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THAYNÁ MENDONÇA AVELINO

DESENVOLVIMENTO E VALIDAÇÃO DE ESFEROIDES MIMÉTICOS DE TECIDO ADIPOSO: UMA NOVA ABORDAGEM NO ESTUDO DA OBESIDADE

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Tese apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências, na área de Fármacos, Medicamentos e Insumos para a Saúde.

Orientadora: Drª Ana Carolina Migliorini Figueira
Coorientadora: Drª Adriana Franco Paes Leme Squina

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"Um homem pode morrer, lutar, falhar, até mesmo ser esquecido; mas sua ideia pode modificar o mundo mesmo tendo passado 400 anos."

Alan Moore - V for Vendetta

RESUMO

A obesidade é mundialmente definida como o acúmulo anormal ou excessivo de gordura que pode resultar em diversas comorbidades, é considerada uma condição pandêmica que quase triplicou nos últimos 45 anos. A maioria dos estudos sobre as origens da obesidade utiliza modelos animais ou cultura de células em monocamada de adipócitos para investigar o tecido adiposo. No entanto, além das abordagens de cultura de células em monocamada não recapitularem totalmente a fisiologia de um organismo vivo, há uma necessidade crescente de reduzir ou substituir os animais na pesquisa. Nesse contexto, o desenvolvimento de estruturas auto-organizadas em 3D, como os esferoides e organoides tem fornecido modelos que reproduzem melhor os aspectos in vitro da fisiologia in vivo em comparação com o cultivo celular tradicional.

Os recentes avanços nas tecnologias ômicas nos permitiram caracterizar essas culturas a níveis de proteoma, metaboloma, fatores de transcrição, ligação ao DNA e a níveis transcriptômicos. Essas duas abordagens combinadas, cultura 3D e ômica, forneceram dados mais realistas sobre uma determinada condição. Sendo assim, o presente estudo concentrou-se no desenvolvimento de um pipeline para estudo da obesidade in vitro, incluindo análise proteômica para validar dois modelos de esferoides derivados de adipócitos. Através da combinação de dados de espectrometria de massa coletados de esferoides 3T3-L1 diferenciados e de tecido adiposo branco murino, em ambiente pró inflamatório e normal, identificamos e quantificamos as proteínas em ambas as amostras.

Por meio de uma análise proteômica abrangente, observamos que o cultivo 3D in vitro de adipócitos diferenciados compartilha importantes vias moleculares com o tecido adiposo real, incluindo a expressão de proteínas envolvidas no processo metabólico central do tecido adiposo. Juntos, nossos resultados mostram uma combinação do desenvolvimento de um método ortogonal para validação de cultivos tridimensionais que os identificou como miméticos do tecido real.

ABSTRACT

Obesity is defined worldwide as the abnormal or excessive accumulation of fat that can result in several comorbidities; it is considered a pandemic condition that has almost tripled in the last 45 years. Most studies on the origins of obesity use animal models or adipocyte monolayer cell culture to investigate adipose tissue. However, monolayer cell culture approaches not fully recapitulating the physiology of a living organism, and there is a growing need to downsize or replace animals in research. In this context, the development of 3D self-assembled structures such as spheroids and organoids have provided models that better reproduce in vitro aspects of in vivo physiology compared to traditional cell culture.

Advances in omics technologies allow us to characterize these cultures at proteome, metabolome, transcription factors, DNA binding and transcriptome levels. These two approaches combined, 3D culture and omics, provide more realistic data about a given condition. Therefore, the present study focused on the development of a pipeline to study obesity in vitro, including proteomic analysis to validate two models of adipocyte-derived spheroids. By combining mass spectrometry data collected from differentiated 3T3-L1 spheroids and murine white adipose tissue, in a pro-inflammatory and normal environment, we identified and quantified proteins in both samples.

Through a comprehensive proteomics analysis, we observed that in vitro 3D culture of differentiated adipocytes shares important molecular pathways with real adipose tissue, including an expression of proteins involved in the central metabolic process of adipose tissue. Together, our results show a combination of the development of an orthogonal method for validation of three-dimensional cultures that identified as mimetic of real tissue.

SUMÁRIO

1 INTRODUÇÃO

1.1 Obesidade uma epidemia global

A obesidade é uma doença crônica multifatorial, definida pelo acúmulo de gordura de forma anormal ou excessiva e que pode resultar em comorbidades sérias (BOLES; KANDIMALLA; REDDY, 2017; VALSAMAKIS; KONSTANTAKOU; MASTORAKOS, 2017). Ela tem sido considerada um dos maiores problemas de saúde pública do século 21, apresentando aumento em sua taxa de crescimento, acometendo crianças e adultos em todo o mundo (EMONT et al., 2015).

O ganho de peso está associado principalmente a um conjunto de quatro componentes que são inter-relacionados e autocontrolados, sendo estes: (1) ingestão de alimentos; (2) a variedade nutricional; e (3) reservas de gordura corporal e (4) termogênese (MARTINEZ, 2000). Mecanismos complexos de regulação fundamentam todos esses quatro elementos (MARTÍNEZ; FRÜHBECK, 1996), no entanto, o peso corporal também é determinado por uma série de fatores complexos como a interação de fatores genéticos, ambientais e psicossociais que atuam através de vários mediadores fisiológicos no consumo de alimentos e gasto de energia (JEBB, 1997).

O desenvolvimento da obesidade é diretamente ligado a múltiplas doenças, como: doenças metabólicas, diabetes tipo 2, hipertensão, dislipidemia, infarto do miocárdio, acidente vascular cerebral, certos tipos de câncer, apneia do sono e osteoartrite (RODGERS; TSCHÖP; WILDING, 2012) (YE, 2013). Além disso, a prevalência de transtornos neuropsiquiátricos, particularmente, demência, depressão e ansiedade, também é aumentada na obesidade (NARAYANASWAMI; DWOSKIN, 2017).

O último levantamento da organização mundial da saúde (OMS), realizado em 2016, apontou que 39% da população mundial está com sobrepeso, ou seja, apresenta índice de massa corporal (IMC) superior a 25Kg/m^2. A população da América do Norte, Argentina, Chile, Venezuela, Reino Unido, Espanha e Oceania supera a média mundial e possui índices alarmantes de sobrepeso em mais de 60% da população. O IMC em crianças e adolescentes de até 19 anos

demonstrou que 18% da população mundial nesta faixa etária apresenta sobrepeso (WHO, 2015, 2016).

Neste informe também foi verificado que 36% da população que apresenta sobrepeso nos países da América do Norte são obesos, ou seja, com IMC maior ou igual a 30Kg/m^2. Assim como os norte-americanos, Líbia, Egito, Turquia e Nova Zelândia possuem mais de 30% da população com sobrepeso e obesa (Figura 1) (WHO, 2015, 2016).

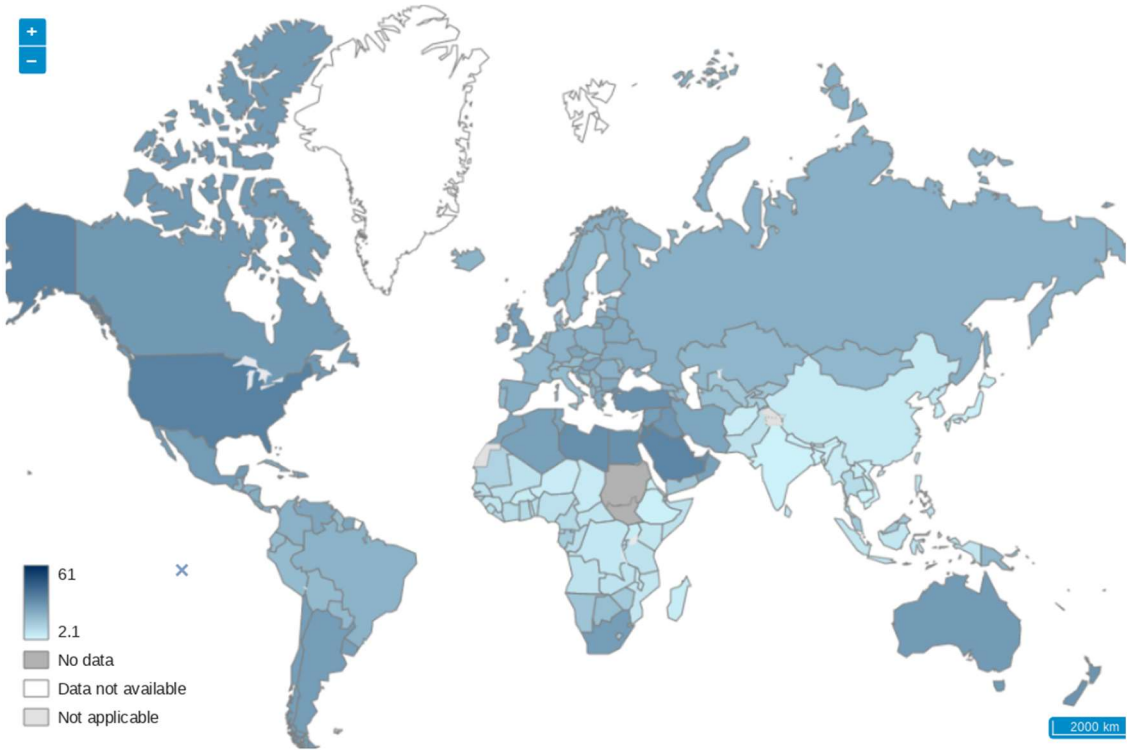

Figura 1: Prevalência da obesidade entre adultos, dados da Organização Mundial de Saúde em seu último levantamento em 2016. Os países representados com tons de azul mais escuro possuem a maior prevalência, enquanto os países de azul mais claro a menor prevalência (WHO, 2015, 2016).

Mundialmente, as complicações provenientes do sobrepeso e da obesidade correspondem a uma parcela significativa dos custos dos sistemas de saúde pública e privada. Estima-se que anualmente os países gastem cerca de 1 trilhão de dólares no tratamento de patologias associadas ao sobrepeso, sendo os maiores gastos concentrados nos tratamentos de diabetes e doenças cardiovasculares (WORLD OBESITY FEDERATION, 2017). Nos Estados Unidos, o Centro de Controle e Prevenção de Doenças (CDC) calculou que o

gasto anual do sobrepeso para o sistema de saúde do país está entre 3,5 e 6,5 bilhões de dólares (LEE et al., 2017).

O Brasil também é afetado pela epidemia da obesidade. Em 2016, a OMS apontou que 56% da população brasileira possuía sobrepeso, da qual, 22% é obesa. O custo para o Sistema Único de Saúde (SUS) com as patologias associadas ao sobrepeso no Brasil ultrapassou 2 bilhões de dólares em 2010, no levantamento realizado pela Associação Brasileira para o Estudo da Obesidade e Doença Metabólica (ABESO) (ABESO, 2015, 2016). Segundo a Federação Mundial da Obesidade, o Brasil é o 6º pais que mais gasta com o tratamento de implicações associadas ao sobrepeso (WORLD OBESITY FEDERATION, 2017).

1.2 O tecido adiposo

Embora multifatorial, a obesidade tem como condição central o acúmulo de gordura desproporcional acompanhado da inflamação sistêmica do tecido adiposo (GONZÁLEZ-MUNIESA et al., 2017) (BOOTH et al., 2015) (BLÜHER, 2009).

O tecido adiposo é dividido em dois tipos, denominados tecido adiposo branco e marrom. O tecido adiposo marrom (Brown Adipocyte Tissue - BAT), é distinguido pela sua capacidade de dissipar energia na forma de calor, desempenhando papel especialmente importante em recém-nascidos, para manutenção de sua temperatura corporal. Este processo - também denominado termogênese não dependente de contração (NST) - é diferente da termogênese derivada do tremor do músculo esquelético (CHEN et al., 2016; GONZÁLEZ-MUNIESA et al., 2017). Para além de suas funções associadas a NST, há ampla evidência apoiando o fato de que o BAT pode atuar como um órgão endócrino único, secretando alguns fatores, chamados de “batoquinas”; em 1980, Silva e Larsen descobriram que a enzima tipo 2 iodotironina deiodinase (DIO2) é especificamente expressa em BAT e converte tiroxina (T4) em triiodotironina (T3) (SILVA; LARSEN, 1983) (SHINDE; SONG; WANG, 2021) , além da função endócrina o BAT tem papel importante na regulação da captação de glicose e no controle dos níveis de insulina (SHINDE; SONG; WANG, 2021).

O tecido adiposo branco (do inglês White Adipocyte Tissue - WAT) representa para os seres humanos o maior estoque de energia e desempenha funções endócrinas, secretando adipocinas e citocinas, como a adiponectina e leptina.

O WAT é considerado um órgão multifuncional que executa intrincadas funções metabólicas em condições fisiológicas. Originalmente WAT era considerado como um depósito para o excesso de calorias ingeridas e reservatório de ácidos graxos livres liberados após a ingestão de alimentos. Entretanto, nas últimas duas décadas, pesquisas demonstraram que ele é um órgão endócrino complexo, atuando como regulador mestre da homeostase sistêmica da energia. Além de secretar inúmeros fatores conhecidos como adipocinas; o WAT também atua na regulação da inflamação e termo regulação. Em humanos é dividido em tecido adiposo subcutâneo e adiposo visceral, sendo que a desregulação deste último está associada ao risco de desenvolvimento de doenças metabólicas (GONZÁLEZ-MUNIESA et al., 2017; PFEIFER; HOFFMANN, 2015).

Os adipócitos que compõem o WAT emergem de um conjunto de células precursoras (pré-adipócitos) que se diferenciam após a ativação de vias que controlam a adipogênese. Neste processo, os pré-adipócitos semelhantes a fibroblastos se diferenciam em adipócitos, sendo este processo dividido em de duas etapas. (GHABEN; SCHERER, 2019) (PROENÇA et al., 2014).

Na primeira etapa, os pré-adipócitos (caracterizado pela expressão do receptor do fator de crescimento derivado de plaquetas-α PDGFRα e/ou PDGFRβ), são formados a partir das células mesenquimais, nessa etapa chamada comprometimento, a linhagem é comprometida a geração de adipócitos, toda essa cascata de sinalizações ocorre sem quaisquer alterações morfológicas, formando então um pré-adipócito. Este comprometimento é então seguido pela diferenciação, na qual os pré-adipócitos específicos sofrem parada do crescimento e divisão celular, acumulam lipídios e formam adipócitos maduros responsivos à insulina (GHABEN; SCHERER, 2019).

Estudos iniciais identificaram que a proteína morfogenética óssea 2 (BMP2) e BMP4 são suficientes para conduzir o comprometimento para

diferenciação de fibroblastos cultivados em adipócitos, e são necessárias para a diferenciação adipogênica in vitro (BOWERS et al., 2006) (WANG et al., 1993). As BMPs se ligam e sinalizam através dos receptores de BMP para ativar o fator de transcrição SMAD4 ativando seus parceiros heterodiméricos SMAD1, SMAD5 e SMAD8 (WANG et al., 1993). O SMAD4 ativado é então capaz de promover a diferenciação terminal estimulando a transcrição do receptor-γ ativado por proliferador de peroxissoma (PPARγ), o principal regulador da adipogênese, iniciando a segunda etapa do processo (WANG et al., 1993).

O PPARγ é um receptor nuclear e foi inicialmente identificado como um fator de transcrição que regula a expressão de um gene específico do tecido adiposo, a proteína de ligação a ácidos graxos 2 (AP2). Ele é caracterizado como regulador mestre da adipogênese, pois é indispensável para a diferenciação dos adipócitos em cultura e in vivo (ROSEN et al., 1999) (BARAK et al., 1999). Numerosos metabólitos lipídicos são propostos como ligantes endógenos do PPARγ, incluindo os ácidos graxos poli-insaturados, eicosanoides e prostaglandinas.

Um dos efeitos downstream mais importantes do PPARγ é a ativação do fator de transcrição C/EBPα (WU et al., 1999), que sinergiza funcionalmente com o receptor para ativar totalmente o programa de adipócitos maduros. Mais de 90% dos sítios de ligação no DNA do PPARγ também são reconhecidos por C/EBPα. Além disso, o PPARγ requer a indução de proteínas da família C/EBP para ativar completamente a transcrição de genes expressos e a diferenciação de pré-adipocitos em adipócitos maduros, como aqueles que codificam o receptor de insulina, AP2 e adiponectina (LEFTEROVA et al., 2008).

O *turnover* de adipócitos é sustentado por um balanço delicado entre adipogênese e apoptose e requer uma constante fonte de pré-adipócitos, sendo o número total de adipócitos em um adulto quase estático, mesmo no contexto da obesidade (GONZÁLEZ-MUNIESA et al., 2017).

Depois de maduros os adipócitos secretam adipocinas atuam como sinalizadores hormonais e nutricionais, dentre elas estão a leptina, adiponectina, respectivamente relacionadas à sinalização de saciedade no hipotálamo, e sensibilização à insulina; além do fator de necrose tumoral (TNF), que promove

uma conexão entre WAT e inflamação (CANNAVINO; GUPTA, 2022) (GHABEN; SCHERER, 2019).

Em condições de normalidade, WAT atua como depósito de energia excedente e sinaliza para que o organismo regule o balanço de ácidos graxos e carboidratos livres, atuando também em centros de saciedade e recompensa no sistema nervoso central. O excesso de nutrientes poderia resultar em hiperplasia de WAT, que teria um efeito protetor sobre alguns órgãos (coração, rim, fígado e pâncreas), evitando a deposição ectópica de gordura nos mesmos (TRAYHURN & BEATTIE, 2001).

Entretanto, como a expansão do tecido adiposo é limitada, em situações de desequilíbrio como na obesidade, as moléculas de triglicérides estocadas irão contribuir inicialmente para a hipertrofia de WAT, até que os adipócitos estejam saturados e impossibilitados de crescer, o que leva à sua ruptura, à invasão do tecido adiposo por macrófagos e ao aumento da liberação de citocinas pró-inflamatórias, com as adipocinas (GONZÁLEZ-MUNIESA et al., 2017). Este fenômeno contribui para um cenário de lipotoxicidade, inflamação e de resistência à insulina. Adicionalmente, o excesso de triglicérides livres irá se alojar em órgãos, resultando na deposição ectópica de gordura principalmente no coração, rim, fígado e pâncreas (GONZÁLEZ-MUNIESA et al., 2017; PFEIFER; HOFFMANN, 2015).

A inflamação é um processo fisiológico caracterizado pelo número elevado de glóbulos brancos ou aumento dos níveis de citocinas pró-inflamatórias na circulação ou tecido; em condições de normalidade a inflamação é uma reação protetora do organismo para controlar os insultos nocivos e iniciar o processo de cicatrização (YE, 2013) (YE; MCGUINNESS, 2013). A reação excessiva da resposta inflamatória geralmente leva a múltiplos efeitos colaterais, como lesão tecidual e disfunção orgânica.

