lead us to think further in other metabolic and inflammatory functions associated with the brown adipose tissue in addition to function of thermogenesis

Human WAT plays a key role in energy homeostasis maintenance, and secrete cytokines and adipokines, which are important for the regulation of the metabolism of lipids and glucose. Brown adipose tissue is a thermogenic organ. BAT mass is inversely correlated with BMI and age that was previously considered physiologically irrelevant in adult humans. However, it was rediscovered in various regions of the adult human body and proved to be active in metabolism. Our results showed in this study that the IL-6 expressed in BAT was associated with hyperglycemia, serum leptin and fibrinogen levels and LDH. As can be observed in this study that IL6 expression in adipose tissue is related inversely with serum levels of LDH and positively correlated with glucose levels showing a possible effect of this cytokine on glucose metabolism. Leptin turn that modulates the hypothalamic level energy expenditure and hunger is higher in groups with higher IL-6 expression in brown adipose tissue which may indicate a connection between this cytokine and the resistance to leptin because both operate in central nervous system by regulating the metabolism. The expression of this cytokine in WAT was associated with hyperglycemia too being that in higher expression group also observed lower serum levels of LHD.This different biological behavior of adipose tissue may be important for more effective therapies and improvement of metabolic disorders related to obesity.

Conflict of interests

The authors declare no conflict of interests.

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FIGURE LEGENDS:

Figure 1

Relationship between sex and IL-6 in white and brown tissue

Table 1

Distribution and analysis of anthropometric and clinical characteristics.

Figure 2

Analysis of serum glycemia in groups according to IL6 expression in brown adipose tissue and white adipose tissue

Figure 3

Expression of IL-6 isolated by tissue

Table 2

Distribution and analysis of leptin, fibrinogen, LDH and ferritin.

Figure 4

Relationship between the serum levels of leptin, fibrinogen and HDL in groups High IL6 White and High IL6 Brown

Supplementary Material – Tables.

Table S1

Distribution and analysis of main laboratorial results

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Figure 1: Relationship between sex and IL-6 in White and Brown tissue

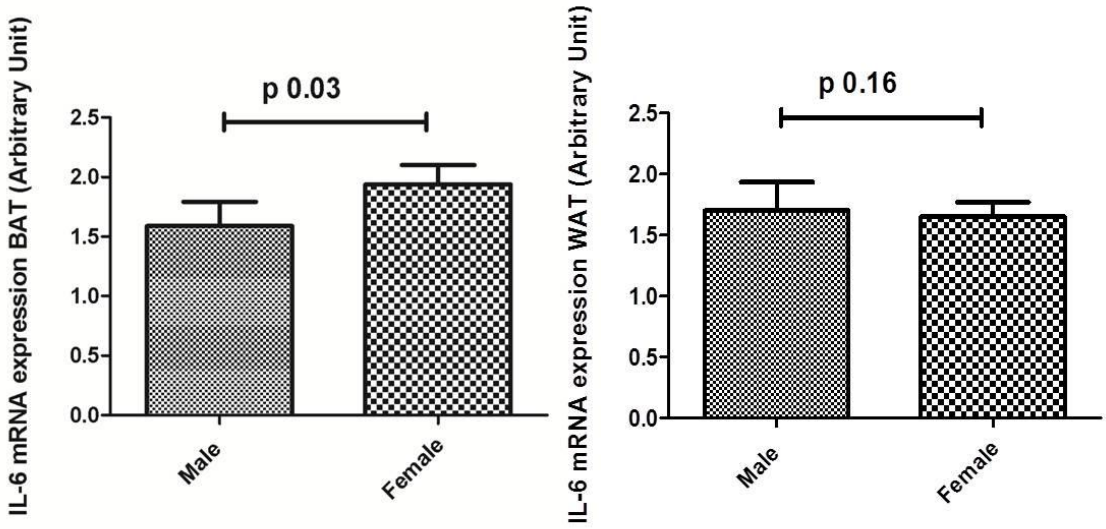

Figure 1: Tests were performed by using chi-square (*p values significant < 0.05). We can observe a significant relationship of IL6 expression in the brown adipose tissue with sex (p 0.03)

Table 1: Distribution and analysis of anthropometric and clinical characteristics

	LowIl6W	HighIL6W	p	Low IL6B	HighIL6B	p
Age (years)	38.84± 4.86	42.33± 7.19	0.57	41.00±5.24	38.87± 6.01	0.65
Weight (Kg)	95.23± 20.44	92.22± 14.69	0.47	97.15±20.28	85.87± 12.66	0.33
WC*** (cm)	108.36±9.37	103.00±7.40	0.33	108.20±10.77	103.02±8.44	0.41
BMI (Kg/m²)	42.93±21.71	33.68±3.00	0.18	36.20±3.69	35.32±4.81	0.57
Percentage of Fat**(%)	31.32±5.71	31.32±4.60	0.53	30.24±3.40	33.28±7.12	0.01
Fat Weight* (Kg)	33.50±6.94	31.03±4.32	0.08	31.89±4.83	33.43±8.89	0.02
Slim Weight (Kg)	73.80±12.77	68.38±8.32	0.16	73.89±11.19	66.55±11.49	0.93

Table1: Tests were performed by using t Student test (*p values significant < 0.05). It was possible to observe a statistically significant difference in the expression of IL-6 in brown adipose tissue of both groups (LowIL6B and HighIL6B) with the percentage of body fat tissue (*p 0.01) and the fat weight of patients (** 0.02). When compared to waist circumference (WC) with the expression of IL-6 in both tissues there was significant relationship in brown adipose tissue (*** p 0.01) however there was no difference between the groups LowIL6B and HighIL6B.

Figure 2: Analysis of serum glycemia in groups according to IL6 expression in brown adipose tissue and white adipose tissue

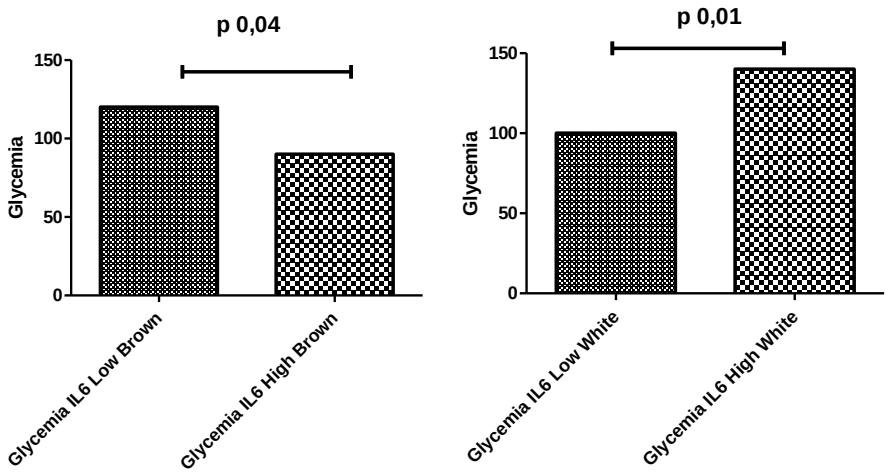

Figure 2: Tests were performed by using t student test * p values significant (p<0.05). We can observe a significant relationship of IL6 expression in white and brown adipose tissue with hyperglycemia (* p 0.01 and p 0.04 respectively).

Figure 3: Expression of IL-6 isolated by tissue

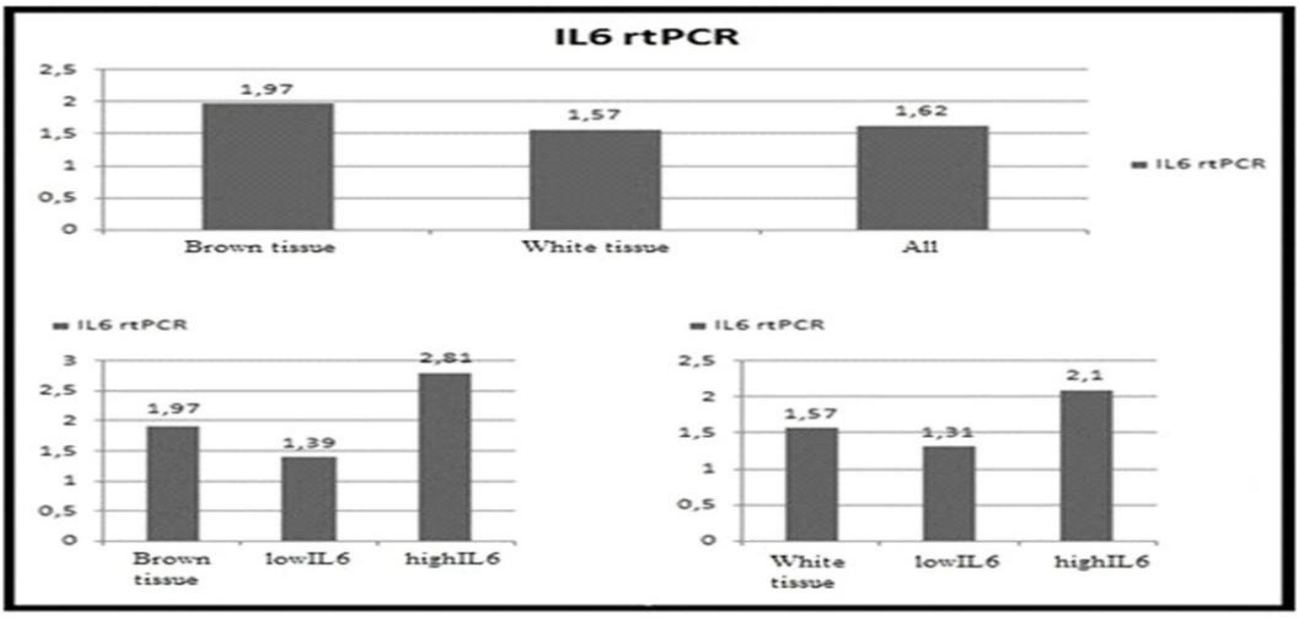

Figure 3: tests were performed by using t Student test. *p values significant (p <0.05). We observe that there was no difference significant relationship of IL6 expression whith in brown and white adipose tissue (p 0.80).

