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Dissertação de Mestrado

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ESTUDO DA EXPRESSÃO DOS GENES *UCP2, NLRP3* E DO MICRORNA-133a NO TECIDO ADIPOSO SUBCUTÂNEO DE PACIENTES COM OBESIDADE E INDIVÍDUOS EUTRÓFICOS

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“Foi o tempo que dedicastes à tua rosa que a fez tão importante”.

(Antoine de Saint-Exupéry)

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Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução geral sobre o assunto da dissertação e na sequência será apresentado o artigo original. Após, serão apresentadas as considerações finais.

Artigo original: Obesity is associated with a downregulation of *UCP2* and miR-133a-3p but not *NLRP3* in subcutaneous adipose tissue

LISTA DE ABREVIATURAS PARA O REFERENCIAL TEÓRICO

ASC	*Inflammasome Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD*
DAMP	*Damage-associated molecular pattern molecule*
DM2	Diabetes mellitus tipo 2
EROs	Espécies reativas de oxigênio
IMC	Índice de massa corporal
IL-18	Interleucina 18
IL-1β	Interleucina-1 beta
LAGB	*Laparoscopic adjustable gastric banding*
miRNA	MicroRNA
NF-κB	*nuclear factor kappa B*
NLRP3	*NLR family pyrin domain containing 3*
PAMP	*Pathogen-associated molecular pattern molecule*
PPAR	*Peroxisome proliferator-activated receptor*
PPAR-α	*Peroxisome proliferator-activated receptor alpha*
PPAR-γ	*Peroxisome proliferator-activated receptor gamma*
siRNA	*Small interfering RNA*
SREBP-1c	*Sterol regulatory element-binding protein 1*
TA	Tecido adiposo
TAS	Tecido adiposo subcutâneo
TAV	Tecido adiposo visceral

THP1	Linhagem humana de monócitos leucêmicos
TLR	*Toll like receptors*
UCP	U*ncoupling proteins*
UTR	*Untranslated region*

LISTA DE ABREVIATURAS PARA O ARTIGO ORIGINAL

ACTB	Beta-actin
AT	Adipose tissue
β2M	Beta-2-Microglobulin
BMI	Body mass index
BP	Blood pressure
DAMP	Danger associated molecular pattern
DXA	Dual energy absorptiometry X-ray
FFA	Fatty free acid
FFM	Fat free mass
FPG	Fast plasma glucose
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment index
IL-18	Interleucine-18
IL-1β	Interleucine-1 beta
IR	Insulin resistance
LDL	Low density lipoprotein
miRNA	MicroRNA
NLRP3	NLR family pyrin domain containing 3
Pre-DM	Pre-diabetes
qPCR	Quantitative polymerase chain reaction

REE	Resting energy expenditure
ROS	Reactive oxygen species
RYGB	Roux-en-Y gastric bypass
SAT	Subcutaneous adipose tissue
STROBE	Strengthening the reporting of observational studies in epidemiology
T2DM	Type 2 diabetes mellitus
THP1	Human cell line of leukemic monocytes
UCP	Uncoupling protein
UTR	Untranslated region
VAT	Visceral adipose tissue

SUMÁRIO

RESUMO

A obesidade é uma doença crônica multifatorial caracterizada por um acúmulo excessivo de gordura corporal resultante de um desequilíbrio energético entre a ingestão e o gasto calórico. Esse desequilíbrio é decorrente da interação entre meio ambiente, suscetibilidade genética e epigenética.

A UCP2 (*uncoupling protein 2*) está localizada na membrana mitocondrial interna e atua dissipando o gradiente de prótons da cadeia respiratória mitocondrial, diminuindo a produção de ATP e, consequentemente, influenciando o gasto energético. Alguns estudos sugerem que a expressão de *UCP2* está diminuída no tecido adiposo (TA) de indivíduos com obesidade; entretanto, os resultados são ainda inconclusivos. Além disso, a UCP2 parece regular a ativação do inflamassoma NLRP3 (*NLR family, pyrin domain-containing 3*). O inflamassoma NLRP3 é um dos principais responsáveis pela produção de citocinas proinflamatórias [interleucina- (IL-1β) e IL-18] no TA e está envolvido na inflamação crônica de baixo grau presente na obesidade. Dessa forma, ambos os genes podem contribuir para a patogênese dessa doença.

Diversos estudos vêm demonstrando a associação de fatores epigenéticos reguladores da expressão gênica, como os microRNAs (miRNAs), em diversos processos patológicos, incluindo na patogênese da obesidade. Estudos recentes *in vitro* e em modelos animais sugerem que o miR-133a-3p regula a expressão de *UCP2* em diferentes tecidos. Além disso, o miR-133a-3p parece também estar envolvido na ativação do inflamassoma NLRP3 através do bloqueio de *UCP2*, mostrando o envolvimento deste miRNA na regulação desses dois genes. Entretanto, até o momento, nenhum estudo avaliou de forma combinada a associação de *UCP2*, *NLRP3* e miR-133a-3p no contexto da obesidade.

Sendo assim, no presente estudo, nós comparamos as expressões de *UCP2*, *NLRP3* e miR-133a-3p no TA subcutâneo (TAS) de pacientes com obesidade e indivíduos eutróficos. Além disso, avaliamos se essas expressões se correlacionavam com parâmetros de composição corporal, taxa metabólica basal, resistência à insulina e perfis glicêmico e lipídico desses indivíduos.

Neste estudo de caso-controle, as expressões dos genes de interesse foram avaliadas no TAS de 61 pacientes, os quais foram divididos em três grupos de acordo com os seus índices de massa corporal (IMC): Grupo 1 (IMC $\leq$ 25kg/m^2; n = 8), Grupo 2 (IMC: 30.0 – 39.9 kg/m^2; n = 24) e Grupo 3 (IMC $\geq$ 40 kg/m^2; n = 29) por meio da técnica de PCR em tempo real. Para este fim, utilizamos biópsias de indivíduos submetidos à cirurgia abdominal eletiva (grupos 1 e 2) ou cirurgia bariátrica (grupo 3).

Como resultado, encontrou-se uma diminuição das expressões de *UCP2* e miR-133a-3p em pacientes com obesidade (Grupos 2 e 3) em comparação com indivíduos eutróficos (Grupo 1). Apesar da expressão de *NLRP3* não ter diferido entre os grupos, ela se correlacionou negativamente com a expressão de *UCP2* e positivamente com a expressão de miR-133a-3p, com valores de circunferência da cintura e de excesso de peso.

Em conclusão, as expressões de *UCP2* e miR-133a-3p estão diminuídas no TAS de pacientes com obesidade. Embora a expressão de *NLRP3* não tenha diferido entre os grupos, ela foi positivamente correlacionada com os valores de circunferência da cintura e excesso de peso. Estes resultados reforçam os papéis da *UCP2*, *NLRP3* e miR-133a-3p-3p na fisiopatologia da obesidade.

ABSTRACT

Obesity is a chronic multifactorial disease characterized by an excessive accumulation of body fat resulting from an energy imbalance between intake and caloric expenditure. This imbalance is due to the interaction between environment, genetic susceptibility and epigenetics.

The uncoupling protein 2 (UCP2) is located in the inner mitochondrial membrane and acts by dissipating the proton gradient from the mitochondrial respiratory chain, decreasing ATP production and, consequently, influencing energy expenditure. Some studies have suggested that *UCP2* expression is decreased in adipose tissue (TA) from obese individuals; however, results are still inconclusive. In addition, UCP2 seems to regulate the activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome. The inflamasomme NLRP3 is one of the main responsible for the production of the proinflammatory cytokines interleukin- (IL-1β) and IL- 18 in the TA and seems to be involved in the chronic low-grade inflammation present in obesity. Thus, both of these genes can contribute to the pathogenesis of this disease.

Several studies have demonstrated the association of epigenetic factors that regulate gene expression, such as microRNAs (miRNAs), in several pathological processes, including in the pathogenesis of obesity. Recent studies in vitro and in animal models have suggested that miR-133a-3p regulates *UCP2* expression in different tissues. Moreover, miR-133a-3p also appears to be involved in the activation of NLRP3 inflammation by blocking *UCP2*, showing the involvement of this miRNA in the regulation of these two genes. However, to date, no study has evaluated the combined association of *UCP2*, *NLRP3* and miR-133a-3p in the context of obesity.

Thus, in the present study, we compared the expressions of *UCP2*, *NLRP3* and miR-133a-3p in subcutaneous AT (SAT) of patients with obesity and eutrophic

individuals. In addition, we evaluated whether their expressions correlate with parameters of body composition, resting energy expenditure, insulin resistance, and glycemic and lipid profiles of these individuals.

In this case-control study, expressions of the genes of interest were evaluated in SAT of 61 patients, which were divided into three groups according to body mass index (BMI): Group 1 (BMI $\leq$ 25 kg /m^2); Group 2 (BMI: 30.0 - 39.9 kg /m^2, n = 24); and Group 3 (BMI $\geq$ 40 kg /m^2; n = 29) using Real Time PCR. For this purpose, we used biopsies of individuals submitted to elective abdominal surgery (groups 1 and 2) or bariatric surgery (group 3).