Diversas alterações contribuem para o início da inflamação crônica no tecido adiposo obesogênico, como estresse do retículo endoplasmático, redução da adiponectina, elevação dos níveis de leptina, morte de adipócitos, infiltração de macrófagos e lipólise (YE; MCGUINNESS, 2013). A inflamação associada à obesidade começa no tecido adiposo e no fígado com elevada infiltração de

macrófagos e expressão de citocinas pró-inflamatórias. As citocinas pró-inflamatórias entram na corrente sanguínea para causar inflamação sistêmica.

Na obesidade, a inflamação tem efeitos benéficos e prejudiciais (YE, 2013) (YE; MCGUINNESS, 2013). Estudos realizados com múltiplas linhagens de camundongos transgênicos sugerem consistentemente que as citocinas pró-inflamatórias (IL-1β, IL-6 e TNF-α) aumentam o gasto de energia e atuam na prevenção da obesidade. A remoção de citocinas pró-inflamatórias por nocaute de genes diminui o gasto de energia e induz obesidade no início da idade adulta.

Em contraste, a elevação de citocinas pró-inflamatórias aumentam o gasto de energia e diminuem o risco de obesidade (YE; MCGUINNESS, 2013). O processo inflamatório do tecido adiposo está altamente relacionado com a resistência à insulina, uma condição em que a captação de glicose induzida pela insulina é prejudicada no tecido sensível ao hormônio, essa falha é resultado da inibição da via de sinalização da insulina (HE et al., 2011) (YE, 2013).

Inúmeras vias são moduladas no ambiente pró-inflamatório da obesidade e da resistência à insulina. A via IKKβ/NF-κB é uma via de sinalização de inflamação dominante que é extensivamente estudada na imunologia. A via da IKKβ foi associada com a inibição da sinalização de insulina por fosforilação de substrato 1 do receptor de insulina (IRS-1) em vários resíduos de serina, incluindo a Ser307 e Ser270 em adipócitos (ZHANG et al., 2008) (GAO et al., 2002)

Outra via envolvida no processo inflamatório do tecido adiposo é a via JNK-AP1, JNK (*c-JUN N-terminal quinase*), uma serina quinase presente na maioria dos tecidos. A atividade de JNK foi encontrada pela primeira vez na patogênese da resistência à insulina pela fosforilação do IRS-1 S307 (S312 em humanos), inibindo a via de sinalização da insulina em resposta ao sinal TNF-α (AGUIRRE et al., 2000) (RUI et al., 2001).

Outros eventos metabólicos são associados ao processo inflamatório e a resistência à insulina no tecido adiposo de indivíduos obesos como: disfunção mitocondrial, hipóxia e hiperinsulinêmia (YE, 2012) (YE, 2009) (LOWELL; SHULMAN, 2005).

1.3 Modelos para estudo da obesidade

O uso de animais para fins científicos é uma prática antiga, que remonta à Grécia antiga (BARRÉ-SINOUSSI; MONTAGUTELLI, 2015). Tanto os humanos como outros mamíferos são organismos altamente complexos, nos quais os órgãos desempenham diferentes funções fisiológicas de forma regulada. As semelhanças anatômicas e fisiológicas entre humanos e animais, principalmente mamíferos, levaram ao uso de animais antes da aplicação de novos compostos em humanos. Ratos e camundongos são os modelos animais pré-clínicos mais amplamente utilizados para estudar a obesidade (BARRÉ-SINOUSSI; MONTAGUTELLI, 2015) (BARRETT; MERCER; MORGAN, 2016).

Esses modelos animais nos ajudam a entender a biopatologia e desenvolver possíveis prevenções e tratamentos contra a obesidade, porém, cada modelo apresenta suas vantagens e desvantagens. Os animais nem sempre reproduzem fidedignamente a fisiologia dos tecidos humanos, são caros, demandam extensivo tempo experimental além de demandarem cuidados para que os experimentos sejam executados seguindo os regulamentos nacionais e internacionais de experimentação animal (LUTZ, 2020).

Embora relevante e necessário, o uso de animais vem sendo revisto e há uma tendência crescente na sua redução em pesquisas científicas desde 1959, quando WMS Russell e RL Burch em uma publicação chamada *The Principles of Humane Experimental Technique* (RUSSELL; BURCH, 1959), apresentaram o conceito dos 3Rs (*Replacement, Reduction and Refinement*). Este conceito visava encontrar alternativas aos testes em animais (substituição), otimizar a quantidade de informações obtidas com menos animais (redução) e adotar métodos que aliviem o sofrimento (refinamento) (RUSSELL; BURCH, 1959) (EBIOMEDICINE, 2022). Esses princípios orientadores visam melhorar a qualidade da ciência e o bem-estar animal, quando o uso dos mesmos é inevitável, e foram adotados por muitos países que garantem que os 3Rs sejam totalmente considerados ao conduzir pesquisas com animais (EBIOMEDICINE, 2022).

No que tange a substituição do uso de animais nas pesquisas, muitos avanços foram feitos no desenvolvimento de novas estratégias de cultura de células, facilitando a substituição parcial dos animais em pesquisa, como por exemplo o uso de tecnologia *lab-on-chip,* de organoides e esferoides para modelar uma grande variedade de tecidos, incluindo o tecido adiposo (MURPHY; LIAW; REAGAN, 2019).

As células cultivadas in vitro sempre são de suma importância para ciência. Com o desenvolvimento de novas metodologias e a demanda pela redução do uso de animais, iniciou-se os cultivos tridimensionais (3D) conhecidos como organoides e esferoides (Figura 2).

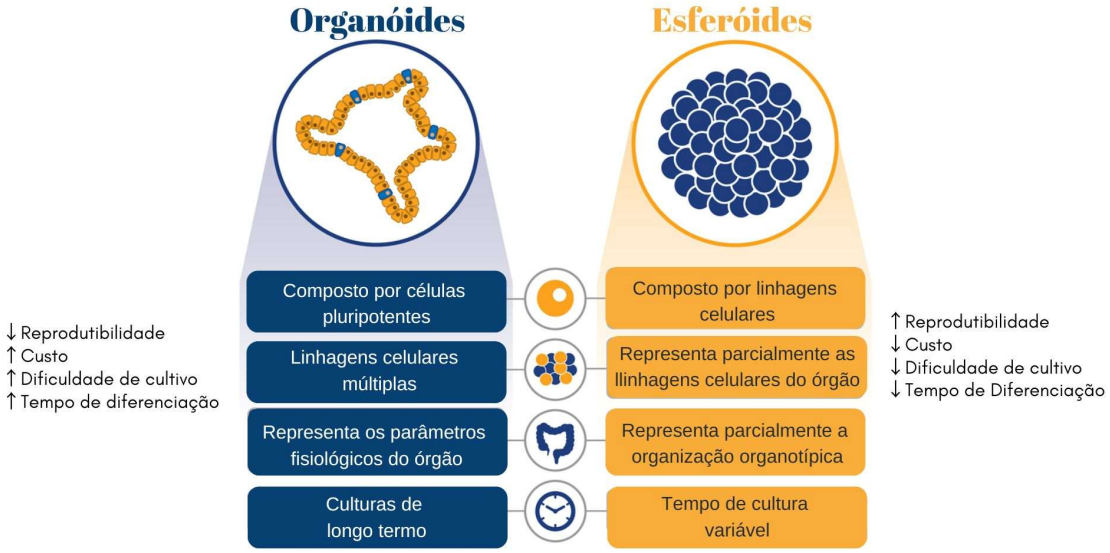

Figura 2: Imagem comparando as vantagens e desvantagens dos cultivos 3D. Imagem adaptada de https://blog.crownbio.com/hubfs/assets/organoid-vs-spheroid.

Os organoides são minúsculas culturas de tecidos tridimensionais auto organizadas derivadas de células-tronco. Essas culturas podem ser criadas para replicar grande parte da complexidade de um órgão ou para expressar aspectos selecionados dele, como produzir apenas certos tipos de células (Zhao et al.,2022). As culturas de organoides exibem heterogeneidade significativa e complexidade variável na composição celular, podendo sofrer morfogênese mal controlada no processo de organização celular e muitas vezes carecem de componentes estromais, vasculares e imunológicos (HOFER; LUTOLF, 2021; YI

et al., 2021). O cultivo de organoides é na maioria dos casos de custo elevado, demandam mais tempo de diferenciação, quando comparado aos esferoides, e não são reprodutíveis em tamanho e em forma, dificultando sua utilização em ensaios de prospecção de novos fármacos e compostos.

Diferente dos organoides, os esferoides são agregados de células, que se auto-organizaram em um ambiente que impede a fixação a uma superfície plana (BIAŁKOWSKA et al., 2020). A formação de esferoides é possível por causa das proteínas de membrana (integrinas) e proteínas da matriz extracelular (BIAŁKOWSKA et al., 2020). Os esferoides são extremamente reprodutíveis, possuem cultivo mais simplificado, a um custo menor comparando-os aos organoides, e acabam sendo a escolha de muitos grupos de pesquisa para serem os modelos em experimentos de descoberta e reposicionamento de novos fármacos (MITTLER et al., 2017) (LANGHANS, 2018).

Esses sistemas mais modernos de cultivo vêm sendo amplamente utilizados nas pesquisas associadas a obesidade. Em um esforço para avaliar a capacidade inflamatória do tecido adiposo em um ambiente 3D, Turner et al. usaram um copolímero de polipeptídeo semelhante à elastina (ELP) e polietilenoimina (PEI) para promover a formação de esferoides de adipócitos. Neste estudo, demonstraram que esferoides de 3T3-L1 diferenciados aumentaram a expressão de proteínas que regulam a captação de ácidos graxos como a CD36, CD40, Pparg e Adipoq quando comparados à cultura 2D da mesma célula (TURNER et al., 2015).

Outro estudo envolvendo o cultivo 3D de adipócitos desenvolveu uma cultura 3D para comparar os depósitos de gordura subcutânea e visceral. Neste caso, devido à diferenciação ineficiente de células-tronco isoladas da gordura visceral, Emont et al. adotaram um sistema de cultura celular baseado em colágeno 3D (EMONT et al., 2015).

Davidenko et al caracterizou as propriedades físicas de um hidrogel 3D baseado em colágeno e ácido hialurônico (HA) para estudo de adipócitos. Os arcabouços de colágeno-HA aumentaram a proliferação de precursores de adipócitos de camundongos (3 T3-L1) e da expressão da adipsina, embora a

expressão de Pparg tenha sido significativamente alterada (DAVIDENKO et al., 2010).

Uma série de outros modelos de culturas tridimensionais utilizando bioimpressão, levitação magnética e com base em arcabouços poliméricos foram propostos para o estudo da obesidade e doenças relacionadas ao tecido adiposo (TSENG et al., 2018) (CHOI et al., 2022) (DUFAU et al., 2021) (MCCARTHY et al., 2020) (DAQUINAG; SOUZA; KOLONIN, 2013) (CHOI et al., 2011) (SHEN et al., 2021).

Para tentar se aproximar ainda mais do ambiente fisiológico do orgão/tecido, os organoides e esferoides podem ser integrados a outros tipos de cultivos em plataformas microfluídicas conhecidas como *lab-on-a-chip*, que, uma vez integradas em um sistema, passa a se chamar *organ-on-a-chip*. Os *organ-on-a-chip* consistem em um sistema biomimético tem como objetivo imitar o ambiente de um órgão fisiológico, com a capacidade de regular parâmetros-chave (WHITESIDES, 2006; WU et al., 2020). Os sistemas microfluídicos são usados para controlar com precisão fluidos (10^{-9} a 10^{-18}L) usando canais que variam em tamanho de dezenas a centenas de mícron (WHITESIDES, 2006) (WU et al., 2020).

Nesse universo dos modelos microfluídicos, um modelo viável e imunocompetente de co-cultura entre adipócitos/PBMCs foi recentemente reportado. Neste sistema, uma cultura fisiologicamente relevante que exibe uma característica chave dos tecidos adiposos diabéticos tipo 2 foi modelada e caracterizada, sendo possível sua aplicação em estudos dos mecanismos da diabetes tipo 2 e em triagem de fármacos antidiabéticos (KONGSUPHOL et al., 2019).

O desenvolvimento e a aplicação de modelos avançados de cultura de células tridimensionais fornecem não apenas uma abordagem atraente para decodificar certos aspectos-chave da sinalização intra e intercelular, mas também a contribuição de fontes não genéticas no contexto de populações geneticamente e epigeneticamente diversas (THOMA et al., 2014) (ZANONI et al., 2016). Os sistemas de cultura de células 3D superam muitas das limitações dos sistemas tradicionais de cultura de células bidimensionais (2D) em

monocamada, imitando mais de perto a complexa heterogeneidade celular e as interações e as condições microambientais.

Apesar do grande esforço empregado no desenvolvimento de modelos celulares 3D do tecido adiposo por vários grupos de pesquisa, permanece a carência de entender como o modelo de cultivo tridimensional está mimetizando a complexidade do tecido adiposo. Nesse cenário o presente estudo teve como um de seus objetivos desenvolver um modelo tridimensional in vitro de tecido adiposo com características obesogênicas e compará-lo com um o tecido adiposo real, para entender com que precisão essas adiposferas remontam o tecido original.

Para tanto foi desenvolvido e detalhadamente caracterizado um esferoide formado por levitação magnética com adipócitos provenientes de células 3T3-L1, além de desenvolvida uma metodologia específica de analise proteômica destas amostras, conforme apresentado no primeiro artigo publicado no decorrer desta tese. Em uma segunda abordagem, os esferoides de tecido adiposo caracterizados foram tratados para o desenvolvimento de uma condição inflamatória almejando mimetizar resistência à insulina ou um estado de obesidade, sendo então comparado a nível proteômico ao tecido adiposo de camundongos obesos e não obesos, conforme apresentado no segundo artigo publicado no decorrer desta tese.

2 DOCUMENTO PUBLICADO

2.1 Artigo I - Mass spectrometry-based proteomics of 3D cell culture: A useful tool to validate culture of spheroids and organoids

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Abstract

Worldwide obesity, defined as abnormal or excessive fat accumulation that may result in different comorbidities, is considered a pandemic condition that has nearly tripled in the last 45 years. The majority of studies on the origins of obesity use animal models or adipocyte monolayer cell culture to investigate adipose tissue. However, in addition to monolayer cell culture approaches do not fully recapitulate the physiology of a living organism, there is a growing need to reduce or replace animals in research. In this context, the development of 3D self-organized structures has provided models that better reproduce the in vitro aspects of the in vivo physiology in comparison to traditional monolayer cell culture.

Besides, recent advances in omics technologies have allowed us to characterize these cultures at the proteome, metabolome, transcription factor, DNA-binding and transcriptomic levels. These two combined approaches, 3D culture and omics, have provided more realistic data about one determined condition. Thereby, here we focused on the development of an obesity study pipeline including proteomic analysis to validate adipocyte-derived spheroids. Through the combination of collected mass spectrometry data from differentiated 3T3-L1

spheroids and from murine white adipose tissue (WAT), we identified and quantified 1732 proteins in both samples. By using a comprehensive proteomic analysis, we observed that the in vitro 3D culture of differentiated adipocytes shares important molecular pathways with the WAT, including expression of proteins involved in central metabolic process of the adipose tissue. Together, our results show a combination of an orthogonal method and an image-based analysis that constitutes a useful pipeline to be applied in 3D adipocyte culture.

INTRODUCTION

Overweight and obesity are defined as abnormal or excessive fat accumulation, an issue that has grown to epidemic proportions causing the death of nearly 4 million people per year due to health complications, according to the global burden of disease 1. In order to find solutions for obesity, the biomedical researchers have been exploring the development of adipocyte cell culture and adipose tissue models2 to understand biological mechanisms with the aim of designing interventions to achieve and maintain healthy metabolism and body weight. Instead of using animals, most of the studies are performed in vitro using adipocytes, being the mouse 3T3-L1 cell line the most common model for this purpose 6,7,8.

For many years researchers have been using animal models to answer important scientific questions aiming to understand obesity and overweight 2,3. The use of animal models could be often costly, time-consuming and present scientific limitations such as poor relevance to human biology 4. The proposed Principles of Human Experimental Technique promulgated the 3Rs of Replacement, Reduction and Refinement5 in science. Since then, researchers have been developing new methods to substitute animal models which can also fill the gaps inherent to them mainly when knowledge must be translated to human physiology. Currently, cell monolayer is widely used, however, they do not represent tissue morphology, organization and physiology; to bring in vitro experiments closer to those performed with animals, several models of 3D cell culture have been developed 6,7,8. The 3D cell culture is an in vitro self-organized and stimulus-sensitive representation of an in vivo tissue being more

realistic than cultures in monolayer 9. The adipocyte spheroids and organoids have been developed to help substitute the animal use in obesity research. Therefore, it is crucial to investigate and characterize these models in comparison with the well-established animal models used in metabolic research and preclinical testing.

Moreover, recent advances in omics technologies, such as mass spectrometry (MS), have become an extensively applied method for 3D cultures characterization. The development of methodologies and improvements in the resolution and sensitivity of MS instruments have allowed it to address a variety of biological questions 10,11. Here we present the development of an obesity study pipeline including proteomic analysis to validate adipocyte-derived spheroids. Our results indicate MS-based proteomics as a method to validate spheroidal 3D cell culture of mouse 3T3-L1 cells to study adipose tissue and its molecular pathways, which correlates with white adipose tissue (WAT).

METHODS

3T3-L1 cell culture and differentiation

3T3-L1 mouse preadipocytes were obtained from American Type Culture Collection (ATCC). 3T3-L1 mouse preadipocytes (passage 10-15) were cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium supplemented with 10% calf serum and 100 U/mL penicillin 100 mg/mL streptomycin at 5% CO2 and 37 °C and harvested before reaching 70% confluence for spheroid assemble. After assembled, the spheroids were differentiated with the use of the induction medium for 48h composed of DMEM, 10% FBS, 1% w/v antibiotics (penicillin and streptomycin), 1 µM dexamethasone, 1 µg/mL of insulin and 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX). After the induction period, medium was aspirated and the maintenance medium composed of DMEM, 10% FBS, 1% w/v antibiotics, 1 µg/mL insulin was added. The maintenance medium was changed every 48 hours until reaching 14 days of differentiation.

Spheroid Assemble

Based on the afore described method8, cells after reaching a 70% confluence were counted and the nanomagnetic particles NanoShuttle ™-PL (Greiner) were added in the proportion of 1 uL for each 1x104 cells. After magnetization, 1,5x104 cells were added to a 96-well culture plate (Greiner Bio-One 655970 - 96 well microplate, PS, well with F/chimney bottom, cell-repellent surface, clear, sterile), (Fig.1). For spheroids formation, a device with 96 magnetic cylinders (Greiner Bio-One 655830) was positioned below the culture plate and the cells remained 24 hours incubated with magnets and kept in a humid incubator at 37 °C and 5 % CO2.