Table 2: Distribution and analysis of Leptin, Fibrinogen, LDH and Ferritin

	LowIl6W	HighIL6W	p	Low IL6B	HighIL6B	p
Leptin*(ng/mL)	36.71±29.22	36.66±22.94	0.35	30.10±22.40	46.06±32.36	0.29
Fibrinogen**(g/L)	11.49±12.93	13.54± 15.61	0.38	10.69±13.90	16.87± 14.77	0.34
LDH *** (U/L)	359.69±99.00	349±123.82	0.23	361.50±109.61	323.75±89.12	0.20
Ferritin (ng/mL)	343.36±291.25	289.17±107.25	0.74	226.35±103.65	195.37±108.31	0.68

Table 2: Tests were performed by using t Student test * p values significant (p <0.05).

Figure 4: Relationship between the serum levels of leptin, fibrinogen and HDL in groups High IL6 White and High IL6 Brown

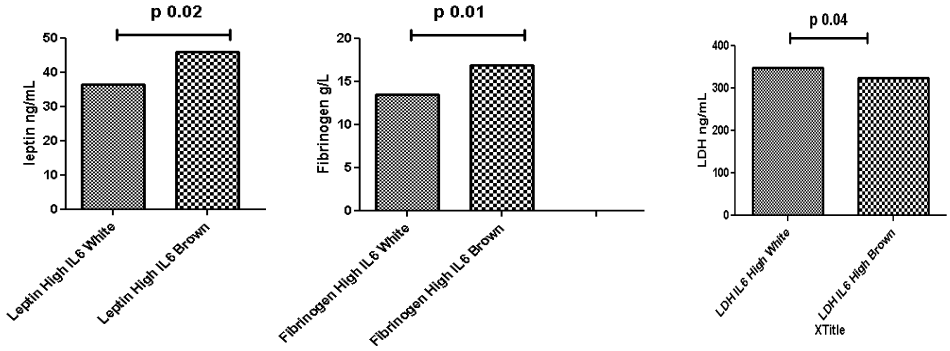

Figure 4: Tests were performed by using t Student test * p values significant (p <0.05) When comparing serum levels of leptin, fibrinogen and Ldh between groups observed significantly higher values of leptin (p 0.02) and fibrinogen (p 0.01) the group High IL6 Brown .When compared the serum levels LDH there was a significantly lower dose in group High IL6 Brown (p 0.04).

Supplementary Material – Tables and Figures.

Table S 1: Distribution and analysis of main laboratorial results

	LowIl6W	HighIL6W	p	Low IL6B	HighIL6B	p
Glucose * (mg/dL)	104.23±40.33	136.00±73.53	0.01	113.75±55.89	94.75±15.02	0.04
Glycated Hemoglobin **(%)	10.35±5.26	6.80±2.09	0.17	6.38±1.73	5.78±0.78	0.42
Post Prandial glucose (mg/dL)	120.58±49.73	136.44±67.43	0.33	116.91±34.50	115.75±51.35	0.25
Insulin (uUI/mL)	16.46±7.89	17.65±8.43	0.81	17.97±8.12	12.10±6.01	0.64
Cholesterol (mg/dL)	206.30±45.31	216.55±44.19	0.98	217.16±43.70	189.00±48.51	0.89
LDL (mg/dL)	127.16±36.51	122.00±36.15	0.80	141.00±36.82	104.50±40.24	0.68
VLDL (mg/dL)	37.50±24.20	38.22±24.06	0.78	29.54±11.94	31.12±23.65	0.22
HDL (mg/dL)	43.07±7.56	46.88±7.40	0.74	45.00±6.36	48.25±10.38	0.07
Triglycerides (mg/dL)	232.53±197.45	202.88±136.97	0.42	207.75±193.11	157.00±123.75	0.35

Table S1: Tests were performed by using t student test * p values significant ($p<0.05$). We can observe a significant relationship of IL6 expression in white and brown adipose tissue with hyperglycemia (* p 0.01 and p 0.04 respectively). When compared glycated hemoglobin with the expression of IL6 in both tissues there was significant relationship in brown adipose tissue (** p 0.01) however there was no difference between the groups LowIL6B and HighIL6B.

4.3 Produto 3

Body mass index and interleukin-6 and tumor necrosis factor-alpha adipose tissue expressions are associated to morphological severity of non-alcoholic fatty liver disease in morbidly obese patients

Antônio Sérgio Barcala Jorge[1, 2]; João Marcus Oliveira Andrade[1]; Alanna Fernandes Paraíso[1], Gislaine Candida Batista Jorge[1]; Christine Mendes Silveira[1,2]; Ludmilla Regina de Souza[1]; Erivelton Pereira Santos[1]; Andre Luiz Sena Guimaraes[1,3]; Sérgio Henrique Sousa Santos[1,3]; Alfredo Maurício Batista De-Paula[1,4].

[1] *Nucleus of Epidemiological and Molecular Research Catrumano. Health Research Laboratory. Health Science Post-graduate Programme. Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil.*

[2] *Department of Physiopathology. Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil.*

[3] *Department of Food Engineering. Universidade Federal de Minas Gerais, 39.404-547, Montes Claros, Minas Gerais, Brazil.*

[4] *Department of Dentistry. Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil.*

Address correspondence to:

Prof. Dr. Alfredo Maurício Batista De-Paula

Programa de Pós-graduação em Ciências da Saúde. Sala 7.

Hospital Universitário Clemente de Faria, Universidade Estadual de Montes Claros.

Avenida Cula Mangabeira, 562. Bairro Santo Expedito. Montes Claros, Minas Gerais, Brazil.

CEP: 39401-001.

Phone: 55-21-38 32248327

e-mail: ambpatologi@gmail.com

Running-title: IL-6 and TNF-α liver expression in morbidly obese patients.

ABSTRACT

Aim Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological spectrum that might range from non-alcoholic hepatic steatosis (NAFL) to non-alcoholic steatohepatitis (NASH) and, ultimately, to cirrhosis, terminal liver failure, and hepatocellular carcinoma. Accumulating evidences indicate that proinflammatory cytokines expression might contribute to NAFLD progression. We investigated the interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in liver and white adipose tissue expressions in samples of NAFL and NASH morbidly obese patients. **Methods and Results** This analytical, cross-sectional study included a sample of individuals with morbid obesity and NAFLD (NAFL = 12 and NASH = 9) submitted to bariatric surgery. A series of clinical, hematological, histopathological, food and nutrients intake, and biochemical factors were assessment in each study subject. IL-6 and TNF-α mRNA expression from fresh/frozen liver and WAT samples were performed by using quantitative real time-polymerase chain reaction. According to our findings, NASH patients showed higher body mass index (BMI) and TNF-alfa and IL-6 mRNA expression in WAT ($p = 0.01$) compared to NAFL patients.**Conclusions** Our results suggest that BMI and TNF-alfa and IL-6 mRNA expression WAT appear to contribute to the severity of NAFLD in individuals with morbid obesity probably by the direct relationship between fat mass and increased production of these cytokines in adipose tissue which may have direct effect on modulation and maintenance of hepatic inflammatory activity.

Key words: morbid obesity, non-alcoholic fatty liver disease, non-alcoholic hepatic steatosis, non-alcoholic steatohepatitis, clinical factors, cytokines, mRNA expression, real time-PCR.

Introduction

Obesity is one of the most common and injurious inflammatory and metabolic human disease (1). This multifactorial inflammatory and metabolic disease is caused by the interaction of a series of genetic/epigenetic and environmental factors (2-4) Obesity has high prevalence in many populations worldwide, affecting over 50% of the adult population and exhibiting high morbidities and premature mortality rates. Morbid obesity (BMI $\geq$ 40 kg/m^2 or BMI $\geq$35 kg/m^2 with at least one obesity-related comorbidity) is associated with both increased susceptibility to a wide range of inflammatory and metabolic diseases and increasing mortality rates(5, 6)

The non-alcoholic fatty liver disease (NAFLD) is worldwide prevalent hepatic disese that represents the manifestation of the metabolic syndrome. NAFLD is a multifactorial hepatic disease in which both environmental factors (sedentary lifestyle, stress, and hypercaloric diet) and a background of genetic/epigenetic host susceptibilities play a role in the pathogenesis and natural history of that disease (7, 8). Typically, NAFLD occurs in individuals with metabolic systemic disturbances such as obesity and metabolic syndrome, but that are not alcohol drinkers, have absence of viral hepatitis infections, do not exhibit autoimmune or drug-induced liver diseases (6, 9). NAFLD comprises a morphological spectrum that varies from an early stage known as non-alcoholic hepatic steatosis (NAFL), to the progressive form known as non-alcoholic steatohepatitis (NASH). With the progression of NASH, it might lead to cirrhosis, hepatocarcinoma, liver failure and, ultimately, death of patients (10-12).

The progression of NAFLD has been hypothesized by the *two hits* theory. The initial condition is related to the accumulation of triglyceride within hepatocytes owing to increased free fatty acids delivery and/or synthesis, decreased export triglycerides by very low density

lipoprotein, and reduced beta-oxidation reaction (“*first hit”*). Posteriorly, with increasing of liver vulnerability caused by *first hit*, occurs a higher vulnerability of the liver to many secondary factors ("*second hit*"), such as oxidative stress promoted by reactive oxygen or/ and nitrogen species, proinflammatory cytokines, adipokines, and mitochondrial dysfunction. These secondary factors cause, direct or indirectly, the reversible and irreversible hepatic injuries (12, 13).

Cytokines play pivotal roles in several local and systemic human inflammatory diseases, including metabolic disorders such as obesity, diabetes *mellitus,* and cardiovascular disease (14). The human tumour necrosis factor-alpha (*TNF-α*) gene is located in the chromosome 6q23-12, in close linkage with the class III region of the major histocompatibility complex (HLA-DR)(15, 16). During inflammation, TNF-α might regulate both innate and adaptative immune responses. This cytokine is primarily produced by mononuclear phagocytes and activated lymphocytes, natural killer cells, endothelial cells, and mast cells in response to exogenous or endogenous immune stimulus. (17). The interleukin-6 (*IL-6*) gene is located in the chromosome 7p21. This gene encodes a homonymous cytokine that has pivotal roles during inflammation and the maturation of B cells (15, 16). IL-6 is mainly produced from mononuclear phagocytic cells but other cells, such as T and B lymphocytes, fibroblasts, endothelial cells, keratinocytes, hepatocytes, and bone marrow cells, may also produce this cytokine. IL-6 has numerous inflammatory roles such as B lymphocytes differentiation, secretion of immunoglobulin in plasma cells, T-cell activation, growth, and differentiation (18-20)

Although the molecular findings are controversial, TNF-α and IL-6 seem to contribute to progression of NAFLD and these cytokines have been considered as potential therapeutic targets (21, 22). In this study, we analyzed the association between anthropometric, clinical,

nutritional, and biochemical factors, as well as, the hepatic and white adipose tissue (WAT) expression of IL-6 and TNF-α in NAFL and NASH patients with morbid obesity.