As a result, *UCP2* and miR-133a-3p expressions were decreased in patients with obesity (Group 2 and 3) compared to eutrophic individuals (Group 1). Although *NLRP3* expression did not differ among groups, it was negatively correlated with *UCP2* expression and positively correlated with miR-133a-3p expression, waist circumference and weigh excess.

In conclusion, *UCP2* and miR-133a-3p expressions are decreased in SAT of patients with obesity. Although *NRLP3* expression did not differ among groups, it was positively correlated with waist circumference and weigh excess values. These results reinforce the roles of UCP2, NLRP3 and miR-133a-3p-3p in the physiopathology of obesity.

CAPÍTULO 1

REFERENCIAL TEÓRICO

1. INTRODUÇÃO

1.1 Obesidade

A obesidade é uma doença crônica multifatorial caracterizada por um acúmulo excessivo de gordura corporal resultante de um desequilíbrio energético entre a ingestão e o gasto calórico, afetando a saúde e reduzindo a qualidade e expectativa de vida dos indivíduos afetados (1, 2). O índice de massa corporal (IMC), calculado pela divisão do peso de uma pessoa (em Kg) pelo quadrado da sua altura (em metros), é o parâmetro mais utilizado para avaliar a adiposidade geral dos indivíduos em ambos os sexos. A ocorrência de sobrepeso em adultos é definida pelo IMC entre 25 e 29,9 Kg/m^2 e obesidade pelo IMC $\geq$ a 30 Kg/m^2 (3).

Segundo dados da Organização Mundial de Saúde (OMS), a prevalência de obesidade praticamente triplicou nas últimas décadas. Em 2016, mais de 1,9 bilhões de adultos estavam com sobrepeso, sendo que, destes mais de 650 milhões tinham obesidade (13% da população acima de 18 anos). Ainda, cerca de 2,8 milhões de pessoas morrem a cada ano devido ao sobrepeso e obesidade (3). No Brasil, a prevalência de obesidade é estimada em 18,9%, sendo a prevalência em Porto Alegre de 19% (4). Além disso, esse aumento está associado com aumento da morbimortalidade e risco aumentado para outras doenças crônicas, como o diabetes mellitus tipo 2 (DM2), doenças cardiovasculares, distúrbios musculoesqueléticos e alguns tipos de cânceres (1).

A obesidade é decorrente da complexa interação entre meio ambiente, suscetibilidade genética e epigenética (2). O aumento na sua prevalência pode ser atribuído a diversas mudanças sociais e ambientais, as quais incluem hábitos alimentares inadequados, sedentarismo, fatores emocionais, urbanização e acesso socioeconômico dependente a uma dieta saudável (1). Além disso, estudos em gêmeos

monozigóticos demonstraram que 50-70% da variância fenotípica dos valores do IMC pode ser explicada por fatores genéticos (5). Adicionalmente, alterações induzidas pela epigenética na expressão gênica emergiram como uma forma alternativa na qual os fatores ambientais podem influenciar a obesidade (1).

1.2 A proteína desacopladora 2 (UCP2) e a obesidade

As mitocôndrias estão localizadas no citoplasma de todas as células eucarióticas e estão envolvidas em diversos processos vitais para a célula, tais como produção de energia, controle redox, homeostase de cálcio e certas rotas metabólicas e de biossíntese, através da capacidade de produzir ATP e controlar a degradação de substratos [revisado em (6, 7)].

Proteínas desacopladoras (*uncoupling proteins* - UCPs) estão presentes na membrana mitocondrial interna e fazem parte de uma superfamília de proteínas transportadoras (8). A proteína UCP2 foi descoberta em 1997 por Fleury *et al*. (9) e possui uma distribuição tecidual bastante ampla, sendo expressa no tecido adiposo (TA) branco e marrom, hipotálamo, músculo esquelético, macrófagos, entre outros [revisado em (6, 10)].

Estudos demonstraram que através do transporte de prótons do espaço intermembranas para a matriz mitocondrial, a UCP2 desacopla a oxidação dos substratos da síntese de ATP, dissipando a energia do potencial de membrana e, consequentemente, diminuindo a produção de ATP pela cadeia respiratória mitocondrial **(Figura 1)**. Esse desacoplamento está associado a funções tecido-específicas como controle da ingestão alimentar, regulação do gasto energético e do metabolismo de ácidos graxos livres, regulação negativa da secreção de insulina pelas células-beta pancreáticas, diminuição da formação de espécies reativas de oxigênio (EROs),

mecanismos associados à patogênese da obesidade e de suas comorbidades como o DM2 [revisado em (6, 10)].

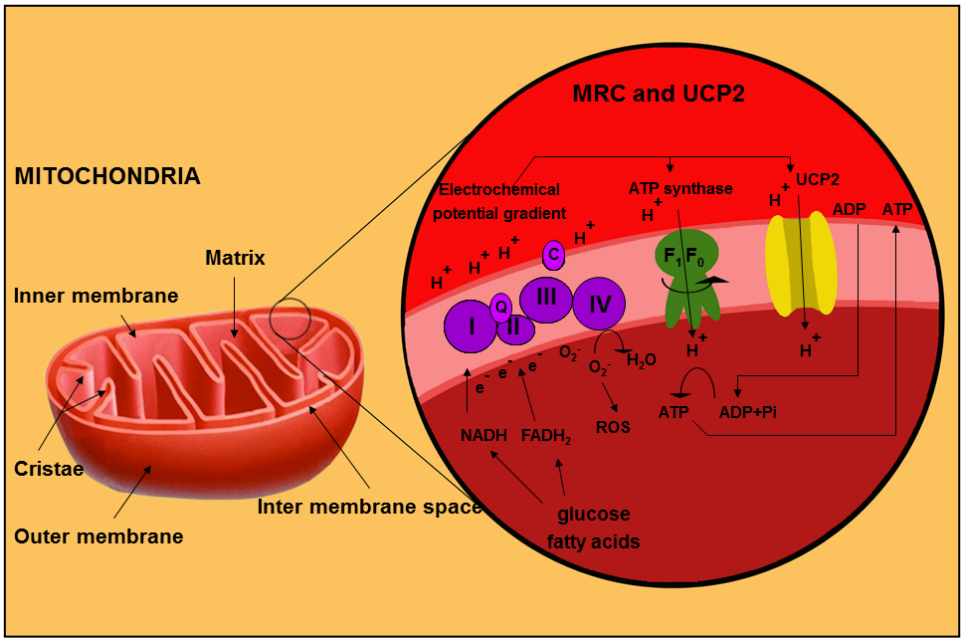

Figura 1. Localização e função da UCP2 na cadeia respiratória mitocondrial. Retirado e adaptado de Souza *et al.* (6)

Em humanos, o gene *UCP2* encontra-se localizado na região 11q13 do cromossomo 11, a qual está ligada à taxa metabólica basal e percentual de gordura corporal (9, 11). Evidências demonstram que apesar de o RNAm da *UCP2* ser encontrado em diversos tecidos, estes níveis não são proporcionais a quantidade de proteína expressa, o que pode ser explicado por diferentes mecanismos de regulação pós-transcricionais e pós-traducionais (7, 12).

Ácidos graxos são importantes reguladores fisiológicos da *UCP2* através da via dos PPAR (*peroxisome proliferator-activated receptor*)-*γ,* PPAR-α e SREBP-1c (*sterol regulatory element-binding protein 1*) de uma maneira tecido-específica (13). Em uma linhagem de adipócitos, ácidos graxos insaturados marcadamente induziram a expressão

de *UCP2* (14). Após a adição de ácidos graxos livres ao meio de cultura, a expressão de *UCP2* também mostrou-se aumentada em linhagens celulares derivadas do fígado e células-beta pancreáticas (14). Além disso, a UCP2 parece atuar no transporte direto de ácidos graxos, quando há um excesso destes dentro da mitocôndria, protegendo as células do dano causado pela peroxidação dos ácidos graxos poli-insaturados [revisado em (15)].

Estudos em humanos sugerem uma diminuição da expressão de *UCP2* em TA subcutâneo (TAS) e omental de adultos com obesidade em comparação com controles eutróficos (16, 17). De acordo com isso, também foi observada a redução de *UCP2* no TAS de crianças com obesidade (18). No entanto, outros estudos não encontraram diferenças na expressão de *UCP2* em TA de indivíduos com obesidade em relação aos indivíduos eutróficos, mostrando que o papel da UCP2 na obesidade ainda é controverso (19, 20).

De Oliveira *et al*. observaram um aumento da expressão de *UCP2* em TAS de pacientes 6 meses após a cirurgia bariátrica em comparação com o período pré-operatório, sugerindo que este aumento de *UCP2* está associado a um maior gasto energético, podendo predizer a perda de peso após a cirurgia (21). Corroborando com esses achados, Heinitz *et al*., mostraram que pacientes com expressão reduzida de *UCP2* no músculo esquelético tiveram uma maior redução do gasto energético durante o período de restrição calórica e perderam menos peso (22), reforçando a importância do papel da UCP2 na obesidade.