Animal model

Male C57BL/6J mice were purchased from Model Organisms Laboratory (LOM) mouse facility at LNBio/CNPEM (Campinas, SP, Brazil). Mice were weaned with 21 days old and were maintained at 22 ± 1 °C on a 12 hours light-dark cycle, with free access to food and water. They were fed a chow diet (Nuvilab CR1) for 12 weeks. The composition of the diet is as follows: crude protein, 220 g/kg; fat, 50 g/kg; carbohydrate, 600 g/kg; crude fiber, 70 g/kg; neutral detergent fiber 200 g/kg (20%), ash, 90 g/kg (9%); energy density, 3860 kcal/kg; calories from protein, 22%; calories from fat, 5%; and calories from carbohydrate, 60%.

At the age of 17 weeks, mice were euthanized in fed state and the perigonadal white adipose tissue (WAT) pads were dissected, washed in PBS and immediately frozen with liquid nitrogen. Later, they were stored at –80 °C until use. The Ethical Committee of CNPEM/LNBio “Comissão de Ética no Uso de Animais” (CEUA-CNPEM) specifically reviewed and approved this study (approval identification 61). The protocols were performed following the guidelines for ethical conduct in the care and use of animals established by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA). Animals were treated humanely and with regard to alleviate suffering.

Spheroid Viability and Size

On the day of assemble (day 0) and days 2, 4, 6, 8, 10, 12 and 14; 12 spheroids were collected per day in order to measure the cell viability through ATP production. The ATP was quantified using a CellTiter-Glo® 3D Cell Viability

Assay (Promega; cat. G9681) following the manufacturer's instructions; the luminescence was recorded in a Glow Max Plate Reader (Promega). The spheroids diameter was measured every 2 days. The images were captured with Operetta High Content Imaging System (Perkin Elmer, Waltham, MA, USA) and quantified using Harmony Software (Perkin Elmer).

Microscopy

The lipid droplets and the nuclei were stained after spheroids were fixed for 1 hour in 4% formalin and washed 3 times in PBS. The LipidSpot (Biotium; cat. Number 70065-T) lipid marker probe and the DAPI (Biotium; cat. Number 40043) were added according with manufacturer's instructions. After staining with the fluorescent probes, the images were obtained using a TCS SP8 (Leica) confocal microscope.

Sample preparation for X-ray microtomography

The 3T3-L1 spheroids were fixed in 4% formalin for 1 hour at room temperature, contrasted with 0.4% osmium solution, and then dehydrated in ethanol baths (70%, 80%, 90%, 95% and 100%). After dehydration, samples were clarified in two xylol baths (30 minutes each) and included in paraplast Plus® (sigma; cat. Number P3683-1KG). The included samples were mounted on a stub in the rotation stage.

X-ray microtomography

Briefly the 3T3-L1 spheroids mounted on a stub were submitted to micro tomography, the images were acquired at the Synchrotron National Light Laboratory (LNLS), on the beamline dedicated to X-ray micro tomography (IMX). More than 2000 X-ray transmission images were obtained by rotating the sample in 360 degrees around a fixed rotation axis, in uniformly spaced angular steps, to produce a stack of synograms that were later computationally transformed into a 3D density map sample electronics 12.

The 3D reconstructed data obtained on the IMX beamline were processed using the Avizo Fire 9.4 software (https://www.fei.com/software/avizo-for-materials-science/). A median filter was applied to all images to remove noise and allow segmentation and quantification of the image. After filtering, an interactive

threshold was applied, in order to separate the signal from the noise. The reconstructed and filtered 3D image was visualized using the 3D rendering function.

Proteomics

Proteolytic Digestion

All samples, WAT, non-differentiated (ND) and differentiated 3T3-L1 spheroids (WA), were submitted to the same protocol of protein extraction and trypsin digestion. Briefly, the samples were homogenized with 8 M urea, 2 M thiourea in 30 mM Tris-HCl pH 8.5, containing 1 mM EDTA, and 1 mM PMSF. The proteins were quantified by Bradford method13 and an aliquot containing 10 µg of proteins was submitted to reduction. Proteins were reduced with 5 mM dithiothreitol (DTT) for 25 minutes at 56 °C and alkylated with 14 mM iodoacetamide (IAA) for 30 minutes at room temperature in the dark. The remaining IAA was removed by the addition of excess DTT. To reduce the final concentration of urea to 1 M, the mixtures were diluted with 50 mM ammonium bicarbonate buffer. Proteins were digested with trypsin (1:50, w/w) for 18 hours at 37°C, and then 1% formic acid (v/v) was added to stop the digestion. The tryptic peptides were desalted with C18 stage tips14. To avoid bias during measurements, all data collection was randomized using the R (v3.4.0) environment.

LC-MS/MS analysis

The peptide mixture (2.0ml) was analyzed using an LTQ Orbitrap Velos (Thermo Fisher Scientific) mass spectrometer coupled to nanoflow liquid chromatography on an EASY-nLC system (Proxeon Biosystems) with a Proxeon nanoelectrospray ion source. Peptides were separated in a 2–35% acetonitrile gradient in 0.1% formic acid using a PicoFrit analytical column (20 cm × ID 75,5 µm particle size, New Objective) at a flow rate of 300 nL/min over 175 minutes, as previously described 15.

Proteomic data analysis

Raw data were processed using MaxQuant v1.5.8 software, and MS/MS spectra were searched against the Mus musculus UniProt database (released on December, 2020, 63,724 sequences, and 28,586,808 residues) using the

Andromeda search engine. A tolerance of 10 ppm was considered for precursor ions, and 1 Da for-fragment ions, with a maximum of two missed cleavages. A fixed modification of carbamidomethylation of cysteine and variable modifications of methionine oxidation and protein N-terminal acetylation were considered. A 1% false discovery rate (FDR) was set for both protein and peptide identifications. Protein quantification was performed using the LFQ algorithm, with a minimal ratio count of 1 and a window of 2 minutes for matching between runs. Data were processed in Perseus v1.6.7.0 software, excluding reverse sequences and those identified “only by site” entries. Protein abundance was calculated based on the normalized spectrum intensity (LFQ intensity) and was log2-transformed. The significance was assessed using Student’s t-test (P-value < 0.05). For data visualization, Volcano plot was performed with fold-change (FC) and p-value threshold value of 2.0 and 0.05, respectively, using the software Metaboanalyst v 5.0.

RESULTS

Spheroids are stable for at least 14 days

Cultured 3T3-L1 pre-adipocytes were assembled in spheroids according to the already proposed methodology16. Spheroids were set up with 1.5x104 3T3-L1 cells and magnetized for 24 hours resulting in a spherical 3D structure (Fig. 1-A, B). After magnetization, the spheroids were kept in culture for 14 days in basal medium and evaluated for their stability and viability. ATP production and diameter were checked every two days, and the data obtained showed that until day 2 the culture seems to be in a period of high metabolic demand, probably because of the change of the type of culture from monolayer to 3D. From day 4 on, the spheroids reached stability on ATP production (Fig. 1-C), suggesting that cell viability was kept.

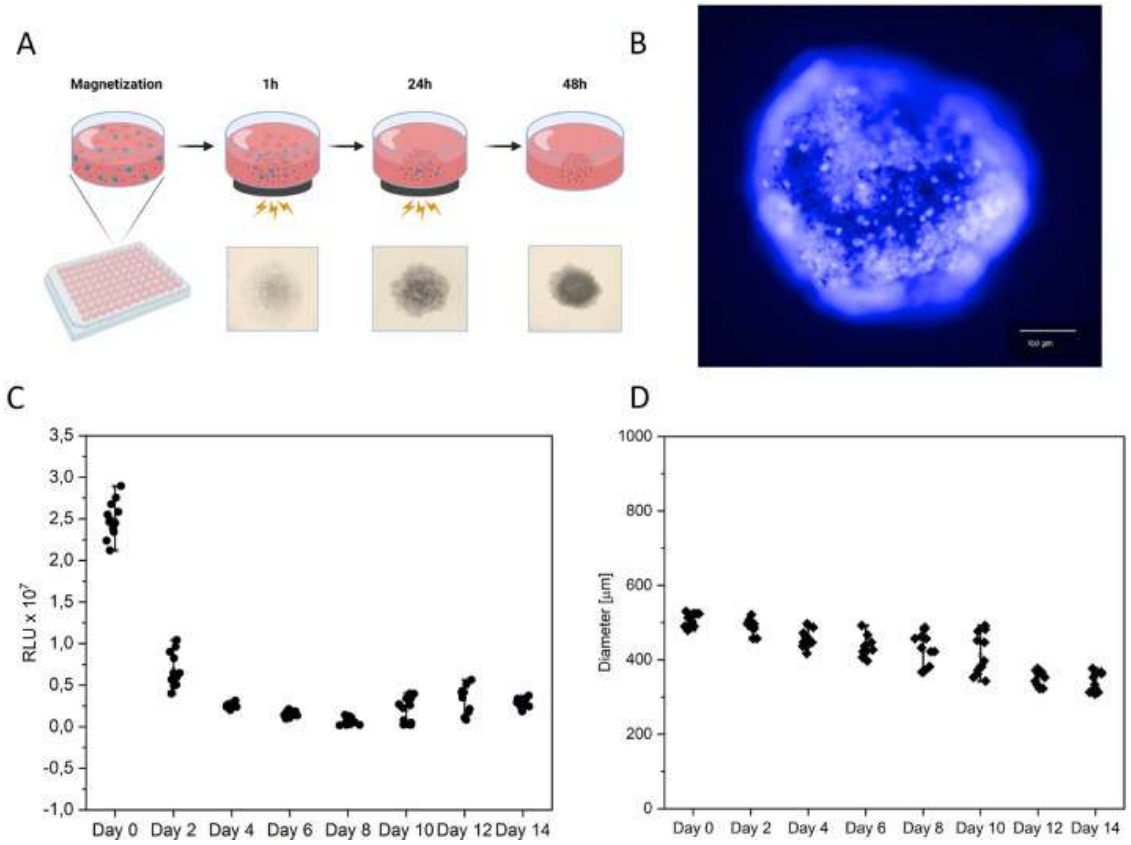

Figure 1: A) Scheme of Scaffold-free generation of spheroids and Phase-contrast image of spheroids, after magnetization the plate was incubated at 37 °C in a humidified chamber for 1 day to allow for magnetic assembly of pre-adipocytes into spheroids. The resultant spheroids were then differentiated for applications. Spheroids were maintained in base media showed no appreciable change in size after 2 weeks of observation. B) fluorescence image of spheroid after assembled, stained with DAPI, measurements realized showed sphericity around 1. C) ATP measurements of spheroids during 14 days. The ATP was quantified with CellTiter-Glo®, luminescence was recorded with one spheroid per well. The dot plots represent the luminescence mean ± SD (n= 12 spheroids per day), the statistical test ANOVA two way, (adjusted p-value < 0.05) indicate that the day of the spheroids were make (day 0) and the day 2 were significantly different from the measurement of other days, from day 4 the spheroids appear to stabilized in ATP production. D) Measurement of spheroids diameter during 14 days, the measurement was performed with n= 12 spheroids/day in an automatized microscopy. The statistical test, ANOVA two way, (adjusted p-value < 0.05) indicate no significance variation during 10 days.

Additionally, spheroid diameter (Fig. 1-D) was measured concomitantly to ATP quantification, exhibiting low variation until day 12, when the quantification indicated a reduction of the diameter, without cell death as founded in ATP

quantification. Finally, to characterize the spheroid shape, the confocal fluorescence measurement indicated that spheroids are compact and well-formed, presenting sphericity around 1(Fig. 1-B).

Micro-tomography Revealed Uniform and Reproducible 3T3-L1 Spheroids

To access the internal structure and morphology, the spheroids (N=24) were submitted to high-resolution synchrotron-based X-ray microtomography (IMX). Spheroids, processed as indicated in method section, were mounted on a stub in the rotation stage for data acquisition (Fig. 2-A).

The 3D reconstructed data obtained on the IMX demonstrated that 3T3-L1 cells formed uniform and stable spheroids, as can be observed through the morphological details on X-ray microtomography images. Cross section images showed the absence of deformed nuclei, which indicates that formed spheroids do not exhibit necrotic centers. Also, it was observed that spheroids present external shape of an elliptical form featuring well-adhered cells and average size of 500μm (Fig. 2B).

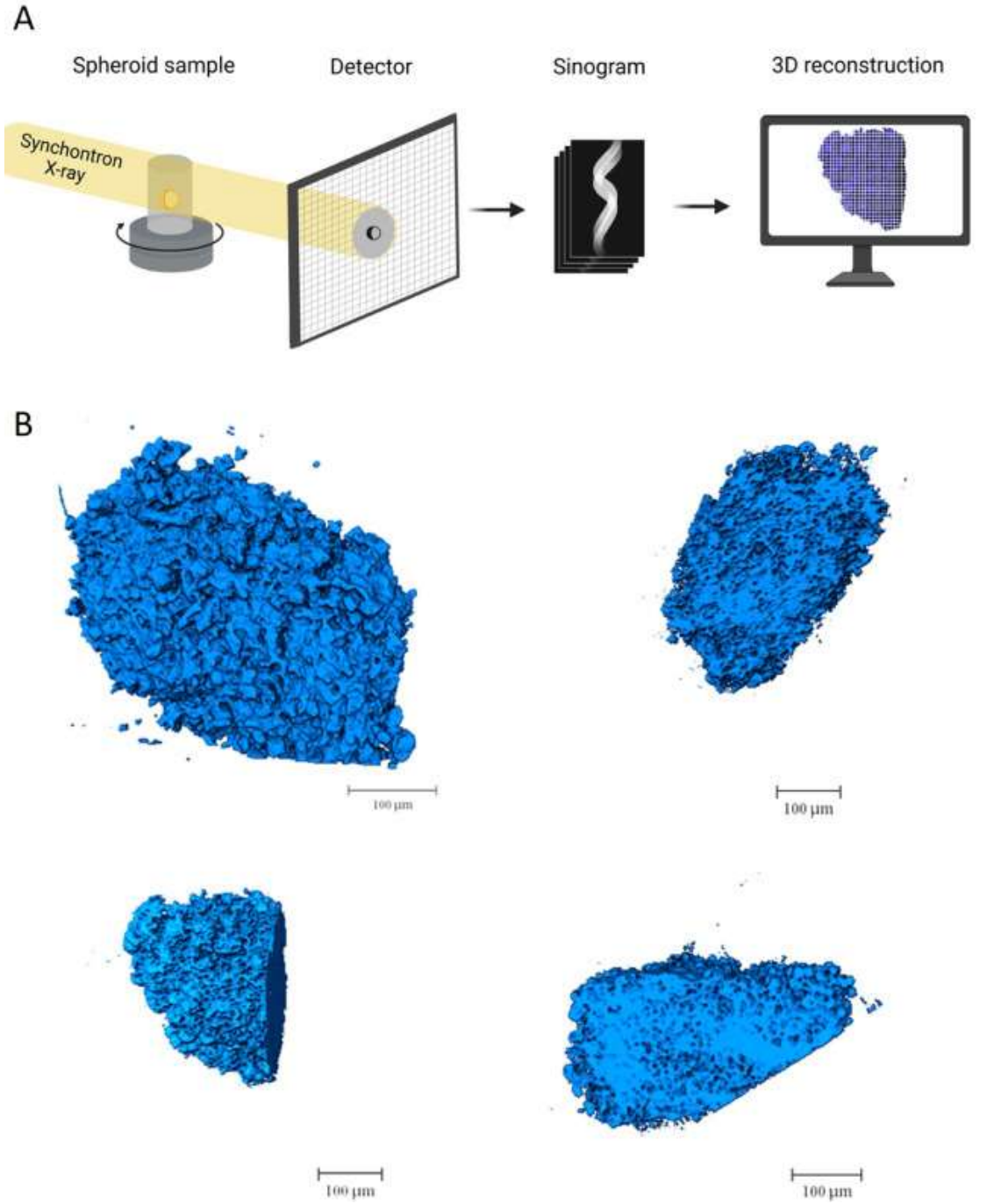

Figure 2: A) Scheme of data acquisition on IMX beam line. The fixed samples were stained and mounted in a stub and exposed to synchrotron x-ray. Images were acquired and recorded as a sinograms; all data were computationally processed and reconstructed using a specific 3D reconstruct software (Avizo) B) X-Ray Micro-tomography characterization of pre-adipocyte spheroids. The image of a whole spheroid is depicted in the superior left, at superior right and inferior left are the reconstructed images of two different cross sections and at inferior right is the center of the spheroid, showing no lack of cell in its nuclei. The spheroids size quantification demonstrated that they retain an elliptical format, with an average diameter of 200µm and 500µm long.

3T3-L1 Spheroids Developed Lipid Droplets on Cells Characterizing Mature Adipocytes

To produce adipocyte-derived spheroids, 3T3-L1-assembled spheroids were differentiated during 14 days. After differentiated, spheroids were stained with LipidSpot (Fig.3) in order to assess the increase of lipid content in the adipocyte cytoplasm. Confocal images of differentiated (WA) and undifferentiated (NDIF) spheroids (Fig. 3) showed an increase in lipid droplets in spheroids treated with differentiation medium (WA), when compared to NDIF spheroids. The presence of lipid droplets indicated a mature state of adipocytes.

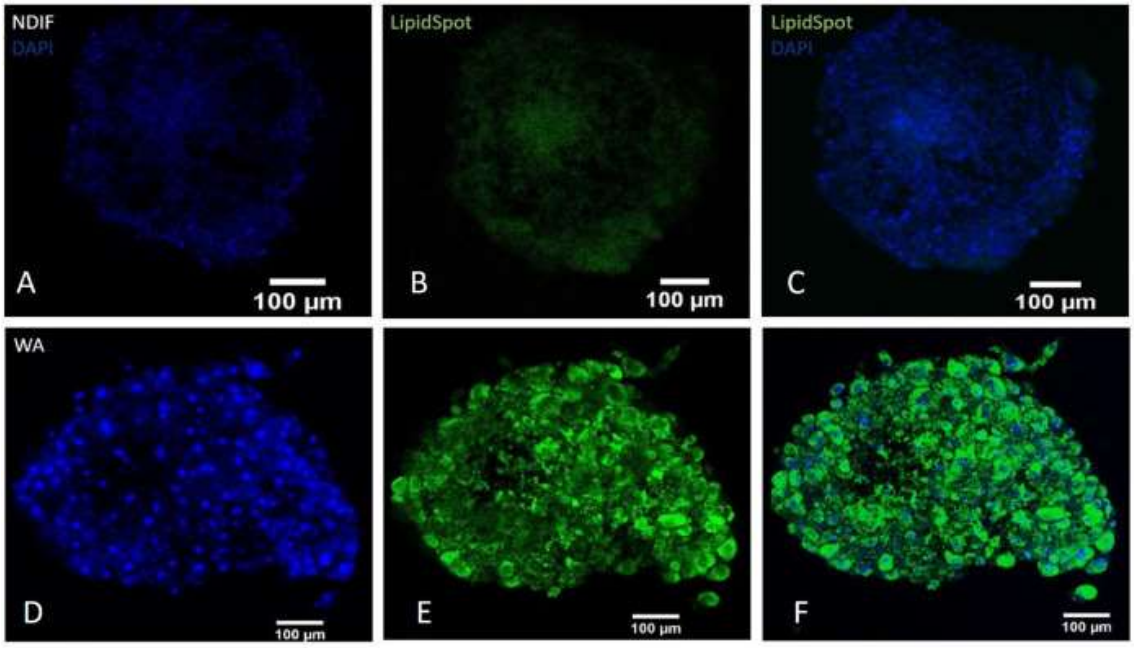

Figure 3: In panel, spheroids no differentiated in the superior line (NDIF, A, B, C) and differentiated spheroids in the inferior line (WA, D, E, F). The cell's nucleus was stained with DAPI (A, D), lipid droplets were stained with LipidSpot (B, E) and both staining were purposed (C, F). Almost the whole space of adipocytes is occupied by lipid droplets after 14 days exposed to differentiated cocktail (WA) when compared with undifferentiated condition (NDIF). Confocal laser scan microscopy was applied to detect DAPI and LipidSpot.