Material and Methods

Ethical aspects

Ethical approval for present study was obtained from a relevant ethic committee (CONEP - 85742/2012). All subjects of this study consent in to participate of this study by signing a consent form.

Study design and sample

This cross-sectional and analytical study enrolled morbid obese individuals (n = 21; male:female ratio: 1:6, mean age: 38.57 ± 11.52 years, ranging from 22 to 63 years) included in a public health service for bariatric surgery in the Montes Claros city, Minas Gerais state, Brazil, during the period from 2013 to 2014.

Clinical evaluations

The diagnosis of NAFLD was microscopically established in all morbid obese patients. Preoperative evaluation of patients was conducted by a multidisciplinary health team that carried out a detailed anamnesis and a series of anthropometrical, nutritional, metabolic, cardiopulmonary, and psychological evaluations and assessments. The patients were subjected to surgical treatment established in national and international guidelines (23, 24).

Inclusion and exclusion criteria

For inclusion in this study were considered morbid obese individuals with age from 18 to 65 years, male:female ratio of 1:6, BMI > 35 kg/m^2, presenting or not any type of

comorbidity that arisen after two-years treatment and follow-up. The main exclusion criteria for selection of individuals were alcohol drinking users; presence of psychiatric or cognitive impairments, severe cardiopulmonary disease, portal hypertension and esophagogastric varices, acute inflammatory disease of upper aerodigestive tract, Cushing's syndrome.

Samples

Peripheral blood samples from all individuals were collected after an overnight fast period. Wedge biopsy of hepatic tissue and white adipose tissue (WAT) of omental were performed surgically and a representative portion of the sample was rinsed in RNA holding and criopreserved at -80 °C. Another representative portion of each tissue was formalin-fixed and paraffin-embedded and submitted to serial sections and staining with hematoxylin & eosin (H&E) and Gomori's trichrome. Posteriorly, these samples were submitted to molecular and morphological analysis.

Nutritional analysis

We performed the 24h dietary recall interview (24-HDR) dietary assessment method in all morbidly obese patients. The 24-HDR questionnaire is literacy and financially accessible, quickly applied, and it obtains a high response rate from subjects with different educational and socio-economic levels (25, 26). Dietary intake records for value caloric (VC), total lipid (TL), saturated fat (SAT), monounsaturated fatty acids (MUFA), and unsaturated fatty acids (PUFA) were measured and the results were expressed as percentage mean value ± standard deviation.

Biochemical analysis

Clinical biochemistry laboratory blood assays were performed for evaluation of hepatic function and integrity (alanine aminotransferase: ALT, aspartate aminotransferase: AST, albumin: A, alkaline phosphatase: ALP, very-low-density lipoprotein: VLDL, high-density lipoprotein-colesterol: HDLc, total cholesterol (TC) and triglycerides (TG). Plasma levels of glucose, C-reactive protein, ferritin, and glycosylated hemoglobin were measured by using enzymatic methods and chromatography. All biochemical factors were performed using Bayer Reagent Packs on an automated chemistry analyzer (Advia 1650 Autoanalyzer; Bayer Diagnostics, Leverkusen, Germany). Enzymatic colorimetric assay was used to measure TC and TG. The homogeneous enzymatic colorimetric test and a selective inhibition method were used to measure LDL-C and HDL-C, respectively. Glucose level was measured by the hexokinase method and serum insulin concentration was measured using an immunoradiometric assay (INS-IRMA; Biosource, Nivelles, Belgium).

Morphological and immunohistochemical analysis

All liver biopsies were reviewed by two trained pathologists (De-Paula, AMB and Mendes, CF). For each sample, diagnosis for NAFL and NASH were reached by using previous morphological criteria (27-29). Morphological analyses were performed by using histopathological 5-µm-thick sections of archived formalin fixed-paraffin embedded- NAFLD tissues were deparaffinized, stained with H&E, and evaluated under a conventional light microscope. Typical morphological findings (hepatic steatosis, ballooning hepatocytes, lobular inflammatory reaction, and staging for hepatic fibrosis) were identified and graded in the liver samples. In all liver samples, the detection of ballooning hepatocytes was immunohistochemically performed by using an anti-human CK-19 monoclonal mouse antibody (Enzo Life Sciences), with a streptavidin-biotin-peroxidase detection system

(LSAB™-Kit Plus Peroxidase®, DakoCytomation, Glostrup, Denmark). Afterwards, the samples were stained with a chromogen (3,3′-diaminobenzidine tetrahydrochloride, DAB), counterstained with Mayer's hematoxylin, cover slipped, and visualized under an optical microscope (Figure S1). Morphological and immunohistochemical analysis of NAFLD samples were filled out by each one of pathologists that were blinded to clinical and biochemical data. More details about the morphological categorization of liver samples in NAFL and NASH groups are exhibited in Supplementary material (Table S1).

RNA isolation and real-time reverse transcriptase PCR analysis

Total cellular RNA was extracted by using Trizol reagent according to the manufacturer's instructions (Invitrogen Corp.®, San Diego, California, USA). cDNAs were synthesized from 1 μg of total RNA by using the moloney murine leukemia virus reverse transcriptase (M-MLV RT) system (Invitrogen™, Life Technologies Corp., USA). The cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) with specific gene primers (Applied Biosystems®, USA) as follows (forward and reverse): Beta actin (5'- CGG CGA CGA CCC ATT CGA AC - 3' and 5'- GAA TCG AAC CCT GAT TCC CCG TC - 3)'; TNF-α (5'- GAG CTG AAC AAT AGG CTG TTC CCA - 3' and 5'- AGA GGC TCA GCA ATG AGT GAC AGT - 3'); IL-6 (5'- AAA TTC GGT ACA TCC TCG ACG GCA - 3'; 5'- AGT GCC TCT TTG CTG CTT TCA CAC - 3'), and SIRT1 (5'- ACT GCA ACA TTC CGG GAC TCT ACT -3'; 5'-AGA GAA CGG CCT TGT CCT TCT TGA - 3'). All qRT-PCR analyses were performed by using the Applied Biosystems StepOne™, Real-Time PCR Systems (Foster City, CA, USA). For each condition, mRNA expression of biomarkers was quantified in duplicate. Beta actin was used as the endogenous control in the comparative cycle threshold (C_T) method.

Statistical analysis

All data were transferred and statistical tests performed with SPSS® 18.0 software (SPSS Inc., Chicago, IL, USA). Association between NAFL and NASH groups according to clinical data were performed by using bivariate statistical tests of Pearson´s Chi-squared and Fisher´s exact tests. The comparisons between NAFL and NASH groups according to anthropometric, nutritional, biochemical, and molecular data were performed by using bivariate statistical tests of Student's t test and Mann-Whitney U test. The level of significance was set at 5% and association with $p < 0.05$ value was considered statistically significant.

Results

The descriptive results of anthropometric, clinical, biochemical, and nutritional findings from patients of this study can be visualized in Tables 1-4.

All NAFLD patients of this study exhibited mean values of weight of 112 Kg (± 15.92, ranging from 90 to 155 Kg); height of 162.7 cm (± 6.73, ranging from 151 to 175 cm), waist circumference of 118.5 cm (± 12.92, ranging from 100 to 157 cm), and BMI of 42.61 kg/cm2 (± 4.08, ranging from 36.8 to 53.6 kg/cm2) (Table 2). According to our clinical and biochemical findings, the majority of NAFLD individuals exhibited hypertension (76.2%) and metabolic syndrome (61.9%). NASH individuals showed a greater occurrence but not significant of diabetes *mellitus*, hypertension, dyslipidemia, and metabolic syndrome compared to NAFL group (Table 3). A high consumption of saturated fatty acids was noted in both NAFLD groups. NASH patients showed a high but not significant consumption of total lipid intake compared to NAFL patients (Table 4). PCR level was positive in 100% of NAFLD patients. NAFL samples exhibited high levels of total cholesterol (limit of 200

μmol/L). Interesting, the level of liver transaminases in both NAFLD groups were considered normal. NAFL individuals showed a high but not significant AST levels compared to NASH individuals. A higher ALT level was detected in NASH individuals. In both NAFLD groups the albumin levels were within the normal range (3.5-5.2 g/dL). However, ferritin was above the normal levels in NASH patients (limit 10-100 ng/mL) (Table 5).

We noted a significant association between NASH individuals and higher BMI ($p = 0.01$) (Table 1). However, it was not identified any association between clinical, nutritional, and biochemical factors and NAFLD groups ($p > 0.05$) (Tables 3, 4 and 5).

Our molecular findings showed that all NAFLD individuals expressed both IL-6 and TNF-α mRNA in liver (0.74 ± 0.31 and 0.68 ± 0.31, respectively) and WAT (1.05 ± 0.50 and 0.92 ± 0.33, respectively) samples. In NAFL individuals, the hepatic and WAT expression of IL-6 was 0.70 ± 0.31 and 0.90 ± 0.22, respectively. The hepatic and WAT expression of TNF-α was 0.65 ± 0.26 and 0.81 ± 0.19, respectively. In NASH individuals, the hepatic and WAT expression of IL-6 was 0.77 ± 0.32 and 1.15 ± 0.60, respectively. The hepatic and WAT expression of TNF-α was 0.70 ± 0.35 and 0.99 ± 0.38, respectively. Our findings did not show any significant association between IL-6 and TNF-α mRNA liver expression and NAFL groups ($p > 0.05$) (Figure 1). In our study we found a positive correlation between IL6-alpha expression in visceral adipose tissue and liver with BMI (p 0:02 p 0:02) and waist circumference (p 0.04 and p.04) respectively, which was not observed with TNF-alpha expression in any of these tissues. In all groups there was a positive correlation between TNF-alpha expression and the expression of IL6 and this was most evident in the NAFL group when we analyze the molecular expression of these cytokines both in adipose tissue and in the liver tissue. (Table 5)

Interesting, a high TNF-α expression was found in hepatic tissue with steatosis grade 0-1 (p = 0.00) (Supplementary material - Table S2). However, in WAT samples, a high IL-6 and TNF-α mRNA expression was found in NASH group compared to NAFL (p = 0.01, for both associations) (Figure 1).

