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ANDRIU DOS SANTOS CATENA

GENOTIPAGEM E ANÁLISE DA EXPRESSÃO DO GENE *TCF7L2* EM PACIENTES COM ALTERAÇÃO DO CRESCIMENTO FETAL E DOENÇAS METABÓLICAS NO ADULTO

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A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera o candidato **ANDRIU DOS SANTOS CATENA** como**: APROVADO.**

Recife, 03 de Fevereiro de 2016

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“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original”.

Albert Einstein

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RESUMO

Segundo a hipótese da programação fetal, alterações metabólicas *in utero* estabelecem padrões fisiológicos que modulam a saúde do ser humano, contribuindo ao desenvolvimento de síndrome metabólica (SMet), obesidade e diabetes tipo 2 (DT2) na vida adulta. O risco de estresse oxidativo é uma condição metabólica que se eleva durante a gravidez, desencadeando a expressão de genes cruciais ao desenvolvimento dessas patologias, como o *TCF7L2*. Portanto, torna-se importante identificar a frequência dos principais polimorfismos desse gene (49080T>C, 103894G>T e 53341C>T) assim como analisar sua expressão em amostras de RNs com peso ao nascer alterado e de pacientes adultos obesos. Desta forma, foram analisadas amostras de 149 indivíduos, subdividas em duas coortes: 98 recém-nascidos da cidade da Paraíba - JP, sendo 11 pequenos para idade gestacional (PIG), 41 grandes para idade gestacional (GIG) e 46 apropriados para idade gestacional (AIG); e 51 adultos atendidos na cidade do Recife - PE, sendo 12 obesos com DT2, 17 obesos sem DT2 e 22 saudáveis (não obesos). Em segundo momento, foram utilizadas ferramentas de bioinformática para compreender as interações biomoleculares envolvendo *TCF7L2* na via de sinalização Wnt. O polimorfismo 49080T>C foi o mais prevalente na população estudada (38,9%) comparado ao 103894G>T (27,7%) e 53341C>T (31,9%). Níveis de mRNA entre as coortes analisadas demonstraram significância estatística (p=0,001). Recém-nascidos PIG apresentaram expressão de *TCF7L2* maior que GIG (1,751 e 1,229, respectivamente) (p=0,017), além de expressão relativa similar com adultos obesos com DT2. Não houve diferença estatística entre a coorte dos adultos (p=0,115). GIG e obesos revelaram forte similaridade (p=0,922). Adicionalmente, análises *in silico* demonstraram que a resposta inflamatória condicionada ao estresse oxidativo durante a gravidez contribui para o aumento de IL-6 e TNFα. Essas citocinas estimulam o aumento de β-catenina, que é translocada ao núcleo para ativar fatores de transcrição como *TCF7L2*. A rede metabólica da TCF7L2 envolve genes e produtos relacionados à via Wnt, como *DKK1, CTNNB1, GCG, APOE, APOC1* e *FTO*. Estas moléculas participam da regulação via Wnt e do metabolismo de carboidratos e lipídeos. Dessa forma, *TCF7L2* parece influenciar o peso ao nascer, o que contribui ao desenvolvimento de SMet e obesidade na vida adulta.

Palavras-chave: *TCF7L2*; programação fetal; alteração do peso ao nascer; síndrome metabólica; obesidade.

ABSTRACT

According to fetal programming hypothesis, metabolic exchange in utero establishes physiological standards that modulate the human health, contributing to common diseases in adulthood development, like metabolic syndrome (MetS), obesity, and type 2 diabetes (T2D). The risk of oxidative stress is a metabolic condition that rises during pregnancy, triggering the expression of critical genes to the development of these pathologies, such as *TCF7L2*. Therefore, it becomes necessary to frequencies identify the main polymorphisms this gene (49080T>C, 103894G>T, and 53341C>T) and analyze their expression in samples of newborns with abnormal birth weight and obese individuals. Thus, 149 subjects of Northeast Brazilian were enrolled in this study, performed in two cohorts: 98 newborns, being 11 with small for gestational age (SGA); 41 large for gestational age (LGA); and 46 appropriate for gestational age (AGA); and 51 adults, being 12 obese with type 2 diabetes (T2D); 17 non-T2D obese; and 22 healthy adults. In the second step, were used bioinformatics tools to understand the molecular interactions involving *TCF7L2* in the Wnt signaling pathway. 49080T>C polymorphism was more prevalent in the population (38.9%) compared to 103894G>T (27.7%) and 53341C>T (31.9%). mRNA levels were showing a statistical difference between newborns and adults cohorts (p=0.001). SGA neonates presented a *TCF7L2* expression higher than LGA (1.751 and 1.229, respectively) (p=0.017), beyond a similar relative expression compared to adults obese DT2. There was no statistical significance in the adult cohort (p=0.115). LGA and obese adult groups revealed high similarity (p=0.922). 53341C>T, 103894G>T, and 49080T>C allelic frequencies were similar to the findings of other studies. Additionally, the *in silico* analysis demonstrated that inflammatory response due oxidative stress during pregnancy contributes to IL-6 and TNFα increases. This cytokine allowed to β-catenin increase, with is translocated to the nucleus for activating of transcription factors such *TCF7L2*. Furthermore, this gene interacts with other genes and products related to Wnt signaling pathway, like *GCG, DKK1, CTNNB1, APOE, APOC1*, and *FTO*. Thus, *TCF7L2* may influence the birth weight, therefore contributing for MetS and obesity in adulthood.