1.3 O inflamassoma NLRP3 e a obesidade

O TA é o principal local de armazenamento do excesso de energia, na forma de triglicerídeos. O crescimento massivo no número e tamanho dos adipócitos contribui

para um estado de inflamação crônica de baixo grau, uma característica da obesidade. Essa inflamação está associada à produção aumentada de citocinas proinflamatórias (23).

Evidências sugerem que o inflamassoma NLRP3 (*NLR family pyrin domain containing 3*) é um regulador chave da inflamação metabólica, possuindo um papel importante na patogênese da obesidade através da produção de citocinas proinflamatórias IL-1β e IL-18 no TA (23-25).

O NLRP3 possui a capacidade de reconhecer uma variedade de sinais de perigo, como padrões moleculares associados a patógenos- ou a danos- (PAMPs ou DAMPs). Os DAMPs incluem moléculas associadas à obesidade, tais como, ácidos graxos livres, glicose, EROS e ceramidas, que podem ativar o complexo inflamassoma. Esse complexo multiproteico é composto pela proteína NLR (*nucleotide-binding domain, leucine rich containing*), pela ASC (*adaptor protein apoptosis-associated speck-like protein containing CARD*) e pela enzima caspase-1 e pode ser ativado por meio de dois sinais. O primeiro sinal, dado através da ativação de NF-κB (*nuclear factor kappa B*) no núcleo, irá gerar NLRP3, pró-IL-1ß e pró-IL-18 inativos via receptores do tipo Toll (TLRs) (26). O segundo sinal, através de EROs e ruptura lisossômica, entre outros, facilitará a oligomerização do NLRP3 inativo com as proteína ASC e procaspase-1, formando o inflamassoma NLRP3:ASC:caspase-1 ativo, o qual irá então clivar as citocinas inativas pró-IL-1β e pró-IL-18, transformando-as em citocinas ativas IL-1β e IL-18 (27) **(Figura 2)**.

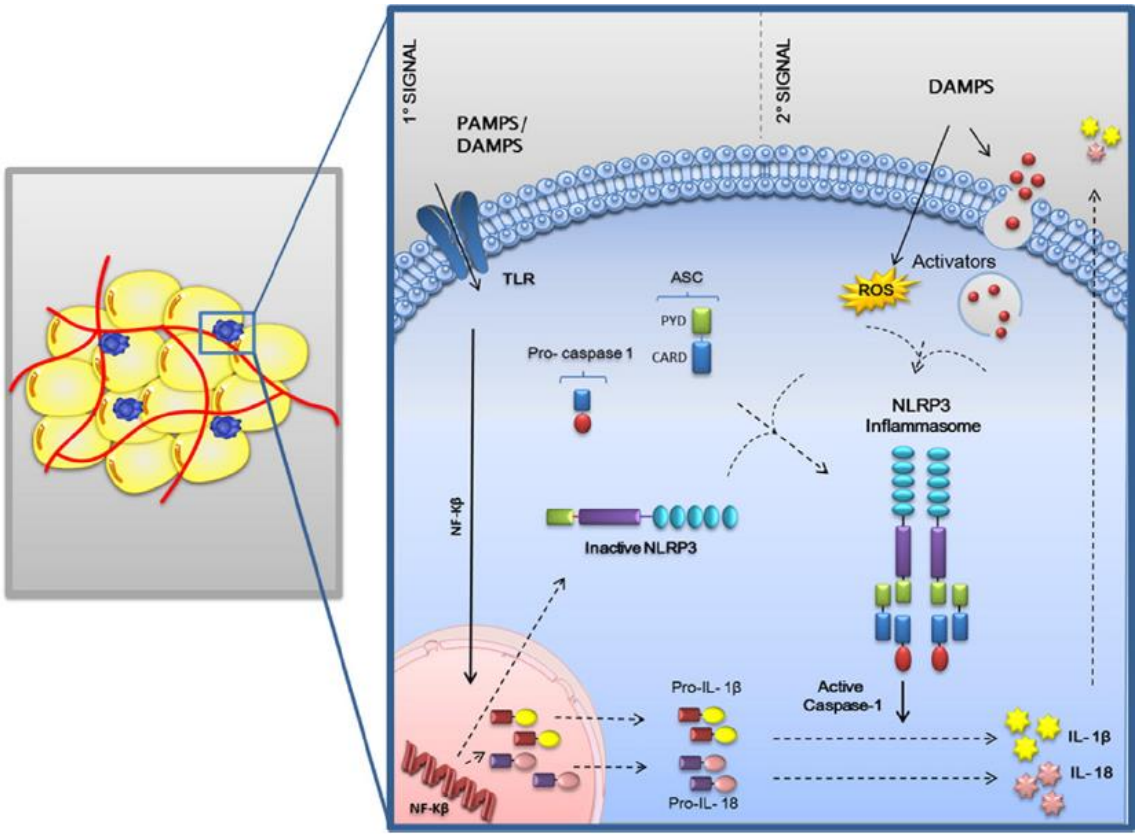

Figura 2. Ativação do inflamassoma NLRP3. Dois sinais são necessários para ativação deste complexo. Em pacientes obesos, macrófagos presentes no tecido adiposo são ativados por exposição a PAMPs ou DAMPs, agindo principalmente através de receptores TLRs. Este primeiro sinal ativa NF-κB no núcleo, levando à transcrição de NLRP3 inativo, pro-IL-1β e pro-IL-18, que, após a tradução, permanecem no citoplasma em suas formas inativas. O segundo sinal, que inclui a geração de EROs e a ruptura lisossômica, entre outros, ativa o inflamassoma NLRP3, facilitando a oligomerização do NLRP3 inativo, a proteína ASC e a procaspase-1. Este complexo ativado, por sua vez, catalisa a conversão da procaspase-1 a caspase-1, que promoverá a conversão de pró-IL-1β em IL-1β e pró-IL-18 em IL-18. Retirado e adaptado de Rheinheimer *et al.* (28).

Uma revisão sistemática realizada recentemente pelo nosso grupo investigou a associação do NLRP3 com obesidade e resistência à insulina. Interessantemente, a maior parte dos estudos em humanos demonstrou um aumento da expressão do *NLRP3*

em TA de indivíduos com obesidade em comparação aos indivíduos magros (**Tabela 1**) (28).

Tabela 1: Estudos que avaliaram a associação entre a expressão do NLRP3 e obesidade em humanos.

1º autor, ano (Ref.)	**Amostra**	**Tecido**	**Resultados**
Bando, 2015 (29)	Pacientes que implantaram um dispositivo cardíaco	TAS	↑ NLRP3 em pacientes com obesidade, dislipidemia e/ou DM. NLRP3 correlacionou-se com IMC (+) e adiponectina (-)
Esser, 2013 (30)	Indivíduos com obesidade metabolicamente não saudáveis, indivíduos com obesidade metabolicamente saudáveis e indivíduos eutróficos	TAS/TAV	↑ NLRP3 em TAV de indivíduos com obesidade metabolicamente não saudáveis *vs.* metabolicamente saudáveis e eutróficos; NLRP3 correlacionou-se com resistência à insulina (+)
Goossens, 2012 (31)	Pacientes com obesidade *vs.* indivíduos eutróficos	SAT	-- NLRP3 foi similar entre os grupos
Kursawe, 2016 (32)	Adolescentes com obesidade	TAS	↑ NLRP3 em TAS de adolescentes com alta razão VAT/VAT + SAT *vs.* grupo de baixa razão
Moschen, 2011 (33)	Pacientes com obesidade grave (antes e depois de LAGB)	TAS/TAV/ fígado	-- NLRP3 não foi afetado pela perda de peso
Serena, 2016	Indivíduos com	Células	↑ NLRP3 em células tronco

(34)	obesidade *vs.* indivíduos eutróficos	tronco derivadas de adipócitos	derivadas de adipócitos de indivíduos com obesidade e/ou DM2 *vs.* indivíduos eutróficos
Vandanmagsar, 2011 (35)	Pacientes com obesidade e DM2 (antes e 1 ano após a perda de peso)	TAS	↓ NLRP3 em TAS de pacientes com DM2 após perda de peso, que foi associada com melhora na sensibilidade à insulina
Yin, 2014 (36)	Mulheres eutróficas *vs.* mulheres com obesidade	TAS	↑ NLRP3 em TAS de mulheres com obesidade *vs.* mulheres eutróficas

TAS, tecido adiposo subcutâneo; TAV, tecido adiposo visceral; IMC, índice de massa corporal; DM, diabetes mellitus; DM2, diabetes mellitus tipo 2; LAGB, *Laparoscopic adjustable gastric banding*. Retirado e adaptado de Rheinheimer *et al*. (28).

Interessantemente, há poucos estudos descrevendo o papel da UCP2 na ativação do inflamassoma NLRP3 (37-39). Estudos realizados em astrócitos e macrófagos de camundongos demostraram que a deficiência de *Ucp2* acentua a produção de EROs e também agrava a inflamação através da ativação do inflamassoma, mostrando a relevância da produção de EROs como DAMP para a ativação do NLRP3 (37-39). Por outro lado, a superexpressão de *UCP2* em macrófagos humanos e de camundongos aumentou significativamente a expressão de *NLRP3* e a sua deficiência ou inibição diminuiu a expressão de *NLRP3*; sugerindo que a UCP2, pelo menos em parte, pode também ativar o inflamassoma NLRP3 através da inibição da via de síntese lipídica ou pelo seu papel de influência na fagocitose (40, 41).