Discussion

A chronic, low-grade inflammation is the central feature of obesity (1) that in turn is an important predictive factor for NAFLD (30-32). Proinflammatory cytokines expressed in liver and WAT seem to participate of molecular signaling pathways that connect obesity and NAFLD. According to our findings, all liver and visceral WAT from NAFL and NASH obese individuals showed mRNA expression for TNF-α and IL-6. Currently, the obesity is considered an inflammatory systemic disease that affect the adipose tissue primarily and other metabolically critical organs such as liver. It has been showed that adipocyte, adipocyte precursor cells, resident macrophage, and reticuloendothelial cells present active genes related to activation of proinflammatory signaling pathways in WAT (1, 20, 33). In parallel, in the liver, mutual damage mechanisms between steatotic hepatocytes, other hepatic cells, and resident and infiltrative inflammatory cells seems to be crucial to trigger the inflammatory responses that promote tissue injuries in liver of obese individuals with NAFLD (21). Since early stages of NAFLD, mutual molecular interactions between normal and steatotic hepatocytes, reticuloendothelial, macrophage-like cells, and infiltrative inflammatory cells are responsible to trigger numerous molecular mechanisms that damage hepatic tissues gradually (21) .

It has been evidenced that both TNF-α and IL-6 are overexpressed inflammatory cytokines in organs such as WAT and liver of obese individuals (34). Moreover, in these

individuals, these cytokines might also contribute to NAFLD progression (21,35). In this study, we did not find any significant differences between liver TNF-α and IL-6 mRNA expressions and NAFLD groups however in patients with NAFL this positive correlation between TNF-alpha and IL-6 expression was more evident both in adipose tissue (β = 0.69) and liver tissue (β = 1.53) denoting perhaps a increased participation of these citocianas in the early stages of the disease (first stage) (12,13). It has been showed that TNF-α produced from inflammatory and liver cells contribute to steatosis hepatic once it stimulates fatty acid synthesis, increases serum triglyceride levels, and stimulates VLDL production from hepatocytes (34,36). Additionally, TNF-α can directly trigger molecular mechanisms that promote cell death of hepatocytes (36). However, the majority of studies with NAFLD individuals have investigated the TNF-α expression in peripheral blood but not in liver tissues (37). In liver, IL-6 activates resident and infiltrative immune cells, hepatocytes, and resident cells. Additionally, some studies have shown that IL-6 might play indirect damage mechanisms on hepatic cells in NAFLD(35). Although the role of IL-6 in NAFLD progression is not established yet, it seems to be a positive correlation between higher morphological aggressiveness of NASH and hepatic IL-6 expression (22).

By the other hand, we showed in this current study that visceral WAT from NASH individuals exhibited high TNF-α and IL-6 mRNA expression. Some studies have reported that visceral fat is a distant key mediator of NAFLD progression, and this intercession is performed by resident and inflammatory cells and numerous chemical mediators. In WAT, TNF-α is known to promote lipolysis and the secretion of free fatty acids that contribute to hepatic steatosis in obesity (38). The IL-6 expression in WAT is associated with high BMI and higher levels of free fatty acids (39). In WAT and liver, IL-6 will exert some proinflammatory activities, such as increasing insulin resistance (40). These activities of IL-6 might also contribute to hepatic injuries in obese patients with NAFLD (40). In this current,

our finding adds more evidence to the hypothesis that centers on ongoing negative feedback loop occurring between WAT and liver in obese patients. Therefore, it is possible that TNF-α and IL-6 WAT expression might trigger damage mechanisms that are promoters of a higher morphological severity of NAFLD in obese patients. However, further experimental prospective studies are necessary to confirm that hypothesis.

In our present study, we showed that morbidly obese individuals with NASH presented high BMI mean compared to NAFL and We found a positive correlation between IL6-alpha expression in visceral adipose tissue and liver with BMI (p 0:02 p 0:02 respectively) and waist circumference (p 0.04 and p 0.04, respectively), which was not observed with expression TNF-alpha in any of these tissues, although some studies have reported controversial findings, BMI is considered a significant predictor of the NAFLD severity frequently (41). Moreover, obese patients have a significantly higher dyslipidemia and insulin resistance and these metabolic disorders may contribute to NAFLD (42, 43).

Some limitations present in this study should be highlighted. Our sample of NAFLD individuals is not large. Moreover, this cross-sectional study does not allow establishing the cause-consequence interrelationship between WAT and hepatic tissues cytokines expressions and NAFLD progression(44). We couldn't assess the WAT samples morphologically due to technical reasons. In both tissues here investigated, liver and WAT, we just analyzed the mRNA expression of TNF-α and IL-6. Data about protein expressions from these cytokines were not investigated in our study.

In conclusion, our findings showed that morbidly obese individuals with NASH have association with high BMI, and TNF-α and IL-6 mRNA expression in visceral WAT. BMI and TNF-alfa and IL-6 mRNA expression WAT appear to contribute to the severity of NAFLD in individuals with morbid obesity probably by the direct relationship between fat mass and increased production of these cytokines in adipose tissue which may have direct

effect on modulation and maintenance of hepatic inflammatory activity. Our findings suggest that dietetic, clinical, and molecular factors might contribute to NAFLD progression.

Competing interests

The authors have declared that no competing interests exist.

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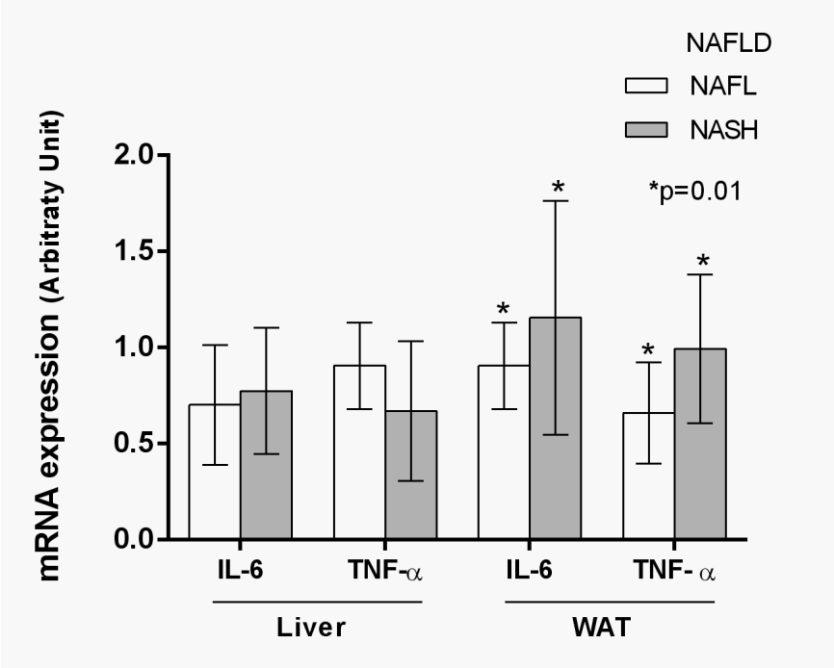

Figure 1. Expression of IL-6 and TNF-α in liver sample of non-alcoholic fatty liver disease (NAFLD) in morbidly obese patients. Our findings showed a significant association between higher TNF-α and IL-6 white adipose tissue (WAT) expression in NASH samples compared to NAFL (p = 0.01, for both). The statistical analyses were performed using Student´s t test or Mann-Whitney U test. The level of significance was set at α = 5% (p< 0.05).

Table 1. Analysis of demographic, anthropometric, and clinical measurements in a sample of non-alcoholic fatty liver disease (NAFLD) individuals with morbid obesity.

Variables	**All**	**NAFL**	**NASH**	**p (NAFL&NASH)**
Age (years)	38.57 ± 11.52	36.25 ± 10.63	40.00 ± 12.27	0.50
Height (cm)	162.71 ± 6.73	159.50 ± 7.17	164.69 ± 5.87	0.39
Weight (Kg)	112.63 ± 15.90	101.72 ± 8.39	119.34 ± 15.85	0.24
Waist circumference (cm)	118.52 ± 12.92	113.56 ± 8.92	121.58 ± 14.32	0.20
BMI (Kg/m²)	42.61 ± 4.08	40.13 ± 1.49	44.14 ± 4.46	0.01*
Systolic pressure (cm/Hg)	13.30 ± 1.44	13.08 ± 0.79	13.48 ± 1.88	0.85
Diastolicpressure (cm/Hg)	7.95 ± 0.54	7.84 ± 0.86	8.05 ± 0.33	0.55

Table 1 Analyses were performed by using Student's t-test and Mann-Whitney U-test (MEDIANA). Results were expressed as mean ± standard deviation (S.D.). * p values significant (p < 0.05). NAFL = non-alcoholic fatty liver. NASH = non-alcoholic steatohepatitis. BMI = body mass index

Table 2. Distribution and analysis of metabolic diseases and family histories in a sample of non-alcoholic fatty liver disease (NAFLD) morbid obese individuals.

Variables	All	NAFL	NASH	p
Diabetes *mellitus*	6 (28.6%)	2 (33.3%)	4 (66.7%)	0.77
Hypertension	16 (76.2%)	6 (37.5%)	10 (62.5%)	0.92
Dyslipidemia	6 (28.6%)	2 (33.3)	4 (66.7%)	0.77
Metabolic syndrome†	13 (61.9%)	4 (30.8%)	9 (69.2%)	0.15

Table 2. Analyses were performed by using Pearson´s chi-square and exact Fisher statistical tests. Results were expressed as sample size and percentage. * p values significant ($p < 0.05$). NAFL = non-alcoholic fatty liver. NASH = non-alcoholic steatohepatitis. † Brazilian Consensus Endrocrinologia and Metabolism 2015

Table 3. Distribution and analysis of food and nutrients intake characteristics according to non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) in obese individuals of this study.