Proteomics of Adipose Tissue Spheroids Demonstrated Similarity to Mouse Adipose Tissue

As an orthogonal method to investigate molecular similarity between murine white adipose tissue (WAT) and differentiated 3T3-L1-derived spheroids (WA), we

characterized the global proteome of WA (n=24) and perigonadal WAT from mice (n=3).

Tissue dissected from mice and differentiated spheroids from three independent experiments were lysated to extract the proteins. The extraction had a protein average yield of 1,5µg/µl and 10µg of protein lysate of each sample (pool of spheroids and perigonadal WAT) were submitted to proteolytic digestion (Fig. 4-A). After proteolytic digestion, the peptide mixture was analyzed using an LTQ Orbitrap Velos (Thermo Fisher Scientific) mass spectrometer coupled to nanoflow liquid chromatography on an EASY-nLC system (Proxeon Biosystems) with a Proxeon nanoelectrospray ion source. The proteomes of WAT and WA spheroids were analyzed using quantitative MS and label-free protein quantitation (LFQ intensity) to compare the relative abundance of proteins.

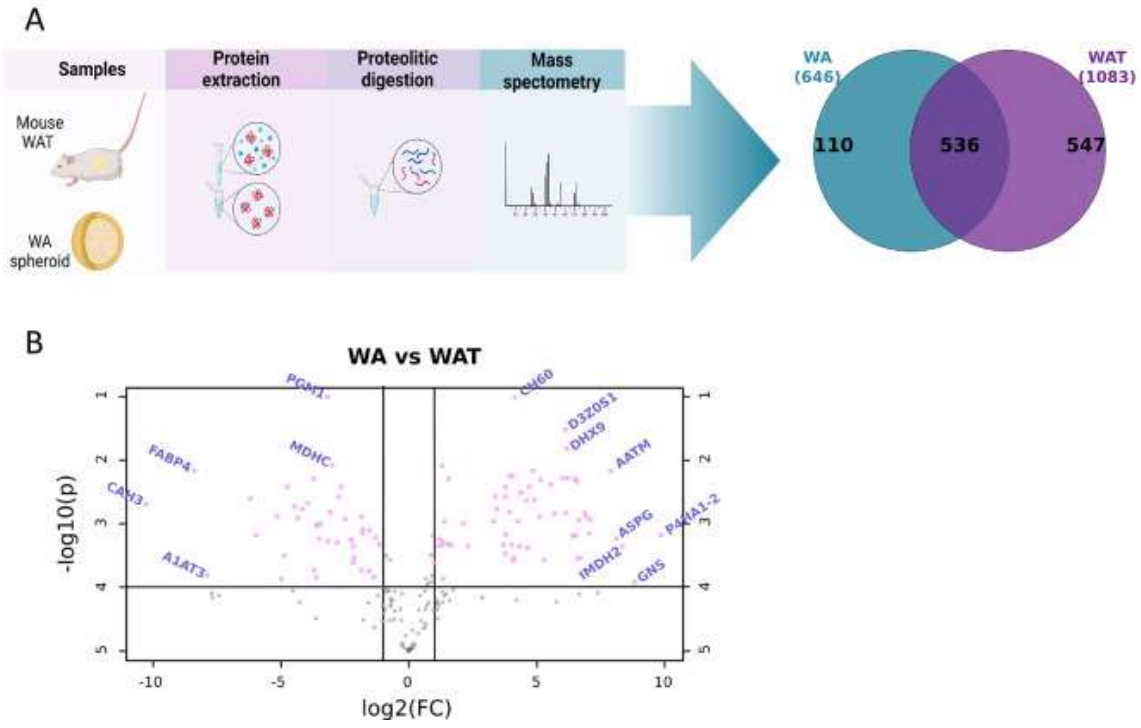

Figure 4: A. Pipeline of mass spectrometry-based proteomics of adipose spheroids and white adipose tissue. The samples (WAT or WA spheroids) were submitted to protein extraction; proteolytic digestion followed by MS analysis. Venn diagram of identified proteins, WA and WAT share 536 common proteins, WA have 110 exclusive proteins and WAT 547 proteins. B. Volcano plots for experimental comparison of WA X WAT in which abundance ratio (Log2) is plotted against the p-value (−log10). There are 63 upregulated proteins and 43 downregulated proteins indicated in plot (adjusted p-value < 0.05), in the right side in pink are the proteins up regulated in

WA and in left the proteins up regulated in WAT, the proteins with major expression difference are named in the graph. The proteins plotted in grey color (101) did not present expression profile differences between the groups, that means the similarity between the groups.

The data shows that for WA, 646 proteins were confidently identified. For the WAT dataset, 1083 proteins were quantified. The proteomic data analysis identified 536 common and exclusive proteins from WA spheroids and WAT (Fig. 4-A). Paired Student's t test (P value < 0.05) indicated 110 proteins that were preferentially expressed in WA, and 547 in WAT. The relatively small number of protein expression identified exclusively in WA spheroids reflects their biological similarity to WAT, and the higher number of identified proteins in WAT reflects the complexity of a real tissue extracted from an organism.

Biological replicates from WA and WAT were combined to investigate proteomic changes. The Volcano plot, in Figure 4-B, shows the up and downregulated proteins, in which 63 proteins were upregulated and 43 downregulated in WAT. Moreover, the 13 proteins with major difference of expression between the groups are named in the graph (Fig. 4-B), most of them are associated with fatty acid metabolism, mitochondrial activity and energy expenditure. On the other hand, 101 proteins (Supplementary material – Table 1) did not present significant difference in expression profile between the groups, indicating that half of the expressed proteins in spheroids and in mouse tissue are the same and presented the same expression rates, which is a strong validation of how our spheroids can mimic the mouse organism.

Enrichment analysis of biological processes of differentiated adipose spheroids

Functional enrichment analysis for the combined up- and downregulated proteins was performed to analyse biological processes. Enriched processes of WA compared to WAT are shown in Figure 5. This classification system uses information on protein sequence to assign a gene to an ontology group on the basis of the Gene Ontology (GO) terms http://www.geneontology.org/.

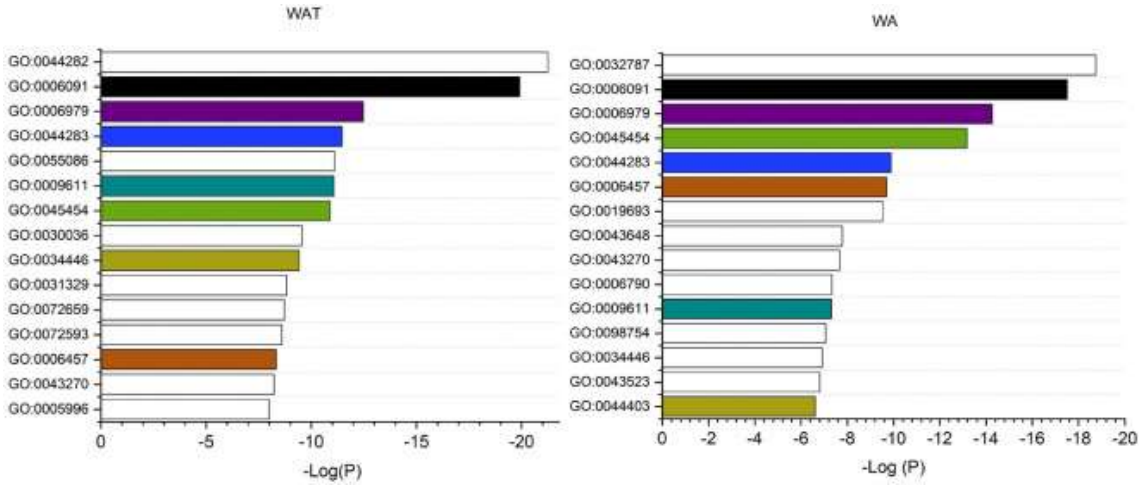

Figure 5: Functional enrichment analysis of murine WAT (left) and WA spheroids (right). A combined list of significantly up- and downregulated proteins was used for analysis, and an adjusted p-value <0.05 was considered statistically significant. Enrichment analysis for GO biological processes was performed using METASCAPE. Bar colors indicate groups of selected processes that appear in the two groups. The biological processes names and IDs, GO:0043270 Positive regulation of ion transport; GO:0006457 Protein folding; GO:0072593 Reactive oxygen species metabolic process; GO:0072659 Protein localization to plasma membrane; GO:0031329 Regulation of cellular catabolic process; GO:0034446 Substrate adhesion-dependent cell spreading; GO:0030036 Actin cytoskeleton organization; GO:0045454 Cell redox homeostasis; GO:0009611 Response to wounding; GO:0055086 Nucleobase-containing small molecule metabolic process ; GO:0044283 Small molecule biosynthetic process; GO:0006979 Response to oxidative stress; GO:0006091 Generation of precursor metabolites and energy; GO:0044282 Small molecule catabolic process; GO:0032787 Monocarboxylic acid metabolic process; GO:0019693 Ribose phosphate metabolic process; GO:0043648 Dicarboxylic acid metabolic process; GO:0006790 Sulfur compound metabolic process; GO:0043270 Positive regulation of ion transport; GO:0043523 Regulation of neuron apoptotic process; GO:0034446 Substrate adhesion-dependent cell spreading; GO:0098754 Detoxification.

The results obtained in functional enrichment featured that 7 of 15 processes are shared between WA and WAT. In general, for similar biological processes found enriched in WAT and WA, the most significantly ones are related to generation of precursor metabolites and energy. The analysis revealed the regulation of various metabolic processes in WA with a central role in adipose tissue, being the response to oxidative stress (represented in purple in Fig. 4) one of the top enriched. Moreover, other metabolic processes were identified in which the most

significant were monocarboxylic catabolic acid process and oxoacid metabolic process.

Regarding the differentially enriched metabolic processes between WA and WAT, WA was enriched in carbohydrate metabolism, detoxification, symbiotic interaction and neuron apoptotic process, while WAT was enriched in plasma lipoprotein particle levels, catabolism, cytoskeleton and mitochondrion organization, and small molecule metabolism, including ROS metabolism. However, despite some of those processes not being identical, they presented some similarities when grouped in a higher order. As an example, protein regulation-related processes, which were presented as protein-binding regulation in WA and as protein stabilizing/membrane-anchoring processing WAT; or carbohydrate metabolism-related process that was more detailed in WA and was restricted to monosaccharide metabolic process in WAT. Additionally, functional enrichment analysis was performed to evaluate the biological processes of non-differentiated (ND) spheroids, comparing ND, WA and WAT. From these group, only one process (GO:0006457 Protein folding) was shared (Supplementary material – S1), the low similarity probably is related to metabolic characteristics of a non-differentiated cells comparing to an adipocyte. In summary, WA and WAT share 7 significantly enriched biological processes indicated by functional analysis.

DISCUSSION

The worldwide pandemic of obesity demands efforts to understand the mechanisms involved in metabolic unbalance caused by overweight being the adipose tissue a central player in obesity treatment and control. Furthermore, the advent of 3D cultures made possible the development of disease and tissue models that can be used to substitute or at least to contribute with studies using traditional animal models in research, by being more realistic and reducing the average number of animals in experimentation. Some studies demonstrated that 3T3-L1 spheroids could be valuable models to investigate obesity and adipogenesis17,18,19,20, contributing with new evidences in biological mechanisms of this condition. Even though adipose spheroids present

morphological similarities to adipose tissue, in order for it to be used as an ally or substitute of animal models, their capacity of recapitulation of other physiological characteristics is also important.

Our data show that differentiated 3T3-L1-derived spheroids produced stable and uniform cultures as shown in details by microtomography. To validate the spheroid model, we proposed a proteomics analysis of WA spheroid in comparison to murine WAT, which provided novel insights about this type of 3D culture and the potential differences between this model and the tissue originated from a living organism. In a quantitative proteomics approach, a total of 1726 unique proteins were identified in both samples. WA and WAT share 536 proteins, representing 83% of the total identified proteins in WA spheroids. This data indicates a high similarity rate between 3D WA model and the tissue dissected from the animal. The proteins recovery for WAT after submitted to protein extraction presented superior yield in comparison with proteins recovery in WA, certainly because the complexity of a tissue, which cannot be reproduced in spheroids and organoids.

Recently, some studies reported the proteomic profile of 3T3-L1 adipocytes in monolayer 21,22. Similar to data obtained from proteomics in monolayer, our protein enrichment analysis data revealed various processes associated with the regulation of metabolism, including those involved in adipogenesis and fatty acid metabolism. Both of these processes are fundamental for the physiology of the tissue that we propose to mimic.

The enrichment analysis of biological processes revealed important pathways shared between WA and WAT. One of them was the "response to the oxidative stress" associated with preadipocyte proliferation increase, adipocyte differentiation, and the size of mature adipocytes 23,24,25,26. In obesity, this biological process is related to a several health disfunctions 26, showing once again the great resemblance of our 3D model with the murine WAT itself. Another enriched process, "generation of precursor metabolites and energy" is on the top of the enriched processes listed in both models and represents an important pathway for adipocytes involved in the regulation of energy consumption and expenditure. This pathway was associated to energy expenditure during exercise

and as a diabetic marker 29,30. All these results indicate that the functional enrichment data was an important tool to identify which biological processes are present in both animal and 3D cell culture model. With this data we exhibit that it is possible to use the 3D models more assertively, with focus on proteins present in keys biological pathways.

In summary, here we proposed a model to validate 3D cultures comparing them to a model organism. Our findings demonstrate that differentiated 3T3-L1 spheroids recapitulate important in vivo properties of murine WAT and provide a framework for future investigations to decipher underlying mechanisms and test therapeutic targets to manage or treat diseases associated with adipose tissue like obesity.

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COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

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3 DOCUMENTO SUBMETIDO PARA PUBLICAÇÃO

3.1 Artigo II - Development of a 3D in vitro adipocyte model for studying obesity related metabolic conditions

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ABSTRACT

Obesity is a major global health problem caused by excessive accumulation of fat can lead to a range of health issues. This condition has become a pandemic that has almost tripled in prevalence over the past 45 years. Adipocytes, which are responsible for energy storage, play a central role in obesity. However, most studies in this field rely on animal models or adipocyte monolayer cell cultures, which are limited in their ability to fully mimic the complex physiology of a living organism. Furthermore, animal models are expensive, time-consuming, and there is a growing push to reduce or replace them in research.

To address these issues, we have developed a 3D in vitro model that more closely mimics the metabolic environment of adipocytes in vivo. Using a workable cell line (3T3-L1), we produced adipocyte spheroids and differentiated them in presence and absence of TNF-α. We compared the proteomic profile of our adipose spheroids with that of adipose tissue from lean and obese C57BL/6J mice, demonstrating the usefulness of our spheroids for studying metabolic conditions. Moreover, our results showed that TNF-α treated spheroids are more similar to obese WAT than the non-treated ones.

Our findings indicate that we have created a simple, reproducible, and cost-effective three-dimensional model that mimics the in vitro metabolic features of real adipose tissue, including insulin resistance and an obesity profile. Developing this model could have a significant impact on the discovery of novel small molecules and biologics for preventing and treating metabolic syndrome and obesity in humans, using in vitro models.

INTRODUCTION

Obesity has become a pandemic-level condition due to the increasing adoption of unhealthy lifestyles by the world population in recent decades. It is a leading cause of mortality in several countries, as it can lead to complications such as cardiovascular, diabetic, hepatic, and neoplastic diseases [1] [2]. Obesity is generally characterized by an excess of body fat resulting from a persistently positive energy balance [1].

Adipocytes play a central role in this scenario, as their ability to store excess calories in the form of triglycerides, which are packaged into large lipid droplets [3]. Recent research has shed light on their profound influence on other aspects of metabolic homeostasis, including a complex interplay with other metabolic processes in the body (Ghaben & Scherer, 2019). Because of the plethora of effects in which adipocytes act, its importance is firmly established, and due to its complexity, adipose tissue is considered essential and highly active metabolic organ, which communicates with nerve tissue, stromovascular cells and immune system, among other tissues and cells, receiving and exporting signaling molecules and responses to the whole organism [5] [6].

During the last 20 years, the functions of adipose tissue were expanded to include its dynamic function as an endocrine organ after the discovery of leptin [7] , followed by adiponectin [4]. Nowadays, it is known that adipose tissue secretes many other proteins and signaling molecules, known as adipokines, that are responsible to regulate the systemic metabolic state, connecting diverse tissues and body functions [8]. Considering the adverse metabolic consequences of the disbalance on this tissue, the better understanding of its divergent functions has been emphasized and urged to lead to more rational therapy proposals, and, in this view, the proposals of new obesity models are a central piece [9].

Currently, there are two main types of obesity models available for research: cellular in vitro models and animal models. Animal models have the advantage of being able to mimic the physiological environment and connections between different organs and systemic responses in the body. However, they are costly and time-consuming and may not always accurately represent the phylogenetic background of humans, limiting the translation of results to human populations.

Additionally, the use of animal models raises ethical concerns related to the welfare of animals used for research purposes [10 11]. The principle of the 3Rs (Replacement, Reduction and Refinement) [10 11] has been proposed as a way to address some of these ethical concerns by promoting the development of alternative methods to minimize animal usage and suffering. While animal models remain an important tool for obesity research, alternative approaches such as in vitro models are being developed to complement or replace animal models where possible.

On the other side, most in vitro methods for studying obesity have been performed using monolayer cell culture, which is a well-established and cost-effective model for large-scale assays that requires less time to be performed. However, it is known that cells in monolayer culture are subject to vastly different microenvironmental and physical conditions than in vivo models [12]. Additionally, fundamental differences in cell behavior have been observed when comparing adipogenesis monolayer and 3D cell cultures. The effects of cell morphology and its 3D organization suggest that 3D cultures of adipocytes may improve the recapitulation of adipogenesis and metabolic function in the spheroid model, highlighting the need to develop and optimize these culture models to better mimic the in vivo microenvironment [913].