Keywords: *TCF7L2*; fetal programming; abnormal birth weight; metabolic syndrome; obesity.

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LISTA DE SIGLAS E ABREVIAÇÕES

A	Adenina
ACTB	*β-catenin*
AGA	*Appropriate for gestational age*
AIG	Apropriado para idade gestacional
APC	Polipose adenomatosa coli
APOC1	*Apolipoprotein C-I*
APOE	*Apolipoprotein E*
AXIN	Proteína axin
BMI	*Body mass index*
C	Citosina
cDNA	*Complementary DNA*
CK1α	Caseína quinase 1α
Ct	*Threshold cycle*
CTNNBI	*Catenin (cadherin-associated protein)*
CVD	*Cardiovascular disease*
DCV	Doença cardiovascular
DKK1	*Dickkopf homolog 1*
DT2	Diabetes *melitus* tipo 2
DNA	Desoxirribonucleic acid
DsH	*Dishevelled*
EDTA	*Ethylenediamine tetraacetic acid*
NOS	*Oxide nitric synthase*
EO	Estresse oxidativo
EROs	Espécies reativas de oxigênio
FFAR4	*Free fatty acid receptor 4*
FTO	*Fat mass and obesity associated*
FZD	*Frizzled*
G	Guanina
GCG	*Glucagon*
GH	Hormônio do crescimento
GIG	Grande para idade gestacional

GLP-1	*Glucagon-like peptide-1*
GSK3β	Glicogênio sintase-quinase 3β
HPA	Hipotálamo-pituitária-adrenal
IGF-1	Fator de crescimento semelhante à insulina
IL-6	Interleucina 6
IMC	Índice de massa corpórea
iNOS	*Oxide nitric synthase inducible*
KEEG	*Kyoto Encyclopedia of Genes and Genomes*
LGA	*Large for gestational age*
LRP	Lipoproteína co-receptora
MetS	*Metabolic syndrome*
mRNA	RNA mensageiro
NCBI	*National Center for Biotechnology Information*
NIH	*National Institutes of Health*
NO	*Nitric oxide*
OMS	Organização Mundial de Saúde
$ONOO^-$	Peroxinitrito
PCR	*Polymerase chain reaction*
PIG	Pequeno para idade gestacional
qPCR	*Quantitative PCR*
RN	Recém-nascido
RNA	*Ribonucleic acid*
RNS	*Reactive nitrogen species*
ROS	*Reactive oxygen species*
SGA	*Small for gestational age*
SMet	Síndrome Metabólica
SNP	*Single-nucleotide polymorphism*
T	Timina
T2D	*Type-2 diabetes mellitus*
TCF4	*Transcription cell factor 4*
TCF7L2	*Transcription cell factor 7-like 2*
TNFα	Fator de necrose tumoral α, do inglês *tumoral factor necrosis α*
$\bullet O_2^-$	Superóxido

LISTA DE SÍMBOLOS

%	Porcentagem
<	Menor que
=	Igual
>	Maior que
©	Copyright
®	Registered trademark
g	Grama
Kb	Kilo base
Kg	Quilograma
m^2	Metro quadrado
ºC	Graus Celsius
™	Trademark
x	Vezes
α	Alfa
β	Beta
ηg	Nano grama
µL	Micro litro

SUMÁRIO

1. INTRODUÇÃO

A obesidade vem aumentando em proporções epidêmicas nas últimas décadas (LAVIE et al., 2014). Essa doença está fortemente associada como fator de risco a diversas patologias sistêmicas, promovendo o aumento da prevalência das doenças cardiovasculares (DCV), dislipidemias, doenças metabólicas, diabetes tipo 2 (DT2) e inflamação (AWAN et al., 2016; LAVIE et al., 2014).