Variables	All	NAFL	NASH	p
VC	188.2% ± 77.54	182.5% ± 68.62	192.4 % ± 86.38	0.95
TL	32.2% ± 6.09	31.3% ± 4.67	32.8% ± 7.11	0.08
SAT	13.1% ± 6.32	11.9% ± 5.14	13.9% 7.18	0.28
MUFA	10% ± 4.54	9.4% ± 4.05	10.5% ± 5.01	0.63
PUFA	4.5% ± 3.2	4.2% ± 3.12	4.7% ± 3.27	0.91

Table 3 Analyses were performed by using Student's t test and Mann-Whiney statistical tests. Results were expressed as percentage mean value ± standard deviation. NAFL = non-alcoholic fatty liver. NASH = non-alcoholic steatohepatitis. VC: value caloric intake; TL: total lipid intake; SAT: saturated fat intake; MUFA/MONSAT: monounsaturated fatty acids intake; PUFA/UNSAT: unsaturated fatty acids intake.

Table 4. Distribution and analysis of main laboratorial findings according to non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) in morbidly obese individuals of this study.

Variables	All	NAFL	NASH	p
Glucose (µmol/L)	97.33 ± 15.68	95.88 ± 21.35	98.23 ± 11.40	0.29
Glycohemoglobin (%)	5.75 ± 1.18	5.66 ± 1.21	5.8 ± 1.22	0.58
Albumin (g/dL)	3.94 ± 0.54	3.78 ± 0.60	4.03 ± 0.50	0.27
Triglycerides (µmol/L)	173.62 ± 24.63	191.25 ± 98.80	162.77 ± 96.81	0.49
Ferritin (ng/mL)	118.18 ± 71.32	91.66 ± 82.19	135.06 ± 61.54	0.18
Total cholesterol (µmol/L)	194.67 ± 37.12	204.63 ± 34.48	188.54 ± 38.68	0.27
HDL-c (µmol/L)	47.90 ± 17.33	48.88 ± 15.42	47.31 ± 19.00	0.74
VLDL-c (µmol/L)	33.24 ± 17.16	37.50 ± 20.66	30.62 ± 14.91	0.49
LDL (µmol/L)	115.05 ± 39.01	121.13 ± 41.26	111.31 ± 38.78	0.53
ALT (IU/L)	18.33 ± 8.00	17.75 ± 7.22	18.69 ± 8.70	0.88
AST (IU/L)	26.76 ± 9.31	31.62 ± 8.10	23.77 ± 8.99	0.07

Table 4 .Analyses were performed by using Student's T test and Mann-Whitney test. Results were expressed as mean ± standard deviation (S.D.). * p values significant ($p < 0.05$). NAFL = non-alcoholic fatty liver. NASH = non-alcoholic steatohepatitis.

CRP = C-reactive protein. HDL-c = cholesterol associated with ApoA-1/high-density lipoprotein particles. VLDL-c = cholesterol associated with ApoA-1/very low-density lipoprotein particles. LDL = low-density lipoprotein. ALT = alanine transaminase. AST = aspartate transaminase.

Table 5. The analysis by linear regression test to evaluate the the correlations of the expression of cytokines IL-6 and TNF-alpha

Variables	R^2	β	p	Low	Upper	DW	Krs
IL6&IMC (Hepatic Tissue)	0.24	0.06	0.02	0.01	0.11	1.36	0.45
IL6&WC (Hepatic Tissue)	0.19	0.02	0.04	0.00	0.03	1.35	0.49
IL6&IMC (Adipose Tissue)	0.24	0.49	0.02	0.00	0.11	1.36	0.61
IL6&WC (Adipose Tissue)	0.19	0.44	0.04	0.00	0.03	1.34	0.60
TNF-alfa&IL6 (Adipose tissue/All)	0.69	0.83	0.00	0.84	1.66	1.63	0.64
TNF-alfa&IL6 (Adipose tissue/NASH)	0.68	0.00	0.82	0.84	1.66	2.00	0.51
TNF-alfa&IL6 (Adipose tissue/NAFL)	0.47	0.69	0.04	0.03	1.06	1.52	0.55
TNF-alfa&IL6 (Hepatic tissue/All)	0.82	1.25	0.00	0.84	1.66	1.63	0.78
TNF-alfa&IL6 (Hepatic tissue/NASH)	0.66	1.12	0.00	0.50	1.69	2.38	0.58
TNF-alfa&IL6 (Hepatic tissue/NAFL)	0.85	1.53	0.00	0.97	2.00	2.54	0.55

Table 5. Analyses were performed by using linear regression test. * p values significant (p < 0.05). WC –wasit circumference BMI –Index Mass Corporal DW – Durbin Watson test Krs – Kolmogorov-Smirnov test

In our study we found a positive correlation between IL6-alpha expression in visceral adipose tissue and liver with BMI (p 0:02 p 0:02) and waist circumference (p 0.04 and p.04) respectively, which was not observed with TNF-alpha expression in any of these tissues. In all groups there was a positive correlation between TNF-alpha expression and the expression of IL6 and this was most evident in the Nat'l group when we analyze the molecular expression of these cytokines both in adipose tissue and in the liver tissue. (Table 5)

Supplementary Material – Tables and Figures.

Table S1. Morphological grading of non-alcoholic fatty liver disease (NAFLD) in liver samples of the morbidly obese patients with metabolic syndrome (MetS).

Samples	NAFLD*		Morphological Parameters															
			Hepatic Esteatosis				Hepatocyte Ballooning			Lobular Inflammation				Hepatic Fibrosis				
	NAFL	NASH	0	1	2	3	0	1	2	0	1	2	3	0	1	2	3	4
1		X			X			X			X			X				
2		X		X				X			X			X				
3	X			X			X			X					X			
4		X			X			X				X		X				
5	X					X	X			X				X				
6		X		X				X				X			X			
7		X		X				X				X			X			
8	X			X			X				X			X				
9	X			X			X				X			X				
10	X			X			X				X			X				
11		X			X			X			X			X				
12		X			X			X				X			X			
13	X				X		X			X				X				
14		X			X			X			X			X				
15		X		X			X					X		X				
16	X			X			X				X			X				
17		X		X				X				X		X				
18		X				X		X				X		X				
19		X		X			X					X			X			
20	X				X		X				X			X				
21		X				X	X					X		X				

Table S2. Analysis of IL-6 and TNF-α liver expression according morfological factors in non-alcoholic fatty liver disease (NAFLD) in morbidly obese patients.

Morphological Variables	**Cytokine mRNA Liver Expression**			
	IL-6	**p**	**TNF-α**	**p**
Hepatic steatosis				
Grades 0-1 (n = 11)	0.74 ± 0.37	0.19	0.75 ± 0.39	0.00
Grades 2-3 (n = 10)	0.74 ± 0.23		0.60 ± 0.17	
Ballooning hepatocytes				
Grade 0 (n = 11)	0.76 ± 0.33	0.95	0.67 ± 0.29	0.93
Grades 1/2 (n = 10)	0.72 ± 0.31		0.69 ± 0.34	
Lobular inflammation				
Grades 0-1 (n = 12)	0.85 ± 0.30	0.64	0.77 ± 0.33	0.43
Grades 2-3 (n = 09)	0.62 ± 0.29		0.58 ±0.27	
Lobular inflammation				
Grade 0 (n = 3)	0.48 ± 0.13	0.17	0.50 ± 0.26	0.67
Grades 1/2/3 (n = 18)	0.78 ± 0.31		0.71 ± 0.31	
Hepatic fibrosis				
Stage 0 (n = 17)	0.82 ± 0.28	0.24	0.73 ± 0.30	0.95
Stages 1-4 (n = 4)	0.38 ± 0.17		0.45 ± 0.26	

Analyses were performed by using Student's T test and Analyses were performed by using Mann-Whitney test. * p values significant ($p < 0.05$).

Figures

Figure S1

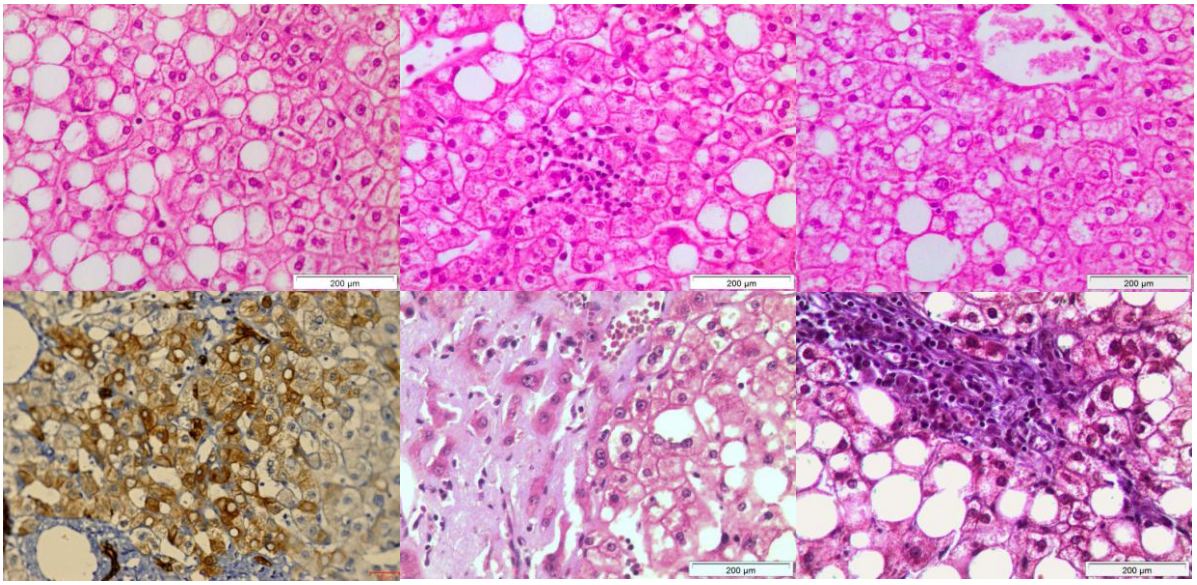

Figure S1. 1A: hepatic steatosis (grade 3) predominantly macrovesicular. 1B: intralobular mild chronic inflammation (grade 2). 1C: ballooned hepatocytes containing intracytoplasmic eosinophilic material consistent with protein degradation. 1D: Immunodetectionn of CK-19 in NASH samples. 1E: Cell death (necrosis) of hepatocytes and extensive focal fibrosis (stage 2). 1F: pericellular fibrosis (stage w) with mild chronic mononuclear inflammation predominantly and ballooned hepatocytes. Magnification: 100X; Staining: Figures 1A-C, E = H&E; Figure 1D: DAB, counterstained with Mayer's hematoxylin; Figures 1F-Gomori´s trichrome.