In this context, here we developed and validated a model of study that involves the cultivation of adipocytes in a 3D cell culture system with an environment more closely of that one encountered in vivo, mimicking certain aspects of a healthy and obese adipose tissue. To achieve this goal, we applied a well stablished cell culturing system based on magnetic levitation [14 15] to bioprinting the pre-adipocytes 3T3-L1 and differentiated them in adipospheres, which were treated with Tumor necrosis factor-alpha (TNF-α). As it is known, TNF-α is a multifunctional cytokine involved in many cellular and biological processes such as immune function, cell differentiation, apoptosis and energy metabolism [16], also, it is well established that TNF-α can induce a state of insulin resistance in adipocytes [17]. The treatment with TNF-α generate a 3D culture in a pro-inflammatory environment, that could reproduce some metabolic similarities to an obese adipose tissue. To validate the efficacy of the developed adipospheres in terms of mimics the animal metabolic conditions, we compared

their proteomic profiles with those of adipose tissue from healthy and obese mice (C57BL/6J). Our results provide a validated, feasible and translatable model to performing large-scale in vitro studies on obesity. Furthermore, our findings highlight some key pathways regulated in the adipose tissue under obese and insulin resistance conditions.

RESULTS

The TNF-α treatment dramatically downregulates the genes involved in adipogenesis in adipospheres.

The 3T3-L1 pre-adipocytes were cultured and assembled in spheroids using a previously stablished methodology [14] [1815]. Specifically, $1.5x10^4$ 3T3-L1 cells were bioprinted into spheroids and magnetized for 24h. Subsequently, the formation of a spherical and compact 3D cell structure was observed. Differentiation of 3T3-L1 spheroids to produce the adipose tissue spheroid model was performed by induction cocktail. In the last 24h of differentiation, some of the spheroids were treated with 2.5 nM of TNF-α (Stephens et al., 1997). TNF-α is a cytokine associated to inflammatory processes, and that had been linked to the induction of insulin resistance in murine adipocytes by reducing the transcriptional activity of GLUT4 gene [19] [20]. Considering this, we investigated if the TNF-α treatment on fully differentiated 3T3-L1 spheroids (Figure 1A) was able to induces a pro-inflammatory environment that generating an insulin resistant adiposphere.

Confocal scanning microscopy was performed to confirm differentiation, and the spheroids were stained with LipidSpot to assess the increase in lipid content within the adipocyte cytoplasm. As shown in Figure 1A, differentiated spheroids (WA and WA-TNF-α) exhibited a greater accumulation of lipids compared to non-differentiated spheroids (NDIF). We observed that the TNF-α treatment did not influence the lipid accumulation but promoted some rearrangement in cells organization on adipospheres compared with WA (Figure 1-A).

The differentiation of pre-adipocytes into mature adipocytes and the effects of TNF-α treatment on the regulation of genes during adipogenesis in the spheroids were further evaluated by relative mRNA expression of Adiponectin

(Adipoq), Lipoprotein lipase (Lpl), Leptin (Lep), Peroxisome proliferator-activated receptor gamma (Ppar γ), though quantitative PCR analysis. All the mRNA expressions were upregulated in WA spheroids in comparison to NDIF, indicating that adipocytes in spheroids achieved differentiation, while the spheroids treated with TNF-α presented a downregulation in the same set of genes expression in comparison to the WA (Figure 1-B). These findings are in line with previous studies, which reported decreased gene expression related to adipogenesis in obese mice with type 2 diabetes mellitus [21].

In addition, adiponectin, an adipocyte-specific protein, which is known to play a key role in the development of insulin resistance and obesity 19. Our results show that adiponectin secreted by fully differentiated spheroids was present in WA and WA- TNF-α spheroids (Figure 1-C). The detection of adiponectin is an additional indicator of adipose tissue differentiation by these 3D cell cultures.

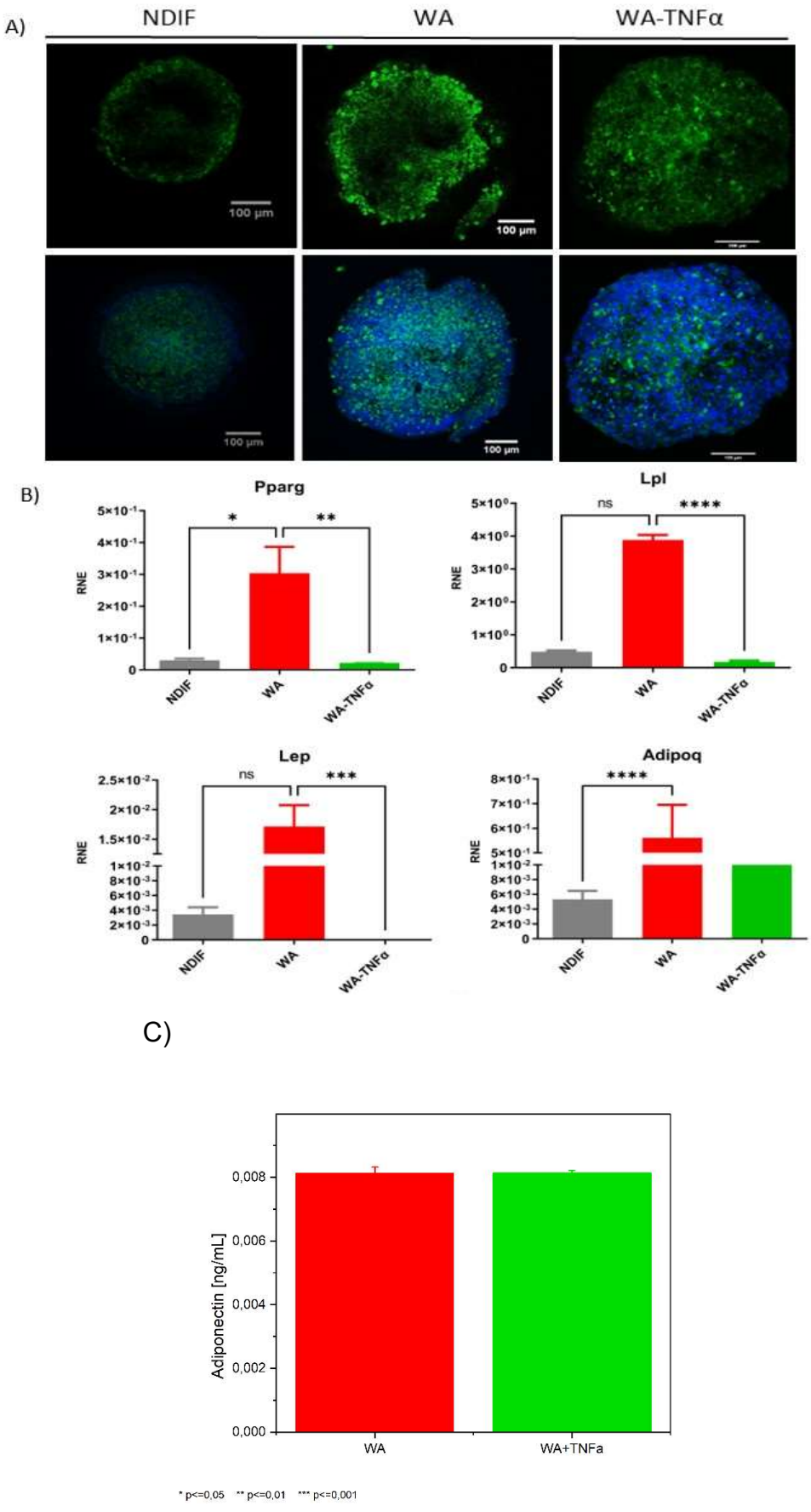
A)
NDIF
WA
WA-TNFα
100 µm
B)
Pparg
Lpl
Lep
Adipoq
RNE
NDIF
WA
WA-TNFα
ns
*
**
***
****
C)
Adiponectin [ng/mL]
0,000
0,002
0,004
0,006
0,008
WA
WA+TNFa
* p<=0,05 ** p<=0,01 *** p<=0,001

Figure 1: In panel, images from confocal scanning microscopy. Spheroid no differentiated in the first column (NDIF) and differentiated spheroids in subsequent columns (WA and WA-TNF-α). The cell's nucleus was stained with DAPI, lipid droplets were stained with LipidSpot and both staining were merged. Almost the whole space of adipocytes is occupied by lipid droplets after 14 days exposed to differentiated cocktail (WA and WA-TNF-α) when compared with undifferentiated condition (NDIF). Confocal laser scan microscopy was applied to detect DAPI and LipidSpot. B) Influence of differentiation cocktail on the expression of adipocyte markers. Graphs display the mRNA expression profile, by qRT-PCR, from Pparγ, Lpl, Lep and Adipoq, of differentiated 3T3-L1 spheroids (WA) and treated with TNF-α. Statistical significance (One-Way ANOVA with Tukey post-test, where ***p<0,0005, **p<0,005 and *p<0,05). Error bars represent standard deviation of means from at least 3 replicates. C)Quantification of secreted adiponectin by differentiated spheroids in culture medium; the measurement was performed after 14 days of differentiation. Statistical significance (One-Way ANOVA with Tukey post-test, where ***p<0,0005, **p<0,005 and *p<0,05). Error bars represent standard deviation of means from at least 3 replicates.

Tumor necrosis factor-alpha induced insulin resistance in 3T3-L1 spheroids altering glucose uptake.

To further investigate the central aspect of insulin resistance, we measured glucose uptake in both treated and non-treated spheroids in the presence and absence of insulin. Insulin-stimulated spheroids (WA-TNF-α + Insulin) showed no significant difference in glucose uptake compared to non-treated spheroid (WA + Insulin), indicating that the TNF-α treatment effectively blocked glucose uptake and induced insulin resistance. In the control spheroids, without TNF-α treatment, the insulin-stimulated spheroids exhibited high glucose uptake levels in comparison to the group not exposed to insulin stimulation (WA). These findings show that WA adipospheres are insulin-sensitive, while 24h TNF-α treatment transforms them into insulin-resistant state (Figure 2), validating our study model of 3D adipose tissue from a metabolically healthy obese individual and an obese individual with insulin resistance.

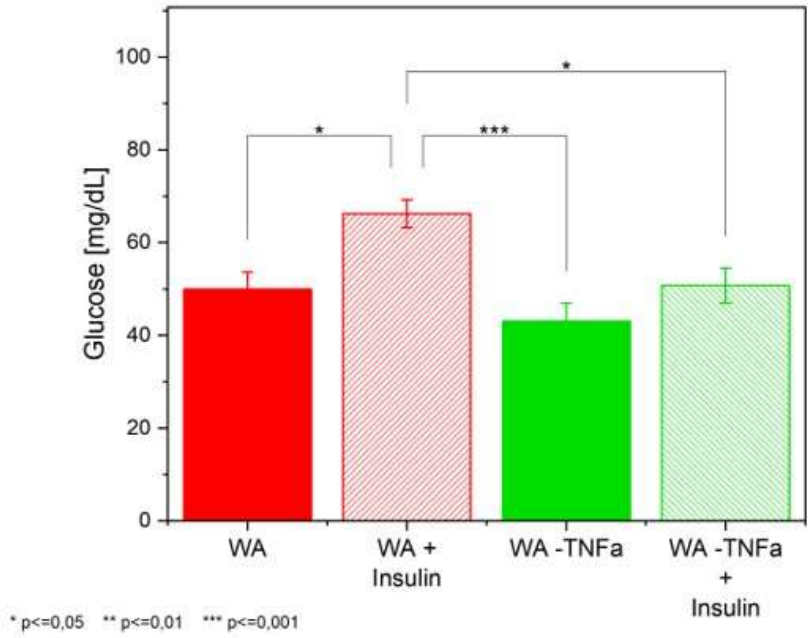

Figure 2: Glucose uptake assay of a metabolically healthy and insulin resistance in vitro model. Statistical significance (One-Way ANOVA with Tukey post-test, where ***p<0,0005, **p<0,005 and *p<0,05). Error bars represent standard deviation of means from at least 3 replicates.

Tumor necrosis factor-alpha treated spheroids presents similarity to mouse adipose tissue in terms of proteome analysis.

The proteome of 3T3-L1 adipose spheroids was evaluated as an orthogonal method to compare the murine white adipose tissue from chow diet feeding mice (WAT), high-fat diet feeding mice (WAT-HDF), and the differentiated 3T3-L1-derived spheroids (WA and WA-TNF-α). Quantitative mass spectrometry (MS) and label-free protein quantitation (LFQ intensity) were used to analyze the protein content, and to compare their relative abundance in our different adipose models (WAT, WAT-HFD, WA, and WA-TNF-α), as previously described [14].

The results show that 842 proteins were confidently identified for WA, while 967 proteins were identified for WA-TNF-α. For the WAT dataset, 1315 and 1330 proteins were quantified for WAT and WAT-HFD, respectively (Figure 3). The higher number of identified proteins in tissues from animals was expected due to their complexity in comparison to 3D adipocyte cultures. Interestingly, 664 proteins were found to be common among all the analyzed groups. These

amounts of common proteins represent the 50-70% of the identified proteins, suggesting similarities among the different adipose spheroids and animal models.

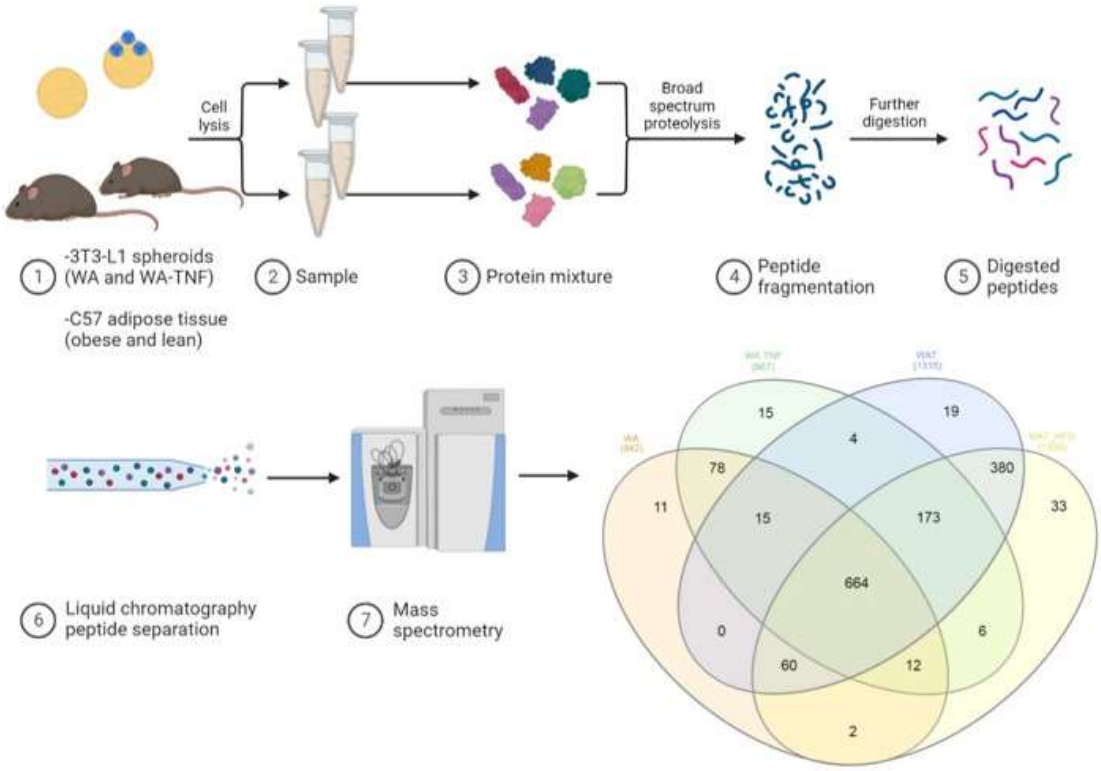

Figure 3: Pipeline of mass spectrometry-based analysis of adipose tissue from lean and obese mice (WAT and WAT-HDF) and from adipose tissue models (WA and WA-TNF); as a representative result, the Venn diagram of mass spectrometry-based proteomics of adipose spheroids (WA and WA-TNFa) and white adipose tissue from mice (WAT and WAT-HDF). The samples were submitted to protein extraction; proteolytic digestion followed by MS analysis. Venn diagram of identified proteins, WA had 842 proteins identified, WA-TNFa 967 proteins, WAT had 1315 and WAT-HFD 1330. WA have 11 exclusive proteins, WA-TNFa have 15, WAT 19 and WAT-HDF 33 exclusive proteins identified.

Pathways enrichment analysis.

To gain a better understanding of the signaling pathways involved in the differentially expressed proteins in the spheroids and mouse tissues, we used MetaboAnalyst 5.0 and Metacore™ software (Calrivate Analytics) to conduct enrichment analysis (EA) using a widely recognized protein-protein signaling database. The EA identified the protein IDs from the WA, WA-TNF-α, WAT, and WAT-HFD sets by the functional ontology function in MetaCore. The possibility

of a random intersection of a gene set and the corresponding ontological entities was assessed using the hypergeometric intersection p-value. A lower p-value indicated that the object was more relevant to the dataset, suggesting a higher rating.

To evaluate changes in proteomic profiles, biological replicates from WA and WAT were combined in the presence and absence of TNF-α (WA) or under chow or high-fat diets (WAT). The resulting heat map (Figure 4-A) displays the differentially expressed proteins with a p-value of 0.05, revealing 600 significant differentially expressed proteins. Protein cluster analysis revealed that WAT and WAT-HFD are more similar to each other than to WA and WA-TNF-α, which is expected given that WAT is a tissue removed from animals and WA is a 3D cell culture from one cell type. However, some proteins displayed a closer relationship between the differentially expressed proteins in WAT/WA and WAT-HFD/WA-TNF-α. The proteins from mitochondrial import inner membrane translocase subunit family (TIM 10 and 13) and Perilipin-2 (PLIN2) were found up regulated in WAT-HDF and WA-TNF-α, indicating an inflammatory and obesogenic environment [2223]. The overexpression of fatty-acid synthase (FAS) is obesity-related [24], our data show the expected-up regulation of this protein in HDF mice tissue but, a down regulation in WA and WA-TNF-α spheroids, this finding is probably related to the fact that the spheroids were not exposed to circulating fat as occurs in animal tissue; all data shows that WA-TNF-α is more closely related to adipose tissue than WA.

Joint pathway analysis of the differentially expressed proteins in both evaluated models and tissues (Figure 4-B) revealed the most impacted pathways (x-axis), which had a higher number of proteins differentially expressed in the comparison among all evaluated samples. Additionally, pathways with few proteins differentially expressed that may impact their correct function were represented by higher p-values (y-axis). Our analysis showed that changes in protein expression affected several metabolic pathways, particularly those related to thermogenesis, glycolysis, pyruvate metabolism, and the PPAR signaling pathway (Figure 4-B). These findings suggest important pathways associated with metabolic disorders that can be modulated and studied using our developed spheroid models.