1.4 MicroRNA 133a-3p como regulador da expressão da *UCP2* e do *NLRP3*

Os microRNAs (miRNAs) são moléculas de RNA fita simples (19–25 nucleotídeos) não codificadores de proteínas, que agem como potentes reguladores pós-transcricionais da expressão gênica em plantas e animais (42). Os miRNAs exercem seus efeitos regulatórios ligando-se aos seus genes ou RNAm alvos, clivando-os e resultando em instabilidade e degradação destes RNAs ou em inibição da tradução **(Figura 3)**. Além disso, o impacto funcional dessa ligação irá depender do grau de complementariedade entre a "seed sequence" (2-8 pares de bases) do miRNA e o RNAm alvo. Dessa forma, este mecanismo permite a redução dos níveis das proteínas de seus genes-alvo, raramente afetando o nível de expressão transcricional (42).

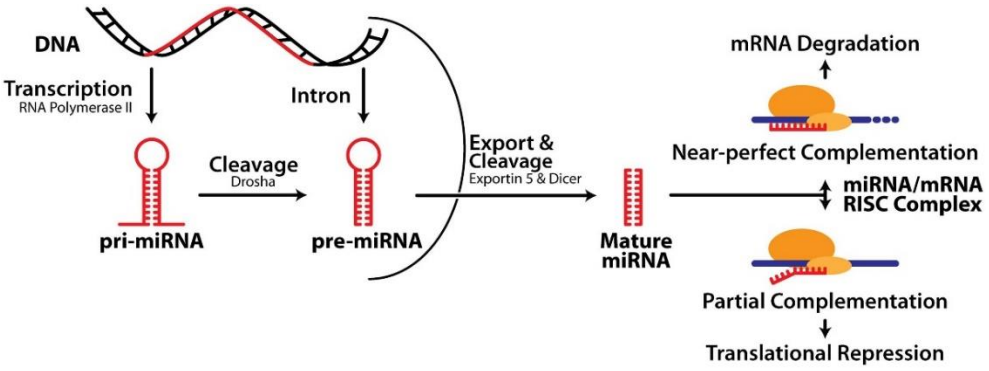

Figura 3. Biogênese e mecanismo de ação dos miRNAs. A biogênese do miRNA consiste em três processos principais: transcrição de um transcrito primário maior (pri-miRNA), seguido por duas clivagens consecutivas feitas pelas enzimas RNAses II, Drosha e Dicer, que irão gerar um miRNA precursor (pré-miRNA) e por fim, a liberação do miRNA maduro. Retirado e adaptado de Ryan *et al*. (43).

Existem evidências de que alterações na expressão de miRNAs possam estar envolvidas em diversos processos patológicos, incluindo na patogênese da obesidade

[revisado em (44, 45)]. O miR-133a-3p foi inicialmente classificado como um miRNA específico de miócitos (*"myomiR"*) e cardiomiócitos (46). Além disso, estudos sugerem que este miR pode ter papel na fibrose tecidual, sepse, desenvolvimento de cânceres, inflamação e resistência à insulina (47-49).

O miR-133a-3p já foi avaliado no TA em modelos animais, onde o bloqueio duplo de miR-133a1 e miR-133a2 em camundongos promoveu a expressão de genes relacionados à termogênese e o *browning* no TAS. Além disso, camundongos *knockout* para miR133a1$^{-/-}$a2$^{+/-}$ tiveram uma maior sensibilidade à insulina e tolerância à glicose em comparação aos animais selvagens (50). De acordo com este achado, a administração de reversina (um inibidor do miR-133a) em camundongos, induziu o *browning* no TAV e atenuou o ganho de peso induzido por dieta rica em gordura, através do aumento da termogênese (51).

Apesar de o miR-133a-3p-3p ainda não ter sido estudado em humanos no contexto da obesidade, ele parece estar envolvido na regulação da *UCP2* e do *NLRP3* (52-54). Alguns estudos *in vitro* e em modelos animais verificaram que o miR-133a-3p regula a expressão de *UCP2* em diferentes tecidos, tais como, músculo esquelético e cardíaco, macrófagos, entre outros (53, 55-57). Um estudo em modelo murino de doença inflamatória intestinal demonstrou que tanto bloquear a *UCP2* (com siRNA) ou aumentar a expressão do miR-133a-3p-1 foram capazes de aliviar a severidade da doença. Além disso, estas modificações também alteraram marcadores de inflamação, estresse oxidativo e metabolismo energético (55).

Bandyopadhyay *et al*. (53) demonstraram o envolvimento do miR-133a-3p na supressão do inflamassoma NLRP3 por meio da diminuição da expressão de *UCP2*. Esses autores relataram que a transfecção de uma linhagem de monócitos humanos (THP1) com o miR-133a-3p-1 diminuiu a expressão de *UCP2.* Além disso, mostraram

que a supressão de *UCP2* aumentou a ativação do inflamassoma, enquanto que a sua superexpressão diminuiu esta ativação (53), mostrando o envolvimento deste miRNA na regulação desses dois genes.

2 JUSTIFICATIVA

Devido ao aumento da epidemia da obesidade, novas alternativas têm-se tornado necessárias para o tratamento dessa doença. Além disso, as comorbidades associadas à obesidade, como o DM2 e danos cardiovasculares, bem como a própria obesidade, comprometem a qualidade de vida e a produtividade dos pacientes gerando grandes custos ao sistema de saúde.

Estudos indicam que a UCP2 parece ter um importante papel na regulação do gasto energético, enquanto que o inflamassoma NLRP3 parece estar envolvido na inflamação crônica de baixo grau presente na obesidade. Dessa forma, ambos poderiam estar contribuindo para a patogênese dessa doença.

Além disso, mais recentemente, as alterações epigenéticas na expressão gênica, através dos miRNAs, emergiram como um caminho alternativo no qual os fatores ambientais podem influenciar no fenótipo da obesidade, tornando-se um amplo caminho a ser pesquisado. Nesse contexto, avaliar a função de miRNAs na regulação de genes envolvidos com o gasto energético e inflamação, presentes na obesidade, é fundamental para uma melhor compreensão da patogênese dessa doença.

Até o momento, poucos estudos avaliaram a expressão de *UCP2* e de *NLRP3* em TAS de indivíduos obesos e eutróficos, com resultados contraditórios. Além disso, nenhum estudo avaliou a expressão do miR-133a-1 e sua relação com a *UCP2* e o *NLRP3* e obesidade.

3 OBJETIVO

3.1 Objetivo Geral

Avaliar as expressões de *UCP2*, *NLRP3* e do miR-133a-3p no tecido adiposo subcutâneo de pacientes com diferentes graus de obesidade e de indivíduos eutróficos.

3.2 Objetivos Específicos

1. Comparar as expressões de *UCP2*, *NLRP3* e do miR-133a-3p no tecido adiposo subcutâneo de pacientes com diferentes graus de obesidade e de indivíduos eutróficos;

2. Correlacionar os níveis de expressão de *UCP2*, *NLRP3* e do miR-133a-3p com características clínicas e laboratoriais relacionadas à obesidade nesses pacientes;

3. Avaliar se as expressões de *UCP2*, *NLRP3* e miR-133a-3p se correlacionam entre si.

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CAPÍTULO 2

ARTIGO ORIGINAL: OBESITY IS ASSOCIATED WITH A DOWNREGULATION OF *UCP2* AND MIR-133a-3p BUT NOT *NLRP3* IN SUBCUTANEOUS ADIPOSE TISSUE

Obesity is associated with a downregulation of *UCP2* and miR-133a-3p but not *NLRP3* in subcutaneous adipose tissue

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KEYWORDS: Obesity; *UCP2*, miR-133a-3p, *NLRP3*, Gene expression; Adipose tissue.

ABSTRACT

Background and Aims: Uncoupling protein 2 (UCP2) plays an important role in the regulation of energy expenditure. NLR family, pyrin domain-containing 3 (NLRP3) inflammasome seems to have a key function in obesity-induced inflammation. In this context, a number of studies have suggested that *UCP2* and *NLRP3* have an important role in obesity, and the microRNA (miR)-133a-3p appears to regulate NLRP3 activation through *UCP2* blockade. Thus, we compared *UCP2*, *NLRP3* and miR-133a-3p expressions in subcutaneous adipose tissue (SAT) of patients with obesity and eutrophic individuals. We also evaluated if these genes correlated with body composition parameters, insulin resistance, and lipid and glycemic profiles.

Methods: SAT biopsies were obtained from 61 individuals who underwent bariatric surgery or elective abdominal surgery. Patients were divided in 3 groups according their body mass indexes (BMI): Group 1 (n= 8; BMI <25.0 kg/m^2), Group 2 (n= 24; BMI 30.0-39.9 kg/m^2) and Group 3 (n= 29; BMI ≥40.0 kg/m^2). All subjects underwent physical and laboratory evaluations. *UCP2, NLRP3* and miR-133a-3p expressions were quantified using qPCR.