5 CONSIDERAÇÕES FINAIS E CONCLUSÕES

O ganho de peso é um problema de saúde pública mundial sendo inegável a ligação entre as taxas crescentes de obesidade e o aumento dos custos com a saúde. Trata-se de uma doença multifatorial causada pela interação de fatores genéticos e ambientais e que pode levar a distúrbios das condições de saúde do organismo representadas pelo aumento de risco de doenças de grande morbi-mortalidade, como diabetes, HAS, dislipidemias, doenças cardiovasculares (DCV), esteato hepatite e câncer (1, 3).

Embora todos os mecanismos envolvidos nesta doença ainda não sejam claros, há algum tempo o tecido adiposo passou a ser visto além de um órgão de armazenamento, hoje tido como órgão dinâmico, metabolicamente associado ao excesso de energia e capaz de sintetizar uma variedade de compostos biologicamente ativos que regulam a homeostase do organismo e que também podem causar um estado de estresse oxidativo prejudicial para a saúde. Dentre estas substâncias biologicamente ativas secretadas pelos adipócitos têm as citocinas, que se caracterizam por serem hormônios protéicos conhecidos como mediadores e reguladores de respostas imunes e inflamatórias que também podem interagir no metabolismo (sensores do balanço energético) (60, 61). Com o aumento no tecido adiposo (hipertrofia e hiperplasia) e a infiltração de macrófagos neste tecido, numerosas citocinas pró-inflamatórias, incluindo TNF-alfa e IL-6 têm suas expressões aumentadas tanto neste tecido como em outros orgãos promovendo um estado de inflamação crônica e de resistência à insulina que, por sua vez, induz a um aumento ainda maior e subsequente da citocinas e de ácidos graxos circulantes levando o organismo a um estado de lipotoxidade (32, 35)

Demonstrar que a expressão destas citocinas IL-6 e TNF-alfa foi significativamente maior em pacientes obesos em comparação com pacientes eutróficos e que estas citocinas se expressam de forma mais acentuada no tecido adiposo visceral de pacientes obesos assim como em pacientes caquéticos (que apresentam também um quadro clínico de estresse oxidativo sabidamente associada a estas citocinas) reforça o pensamento sobre o papel e a importância destas citocinas no quadro de inflamação crônica presente no paciente obeso (103, 104).

A ocorrência de complicações da obesidade depende não apenas do excesso de peso, mas também da distribuição da gordura corporal onde as consequências adversas para a saúde

estão menos relacionadas com a deposição de gordura corporal total do que com a distribuição desta gordura no corpo. Em mamíferos existem dois tipos principais de tecido adiposo: tecido adiposo branco e o tecido adiposo marrom sendo que o tecido adiposo branco desempenha um papel fundamental na manutenção da homeostase energética e na secretação de citocinas e adipocitoquinas (41). Já o tecido adiposo marrom é um órgão termogênico, inversamente correlacionada com o IMC e com a idade dos indivíduos tendo sido considerado fisiologicamente irrelevante em humanos adultos até pouco tempo, no entanto, a sua detecção em várias regiões do corpo humano adulto numa forma metabolicamente ativa tem mudado este conceito (43, 44).

Procurar entender o comportamento do tecido adiposo branco e marrom, avaliando a expressão de citocina IL-6 de forma individualizada nestes dois tecidos e analisando como isto pode interferir em algumas fases do metabolismo demonstram-se relevantes. Os resultados encontrados mostraram que a expressão de IL-6 no tecido adiposo branco abdominal foi associada significativamente com níveis de glicemia, leptina, fibrinogénio e LDH do soro. Como pode ser observado, a expressão de IL-6 no tecido adiposo foi relacionada inversamente com os níveis séricos de LDH e positivamente correlacionada com os níveis de glicose o que confirma, de certo modo, o efeito desta citocina no metabolismo da glicose tanto na captação, como na oxidação de carboidratos. Por sua vez, os níveis de leptina sérica, substância esta que se relaciona com a modulação do gasto energético e do centro da fome a nível hipotalâmico, foi maior no grupo com maior expressão de IL-6, o que pode indicar uma associação entre essa citocina e a resistência hipotalâmica à leptina já descrita nos pacientes obesos (85,87,91, 105).

No tecido adiposo marrom a expressão de IL-6 parece estar relacionada a um estímulo pelo incremento da gordura corporal (principalmente abdominal), sendo que neste tecido os níveis de IL-6 foram também associados com elevação dos níveis séricos de glicose. Tem sido observado de forma consistente na literatura que as vias inflamatórias ativadas em indivíduos com diabetes Tipo 2 e obesidade estão intimamente associados com um número de diferentes manifestações clínicas, incluindo a hipertensão arterial sistêmica, hipertrigliceridemia, HDL baixo e disfunção endotelial que favorecem o processo de aterosclerose e disfunção cardiovascular (8, 14,106,107).

A doença hepática gordurosa não alcoólica (DHGNA) é uma importante complicação da síndrome metabólica, que está se tornando uma causa cada vez mais comum de doença

hepática crônica (96). O seu espectro clínico-patológico varia desde um quadro de esteatose hepática não-alcoólica (NAFL) para esteato-hepatite não alcoólica (NASH) e, finalmente, podendo evoluir para a cirrose e ou adenocarcinoma. Evidências recentes mostraram a participação fundamental de citocinas pró-inflamatórias na progressão desta doença (94, 95).

O objetivo do presente estudo foi analisar dados clínicos, bioquímicos e antropométricos e a expressão de IL-6 e TNF-alfa em pacientes portadores de NAFL e NASH. Após análise histológica e imunohistóquimica do tecido hepático para gradação da doença e estudo da expressão de IL-6 e TNF-alfa no tecido hepático e adiposo observou-se que a apesar de não haver diferença significativa entre os grupos, os pacientes do grupo NASH apresentaram percentualmente uma maior incidência de comorbidades ligadas a obesidade (diabetes, HAS, dislipidemia e síndrome metabólica) isto talvez, por uma associação ao quadro inflamatório de maior intensidade presente nestes pacientes em comparação aos portadores de NAFL. O IMC, este sim, foi diretamente associado com a gravidade da doença.

A influência dos ácidos graxos ingeridos nas concentrações plasmáticas de lípideos e lipoproteínas é fato, assim como o consumo elevado de ácidos graxos saturados, que aumenta a concentração de colesterol no plasma. Em ambos os grupos estudados, a ingestão de gordura na dieta excedeu o recomendado pela literatura (ideal 30% do valor total de calorias ingeridas) (108), o que pode ter refletido nos níveis séricos elevados de triglicerídeos também encontrado em ambos os grupos. Este detalhe parece relevante, pois é sabido que o percentual de ingestão de ácidos graxos na dieta participa diretamente e indiretamente na patogênese da esteatose (95,96).

Como já demonstrado na literatura, na maioria das vezes o diagnóstico clínico laboratorial desta doença, assim como de sua gravidade se torna falho quando associa-se alterações laboratoriais com a histológica (padrão ouro). Neste estudo não foi observado diferença estatística entre as dosagens de transaminases, bilirrubinas e albumina sérica com os dois grupos de pacientes estudados (109). Quanto à avaliação do perfil inflamatório, do ponto de vista da expressão destas citocinas foi possível observar que tanto IL-6 e TNF-alfa foram significativamente mais expressas no tecido adiposo em pacientes com NASH (p 0,01) assim como no tecido hepático de pacientes com NASH e diagnosticados como portadores de síndrome metabólica (IL-6 p 0,01 e TNF-alfa p 0,04) confirmando pelo menos em parte a teoria dos *hits* onde estas citocinas são as responsáveis, pelo menos em parte pela manutenção e potencialização do estado inflamatório, tanto de forma apócrina no tecido hepático, como de

forma endócrina em outros tecidos (97,98, 102). A observação de níveis mais elevados de ferritina sérica (acima de 100 ng/ml) nos pacientes portadores de NASH, embora não significativamente estatístico, também pode demonstrar de forma indireta uma maior atividade inflamatória no tecido hepático destes pacientes, pois a sobrecarga de ferro, que é observada em 15% dos pacientes com síndrome metabólica e em pelo menos metade dos pacientes com esteatose hepática é um catalisador potente do estresse oxidativo atuando diretamente sobre a peroxidação lipídica (110) .

Concluindo, tem sido observado de forma consistente que as vias inflamatórias ativadas em indivíduos obesos estão intimamente associadas com um número de diferentes manifestações clínicas, onde o tecido adiposo é caracterizado pelo aumento da produção e secreção de um amplo painel de moléculas inflamatórias (citocinas) que tem efeitos locais sobre a fisiologia deste tecido alterando a sua função, além de efeitos potenciais sobre outros órgãos e tecidos. Resultando-se assim, em um estado metabólico alterado de todo o organismo. Compreender a fisiopatologia deste estado de stress oxidativo é importante para o desenvolvimento de intervenções eficazes, tanto no campo preventivo detectando grupos de maior risco e suceptibilidade às complicações da doença, bem como também na terapêutica, modulando e alterando a resposta inflamatória destes indivíduos previnindo os danos causados por este estresse metabólico causado pela lipotoxidade presente nestes indivíduos.

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APÊNDICE

APÊNDICE A – Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE ESTADUAL DE MONTES CLAROS

HOSPITAL UNIVERSITÁRIO CLEMENTE DE FARIA

Unimontes

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM PESQUISA

Título da pesquisa: Análises moleculares e corporais em seres humanos: uma comparação da composição química e tecidual de pacientes eutróficos, caquéticos e obesos com foco nas alterações no metabolismo lipídico, glicídico e inflamatório

Instituição promotora: Universidade Estadual de Montes Claros – Unimontes

Pesquisador: Antonio Sérgio Barcala Jorge; Gislaine Cândida Batista Jorge; João Marcus Oliveira Andrade; Thaísa Soares Crespo

Orientador: Dr. Sérgio Henrique Sousa Santos

Atenção: Você está sendo convidado (a) para participar, como voluntário, de uma pesquisa. Antes de aceitar participar, é importante que você leia e compreenda a explicação sobre os procedimentos. Após ser esclarecido (a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que tem duas vias. Uma delas é sua e a outra é o pesquisador responsável.