The Figures 4-C and 4-D illustrate the enriched metabolic processes of WA x WAT and WA-TNF-α x WAT-HDF comparisons, respectively, which revealed the top ten differentially expressed proteins upregulated in the adipose tissue of mice and spheroid samples. The common processes observed in all samples were related to insulin metabolism, fenofibrate action in type 2 diabetes (T2D), oxidative stress in T2D, adiponectin pathogenesis in T2D, regulation of metabolism and role of adiponectin, transcription of HIF1 targets, TCA cycle and cytoskeleton remodeling. These findings suggest that the characteristic of the adipose tissue is mimicked by the differentiated adipospheres, indicating their capacity to resemble the adipose tissue.

Intriguingly, the upregulation of several proteins related to fatty acid metabolism and mitochondrial respiration, including FABP5, TIM13, PLIN2, and PLIN5, was observed in WAT-HFD and WA-TNF-α, while FABP4 and FAS were upregulated in WAT and WAT-HFD but downregulated in both spheroid models (see Figure 4-C and 4-D). These results provide further evidence that our spheroid models may recapitulate some of the characteristics of real tissues; showing a greater similarity to mouse tissue in our TNF-α-treated spheroid model.

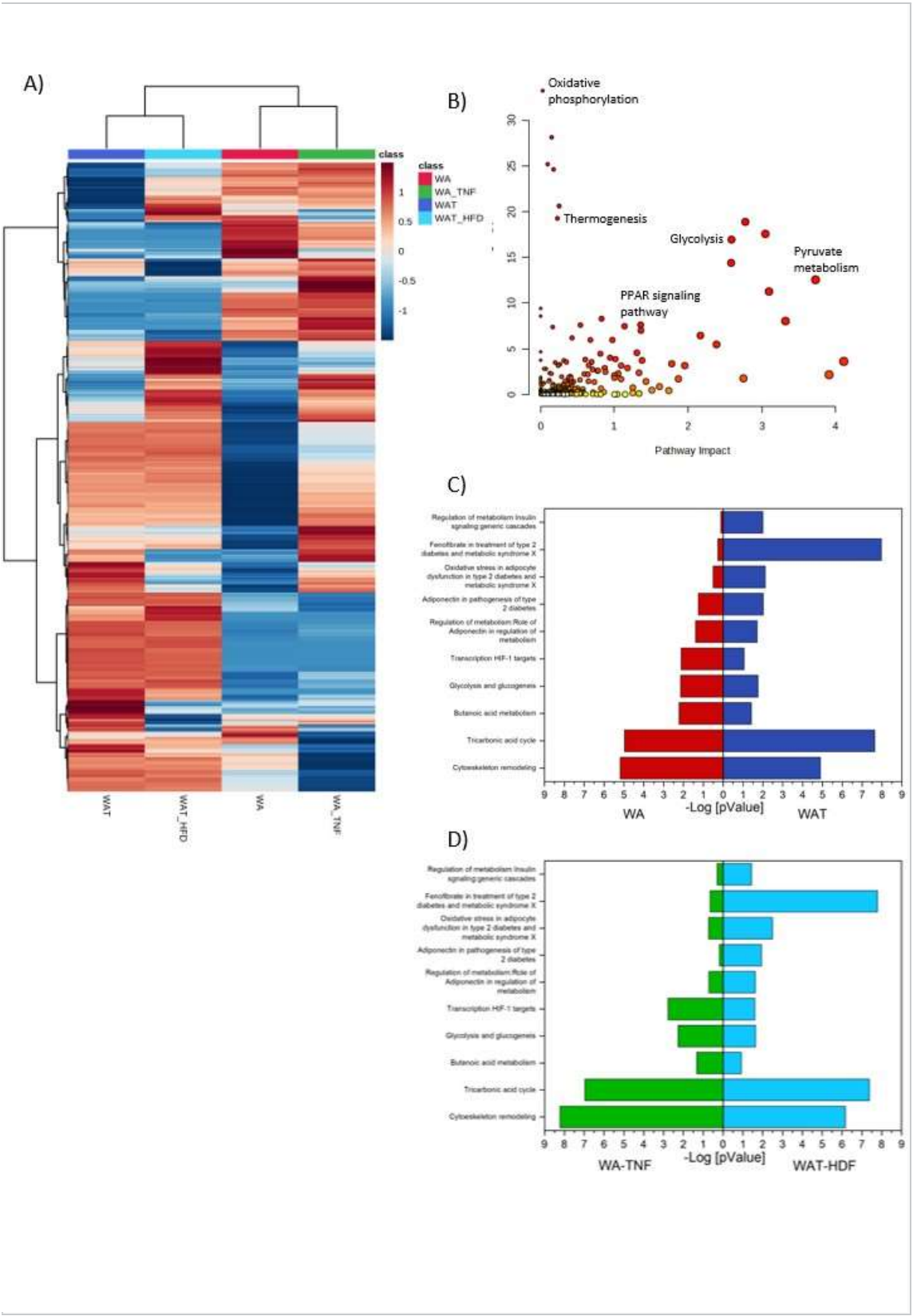

Figure 4: A) Heat map of mass spectrometry-based proteomics of adipose spheroids showing a comparative proteomic profile among adipose spheroids and adipose tissue from mice. B) Joint pathway analysis, the graph is showing the pathways that are enrichment. C) top 10 metabolic

process networks; in red the process from WA and in dark blue from WAT. D) top 10 metabolic process networks; in green the process from WA-TNF-α and in dark blue from WAT-HDF.

Considering the two most similar enriched metabolic processes – (i) regulation of metabolism and the role of adiponectin in regulating metabolism, and (ii) glycogenolysis - we performed further analysis using GeneGo Metacore™ enrichment analysis to produce pathway maps (refer to Figure 5). The graphical representation of the pathway map is based on the distribution of protein enrichment, where well-characterized proteins or protein complexes are displayed as individual symbols, and the data collected from all experiments are shown and linked on the maps as thermometer-like symbols. An upward-facing red thermometer indicates upregulated proteins. We identified several crucial hubs, including proteins involved in mitochondrial biogenesis, fatty acid uptake, and cell energy maintenance, all of which are regulated directly or indirectly by adiponectin (Figure 5-A). In particular, we found that among all the upregulated proteins, those involved in adiponectin signaling pathway were most involved in mitochondrial biogenesis, inhibition of ROS production, and fatty acid oxidation - essential functions of adipose tissue. In Figure 5-B, we highlight the upregulated proteins orchestrating the glycogenolysis process, with proteins involved in the electron transport chain, pyruvate metabolism, and ATP metabolism, being the most upregulated proteins encountered in spheroids (WA and WA-TNF-α) proteome, similar to the results from mice adipose tissue (WAT and WAT-HDF).

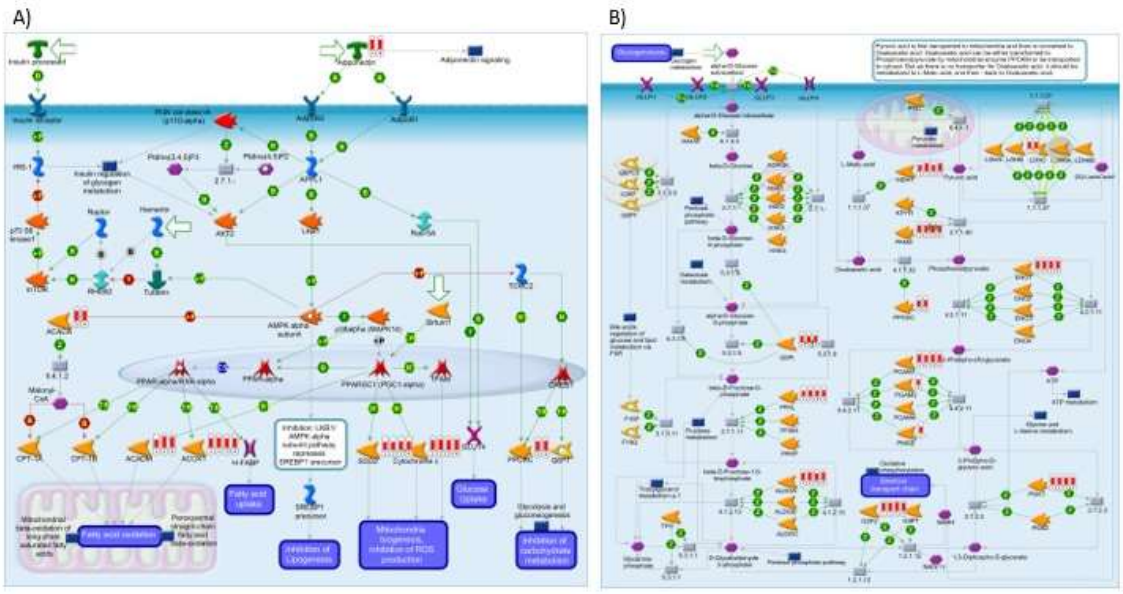

Figure 5: A) Regulation of metabolism, the role of Adiponectin in regulation of metabolism. B) Glycogenolysis 1) WA, 2) WAT, 3) WA-TNF-α and 4) WAT-HDF. The pathway images were generated by GeneGo Metacore™ enrichment analysis. Well-characterized proteins or protein complexes are shown as individual symbols within the image; experimental data from all the records are connected and depicted as thermometer-like figures on the maps. Upward-facing thermometers are shown in red and indicate more abundant proteins. The linkage of proteins by arrows depicts the stimulatory and inhibitory effects or interaction of the encoded protein on the desired protein. Further explanations are provided at https://portal.genego.com/help/MC_legend.pdf.

DISCUSSION

Over the last 50 years, obesity has reached pandemic proportions worldwide, causing significant economic and societal consequences [2526]. This condition is characterized by an imbalance in energy expenditure and accumulation, which is associated with a persistent inflammatory condition often referred to as a sterile inflammatory process due to the absence of infectious agents.

Currently, obesity research relies heavily on animal models and two-dimensional cell culture models *in vitro.* However, animal models have ethical implications and limitations, and cell culture monolayers fail to represent the real structure and physiology of the tissues. Therefore, there is a growing demand for more refined models that can reproduce human and mouse white and brown/beige adipose tissues in vitro with physiological accuracy [25].

To develop an in vitro a model of adipose tissue, capable to recapitulate its physiologic aspects, we produce spheroids of a widely used 3T3-L1 cell lineage in obesity research and differentiated them in presence or absence of TNF-α treatment (respectively named as WA-TNF-α, and WA). It is known that adipocytes treated with TNF-α can replicate a wide range of insulin resistance transcriptional changes associated with metabolism [2720], and our data show that TNF-α treated adipospheres are insulin resistant.

The insulin resistance induced in the 3D models of mature 3T3-L1 cells (WA-TNF-α spheroids) has been first evidenced by changes in the expression of four adipocyte marker genes (Adipoq, Lep, Pparg, and Lpl), which were downregulated in WA-TNF-α spheroids compared to WA and NDIF. As previously reported, Lpl, which regulates adipose tissue, is significantly affected in insulin-resistant individuals, especially in the postprandial period [28]. Furthermore, PPARγ activation in mature adipocytes improves insulin sensitivity by inducing the expression of several genes involved in the insulin signaling cascade [29], and its downregulation is associated with insulin resistance. The role of leptin in the insulin metabolism remains unclear, with evidence suggesting that it can act as both insulin-sensitizing agent and inductor of insulin-resistant phenotype. In vitro observations suggest leptin plays an inhibitory role on glucose metabolism, while in vivo, it tends to play an insulin-sensitizing role due to central mechanisms [30]. Thus, our model of insulin-resistant spheroids by exposure to TNF-α for 24h reproduces the gene effects found in individuals resistant to this hormone.

The secretion of adiponectin in mature adipocytes is associated with insulin resistance and adipocyte differentiation phenotypes of our *in vitro* obesity model, we measured adiponectin levels (Figure 1). Our findings showed that the adipokine, adiponectin (Adipoq), was secreted in our adiposphres and the mRNA expression was decreased in WA-TNF-α. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation [31], and its high levels are associated with adipocyte differentiation, which tends to decrease with increasing adiposity in visceral adipose tissue [32]. Low levels of adiponectin in circulation are linked to insulin resistance and obesity, altering glucose metabolism and insulin sensitivity [33] [19]. Based on this, our data indicate that both WA and WA-TNF-α adipospheres achieved the expected differentiation stage for adipocytes, and

WA-TNF-α as it were downregulated at the end of differentiation, indicating that they may have an insulin-resistant phenotype.

When we studied the functionality of our adipospheres (WA and WA-TNF-α) through the uptake of glucose after a stimulus or not of insulin, our results show that WA-TNF-α spheroids were not able to uptake glucose like the non-treated ones (WA), even in the presence of insulin. These results agree with similar ones reported in stem cell–derived adipocytes models [34], and show further evidence that our TNF-α treated adipospheres are insulin resistant, while WA is still insulin sensitive. In this way, our adipospheres models mimic the adipose tissue from a metabolically healthy obese individual and an obese individual with insulin resistance.

The most thorough validation of adiposphere models was performed by the comparison among them and with the real mouse tissue in a proteomic context. Proteomic studies of adipose tissue have previously been conducted to understand obesity and the regulation of gene expression, signaling, and metabolic changes in adipose tissue [35]. These studies have revealed gender-specific hallmarks related to redox status, immune responses, adipose tissue accumulation, and mitochondrial remodeling associated with aging and T2D [36]. In addition, comparison of subcutaneous and visceral adipose tissue identified novel protein species that may be involved in the obesity development in humans [37] and the analysis of distinct cellular components of adipose tissue shows that stroma vascular fraction, including pre-adipocytes, perivascular cells and blood cells are also important to secretion of adipokines and to response to regulatory signals [38].

Regarding the differences found in the number of identified proteins in our adipospheres models and in the mice tissues, which is explained by the complexity of the entire organ on face of a 3D culture composed by only one cell type, our data showed that proteins involved in the fatty acid and mitochondrial pathways are differently expressed in spheroids and in mouse adipose tissue. However, there is more similarity between WAT and WA model and WAT-HFD and WA-TNF model, suggesting two distinct models for evaluating adipose tissue, a lean and an insulin resistance one.

Our proteomic analysis provided valuable insights into protein interactions and cluster formations, allowing us to construct a directed network that elucidated crucial processes regulated in both adipose tissues and 3D models. These findings underscore the potential of these models in mimicking real tissues. Of note, among the top ten positively regulated metabolic processes, the role of adiponectin in metabolism was particularly noteworthy, especially for WAT-HDF and WA-TNF-α. Adiponectin, secreted by adipocytes, has been implicated in the development of insulin resistance [39]; our data showed that TNF-α-treated spheroids downregulated the relative mRNA expression of adiponectin and reduced the amount of secreted protein. Pathway analysis revealed that the pathway centrally regulated by adiponectin was enriched in our models.

Furthermore, our findings revealed that the translocase of the inner membrane complex (TIM13) was upregulated in both WAT-HDF and WA-TNF-α, consistent with previous studies that demonstrated its association with cardiac dysfunction in high-fat diet-induced obesity models [40]. We also observed upregulation of perilipin family proteins (PLIN 2 and PLIN 5) in both our WA-TNF-α and WAT-HDF, mirroring the expression pattern seen in obese mice. Perilipin proteins are known to be upregulated in obesity and have been linked to the regulation of lipid storage effects in obesity and insulin resistance-related non-alcoholic fatty liver disease [23 41]. Taken together, our results demonstrate that our adiposphere models effectively recapitulate key molecular and functional changes observed in real adipose tissue, thus serving as a valuable tool for studying obesity-related metabolic disorders.

Our analysis yielded interesting findings indicating that the glycogen metabolism pathway is significantly regulated in both adipose tissue and adipose spheroids. We observed upregulation of several proteins (MDH1, PKM2, ENO1, TGAM1, PFKL, ALDOA, G3Q2), which play crucial roles in this metabolic pathway. This finding is consistent with previous research suggesting that glycogen metabolism is critical in maintaining energy homeostasis, and that adipose tissue may contain glycogen stores [42,43]. Moreover, the accumulation of excess glycogen in adipose tissue has been proposed as a key feature of inflammatory-related metabolic stress in human obesity [42–44].

In summary, here we present WA and WA-TNF-α models are suitable for investigating metabolic pathways associated with obesity, including insulin resistance, adiponectin regulation, and glycogenolysis. Additionally, our comparison of these models with adipose tissue from mice indicates that they accurately replicate key aspects of adipose tissue in vitro, including morphology, gene expression, and proteomic profile. Overall, our findings suggest that these models could be valuable tools for further studying the complex metabolic processes involved in obesity and related metabolic disorders.

CONCLUSION

We have developed two 3D adipose tissue models, that closely mimic mouse adipose tissue, making them useful models for investigating metabolic pathways associated with obesity. Additionally, we also developed the TNF-α treated model, which mimics a phenotype and metabolic characteristics of an insulin resistant adipose tissue. These models replicate crucial aspects of adipose tissue, such as morphology, gene expression, and proteomic profile, indicating their high potential for studying obesity-related metabolic pathways. Through proteomic analysis, we identified key proteins involved in lipid storage regulation, insulin resistance, and obesity condition. Our findings demonstrate the capability of these models to mimic real tissue and enhance our understanding of the mechanisms underlying obesity-related metabolic disorders in special the insulin resistance and open an opportunity to be a feasible model to substitute the use of animals in the pre-clinical trials studies.

METHODS

3T3-L1 cell culture, spheroid assemble, and differentiation.

3T3-L1 mouse preadipocytes were obtained from American Type Culture Collection (ATCC). 3T3-L1 mouse preadipocytes (passage 10-15) were cultured in high glucose (4.5 g/L) Dulbecco’s Modified Eagle Medium supplemented with 10% calf serum and 100 U/mL penicillin–100 mg/mL streptomycin at 5% CO2 and 37°C and harvested before reaching 70% confluence for spheroid assemble.

After reaching confluence, cells were counted and the nanomagnetic particles NanoShuttle ™ -PL (Greiner) was added in the proportion of 1uL for each $1x10^4$ cells. After magnetization, cells were added to a 96-well culture plate (Greiner Bio-One 655970 - 96-well microplate, PS, well with F / chimney bottom, cell-repellent surface, clear, sterile), in the amount of $1,5x10^4$ cells. For spheroids formation, a device with 96 magnetic cylinders (Greiner Bio-One 655830) was positioned below the culture plate, the cells remain for 24 hours incubated with magnets and kept in a humid incubator at 37 ° C and 5% CO_2.