Results: *UCP2* and miR-133a-3p expressions were decreased in SAT from patients with obesity (Group 2 + Group 3) compared to Group 1 patients, while *NLRP3* expression did not differ among groups. *UCP2* expression was negatively correlated with *NLRP3* expression. Moreover, *NLRP3* expression was positively correlated to miR-133a-3p expression, waist circumference and weight excess.

Conclusions: Our results suggests that *UCP2* and miR-133a-3p are downregulated in patients with obesity. Although *NRLP3* expression did not differ among patients from different BMI categories, it correlated with waist circumference and weight excess.

1. INTRODUCTION

Obesity is a chronic multifactorial disease that affects more than 650 million adults and over than 124 million children and adolescents worldwide (1, 2). It is characterized by an excessive accumulation of body fat resulting from an unbalance between energy intake and energy expenditure (3). In adipose tissue (AT), excess of energy is stored inside cytosolic lipid droplets as triglycerides (4). The massive adipocyte expansion in number and size contributes to a chronic low-grade inflammation state, a hallmark of obesity. This inflammation can lead to complications in other metabolic tissues through lipotoxicity and inflammatory pathways, leading to comorbidities such as insulin resistance (IR) and type 2 diabetes mellitus (T2DM) (5, 6).

Obesity is caused by a complex interaction between genetic, epigenetic and environmental factors (7). Recent studies have suggested that uncoupling protein 2 (*UCP2*) gene expression is reduced in omental and subcutaneous adipose tissue (SAT) in adults and children with obesity (8-10); however, other studies reported contradictory results (11, 12). UCP2 is located in the inner mitochondrial membrane and is widely expressed throughout the body tissues, including in the human white adipose tissue (WAT). It mildly uncouples substrate oxidation from ATP synthesis, thereby dissipating the membrane potential energy and, consequently, decreasing ATP production. The uncoupling thus leads to tissue-specific functions such as regulation of lipid metabolism, inhibition of insulin secretion from pancreatic beta cells, decreasing reactive oxygen species (ROS) formation by mitochondria and, importantly, energy expenditure regulation [reviewed in (13-15)], which might explain UCP2 association with obesity.

Compelling evidence suggests that the NLR family pyrin domain containing 3 (NLRP3) activation by danger-associated molecular patterns (DAMPs) has a key role in

obesity-induced inflammation (5, 16-18), with a number of studies showing increased NLRP3 expression in AT from humans and mice [reviewed in (18)]. DAMPs include obesity-associated danger signals, such as ROS, free-fatty acids (FFAs), and ceramides, which will activate NRLP3 inflammasome-caspase 1; thus leading to maturation of the pro-inflammatory cytokines IL-1β and IL-18 (5, 16, 17). Interestingly, UCP2 seems to have a role in the activation of NLRP3 inflammasome (19-22). Few studies have shown that *Ucp2* deficiency exacerbates ROS production and also aggravates inflammation through Nlrp3 inflammasome activation in astrocytes and macrophages from mice (19-21). In contrast, *UCP2* overexpression in human macrophages significantly upregulated *NLRP3* expression (23).

Regarding epigenetic factors, microRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression mainly by translation repression or transcript degradation (24); thus, dysregulation in their expressions have been linked to many diseases, including obesity (25). Previous studies in animal models reported that miR-133a-3p regulates *Ucp2* expression in different tissues, such as colonic (26), skeletal and cardiac muscles (27), and also in human breast cancer cells (28). In this context, Bandyopadhyay *et al*. (29) showed that this miRNA also has a role in NLRP3 inflammasome activation by suppressing expression of its direct target *UCP2* in human THP1 cell line derived from monocytes.

To date, no study has evaluated the combined association of *UCP2*, *NLRP3* and miR-133a-3p with obesity. Therefore, in the present study, we compared *UCP2*, *NLRP3* and miR-133a-3p expressions in SAT of patients with different degrees of obesity and in eutrophic individuals. Moreover, we evaluated if their expressions correlate with parameters of body composition, resting energy expenditure (REE), IR, glycemic and lipid profiles of these subjects.

2. METHODS

2.1. Study subjects

This case-control study was designed in accordance with STROBE guidelines for reporting of association studies (30). The sample population included 61 patients recruited from the outpatient clinic at Hospital de Clínicas de Porto Alegre (HCPA) between August 2013 and August 2016. Patients were divided into 3 groups according to body mass index (BMI) categories, as follows: 1) 8 lean patients with BMI between 18.5 and 25.0 kg/m^2 (Group 1); 2) 24 patients with BMI 30.0 – 39.9 kg/m^2 (Group 2: obesity degrees 1 or 2), and 3) 29 patients with BMI ≥40.0 kg/m^2 (Group 3: obesity degree 3). Obesity was classified following World Health Organization guidelines (2).

Biopsies of SAT from groups 1 and 2 were collected during elective laparoscopic abdominal surgeries, while for group 3, they were obtained during an open Roux-en-Y gastric bypass (RYGB) surgery. Eligible participants had to be 18-years old or older. Patients with presence of systemic infection, impaired thyroid function, any acute inflammatory disease, cancer, current treatment with systemic corticosteroids, pregnancy or use of any medication known to influence REE, as well as smokers were excluded from the study. Clinical, body composition, REE, and biochemical measurements were assessed preoperatively. The study protocol was approved by the Ethical Committee of HCPA, and all subjects gave assent and written informed consent prior to participation.

2.2. Clinical, body composition and biochemical measurements

A standard questionnaire was used to collect information concerning socio-demographic status, lifestyle habits (physical activity, smoking status, and alcohol consumption), and actual and previous health history and use of medications. All patients underwent physical and laboratory evaluations. Briefly, they were weighted unshod, wearing light clothes and their height was measured. BMI was calculated as weight (kg)/height^2 (meters), and the body composition [fat mass and fat-free mass (FFM)] was evaluated using dual-energy absorptiometry X-ray (DXA; Lunar Prodigy Advance; GE Medical Systems, Madison, WI, USA). REE was assessed by an open-circuit indirect calorimetry (Korr Medical Technologies, Model 7100, Salt Lake City, UT, USA). Respiratory data were collected over a 30-minute period, in the morning, after 12 hours of fasting.

Office blood pressure (BP) was measured in the sitting position, on the left arm, after a 5-min rest, with a digital sphygmomanometer Onrom (HEM-705CP). The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP. Systemic arterial hypertension was defined as BP ≥140/90 mmHg in two occasions or use of antihypertensive medications. Diagnosis of T2DM and pre-diabetes (pre-DM) was established according to the American Diabetes Association guidelines (31).

Fasting peripheral blood samples were collected from all patients before surgery for laboratory analyses. Total plasma cholesterol, HDL cholesterol and triglycerides were measured using enzymatic methods, and LDL cholesterol was calculated with the Friedewald equation. Fasting plasma glucose (FPG) levels were determined using the glucose oxidase method. Glycated hemoglobin (HbA1c) measurements were performed by high performance liquid chromatography of ion-exchange (Variant II Turbo; Bio-Rad, USA). Serum insulin was quantified by radioimmunoassay (Elecsys R Systems 1010/2010/ modular analysis E170 - ROCHE), and IR was estimated using the

homeostasis model assessment (HOMA-IR) index = fasting insulin (μUI/ml) x fasting plasma glucose (mmol/L) / 22.5 (32). Leptin and adiponectin levels were quantified in plasma using ELISA kits (Thermo Fisher Scientific; DE, USA), following the manufacturer's instructions.

2.3. RNA extraction and quantification of *UCP2*, *NLRP3* and miR-133a-3p expressions in SAT by qPCR

Immediately following SAT biopsy collection, samples were placed in RNAlater solution (Thermo Fisher Scientific) and stored at -80ºC until gene expression analyses. Total RNA was extracted from ≅100 mg of SAT using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Concentrations and quality of RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Fisher Scientific). Only RNA samples which achieved adequate purity ratios (A260/A280 = 1.9-2.1) were used for subsequent analyses. In addition, RNA integrity and purity were checked in agarose gels containing GelRed Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA, USA). The mean RNA concentration [± standard deviation (SD)] isolated from SAT was 80 ± 31 ng/μL.

For *UCP2* and *NLRP3* genes, reverse transcription of 250 ng of RNA into cDNA was carried out using the SuperScript VILO Master Mix (Thermo Fisher Scientific), following the manufacturers protocol for the random primer method. Then, cDNA was amplified by quantitative real-time PCR (qPCR). qPCR experiments were performed by monitoring in real-time the increase in fluorescence of the SYBR® Green dye (33). Primers for *UCP2*, *NLRP3* and their respective reference genes [beta-actin (*ACTB*) and beta2-microglobulin (*β2M*)] were designed using published human gene sequences and

the Primer Express 3.0 Software (Thermo Fisher Scientific), and are shown in the **Supplementary Table 1**.