1 - Objetivo: Avaliar o metabolismo glicídico, lipídico e inflamatório de pacientes obesos, caquéticos e eutróficos comparando a expressão de vários marcadores moleculares no tecido adiposo visceral e abdominal, tecido muscular e gastrointestinal assim como a relação qualitativa e quantitativa destes marcadores com variáveis relacionadas a estilo de vida.

2 – Metodologia/Procedimentos: Serão realizadas entrevistas, exames e análise de tecidos corporais. Os exames de sangue serão coletados com técnicas adequadas por profissional médico no momento do ato cirúrgico ou endoscópico (as amostras serão submetidas à análises laboratoriais). Serão realizadas avaliações de medidas antropométricas (peso, altura e circunferência abdominal e bioimpedância) e avaliação nutricional. Serão analisados os tecidos adiposos da parede abdominal e intra-abdominal, tecido muscular e gastrointestinal. Estas análises permitirão

identificar algumas condições de saúde dos participantes, sendo que nenhum procedimento trará prejuízo à saúde dos participantes.

3 - Justificativa: É sabido que a obesidade, caquexia e as suas comorbidades estarão entre as principais causas de morte deste século, e, portanto, é necessário que entendamos bem a fisiologia e as propensões genéticas para estas doenças a fim de que possamos propor modelos de prevenção e tratamento adequados para este grupo da população. Este estudo se baseia na interrelação dos hábitos nutricionais, estilo de vida e a predisposição genética dos indivíduos.

4 - **Benefícios**: Você estará contribuindo para a compreensão do fenômeno estudado e para a produção de conhecimento científico.

5 - **Confidencialidade das informações**: Será mantido o sigilo quanto à identificação dos participantes. As informações/opiniões emitidas serão tratadas anonimamente no conjunto dos entrevistados e serão utilizadas apenas para fins de pesquisa.

6 - **Compensação**: A participação é voluntária, portanto, não é passível de remuneração.

7 - **Informações adicionais**: Será garantida ao participante a liberdade de recusar ou retirar o consentimento sem penalização em qualquer etapa da pesquisa.

8 - **Consentimento**: Li e entendi as informações precedentes. Tive oportunidade de fazer perguntas e todas as minhas dúvidas foram respondidas a contento. Este formulário está sendo assinado voluntariamente por mim, indicando meu consentimento para participar nesta pesquisa, até que eu decida o contrário. Receberei uma cópia assinada pelo pesquisador deste consentimento.

_____	_____	_____
Nome do participante	Assinatura do participante	Data
_____	_____	_____
Nome do coordenador	Assinatura do coordenador	Data

Orientador: Prof. Sérgio Henrique Sousa Santos

Avenida Cula Mangabeira, nº 567, Santo Expedito. CEP 39400-000 - Montes Claros - MG. Fone: (38) 3224-8382 – Hospital Universitário Clemente de Faria

APÊNDICE B - Capítulo de livro escrito

Jorge ASB, Crespo T, Jorge GCB. Non-Alcoholic Fatty Liver Disease:Clical, Laboratory and Imaging Diagnostic Methods. In: Santos SHS.HEPATIC STEATOSIS Clinical Risk Factors, Molecular Mechanisms and Treatment Outcomes. 01ed.New York: Nova Biomedical, 2014, v. 01, p. 21-40.

APÊNDICE C - Apresentações e publicações em Anais de Congresso

1- ANDRADE, J. M. O. ; PARAISO, A. F. ; CRESPO, T. S. ; **JORGE, A. S. B.** ; JORGE, G. C. B**.** ; SANTOS, S. H. S. . Resveratrol induces browning program, up-regulate thermogenesis and mitochondrial function by interaction between metabolic organs im mice fed with high-calorie diet. In: 5th World Congress on Diabetes & Metabolism, 2014, las Vegas, USA. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 05. p. 131-131.

2- ANDRADE, J. M. O. ; PARAISO F, A. ; CRESPO, T. S. ; **JORGE, A. S. B.** ; JORGE, G. C. B**.** ; SANTOS, S. H. S. . Resveratrol incrases brown adipose tissue thermogenesis markers by increasing SIRT1 and energy expenditure and decreasing fat accumulation in adipose tissue of mice fed a standart diet. In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 05. p. 130-130.

3- LELIS, D. F. ; PINHEIRO, T. ; ANDRADE, J. M. O. ; PINHEIRO, T. ; PARAISO F, A. ; CRESPO, T. S. ; **JORGE, A. S. B**. ; JORGE, G. C. B**.** ; SANTOS, S. H. S. . Caloric restriction versus malnutrition:Modulation of inflammatory cytokines and renin-angiotensin systemexpression in adipose tissue. In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP. v. 05. p. 132-132.

4- ANDRADE, J. M. O. ; LEMOS, F. O. ; PIRES, S. ; PARAISO, A. F. ; CRESPO, T. S. ; **JORGE, A. S. B**. ; JORGE, G. C. B**.** ; LOPES, M. T. ; SANTOS, S. H. S. . Proteomic white adipose tissue analysis of obese mice fed with a high-fat diet and treated with oral angiotensin (1-7). In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 5. p. 133-133.

5- CRESPO, T. S. ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B**.** ; **JORGE, A. S. B.** ; SANTOS, S. H. S. . Effects of sleeve gastrectomy with/without omentectomy in body and metabolic profile of diabetic rats. In: 5th World Congress on Diabetes & Metabolism,

2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 05. p. 134-134.

6- **JORGE, A. S. B**. ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B**.** ; CRESPO, T. S. ; VIEIRA, C. A. ; PAULA, A. M. B. ; SANTOS, S. H. S. . Comparative expression of inflammatory-related genes in visceral adipose tissue of normal weight, cachectic, and morbidly obese subjects. In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 5. p. 135-135.

7- **JORGE, A. S. B.** ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. ; CRESPO, T. S. ; SILVEIRA, C. M. ; SANTOS, E. P. ; PAULA, A. M. B. ; SANTOS, S. H. S. . Analysis of clinical variables associated with TNF-alpha and IL-6 expression in visceral adipose tissue. In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 5. p. 136-136.

8- **JORGE, A. S. B.** ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. ; CRESPO, T. S. ; SILVEIRA, C. M. ; SANTOS, E. P. ; SANTOS, S. H. S. ; PAULA, A. M. B. . Analysis of dietary intake, clinical, biochemical, and non-alcoholic hepatic steatosis and non-alcholic steatohepatitis in morbidly obese patients. In: 5th World Congress on Diabetes & Metabolism, 2014, Las Vegas. Journal of Diabetes & Metabolism/5th World Congress on Diabetes & Metabolism. Los Angeles: OMICS PUBLISHING GROUP, 2014. v. 05. p. 137-137.

9- JORGE, G. C. B. ; **JORGE, A. S. B.** ; ANDRADE, J. M. O. ; PARAISO, A. F. ; GUIMARAES, A. L. ; SANTOS, S. H. S. . ABSENCE OF BREAKFAST ASSOCIATED WITH A HIGHTER RISK OF OVERWEIGHT AND OBESITY. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 117-117.

10- **JORGE, A. S. B**. ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. . INVOLVED MECHANISMS OF THE INFLAMMATORY RESPONSE INDUCED BY OBESITY. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 74-74.

11- ANDRADE, J. M. O. ; PARAISO, A. F. ; BARROS, L. O. ; **JORGE, A. S. B**. ; JORGE, G. C. B**.** . CROSS TALK BETWEEN ANGIOTENSI (1-7)/MAS AXIS AND SIRTUINS IN ADIPOSE TISSUE. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 74-74.

12- PINHO, L. ; ANDRADE, J. M. O. ; PARAISO, A. F. ; **JORGE, A. S. B**. ; JORGE, G. C. B. ; SANTOS, S. H. S. . DIET COMPOSITION MODULATE expression OF SIRTUINS AND RENIN-ANGIOTENSIN SYSTEM COMPONENTS IN ADIPOSE TISSUE. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 75-75.

13- PINHO, L. ; ANDRADE, J. M. O. ; BARROS, L. O. ; JORGE, G. C. B. ; **JORGE, A. S. B.** ; SANTOS, S. H. S. . DEVELOPMENT AND VALIDITY OF A QUESTIONNAIRE TO TEST THE KNOWLEDGE OF PRIMARY CARE PERSONNEL REGARDING NUTRITION IN OBESE ADOLESCENTS. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 163-163.

14- BARROS, L. O. ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. ; **JORGE, A. S. B**. ; SANTOS, S. H. S. . ORAL FORMULATION OF ANGIOTENSIN (1-7) IMPROVES LIPID METABOLISM AND PREVENTS HIGH-FAT-DIET-INDUCED HEPATIC STEATOSIS AND INFLAMMATION IN MICE. In: 20 th European Congress on Obesity, 2013, Liverpool, UK, 12-15 May. OBESITY FACTS The European Journal of obesity. LIVERPOOL: KARGER, 2013. v. 06. p. 83-83.

15- CRESPO, T. S. ; **JORGE, A. S. B.** ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. . CHARACTERIZATION OF INFLAMMATORY PROFILE IN ADIPOSE TISSUE OF OBESE AND CACHECTIC PATIENTS. In: IX International Symposium on Vasoactive Peptides, 2013, Belo Horizonte. IX International Symposium on Vasoactive Peptides May 2-5,2013. Belo Horizonte, 2013. p. 120-120.

16 - LULA, J. ; **JORGE, A. S. B.** ; ANDRADE, J. M. O. ; PARAISO, A. F. ; JORGE, G. C. B. ; SANTOS, S. H. S. . ANALYSIS OF INFLAMMTORY CYTOKINES EXPESSION IN HEPATIC TISSUE OF MORBIDLY OBESE PATIENTS. In: IX International Symposium on Vasoactive Peptides, 2013, Belo Horizonte. IX International Symposium on Vasoactive Peptides May 2-5,2013. Belo Horizonte, 2013. p. 114-114.