After assembled, the spheroids were differentiated into adipocytes through the induction cocktail composed of DMEM, 10% FBS, 1% w/v antibiotics (penicillin and streptomycin), 1µM dexamethasone, 1µg / mL of insulin and 0.5mM of 3-isobutyl-1-methylxanthine (IBMX), for 48h. After induction, the medium was aspirated and the maintenance medium, composed of DMEM, 10% FBS, 1% w/v antibiotics, 1 µg/mL insulin was added. The maintenance medium was changed every 48 hours until reach 14 days of differentiation. In last 24h of differentiation, the maintenance medium was changed in the WA-TNF-α spheroids; DMEM supplemented with 0,5 BSA (Roche) and 2,5nM of TNF-α (Gibco);

Animal model

Male C57BL/6J mice were purchased from Model Organisms Laboratory (LOM) mouse facility at LNBio/CNPEM (Campinas, SP, Brazil). Mice were weaned with 21 days old, being maintained on a photoperiod of 12:12 light/dark cycle, at 21-24°C, with free access to food and water and 3 animals per cage. They were randomly separated into two different groups: control group was fed a chow diet (Nuvilab CR1), and high fat diet (HFD) group was fed a 60% fat diet for 12 weeks (diet compositions are described in supplemental material; Pragsolucoes Biociencias, Jau - SP, Brazil). At the age of 17 weeks, mice were sacrificed in fed state and the perigonadal white adipose tissue (WAT) and brown adipose tissue (BAT) were dissected and immediately frozen with liquid nitrogen. Later, they were stored at –80 C until use.

The Ethical Committee of CNPEM/LNBio “Comissão de Ética no Uso de Animais” (CEUA-CNPEM) specifically reviewed and approved this study

(approval identification 61). The protocols were done conformed to the guidelines for ethical conduct in the care and use of animals established by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA). Animals were treated humanely, regarding to alleviate suffering.

Microscopy

The lipid droplets and the nuclei were stained after spheroids were fixed for 1 hour in 4% formalin and washed 3 times in PBS. The LipidSpot (Biotium; cat. Number 70065-T) lipid marker probe and the DAPI (Biotium; cat. Number 40043) were added according with manufacturer's instructions. After staining with the fluorescent probes, the images were obtained using a TCS SP8 (Leica) confocal microscope.

Gene expression

Spheroids were homogenized in 0,5 ml of TRIzol Reagent (Invitrogen) in order to extract the RNA according with manufacturing protocol. A minimum of 20 spheroids was used to make RNA for each condition. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative PCR reactions using SYBR-green master mix (Applied Biosystems) were run on Applied biosystem termocycler. After verifying its suitability, rpl27 and tbp was used to normalize gene expression. Fold changes were calculated using the 2−DDCt method as described [45]. Primers used for the various genes are listed in Table S1.

Glucose uptake assay

*After s*pheroids formation and differentiation in WA, the spheroids were cleaned with PBS and they were maintained for 24 hours without insulin stimulation in complete DMEM high glucose. Immediately later, was done a serum starvation with DMEM low glucose during 1 hour. The next step was stimulated the spheroids with or without insulin (100 nM) in DMEM completed with 10% charcoal serum and 1% penicillin/streptomycin. After 30 minutes of stimulation, the medium was taken and the glucose content was quantified by colorimetric method (GOD-PAD; LABORLAB, SP, Brasil). The absorbance of the samples was measured by Clariostar (BGM LABTECH).

Adiponectin quantification

Secreted adiponectin was measured with ELISA (Crystal Chem #80569), the quantification was performed according to manufacturer's protocol with 10uL of spheroid medium; The absorbance of the samples was measured by Clariostar (BGM LABTECH).

Proteomics

Proteolytic Digestion

All samples were submitted to the same protocol of protein extraction and trypsin digestion as previously described [14]. Briefly, the samples were homogenized with lysis buffer (8M urea, 2M thiourea in 30mM Tris-HCl pH 8.5, containing 1mM EDTA, and 1mM PMSF). The proteins were quantified by Bradford method and an aliquot containing 10 µg of proteins was submitted to reduction with 5 mM dithiothreitol (DTT), for 25 minutes, at 56 °C, and alkylated with 14 mM iodoacetamide (IAA), for 30 minutes, at room temperature, in the dark. The remaining IAA was removed by the addition of excess DTT. To reduce the final concentration of urea to 1 M, the mixtures were diluted with 50 mM ammonium bicarbonate buffer. Proteins were digested with trypsin (1:50, w/w), for 18 hours, at 37°C, and then, 1% formic acid (v/v) was added to stop the digestion. The tryptic peptides were desalted with C18 stage tips. To avoid bias during measurements, all data collection was randomized using the R (v3.4.0) environment.

LC-MS/MS analysis

The peptide mixture (2.0ml) was analyzed using an LTQ Orbitrap Velos (Thermo Fisher Scientific) mass spectrometer coupled to nanoflow liquid chromatography on an EASY-nLC system (Proxeon Biosystems) with a Proxeon nanoelectrospray ion source. Peptides were separated in a 2–35% acetonitrile gradient, in 0.1% formic acid using a PicoFrit analytical column (20 cm × ID 75,5 µm particle size, New Objective), at a flow rate of 300 nL/min, over 175 minutes, as previously described [46].

Proteomic data analysis

Raw data were processed using MaxQuant v1.5.8 software, and MS/MS spectra were searched against the Mus musculus UniProt database (released on December, 2020, 63,724 sequences, and 28,586,808 residues) using the Andromeda search engine. A tolerance of 10 ppm was considered for precursor ions, and 1 Da for-fragment ions, with a maximum of two missed cleavages. A fixed modification of carbamidomethylation of cysteine and variable modifications of methionine oxidation and protein N-terminal acetylation were considered. A 1% false discovery rate (FDR) was set for both protein and peptide identifications. Protein quantification was performed using the LFQ algorithm, with a minimal ratio count of 1 and a window of 2 minutes for matching between runs. Data were processed in Perseus v1.6.7.0 software, excluding reverse sequences and those identified “only by site” entries. Protein abundance was calculated based on the normalized spectrum intensity (LFQ intensity) and was log2-transformed. The significance was assessed using Student’s t-test (P-value < 0.05). Data visualization, pathway maps, and enrichment analysis was performed with fold-change (FC) and p-value threshold value of 2.0 and 0.05, respectively, using the software Metaboanalyst v 5.0 and MetaCore (Clarivate).

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AUTHOR CONTRIBUTIONS

TMA designed the research, article, experiments and developed them; MGA designed and performed animal experiments, discussed results, and revised the article. MFT performed the adiponectin quantifications; PSLO helped with Metacore analysis; RRD and AFPL helped with proteomic experiments and data analysis. FRT helped with primers development and standardization, and qPCR analysis. ACMF designed the research, provided funding, corrected, and revised the article. All authors contributed to the article and approved the submitted version.

COMPETING INTERESTS

The author(s) declare no competing interests.

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Supplemental material

Development of a 3D In Vitro Adipocyte Model for Studying Metabolic Conditions and Obesity.

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Table 1 – Diet Composition

Ingredient	Kcal (%)	Quantity g/ml
Cornstarch	0	0
Casein	19.8	258.5
L-Cystine	0.3	3.9
Dextrinized cornstarch	12.4	161.5
Sucrose	7.2	94.1
Soybean oil	5.6	32.3
Lard	54.6	316.6
fiber	0	64.6
Mineral mix PSB10026B*	0	64.6
Vitamin mix AIN A 10X*	0.1	1.3
Choline chlorhydrate	0	2.6

This diet provides 5217.3 kcal/kg; the composition in percentage of kcal is 20.1% protein, 19.7% carbohydrates and 60.2% fat. *For details, see Reeves et al. (Reeves, P. G., Nielsen, F. H., Fahey, G. C., Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 1993, 123, 1939–1951.)

4 DISCUSSÃO

O presente estudo foi sumarizado em dois artigos, sendo o primeiro deles (Artigo I) focado na detalhada caracterização dos esferoides de adipócitos, aliando técnicas de imagem, como microscopia confocal e micro-tomografia de raios-X, a proteômica; o segundo artigo (Artigo II) versa sobre o desenvolvimento de um modelo in vitro mimético de tecido adiposo com fenótipo de resistente à insulina, com foco na análise proteômica detalhada das vias enriquecidas no esferoide adiposo modulado pela citocina pró-inflamatório TNF-α.

A cultura de células bidimensionais (2D) tem sido usada na maioria das pesquisas biológicas das últimas décadas, no entanto, apresenta limitações como a incapacidade de expressar o microambiente da biologia in vivo que afeta as propriedades fisiológicas das células. Estudos demonstraram a importância da matriz extracelular (ME) no comportamento celular, sendo que a cultura de células 3D pode representar um ambiente mais mimético do in vivo com relação aos sistemas de cultura de células 2D (RAVI et al., 2015) (PAMPALONI; REYNAUD; STELZER, 2007) (CHOI et al., 2022).

Os esferoides obtidos possuem forma e tamanho com pouca variação como evidenciado nos experimentos de *high content image* e nas mensurações de microtomografia de raios-X relatados no artigo I. A uniformidade dos cultivos tridimensionais é uma variável importante quando consideramos a utilização desses modelos em experimentos para descoberta de novos fármacos (SIVITILLI et al., 2020) , o método escolhido para sua produção propicia essa uniformidade, gerando cultivos estáveis e padronizáveis (Souza et al., 2010).

Além da forma e tamanho, a estabilidade e viabilidade dos esferoides são de suma importância; cultivos 3D são mais susceptíveis a hipóxia e a formar centros necróticos em virtude de sua conformação tridimensional (PAMPALONI; REYNAUD; STELZER, 2007) (EDMONDSON et al., 2014) (RAVI et al., 2015) . Os esferoides diferenciados tiveram sua viabilidade acompanhada ao longo do tempo por meio da quantificação do ATP, e se mostraram estáveis ao longo do período de cultivo (14 dias). Não foram observadas mudanças estatisticamente significativas no tamanho dos esferoides durante o período de acompanhamento; a micro tomografia de raios-X também evidenciou a formação de culturas livres de centros necróticos, mostrando esferoides coesos e sem núcleos amorfos ou com orifícios.

Uma vez formados, os esferoides foram tratados em meio suplementado com fatores indutores de diferenciação, e após 14 dias de diferenciação, os esferoides passaram por avaliações quantitativas e qualitativas. Para checar o acúmulo lipídico no citoplasma, os esferoides foram corados com marcador específico para lipídios (LipidSpot) e submetidos à microscopia de fluorescência

confocal, os esferoides diferenciados (WA) mostraram maior acúmulo lipídico quando comparado ao não diferenciado (NDIF).

A linhagem celular 3T3-L1 é um dos modelos mais bem caracterizados para estudos envolvendo adipócitos em cultura. Em estudo anterior quando injetados em camundongos, os pré-adipócitos 3T3-L1 se diferenciam e formam bolsas de gordura indistinguíveis do tecido adiposo normal (GREEN; KEHINDE, 1979). Já foi relatado inúmeras vezes que em cultura, os pré-adipócitos 3T3-L1 diferenciados possuem a maioria das características estruturais dos adipócitos de tecido animal (NOVIKOFF et al., 1980), sendo que a formação e aparência de gotículas de gordura em desenvolvimento imitam o tecido adiposo vivo (Green & Meuth, 1974).

A diferenciação de pré-adipócitos em adipócitos maduros e os efeitos do tratamento com TNF-α na regulação de genes durante a adipogênese foram avaliados por meio da expressão relativa de mRNA de Adiponectina (Adipoq), Lipoproteína lipase (Lpl), Leptina (Lep), Receptor-γ ativado por proliferador de peroxissoma (Pparg), por meio de análise de PCR quantitativa. Todas as expressões relativas de mRNA foram reguladas positivamente nos esferoides WA em comparação ao NDIF, indicando que os adipócitos nos esferoides se diferenciaram (Artigo II).

Expressão da lipoproteína lipase é considerado um evento precoce na diferenciação de adipócitos que precede o mRNA de Lpl e tem sido frequentemente citado como um sinal precoce de mudanças evidentes na morfologia e na expressão da diferenciação de adipócitos (AILHAUD et al., 1985; GREGOIRE; SMAS; SUL, 1998), assim como evidenciamos nos nossos dados a expressão aumentada do Lpl em WA atrelado as mudanças morfológicas verificadas.

Assim como o Lpl, foi verificado o aumento da expressão relativa de mRNA do PPARγ no WA; o PPARγ é considerado o principal regulador da adipogênese, uma vez que a expressão ectópica do PPARγ isoladamente em fibroblastos é capaz de iniciar o processo de adipogênese e nenhum outro fator conhecido poderia induzir a adipogênese sem a presença do PPARγ (FARMER, 2006; TONTONOZ; HU; SPIEGELMAN, 1994). Estudos in vivo revelam que o

PPARγ é vital no controle da adipogênese e no metabolismo lipídico/glicose. Em camundongos adultos, a ablação de PPARγ nos tecidos adiposos com sistema de recombinação Cre-ER (T2) dependente de tamoxifeno leva à morte de adipócitos e subsequente renovação, sugerindo que o PPARγ também é necessário para a sobrevivência de adipócitos maduros e não somente durante a indução da adipogênese como acreditava-se (VIDAL-PUIG et al., 1997) (IMAI et al., 2004) (MA et al., 2018) (SCHOONJANS; STAELS; AUWERX, 1996).

As adipocinas também são centrais na regulação dos adipócitos além de serem marcadores de maturidade (OUCHI et al., 2011) (doi: 10.1038/nri2921), as expressões relativas das adipocinas Lep e Adipoq foram encontradas reguladas positivamente nas amostras WA. A leptina atua principalmente no sistema nervoso central, produzindo efeitos anorexígenos e estimulando o gasto energético (FLIER, 1998) (OLIVEIRA et al., 2015); já a adiponectina está envolvida em importantes efeitos metabólicos, como estimular a oxidação de ácidos graxos, reduzir a gliconeogênese e aumentar a termogênese (FRUEBIS et al., 2001). Dessa forma podemos inferir que os adipócitos WA atingiram um estágio avançado de diferenciação e maturidade.

Uma vez que os esferoides WA foram devidamente caracterizados (Artigo I e II), um tratamento com fator de necrose tumoral-alfa (TNF-α) foi aplicado ao fim do período de diferenciação para atuar como modulador pró-inflamatório (Artigo II); o TNF-α é conhecido por ser uma citocina multifuncional que pode regular muitos processos celulares e biológicos, como função imunológica, diferenciação celular, proliferação, apoptose e metabolismo energético (CAWTHORN; SETHI, 2008). É bem estabelecido que o TNF-α pode induzir um estado de resistência à insulina nos adipócitos (RUAN; LODISH, 2003), como o objetivo era reproduzir in vitro um ambiente pró-infamatório que levasse ao fenótipo de resistência à insulina, o TNF-α foi utilizado como indutor para tal.

Os esferoides tratados com TNF-α (WA-TNF-α) apresentaram uma regulação negativa no mesmo conjunto de genes (Lep, Adipoq, Pparg e Lpl) em comparação com o WA (Artigo II).

A supressão dos níveis de mRNA do PPARγ também foi observada após o tratamento com TNF-α, esse fenômeno tem sido relacionado a mecanismos

ainda não compreendidos totalmente; elucida-se que a própria expressão do PPARγ pode ser regulada positivamente pela atividade do mesmo. Assim, em adipócitos tratados com TNF-α, é provável que a regulação negativa da atividade de PPARγ também resulte em níveis reduzidos de mRNA (JAIN; PHELPS; PEKALA, 1999). Além disso, a supressão da expressão de GLUT4 também poderia ser mediada por TNF-α, por meio de um mecanismo dependente de PPARγ e C/EBPα (CAWTHORN; SETHI, 2008). Essa supressão do GLUT4 está atrelada à resistência da captação de glicose na presença de insulina pelo tecido adiposo como observada na diabetes tipo 2, associando essa regulação negativa do mRNA de PPARγ a quadros de hiperinsulinemia e obesidade (FLORES-RIVEROS et al., 1993).

A produção da maioria das adipocinas é aumentada no estado obeso, e essas proteínas pró-inflamatórias normalmente atuam promovendo doenças metabólicas ligadas à obesidade. Além da leptina, adiponectina, TNF e IL-6, outras adipocinas foram mais recentemente identificadas como facilitadoras do processo inflamatório: resistina, proteína de ligação ao retinol 4 (RbP4), lipocalina 2, IL-18, proteína semelhante à angiopoietina 2 (ANGPTL2), ligante de quimiocina CC 2 (CCL2), CXC-quimiocina ligante 5 (CXCL5) e nicotinamida fosforibosiltransferase (NAmPT) (OUCHI et al., 2011).

Outros estudos relataram diminuição da expressão gênica de genes associados à adipogênese em camundongos obesos e com diabetes mellitus tipo 2 (NADLER et al., 2000), especialmente do mRNA algumas adipocinas como observamos em nosso conjunto de dados com a redução relativa da expressão de Lep e Adipoq.

Estudos anteriores demonstraram que a adiponectina no plasma e no tecido adiposo estão diminuídos em indivíduos obesos em comparação com indivíduos magros. Consistente com isso, a produção de adiponectina pelos adipócitos é inibida por fatores pró-inflamatórios, como TNF e IL-6 (RYO et al., 2004) (OUCHI et al., 2003), corroborando com nossos achados dos níveis reduzidos de expressão da adiponectina.

Nossos dados mostraram também uma regulação negativa da leptina no esferoide tratado com TNF-α. Embora a literatura relate um aumento da leptina

em ambiente obesogênico; o papel da leptina no metabolismo da insulina permanece incerto, com evidências sugerindo que ela pode atuar tanto como agente sensibilizador da insulina, quanto indutor do fenótipo de resistência à insulina (CEDDIA et al., 2002) (OUCHI et al., 2011) (MARTÍNEZ-SÁNCHEZ, 2020).

Os dados de expressão gênica indicaram que os esferoides WA-TNF-α estavam em um estado pró-infamatório, com fenótipo bastante consistente de resistência à insulina; nesse cenário investigamos a responsividade a insulina nesse modelo, por meio do ensaio de captação de glicose (Artigo II). Em nossos experimentos, os esferoides sensibilizados com TNF-α não foram responsivos à insulina, apresentando redução na captação de glicose na presença e ausência de insulina quando comparado aos esferoides não tratados (WA).

É sabido que o tecido adiposo atua como um regulador chave do metabolismo sistêmico de carboidratos e pode desempenhar um papel importante na detecção de glicose (ABEL et al., 2001). Sabe-se também que adipócitos tratados com TNF-α podem replicar uma ampla gama de alterações transcricionais de resistência à insulina associadas ao metabolismo (LO et al., 2013) (KRAKO JAKOVLJEVIC et al., 2021). Neste estudo, demonstramos que as adiposferas tratadas com TNF-α apresentam esse perfil ao não responder à insulina no ensaio de captação de glicose.