PCR reactions were performed using 5 μL of 1X Fast SYBR Green Master Mix (Thermo Fisher Scientific), 0.5 μL (1 ng/μL) of forward and reverse primers for each gene and 1 μL of cDNA template (12.5 ng/μL for *UCP2* and 250 ng/μL for *NLRP3*), in a total volume of 10 μL. Then, cDNA was amplified by qPCR in the ViiA7 Real Time PCR System (Thermo Fisher Scientific). qPCR specificity was determined using melting curve analyses and the primers generated amplicons that produced a single sharp peak during the analyses. Each sample was assayed in triplicate and a negative control was included in each experiment. Quantification of *UCP2* and *NLRP3* expressions was performed by relative quantification using the comparative ΔΔCq method, and are expressed relative to *ACTB* or *β2M* genes, respectively. The ΔΔCq method calculates changes in gene expression as relative fold differences (n-fold changes) between an experimental and an external calibrator sample (34).

For hsa-miR-133a-3p expression, reverse transcription of 2 ng/μl of RNA into cDNA was carried out using TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific; assay reference number: 002246) and the small nuclear RNA, C/D box 48 (*SNORD48*) was used as reference gene (assay reference number: 001006). Next, qPCR experiments were performed using 5 μL of 1X TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific), 0.5 μL (1 ng/μL) of TaqMan MicroRNA Assays (Thermo Fisher Scientific), containing the specific primers for each target, and 1 μL of cDNA template (10 ng/μL), in a total volume of 10 μL. Each sample was assayed in triplicate and a negative control was included in each experiment. Quantification of hsa-miR-133a-3p was also performed in a ViiA7 Fast Real-Time PCR System using the comparative ΔΔCq method, as described above.

2.4. Statistical analyses

Normal distribution of variables was checked using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Variables with normal distributions are shown as mean ± SD. Variables with skewed distribution were log-transformed before analyses and are shown as median (25^{th} –75^{th} percentiles). Categorical data are shown as percentage. Clinical and laboratory characteristics and *UCP2, NLRP3* and mir133a-3p expressions were compared among groups by using unpaired Student's t-test, one-way ANOVA or χ^2 tests, as appropriate. When variables did not achieve a normal distribution after log-transformation, Kruskal-Wallis non-parametric test was used for comparisons. Correlations between quantitative variables were calculated using Pearson's or Spearman`s correlation coefficients, as appropriate, without and with adjustment for BMI. All statistical analyses were performed using SPSS version 18.0 (IBM SPSS Statistics, Chicago, IL, USA), and P values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Sample description

Sixty-one patients were included in the study, most of them female (77%, n = 47), white (83.6% n = 51), and with a mean age of 45.1 ± 13.4 years old. Moreover, 62.3% (n = 38) of them had systemic arterial hypertension, while 11.5% (n = 7) had pre-DM and 21.3% (n = 13) had T2DM. Clinical and laboratorial characteristics of patients according to BMI categories are shown in **Table 1**. Age, gender, ethnicity, total cholesterol, HDL and LDL levels, systolic BP and pre-DM frequency did not differ significantly among groups. Patients from Group 3 had increased HbA1c and

triglycerides levels, HOMA-IR values, diastolic BP, REE and prevalence of HAS compared to patients from Group 1. Fat mass and FFM were differently distributed among the three study groups. Adiponectin levels seem to be decreased in Group 2 and 3 patients compared to Group 1, while leptin levels were similarly increased in Group 2 and 3 patients compared to Group 1 subjects (**Table 1**).

3.2. *UCP2, NLRP3* and miR-133a-3p expressions in SAT from patients according to different BMI categories

No significant differences were observed when *UCP2*, *NLRP3* and miR-133a-3p expressions were compared between white and non-white patients (*UCP2*: P= 0.880, *NLRP3*: P= 0.853, and miR-133a-3p: P= 0.668); therefore, both ethnicities were further analyzed together.

Expressions of *UCP2, NLRP3* and miR-133a-3p were investigated in SAT of patients with different BMI categories (**Figure 1**). Patients with obesity (Group 2 + Group 3) showed decreased *UCP2* expression in SAT compared to eutrophic individuals [Group 3: 31.1 median (0.9 – 1.6, 25^{th} –75^{th} percentiles); Group 2: 1.1 (0.9 – 1.4); and Group 1: 2.2 (1.6 – 2.5) n-fold changes; P= 0.031]; although, the comparison of Group 3 with Group 1 did not reach formal statistical significance (P= 0.057; **Figure 1A**).

NLRP3 expression did not differ significantly among groups (P= 0.238, **Figure 1B)**. Similarly to *UCP2*, miR-133a-3p seems to be downregulated in patients with obesity compared to Group 1 [Group 3: 1.6 (0.9 – 3.0); Group 2: 0.9 (0.5 – 8.5); and Group 1: 13.5 (3.5 – 33.2)] n-fold changes; P= 0.029]; although, the comparison of Group 3 with Group 1 also did not reach statistical significance (P= 0.09; **Figure 1C**).

3.3. Correlations between *UCP2, NLRP3* and miR-133a-3p expressions and clinical and laboratory characteristics

Correlations analyses between *UCP2, NLRP3* and miR-133a-3p expressions in SAT and clinical and laboratory characteristics for the whole sample are shown in **Table 2**. *UCP2* expression was negatively correlated with *NLRP3* expression (*r*= -0.307, P= 0.024), which seems to remain correlated after BMI adjustment (P= 0.051). *NLRP3* was positively correlated with miR-133a-3p expression (*r*= 0.353, P= 0.013), which was maintained after BMI adjustment (P= 0.003). *UCP2* expression did not correlate with miR-133a-3p expression (*r*= 0.11, P= 0.941) (**Table 2**).

Moreover, *UCP2* expression was negatively correlated with REE (*r*= -0.274, P= 0.045) and systolic BP (*r*= -0.291, P= 0.033); however, these correlations were lost after BMI adjustment. *NLRP3* expression showed a significant positive correlation with waist circumference (*r*= 0.268, P= 0.037) and weight excess (*r* = 0.355, P= 0.010). The expression of this gene was also positively correlated with triglycerides (*r*= 0.310, P= 0.017) and REE (*r*= 0.261, P= 0.048) while was negatively correlated with adiponectin levels (*r* = -0.264, P= 0.049), but these correlations were lost after BMI adjustment. In addition, miR-133a-3p expression was negatively correlated with HbA1c levels (*r*= -0.338, P= 0.018), but this was not maintained after BMI adjustment (P= 0.066). This miRNA was also correlated with age after adjusting for BMI (*r*= -0.288, P= 0.047) (**Table 2**).

4. DISCUSSION

A number of studies have suggested that *UCP2* and *NLRP3* have an important role in obesity (8-10, 35, 36), and miR-133a-3p appears to regulate NLRP3 activation through *UCP2* blockade (29). However, to date, no study evaluated the combined association of *UCP2*, *NLRP3* and miR-133a-3p in obesity. Therefore, in this study, we verified a decrease in *UCP2* and miR-133a-3p expressions in SAT from patients with obesity when compared to eutrophic patients, while *NLRP3* expression was not different among groups.

UCP2 is expressed in a wide range of tissues and plays a crucial role in controlling energy expenditure due to the membrane potential energy dissipation [reviewed in (13-15)]. Our present results showing *UCP2* downregulation in patients with obesity is in accordance with this role. Hence, we hypothesized that this downregulation may result in a decreased production of UCP2 protein, consequently, decreasing energy expenditure and, thereafter, increasing accumulation of fat. Accordingly, Heinitz *et al*. (37) showed a significant *UCP2* downregulation in skeletal muscle from patients with obesity submitted to long-term caloric restriction, which was associated with lower energy expenditure and less weight loss. Moreover, our results are in agreement with previous studies showing decreased *UCP2* expression in SAT from adults and children with obesity (8-10). In contrast, Vidal Puig *et al.* (12) did not report any difference in *UCP2* expression in SAT from individuals with obesity and lean individuals. Hence, the association between *UCP2* and obesity is still under debate. Of note, polymorphisms in this gene have been associated with susceptibility for obesity and BMI differences in a number of ethnicities [reviewed in (38, 39)].

Obesity-associated signals derived from cellular stress, such as FFAs, glucose, and ROS can activate the NLRP3 inflammasome complex, leading to IL-1β and IL-18 production; thus, increasing obesity-induced inflammation. Accordingly, few studies

have shown increased *NLRP3* expression in AT from patients with obesity compared to lean subjects (36, 40, 41). In addition, *NLRP3* expression was upregulated in obese adolescents with high visceral adipose tissue (VAT)/VAT + SAT ratio compared to obese adolescents with low ratio (36). Bando *et al*. (40) found higher *NLRP3* expression in patients with obesity compared to lean subjects, which was positively correlated with BMI and negatively correlated with adiponectin levels. Accordingly, Esser *et al.* (41) showed that *NLRP3* expression was upregulated in VAT from metabolically unhealthy obese patients compared to metabolically healthy obese patients. In these subjects, *NLRP3* expression was positively correlated with IR values. However, in our study, we could not find an association between *NLRP3* expression and obesity. In agreement with our data, Goossens *et al.* (42) also did not find differences in *NLRP3* expression in SAT from individuals with obesity and lean subjects. In addition, *NLRP3* expression was not affected by bariatric-induced weight loss in patients with obesity (43). Although we did not find differences in *NLRP3* expression among the analyzed groups, we observed positive correlations of this gene with waist circumference and weight excess, indicating that it may play a role in fat accumulation.