ANEXO A - Parecer favorável do Comitê de ética Pesquisa

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

Plataforma Brasil

PROJETO DE PESQUISA

Título: Avaliação metabólica e corporal em seres humanos: uma comparação da composição química e tecidual de pacientes obesos mórbidos e eutróficos com foco nas alterações no metabolismo lipídico e glicídico relacionadas à obesidade

Área Temática:

Versão: 2

CAAE: 01987912.0.0000.5146

Pesquisador: Sérgio Henrique Sousa Santos

Instituição: Universidade Estadual de Montes Claros - UNIMONTES

PARECER CONSUBSTANCIADO DO CEP

Número do Parecer: 85742

Data da Relatoria: 13/07/2012

Apresentação do Projeto:

De acordo com o Projeto a obesidade, nedieze ou pimelose é uma doença, na qual a reserva natural de gordura aumenta até o ponto em que passa a associar-se com problemas de saúde ou ao aumento da taxa de mortalidade. Apesar de se tratar de uma condição clínica individual, esta é vista, cada vez mais, como um sério e crescente problema de saúde pública. Nos últimos anos a prevalência da obesidade vem crescendo em quase todas as comunidades do mundo. Segundo a Organização Mundial da Saúde (OMS), há 300 milhões de obesos no mundo e, destes, um terço está nos países em desenvolvimento. O projeto destaca que o número crescente de indivíduos obesos e com síndrome metabólica, nos últimos anos, é decorrente de alterações na composição da dieta, associadas a mudanças econômicas, sociais, demográficas e comportamentais.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar o metabolismo glicêmico e lipídico de pacientes obesos mórbidos e eutróficos comparando a expressão de marcadores moleculares no tecido adiposo visceral, abdominal e fígado assim como a relação da expressão desses marcadores com dados antropómetricos, bioquímicos e clínicos.

Objetivo Secundário:
Comparar a expressão de sirtuinas e componentes do sistema renina-angiotensina nos tecido adiposo visceral e abdominal e fígado de pacientes obesos e não obesos. Comparar o padrão de expressão dos marcadores inflamatórios PAF, IL1-ß, IL-6, TNF-¿ e TGF-ß e as suas relações com o ganho de peso, ingestão alimentar, morfologia dos adipócitos, perfil lipídico e nível glicêmico. Comparar o padrão de expressão de adipocinas (leptina, resistina e adiponectina). Mensurar e comparar os níveis plasmáticos lipídicos, dosando especificamente os níveis de triglicérides, colesterol total e HDL. Mensurar e comparar os níveis plasmáticos glicêmicos, dosando especificamente os níveis de glicose sérica e hemoglobina glicosilada.

Avaliação dos Riscos e Benefícios:

A retirada dos tecidos e do sangue não acarretará em prejuízos à saúde dos sujeitos envolvidos no estudo, visto que são indicados nas técnicas cirúrgicas adotadas. Os demais testes, como a avaliação nutricional e a bioimpedância são considerados não invasivos, não acarretando desse modo riscos ao paciente. Os resultados do estudo permitirão conhecer o papel do tecido adiposo na gênese da

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:** (383)229--8103 **E-mail:** vaniasvb@unimontes.br

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

síndrome metabólica e das alterações decorrentes dela, das alterações inflamatórias, dos possíveis fatores de risco para essas alterações, do diabetes e da obesidade e contribuirão para o melhor entendimento do papel fisiológico desta patologia nessas alterações metabólicas. Além disso, subsidiará futuras pesquisas sobre possíveis associações entre a obesidade e as comorbidades associadas a ela, podendo ainda contribuir para o desenvolvimento de possíveis métodos terapêuticos para a intervenção nessas alterações metabólicas.

Comentários e Considerações sobre a Pesquisa:

A obesidade é um problema sério de saúde pública, pode estar relacionada ao aparecimento de doenças cardiovasculares e diabetes. O presente trabalho pode representar uma estratégia de melhoria da qualidade de vida dos pacientes obesos além de possibilitar mecanismos de prevenção a doenças relacionadas com esse processo.

Considerações sobre os Termos de apresentação obrigatória:

Os termos apresentados no projeto cumprem todas as exigências legais do comitê.

Recomendações:

Recomendo aprovação do projeto sem restrições.

Conclusões ou Pendências e Lista de Inadequações:

Não há nenhuma pendência, ou inadequação no projeto.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O comitê de ética em pesquisa da Unimontes entende que o projeto atende aos preceitos éticos da pesquisa em seres humanos. Sendo assim aprovado.

MONTES CLAROS, 30 de Agosto de 2012

_____

Assinado por:
Maisa Tavares de Souza Leite

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Daroy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (383)229--8103 **E-mail:** vaniasvb@unimontes.br

ANEXO B – Normas da revista para submissão do artigo

Artigo 1

Author Guidelines/Journal of Obesity

Article Processing Charges

Journal of Obesity is an open access journal. Open access charges allow publishers to make the published material available for free to all interested online visitors.

Units of Measurement

Units of measurement should be presented simply and concisely using System International (SI) units.

Title and Authorship Information

The following information should be included

- Paper title
- Full author names
- Full institutional mailing addresses
- Email addresses

Abstract

The manuscript should contain an abstract. The abstract should be self-contained and citation-free and should not exceed 200 words.

Introduction

This section should be succinct, with no subheadings.

Materials and Methods

This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may each be divided by subheadings or may be combined.

Conclusions

This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

References

Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

Ethical Guidelines

In any studies that involve experiments on human or animal subjects, the following ethical guidelines must be observed. For any experiments on humans, all work must be conducted in accordance with the Declaration of Helsinki (1964). Papers describing experimental work which carries a risk of harm to human subjects must include a statement that the experiment was conducted with the human subjects’ understanding and consent, as well as a statement that the responsible Ethical Committee has approved the experiments. In the case of any animal experiments, the authors must provide a full description of any anesthetic or surgical procedure used, as well as evidence that all possible steps were taken to avoid animal suffering at each stage of the experiment.

Artigo 2

Instructions for authors/ Journal of Inflammation

Submission process

Manuscripts must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The submitting author takes responsibility for the article during submission and peer review. Please note that Journal of Inflammation levies an article-processing charge on all accepted.

Title page

The title page should:

- provide the title of the article
- list the full names, institutional addresses and email addresses for all authors
- indicate the corresponding author

Abstract

The Abstract of the manuscript should not exceed 350 words and must be structured into separate sections: **Background,** the context and purpose of the study; **Methods**, how the study was performed and statistical tests used; **Results**, the main findings; **Conclusions**, brief summary and potential implications. Please minimize the use of abbreviations and do not cite references in the abstract. **Trial registration**, if your research reports the results of a controlled health care intervention, please list your trial registry, along with the unique identifying number (e.g. **Trial registration**: Current Controlled Trials ISRCTN73824458). Please note that there should be no space between the letters and numbers of your trial

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state - and, if helpful, illustrate - the background to the research and its aims. Reports of clinical research should, where appropriate, include a summary of a search of the literature to indicate why this study was necessary and what it aimed to contribute to the field. The section should end with a brief statement of what is being reported in the article.

Methods

The methods section should include the design of the study, the setting, the type of participants or materials involved, a clear description of all interventions and comparisons, and the type of analysis used, including a power calculation if appropriate. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses in the Methods section.

For studies involving human participants a statement detailing ethical approval and consent should be included in the methods section.

Results and discussion

The Results and discussion may be combined into a single section or presented separately. Results of statistical analysis should include, where appropriate, relative and absolute risks or

risk reductions, and confidence intervals. The Results and discussion sections may also be broken into subsections with short, informative headings.

Conclusions

This should state clearly the main conclusions of the research and give a clear explanation of their importance and relevance. Summary illustrations may be included.

References

All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. Each reference must have an individual reference number. Please avoid excessive referencing. If automatic numbering systems are used, the reference numbers must be finalized and the bibliography must be fully formatted before submission.

Figure legends

The legends should be included in the main manuscript text file at the end of the document, rather than being a part of the figure file. For each figure, the following information should be provided: Figure number (in sequence, using Arabic numerals - i.e. Figure 1, 2, 3 etc); short title of figure (maximum 15 words); detailed legend, up to 300 words.

Artigo 3

Author Guidelines/Hepatology

Manuscripts describing original research must be no longer than 6,000 words (including references), abstract of 275 words, no more than 50 references, and a title with no more than 120 characters (excluding spaces). Please include all relevant portions of the Methods section in the main manuscript. See Submission elements below. No more than 8 figures and tables, with a maximum of 6 panels per figure. We encourage you to submit additional methodological details, nonessential figures or portions of your manuscript as supplementary material for online publication only.

Submission Elements

Submissions must include the following:

- Complete manuscript (title page, author information, abstract, and text) as one Microsoft Word document
- Tables submitted separately in one Microsoft Word document
- Figures submitted separately in TIFF format with a resolution of 300 dpi (dots per inch)

- No more than 8 figures and tables, with a maximum of 6 panels per figure. We encourage you to submit non-essential figures or portions of your manuscript as supplementary material for online publication only
- Any material that could constitute prior or concurrent publication of similar data by any of the authors, including symposium proceedings, book chapters, invited papers, and the like
- The names of reviewers whose expertise qualifies them to review the work
- A description of any commercial affiliation or consultancy of an author that could be construed as a conflict of interest (this information will be kept confidential unless the Editor recommends disclosure in a footnote if an appearance of a conflict is perceived)
- An author agreement signed by every contributing author. It is assumed, however, that the corresponding author speaks for his or her coauthors and certifies that all listed authors participated meaningfully in the study and that they have seen and approved the final manuscript.
- Editorial formatting (use of italics, superscripts, Greek letter, etc.) should be consistent. Typographical formatting (column widths, type styles, etc.) should not be used. Text should be flush left; paragraphs should not be indented and should be separated by two hard returns. Do not use hard returns within paragraphs. Do not use the program's indenting or margin-setting features; these will be added during typesetting.
- Submissions may include the names of any reviewers the authors wish to exclude along with the reason for exclusion for the Editors to consider.
- Requests to exclude an Associate Editor can only be considered if a clear conflict of interest is explained in the cover letter.