Uma vez que os modelos de tecido adiposo fisiológico (WA) e pró-inflamatório foram estabelecidos e mostraram ser biologicamente relevantes, com o objetivo de validá-los desenvolvemos um *pipeline experimental*, para validação dos modelos e para compreender o quanto os modelos mimetizam o tecido biológico utilizando a proteômica (Artigo I).

Os avanços nas tecnologias *"ômicas"*, como espectrometria de massas (MS), tornaram-se um método amplamente aplicado para caracterização de culturas 3D. Além disso, o desenvolvimento de metodologias e melhorias na resolução e sensibilidade dos instrumentos de MS permitiram abordar uma variedade de questões biológicas (MIGISHA NTWALI et al., 2020) (GONNEAUD et al., 2017). No entanto, ainda que os avanços no campo da proteômica tenham sido significativos nos últimos anos, há uma dificuldade em obter resultados

robustos de culturas tridimensionais; fatores como a disponibilidade de amostra e rendimento nos processos de extração proteica demandam uma série otimizações metodológicas para a obtenção de resultados confiáveis, nosso trabalho focou-se em otimizar e propor uma metodologia robusta para tal análise (Artigo I).

Para desenvolvimento do ensaio de protêomica de culturas tridimensionais, 7 pilotos experimentas foram realizados para otimizar o processo de extração proteica, lise celular, recuperação proteica e análise de dados. Na primeira fase do experimento comparamos o proteoma total dos esferoides WA com o WAT (Artigo I).

Em uma abordagem proteômica quantitativa (Artigo I), um total de 1726 proteínas únicas foram identificadas em ambas as amostras. WA e WAT compartilham 536 proteínas, representando 83% do total de proteínas identificadas nos esferoides WA. Esses dados indicam uma alta taxa de similaridade entre o modelo WA 3D e o tecido dissecado do animal. A recuperação de proteínas para WAT após submetida à extração proteica apresentou rendimento superior em comparação com a recuperação de proteínas em WA, certamente pela complexidade de um tecido, que não pode ser reproduzido em esferoides e organoides.

Dois estudos relataram o perfil proteômico de adipócitos de 3T3-L1 em monocamada (CHAN et al., 2019) (CHOI; GOSWAMI; SCHMIDT, 2020) , esses estudos realizados em adipócitos cultivados em monocamada demonstram alguns dados semelhantes a nossos dados de cultivo 3D para as análises de enriquecimento de vias revelando processos associados à regulação do metabolismo, incluindo aqueles envolvidos na adipogênese e no metabolismo de ácidos graxos, processos fundamentais para o funcionamento dos adipócitos. Entretanto, o pipeline proposto no Artigo I recuperou uma quantidade maior de proteínas totais e mostrou o envolvimento dessas vias associando-as ao metabolismo energético, que não foi relatado nos estudos em monocamada. Com base nesses resultados é possível inferir que o cultivo 3D favoreceu para que as células mimetizassem melhor um adipócito proveniente de um tecido

adiposo real e o método por nós estabelecido demonstrou ser mais robusto com relação à quantidade de proteínas recuperadas.

Uma vez padronizado, o *pipeline* de análise de espectrometria de massas foi aplicado em modelos mais complexos, com intuito de avaliar até que ponto conseguimos reproduzir um tecido adiposo com características do tecido adiposo obeso in vitro. Para tanto, comparamos os esferoides WA-TNFα com tecido adiposo de camundongos que receberam dieta hiperlipídica (WAT-HFD) (Artigo II) e mantivemos os tecidos normais para controle (WA e WAT). Os resultados mostram que 842 proteínas foram identificadas com segurança para WA, enquanto 967 proteínas foram identificadas para WA-TNFα. Para o conjunto de dados WAT, 1315 e 1330 proteínas foram quantificadas para WAT e WAT-HFD, respectivamente; 664 proteínas foram encontradas em comum entre todos os grupos analisados, sugerindo semelhanças entre eles.

Para obter uma melhor compreensão das vias de sinalização envolvidas nas proteínas diferencialmente expressas nos esferoides e tecidos de camundongos, usamos o MetaboAnalyst 5.0 e o software Metacore™ (Calrivate Analytics) para conduzir a análise de enriquecimento (EA) usando um banco de dados de sinalização proteína-proteína amplamente reconhecido.

O EA identificou os IDs das proteínas dos conjuntos WA, WA-TNFα, WAT e WAT-HFD pela função de ontologia funcional no MetaCore. A possibilidade de uma interseção aleatória de um conjunto de genes e as entidades ontológicas correspondentes foram avaliadas usando o valor p de interseção hipergeométrica. Um valor de p mais baixo indicou que o objeto era mais relevante para o conjunto de dados, sugerindo uma classificação mais alta.

A análise de agrupamento de proteínas (*heatmap*) revelou que WAT e WAT-HFD são mais semelhantes entre si do que WA e WA-TNF-α. este resultado era esperado visto que WAT é um tecido removido de animais e WA é uma monocultura de células 3D, que comparado ao tecido não representa a complexidade celular do tecido real. No entanto, algumas proteínas apresentaram uma relação mais próxima entre as proteínas diferencialmente expressas em WAT/WA e WAT-HFD/WA-TNF-α, sobre essas irei discorrer nos próximos parágrafos.

As proteínas da família de subunidades de translocase de membrana interna de importação mitocondrial (TIM 10 e 13) foram reguladas positivamente em WAT-HDF e WA-TNF-α, consistente com estudos anteriores que demonstraram sua associação com disfunção cardíaca em modelos de obesidade induzida por dieta rica em gordura (HUNG et al., 2017), conferindo ao nosso modelo mais um indicativo de replicação de vias importantes reguladas no tecido adiposo obeso.

Também descobrimos que as proteínas da família da perilipina (PLIN 2 e PLIN 5) foram reguladas positivamente tanto em nosso WA-TNF-α, quanto em WAT de camundongos obesos (WAT-HDF). Sabe-se que as proteínas perilipina são reguladas positivamente na obesidade e têm sido associadas à regulação dos efeitos do armazenamento de lipídios na obesidade, na resistência à insulina e na doença hepática gordurosa não alcoólica (MIYOSHI et al., 2010) (ORLICKY et al., 2019).

Identificamos várias vias cruciais, incluindo as vias orquestradas por proteínas envolvidas na biogênese mitocondrial, captação de ácidos graxos e manutenção da energia celular, todas reguladas direta ou indiretamente pela adiponectina (Figura 5-A – Artigo II). Em particular, descobrimos que entre todas as proteínas reguladas positivamente, aquelas envolvidas na via de sinalização da adiponectina foram as mais envolvidas na biogênese mitocondrial, inibição da produção de ROS e oxidação de ácidos graxos - funções essenciais do tecido adiposo. Na Figura 5-B do artigo II, destacamos as proteínas supra reguladas orquestrando o processo de glicogenólise, com as proteínas envolvidas na cadeia de transporte de elétrons, metabolismo do piruvato e metabolismo do ATP, sendo as proteínas mais suprareguladas encontradas no proteoma dos esferoides (WA e WA-TNF-α), semelhante aos resultados do tecido adiposo de camundongos (WAT e WAT-HFD).

Além das vias discutidas anteriormente, nossa análise revelou que o metabolismo do glicogênio também é significativamente regulado tanto no tecido adiposo, quanto nos esferoides adiposos. Esse achado está de acordo com estudos anteriores que mostraram que o metabolismo do glicogênio desempenha um papel crucial no controle da homeostase energética e

sugeriram que o tecido adiposo pode conter reservas de glicogênio (CEPERUELO-MALLAFRÉ et al., 2016). Além disso, o excesso de glicogênio no tecido adiposo foi proposto como uma característica chave potencial do estresse metabólico relacionado à inflamação na obesidade humana (CEPERUELO-MALLAFRÉ et al., 2016).

5 CONCLUSÃO

Atualmente é sabido que as abordagens experimentais utilizando a cultura de células em monocamada não recapitulam totalmente a fisiologia de um organismo vivo. Juntamente a esse fato há uma necessidade crescente de reduzir ou substituir os animais na pesquisa. Nesse contexto, o emerge a necessidade de desenvolvimento de modelos experimentais in vitro que reproduzam melhor os aspectos da fisiologia in vivo em comparação com o cultivo celular tradicional em monocamada.

Sendo a obesidade um problema de saúde global, esforços são requeridos para descoberta de novas tecnologias para estudo dessa condição. Nesse contexto, nos desafiamos a desenvolver modelos de cultivo in vitro que fossem capazes de mimetizar parte dos aspectos fisiológicos e metabólicos do organismo obeso.

No geral, nossos resultados demonstram que nossos modelos de adiposferas podem efetivamente recapitular as principais alterações moleculares e funcionais observadas no tecido adiposo real, tornando-os uma ferramenta valiosa para estudar distúrbios metabólicos relacionados à obesidade. Além disso, os resultados indicam a proteômica baseada em MS como um método para validar a culturas de células em 3D.

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7 APÊNDICES

7.1 APÊNDICE A – Artigo publicado em colaboração: pH and the Breast Cancer Recurrent Mutation D538G Affect the Process of Activation of Estrogen Receptor α Biochemistry

› Biochemistry. 2022 Mar 15;61(6):455-463. doi: 10.1021/acs.biochem.1c00806. Epub 2022 Mar 3.

pH and the Breast Cancer Recurrent Mutation D538G Affect the Process of Activation of Estrogen Receptor α

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Affiliations + expand

PMID: 35238537 DOI: 10.1021/acs.biochem.1c00806

Abstract

Estrogen receptor α (ERα) is a regulatory protein that can access a set of distinct structural configurations. ERα undergoes extensive remodeling as it interacts with different agonists and antagonists, as well as transcription activation and repression factors. Moreover, breast cancer tumors resistant to hormone therapy have been associated with the imbalance between the active and inactive ERα states. Cancer-activating mutations in ERα play a crucial role in this imbalance and can promote the progression of cancer. However, the rate of this progression can also be increased by dysregulated pH in the tumor microenvironment. Many molecular aspects of the process of activation of ERα that can be affected by these pH changes and mutations are still unclear. Thus, we applied computational and experimental techniques to explore the activation process dynamics of ER for environments with different pHs and in the presence of one of the most recurrent cancer-activating mutations, D538G. Our results indicated that the effect of the pH increase associated with the D538G mutation promoted a robust stabilization of the active state of ER. We were also able to determine the main protein regions that have the most potential to influence the activation process under different pH conditions, which may provide targets of future therapeutics for the treatment of hormone-resistant breast cancer tumors. Finally, the approach used here can be applied for proteins associated with the proliferation of other cancer types, which can also have their function affected by small pH changes.

7.2 APÊNDICE B – Artigo publicado em colaboração:AM-879, a PPARγ non-agonist and Ser273 phosphorylation blocker, promotes insulin sensitivity without adverse effects in mice

› Metabol Open. 2022 Dec 21;17:100221. doi: 10.1016/j.metop.2022.100221. eCollection 2023 Mar.

AM-879, a PPARγ non-agonist and Ser273 phosphorylation blocker, promotes insulin sensitivity without adverse effects in mice

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PMID: 36588655 PMCID: PMC9800205 DOI: 10.1016/j.metop.2022.100221

Free PMC article

Abstract

Obesity is one of the main risk factors for type 2 diabetes, and peroxisome proliferator-activated receptor γ (PPARγ) is considered a promising pathway on insulin sensitivity and adipose tissue metabolism. The search for molecules acting as insulin sensitizers have increased, especially for molecules that block PPARγ-Ser273 phosphorylation, without reaching full agonism. We evaluated the *in vivo* effects of AM-879, a PPARγ non-agonist, and found that AM-879 exerts different effects in mice depending on the dose. At lower doses, this ligand decreased BAT, increased leptin and Crh expression. However, at a higher dose, it promoted improvement on insulin sensitivity, ameliorates expression of metabolism-related genes, decreased the expression of genes related to liver toxicity, maintaining body weight and adipocyte size. These results present a new lead molecule to ameliorates insulin resistance and confirm AM-879 as a PPARγ non-agonist which blocks Ser273 phosphorylation as a good strategy to modulate insulin sensitivity without developing the adverse effects promoted by PPARγ full agonists.

Keywords: Diabetes; Non-agonist; Obesity; PPARγ; Phosphorylation.

7.3 APÊNDICE C – Artigo publicado em colaboração: Green tea extract increases adiponectin and PPAR α levels to improve hepatic steatosis.

Green tea extract increases adiponectin and PPAR α levels to improve hepatic steatosis

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Affiliations + expand

PMID: 35134507 DOI: 10.1016/j.jnutbio.2022.108957

Abstract

We postulated that Green tea (GT) improvements in non-alcoholic fatty liver disease (NAFLD) are dependent on adiponectin action in the liver. Male wild-type and adiponectin knockout (adipoKO) mice were induced to obesity for 8 weeks with a high-fat diet and then treated with GT for the last 12 weeks of the experimental protocol. Glucose and insulin tolerance tests, indirect calorimetry, histologic analysis of liver sections, and quantification of mRNA of hepatic genes related to glucose or fatty acid metabolism were performed. In vitro, we assessed the mechanism by which GT catechins act to improve hepatic steatosis by measuring lipid accumulation, and transcript levels of lipogenic genes in HepG2 cells treated with GT in the presence of a PPAR antagonist. Additionally, we performed a PPAR transactivation assay in 293T cells to test if catechins could activate PPARs. Different from wild-type mice, adipoKO animals treated with GT and fed a HFD gain body weight and fat mass, that were associated with a decrease in energy expenditure, were insulin resistant, and had no improvements in hepatic steatosis. Increased lipid levels were associated with no modulation of PPARα levels in the liver of adipoKO mice treated with GT. In vitro, we demonstrated GT catechins act to reduce hepatic steatosis in a PPARα-dependent manner, and especially epigallocatechin and epicatechin can indirectly activate PPARα, although it seems they are not direct ligands. By providing the mechanisms by which GT catechins act in the liver to improve steatosis, our data contribute to the discovery of novel therapeutic agents in the management of NAFLD.

Keywords: Adiponectin; Catechins; Green Tea; Liver; Obesity; Steatosis.

7.4 APÊNDICE D – Artigo publicado em colaboração:GQ-130, a novel analogue of thiazolidinedione, improves obesity-induced metabolic alterations in rats: Evidence for the involvement of PPARβ/δ pathway

GQ-130, a novel analogue of thiazolidinedione, improves obesity-induced metabolic alterations in rats: Evidence for the involvement of PPARβ/δ pathway

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Affiliations + expand

PMID: 31909493 DOI: 10.1111/1440-1681.13252

Abstract

The present investigation aimed to characterize the effect of a short-time treatment with a new thiazolidinedione (TZD) derivative, GQ-130, on metabolic alterations in rats fed a high-fat diet (HFD). We investigated whether metabolic alterations induced by GQ-130 were mediated though a mechanism that involves PPARβ/δ transactivation. Potential binding and transactivation of PPARα, PPARβ/δ or PPARγ by GQ-130 were examined through cell transactivation, 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence quenching assays and thermal shift assay. For in vivo experiments, male 8-week-old Wistar rats were divided into three groups fed for 6 weeks with: (a) a standard rat chow (14% fat) (control group), (b) a HFD (57.8% fat) alone (HFD group), or (c) a HFD associated with an oral treatment with GQ-130 (10 mg/kg/d) during the last week (HFD-GQ group). In 293T cells, unlike rosiglitazone, GQ-130 did not cause significant transactivation of PPARγ but was able to activate PPARβ/δ by 153.9 folds in comparison with control values (DMSO). Surprisingly, ANS fluorescence quenching assay reveals that GQ-130 does not bind directly to PPARβ/δ binding site, a finding that was further corroborated by thermal shift assay which evaluates the thermal stability of PPARβ/δ in the presence of GQ-130. Compared to the control group, rats of the HFD group showed obesity, increased systolic blood pressure (SBP), insulin resistance, impaired glucose intolerance, hyperglycaemia, and dyslipidaemia. GQ-130 treatment abolished the increased SBP and improved all metabolic dysfunctions observed in the HFD group. Oral treatment with GQ-130 was effective in improving HFD-induced metabolic alterations probably through a mechanism that involves PPARβ/δ activation.

7.5 APÊNDICE E – Capítulo de livro publicado: PPAR Modulation Through Posttranslational Modification Control

Nuclear Receptors pp 537–611 | Cite as

PPAR Modulation Through Posttranslational Modification Control

Natália B. Videira, Marieli M. G. Dias, Maiara F. Terra, Vinícius M. de Oliveira, Marta García-Arévalo, Thayná M. Avelino, Felipe R. Torres, Fernanda A. H. Batista & Ana Carolina M. Figueira

Chapter | First Online: 29 September 2021
609 Accesses | **2** Citations

Abstract

The peroxisome proliferator-activated receptors (PPAR) are transcription factors modulated by ligands and members of the nuclear receptor superfamily. There are three different human PPAR isotypes: PPARα, PPARδ/β, and PPARγ, which regulate the transcription of their target genes involved with energy metabolism, inflammatory process, and cellular differentiation in different human tissues. Because of these activities, PPARs are considered important targets for drugs to treat metabolic diseases, including diabetes, dyslipidemia, and obesity. Besides ligand modulation, PPARs activities can be modulated by posttranslational modifications (PTM), such as phosphorylation, SUMOylation, ubiquitination, acetylation, and O-GlcNAcylation. The understanding of PTMs modulation of PPARs function could contribute for the development of metabolic diseases treatment with more specificity and fewer side effects. Therefore, in this chapter, we present an overview of PTMs that modulate the activity of each PPAR isotype and strategies to modulate these PTMs and thus regulate PPARs action.

8 ANEXOS

8.1 ANEXO A - Autorização para experimentação Animal

CNPEM

Committee on Ethics in the Use of Animals

CEUA/CNPEM

We certify that the project entitled "Development of adipose tissue mimetic organoids: A new approach in the assessment of adipogenesis" (protocol no. 61), under the responsibility of **Ana Carolina Migliorini Figueira** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except Man), for scientific research purposes- is in accordance with the provisions of Law No. 11,794, of October 8th, 2008, of Decree No. 6,899, of July 15th, 2009, and with the rules issued by the National Council for the Control of Animal Experimentation (CONCEA) and ARRIVE guidelines, and was approved by the Committee on Ethics in the Use of Animals (CEUA-CNPEM), of the National Center for Research in Energy and Materials, in a meeting on 02/07/2019.

Project term	03/01/2019 a 02/01/2020
Species	C57Bl/6J
Number of animals	6
Weight/age	10 – 15 g / 21 days
Gender	Males
Source	LNBio bioterium-CNPEM

Campinas, February 15th, 2019.

Rafael Elias Marques

Dr. Rafael Elias Marques
Vice coordinator

Ângela Saito

Dra. Ângela Saito
Alternate Member

8.2 ANEXO B - Autorização da editora para inserção na tese de artigo publicado

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Mass spectrometry-based proteomics of 3D cell culture: A useful tool to validate culture of spheroids and organoids

Author:
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