Since the main source of cellular ROS is the mitochondria, a decrease in *UCP2* expression observed in patients with obesity and T2DM might increase cellular ROS, which, in turn, could act as a DAMP for activation of the NLRP3 inflammasome (44). In agreement with this hypothesis, some studies have suggested that UCP2 is an important regulator of the NLRP3 inflammasome (19-23). *Ucp2* deficiency in mice exacerbated ROS production and aggravated inflammation through increased Nlrp3 inflammasome activation, showing the relevance of mitochondrial ROS production for NLRP3 activation (19-21). Moreover, Moon *et al*. (22) showed that *Ucp2* deficient macrophages from mice had suppressed NLRP3-mediated caspase-1 activation

associated with inhibition of lipid synthesis. Hence, UCP2 also seems to regulate NLRP3 inflammasome activation through increased production of DAMPs derived from lipid synthesis. In agreement with these studies, Rajanbabu *et al*. (23) showed that *UCP2* overexpression in THP1 cells increased *NLRP3* expression, while cells treated with genipin (an UCP2 inhibitor) had suppressed *NLRP3* expression. The present study shows that *UCP2* expression in SAT was negatively correlated with *NLRP3* expression after adjustment by BMI, corroborating that UCP2 can be an NLRP3 regulator (29).

MiRNAs have been recently discovered as biological regulators with potential to control inflammation through several pathways (45, 46). In the present study, we showed for the first time that miR-133a-3p is downregulated in SAT from patients with obesity compared to lean individuals. Despite the lack of studies evaluating the association between miR-133a-3p expression and obesity in humans, Liu *et al*. (47) reported that double knockout of miR-133a1 and miR-133a2 in mice promoted thermogenic gene programs and browning of SAT. Furthermore, they showed that miR133a1$^{-/-}$a2$^{+/-}$ deficient mice exhibited increased insulin sensitivity and glucose tolerance compared to wildtype mice. Accordingly, a recent study showed that intraperitoneal administration of reversine, a miR-133a-3p inhibitor, induced browning of VAT and attenuated diet-induced weight- gain through enhanced thermogenesis in mice (48). Moreover, treatment with high-fat diet decreased miR-133a expression in soleus and gastrocnemius muscles from mice (49), reinforcing the regulatory role of miR-133a-3p in AT.

As already mentioned, previous studies *in vitro* and in animal models reported that miR-133a-3p regulates *UCP2* in different tissues (26-28). Moreover, miR-133a-3p seems to be involved in NLRP3 inflammasome activation. Bandyopadhyay *et al.* (29) reported that transfection of THP1 cells with miR-133a-3p decreased *UCP2* expression and

increased NLRP3 activation. In addition, they showed that suppression of *Ucp2* increased inflammasome activation, whereas its overexpression decreased this activation. Here, we did not find any correlation between *UCP2* and miR-133a-3p expressions. Nevertheless, is important to considerate that miRNAs negatively regulate gene expression by translation repression or transcript degradation, consequently decreasing protein levels rather than mRNA content. In this study we evaluated only *UCP2* mRNA; hence, we cannot exclude the possibility that this miRNA is decreasing UCP2 protein levels.

Despite the relevant results, this study has few limitations. First, the number of patients included in each group is relatively small to perform stratification analyses; however, our observation of significant correlations even with this sample size makes our data more compelling. Second, our results are limited to SAT depot. We acknowledge that VAT may be more metabolically active than SAT; nevertheless, VAT biopsies were not possible during laparoscopic abdominal surgeries, which was the case for lean subjects and patients with obesity degree 2. Finally, because of shortage of SAT samples, it was not possible to performed Western Blot analysis to verify UCP2 protein content.

In conclusion, *UCP2* and miR-133a-3p expressions are decreased in SAT from patients with obesity. Although *NRLP3* expression did not differ among patients stratified by BMI categories, it was positively correlated with weigh excess and waist circumference. These results strengthen the roles of UCP2, NLRP3 and miR-133a-3p-3p in obesity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure Legend

Figure 1. *UCP2*, *NLRP3* and miR133a-3p expressions according to BMI categories: Group 1 (BMI <25.0 kg/m^2), Group 2 (BMI 30.0 – 39.9 kg/m^2) and Group 3 (BMI 40.0 kg/m^2). Relative expressions of **A)** *UCP2*, **B)** *NLRP3* and **C)** miR133a-3p were evaluated using qPCR. P values were obtained using Kruskal-Wallis tests with post-hoc analyses. * P< 0.050 and # P< 0.100. Results are expressed as n-fold changes. The horizontal lines inside each box indicate medians, the top and bottom of the boxes indicate interquartile ranges, whiskers indicate the 5th and 95th percentiles, and circles indicate outlier values.

TABLE 1. Clinical and laboratory characteristics of patients included in the study according to BMI categories

	Group 1 **(n = 8)**	**Group 2** **(n = 24)**	**Group 3** **(n = 29)**	**P value**
Age (years)	43.0 (29.0 –59.2)	46.5 (33.0 – 58.0)	42.0 (33.5 – 53.0)	0.831
Ethnicity (% non-white)	37.5	12.5	13.8	0.304
Gender (% male)	12.5	33.3	17.2	0.288
BMI (kg/m^2)	21.4 ± 1.9	33.6 ± 3.3	47.8 ± 9.6	-
HOMA-IR	3.4 (2.6 – 5.3) [a]	9.1 (4.7 – 18.9) [ab]	11.3 (7.2 – 19.6) [b]	**0.028**
HbA1c (%)	5.2 (4.9 – 5.6) [a]	5.5 (5.3 – 6.3) [ab]	6.0 (5.5 – 7.0) [b]	**0.017**
REE (kcal/d)	1247.5 ± 265.6 [a]	1820.4 ± 616.1 [ab]	2182.8 ± 664.7 [b]	**0.001**
Fat mass (kg)	18.1 ±3.8 [a]	36.3 ± 9.8 [b]	61.7 ± 13.0 [c]	**< 0.001**
Fat-free mass (kg)	38.5 (32.5 – 40.6) [a]	52.0 (45.1 – 56.6) [b]	60.9 (53.5 – 68.0) [c]	**< 0.001**
Systolic BP (mmHg)	119.7 ± 27.3	127.8 ± 13.3	133.5 ± 14.7	0.096
Diastolic BP (mmHg)	67.5 ± 7.8 [a]	75.9 ± 11.7 [ab]	81.9 ± 10.1 [b]	**0.003**
Hypertension (%)	12.5[a]	58.3[ab]	79.3[b]	**0.002**

Total cholesterol (mg/dL)	149.5 (147.0 – 162.0)	176.5 (160.0 – 221.2)	174 (152.5 – 212.0)	0.163
Triglycerides (mg/dL)	41.0 (25.7 – 98.7) [a]	94.0 (65.7 – 149.2) [ab]	129.0 (100.5 – 233.5) [b]	**0.001**
LDL-cholesterol (mg/dL)	142.1 (135.4 – 170.0)	153.2 (139.4 – 181.7)	136.2 (117.9 – 168.9)	0.185
HDL-cholesterol (mg/dL)	47.6 ± 13.7	41.9 ± 9.6	38.3 ± 9.9	0.074
T2DM (yes, %)	12.5 [ab]	4.2 [a]	37.9 [b]	**0.009**
Pre-diabetes (yes, %)	12.5	8.3	13.8	0.821
Adiponectin (μg/ml)	21.1 (14.2 – 39.7) [a]	13.2 (11.1 – 16.6) [b]	13.9 (10.0 – 19.2) [b]	**0.036**
Leptin (ng/ml)	13.7 (6.2 – 25.4) [a]	44.5 (25.7 – 65.9) [b]	98.7 (32.8 – 140.4) [b]	**< 0.001**

Abbreviations: BMI, body mass index; BP, blood pressure; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; REE, rest energy expenditure; T2DM, type 2 diabetes mellitus. Data are expressed as mean ± SD, median (25th - 75th percentiles), or %. P values were obtained with χ^2 tests, ANOVA or Kruskal Wallis, followed by post-hoc tests as appropriate. Variables with equal letters do not differ significantly in the statistical tests; those denoted with different letters were statistically different.

TABLE 2. Correlations between *UCP2, NLRP3* and miR-133a-3p expressions and clinical and laboratory characteristics

	UCP2 **Unadjusted**	***UCP2*** **BMI adjusted**	***NLRP3*** **Unadjusted**	***NLRP3*** **BMI adjusted**	**miR-133a-3p Unadjusted**	**miR-133a-3p BMI adjusted**
	***r* / P value**	***r* / P value**	***r* / P value**	***r* / P value**	***r* / P value**	***r* / P value**
UCP2 (n-fold)	-	-	**-0.307 / 0.024**	**-0.269 / 0.051**	0.011 / 0.941	-0.072 / 0.632
NLRP3 (n-fold)	**-0.307 / 0.024**	**-0.259 / 0.051**	-	-	**0.353 / 0.013**	**0.418 / 0.003**
miR-133a-3p (n-fold)	0.011 / 0.941	-0.072 / 0.632	**0.353 / 0.013**	**0.418 / 0.003**	-	-
Age (years)	0.021 / 0.879	0.002 / 0.987	-0.168 / 0.195	-0.238 / 0.067	-0.238 / 0.098	**-0.288 / 0.047**
BMI (kg/m^2)	-0.199 / 0.150	-	0.226 / 0.080	-	-0.158 / 0.280	-
Waist circumference (cm)	-0.218 / 0.113	-	**0.268 / 0.037**	-	-0.204 / 0.159	-
Fat mass (kg)	-0.108 / 0.455	0.133 / 0.362	0.143 / 0.296	-0.071 / 0.609	0.074 / 0.686	0.155 / 0.315
Fat-free mass (kg)	-0.203 / 0.145	-0.087 / 0.541	0.155 / 0.240	0.038 / 0.775	-0.068 / 0.645	-0.024 / 0.875
Weight Excess	-0.177 / 0.243	-	**0.355 / 0.010**	-	-0.160 / 0.323	-
REE (kcal)	**-0.274 / 0.045**	-0.192 / 0.167	**0.261 / 0.048**	0.183 / 0.174	-0.052 / 0.722	0.080 / 0.590
HOMA-IR	<0.001 / 0.998	0.055 / 0.703	-0.006 / 0.964	-0.038 / 0.778	-0.201 / 0.175	-0.151 / 0.316
HbA1c (%)	0.071 / 0.608	0.209 / 0.134	-0.060 / 0.648	-0.128 / 0.328	**-0.338 / 0.018**	-0.267 / 0.066
Total cholesterol (mg/dL)	-0.122 / 0.388	-0.106 / 0.460	0.215 / 0.102	0.191 / 0.152	-0.137 / 0.359	0.002 / 0.992
Triglycerides (mg/dL)	-0.202 / 0.152	-0.212 / 0.135	**0.310 / 0.017**	0.242 / 0.068	-0.276 / 0.061	-0.194 / 0.196

HDL-cholesterol (mg/dL)	0.120 / 0.394	0.003 / 0.984	-0.139 / 0.291	0.038 / 0.773	0.129 / 0.382	0.241 / 0.102
Leptin (μg/ml)	-0.070 / 0.629	0.006 / 0.969	0.075 / 0.588	0.084 / 0.551	-0.163 / 0.279	-0.132 / 0.387
Adiponectin (μg/ml)	0.210 / 0.136	0.188 / 0.187	**-0.264 / 0.049**	-0.165 / 0.227	0.246 / 0.096	0.162 / 0.282
Systolic BP (mmHg)	**-0.291 / 0.033**	-0.251 / 0.070	0.038 / 0.769	-0.111 / 0.399	-0.100 / 0.496	0.006 / 0.969
Diastolic BP (mmHg)	-0.235 / 0.087	-0.242 / 0.081	0.146 / 0.262	0.113 / 0.389	0.077 / 0.599	0.160 / 0.278

Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin; REE, rest energy expenditure; HOMA-IR, homeostasis model assessment-insulin resistance; BP, blood pressure.

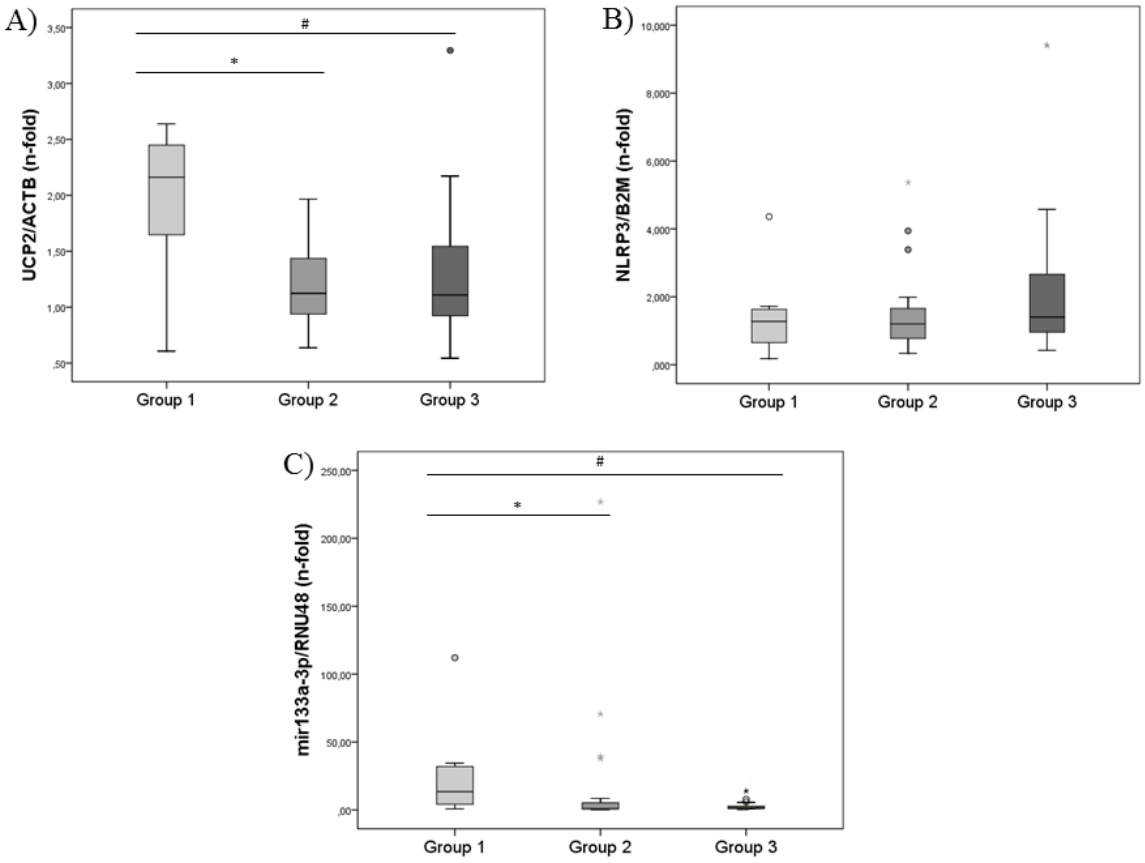

Figure Legend

Figure 1. *UCP2*, *NLRP3* and miR133a-3p expressions according to BMI categories: Group 1 (BMI <25.0 kg/m^2), Group 2 (BMI 30.0 – 39.9 kg/m^2) and Group 3 (BMI 40.0 kg/m^2). Relative expressions of **A)** *UCP2*, **B)** *NLRP3* and **C)** miR133a-3p were evaluated using qPCR. P values were obtained using Kruskal-Wallis tests with post-hoc analyses. * P< 0.050 and # P< 0.100. Results are expressed as n-fold changes. The horizontal lines inside each box indicate medians, the top and bottom of the boxes indicate interquartile ranges, whiskers indicate the 5th and 95th percentiles, and circles indicate outlier values.

Supplementary table 1. Sequences of primers used for qPCR analyses

Gene	Primer sequence (R) 5' – 3'	Primer sequence (F) 5' – 3'
UCP2	TTGGGTTCAAGGCCACAGAT	CCAGCCCCAAGAAACTTCAC
ACTB	ACAGCCTGGATAGCAACGTACA	AGGCCAACCGCGAGAAG
NLRP3	GCCATCTCCACGGAATCAAA	CCTTTCCAAAGACCCGTCAAG
β2M	ACAAGTCTGAATGCTCCACT	CTATCCAGCGTACTCCCAAG

UCP2, Uncoupling protein 2; *ACTB*, Beta-actin; *NLRP3*, NLR family pyrin domain 3; *β2M*, beta2-microglobulin.

CONSIDERAÇÕES FINAIS

As principais conclusões deste estudo são:

- As expressões gênicas de *UCP2* e do miR-133a-3p estão diminuídas no TAS de pacientes com obesidade quando comparados ao grupo de pacientes eutróficos;
- A expressão gênica de *NLRP3* foi similar entre os grupos; entretanto se correlacionou com valores de circunferência da cintura e de excesso de peso dos indivíduos avaliados;
- A expressão de *NLRP3* se correlacionou negativamente com a expressão de *UCP2* e positivamente com a expressão de miR-133a-3p;

Este foi o primeiro estudo que avaliou a associação entre a expressão de *UCP2*, *NLRP3* e miR-133a-3p e obesidade, reforçando a importância destes genes nessa doença. Entretanto, estudos adicionais são necessários para confirmar os papeis destes genes e seus reguladores na obesidade.

OUTRAS PRODUÇÕES BIBLIOGRÁFICAS NO PERÍODO DO MESTRADO

Manuscritos desenvolvidos no período do mestrado e em fase de finalização:

- **The role of uncoupling proteins 1, 2 and 3 in weight loss after bariatric surgery: a systematic review**

  Oliveira M.S., Nique P.S., Crispim D., Souza B.M.

- **Polymorphism -866G/A in UCP2 gene is associated with a lower weight loss in patients with severe obesity submitted to bariatric surgery**

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  Nique P.S., Oliveira M.S., Souza B.M., Assmann T.S., Recamonde-Mendoza, M., Crispim D., Bauer A.C., Canani L.H.

- **miR-126 and miR-200b expression are associated to choroid and retinal thickness in patients with type 2 diabetes mellitus**

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