O tecido adiposo, presente em quantidade significativa na obesidade, promove a secreção de adipocitocinas pró-inflamatórias (HABIB et al., 2015). Estas citocinas podem desencadear o desenvolvimento da Síndrome Metabólica (SMet) (BAYS et al., 2013), doença que afeta um em cada cinco adultos e emerge como a epidemia do novo milênio, impactando a vida de milhões de pessoas ao redor do mundo (BHATNAGAR et al., 2011). Evidências clínicas e experimentais demonstram que a ocorrência de muitas doenças não transmissíveis, incluindo a obesidade e a SMet, podem ser influenciadas pelo ambiente intrauterino (FERNANDEZ-TWINN; OZANNE, 2010).

O ambiente intrauterino contribui para a manutenção da saúde da prole ao longo do seu desenvolvimento (SIMMONS, 2009). Os modelos de programação fetal (BARKER, 1995) e as origens do desenvolvimento das doenças (GLUCKMAN et al., 2008) postulam que, durante períodos de rápido desenvolvimento como a vida pré-natal e primeira infância, o organismo é susceptível a fatores ambientais e genéticos com influência persistente sobre o risco do desenvolvimento às doenças metabólicas (STOUT et al., 2015).

O acúmulo de gordura e hiperlipidemia são alterações do metabolismo lipídico que estão associadas à gestação, promovendo o desenvolvimento de estresse oxidativo (EO) (HERRERA; ORTEGA-SENOVILLA, 2010). O EO apresenta mecanismos fisiopatológicos comuns no desenvolvimento de diferentes patologias não transmissíveis, podendo afetar a programação fetal (RODRÍGUEZ-RODRÍGUEZ et al., 2015).

O perfil genético do embrião também pode contribuir para o risco de desenvolvimento de doenças metabólicas de acordo com as condições de vida no período intrauterino (ALEXANDRE-GOUABAU et al., 2012; HAY, 2013). Alterações no peso e na composição corporal ao nascer, sejam na faixa superior do normal para a idade gestacional (grandes para a idade gestacional, GIG) ou reduções significativas na altura do nascimento e peso (pequenos para a idade gestacional, PIG) podem levar a sequelas metabólicas na vida adulta (RAMÍREZ-VÉLEZ, 2012).

O exato papel dos genes na variação normal do peso ao nascimento ainda é pouco compreendido. Genes que promovem diabetes ou que alteram a glicose em jejum são bons candidatos para os genes que influenciam o peso ao nascimento (FREATHY et al., 2007). Assim surge o gene *TCF7L2*, que apresenta uma forte tendência ao desenvolvimento de DT2 por alterar a secreção de insulina (WEAVER; TURNER; HALL, 2012).

Pertencente à família do fator de transcrição 4 (TCF4), *TCF7L2* é ativado pela via de sinalização Wnt, o qual controla praticamente todos os aspectos do desenvolvimento embrionário (CLEVERS; NUSSE, 2012). Evidências epidemiológicas sugerem que a via de sinalização Wnt pode também desempenhar um papel na promoção de desordens metabólicas pela ativação desse gene (BOJ et al., 2012).

Portanto, levando em consideração o modelo de programação fetal e o desenvolvimento de doenças metabólicas na vida adulta a partir de desordens no ambiente uterino, torna-se importante avaliar os principais polimorfismos e a expressão do gene *TCF7L2*. Através de análises moleculares de recém-nascidos com alteração do crescimento fetal e de pacientes obesos, permite-se traçar o mecanismo de ação desse gene a partir de situações envolvendo estresse oxidativo.

2. REVISÃO DE LITERATURA

2.1. Biologia do crescimento intrauterino

Crescimento e desenvolvimento fetal são processos dinâmicos que dependem de interações síncronas entre mãe, placenta e feto, a fim de permitir adequadas condições de crescimento e sobrevivência da prole. Governada por uma constituição genética própria, a trajetória de crescimento fetal só pode ser cumprida se a nutrição materna e função placentária forem suficientes para manter sua proliferação, crescimento e diferenciação (WARNER; OZANNE, 2010). Numerosos estudos epidemiológicos, clínicos e experimentais demonstram claramente que um ambiente intrauterino comprometido pode ter impacto sutil ou drástico ao longo da vida do recém-nascido (RN) (LI; GONZALEZ; ZHANG, 2012).

O crescimento humano é um processo que se estende desde a vida intrauterina até a idade adulta. A multiplicidade de fatores envolvidos neste processo permite que sua avaliação constitua um indicador do estado de saúde e bem-estar (CARRASCOSA et al., 2004). De fato, o período desde a concepção até o nascimento é caracterizado por elevado crescimento e desenvolvimento tecidual (Figura 1). Neste período, observa-se a maior velocidade de crescimento de toda a vida. De uma única célula fertilizada ocorre a diferenciação em mais de 200 tipos celulares. Isto significa para um aumento em comprimento de cerca de 5.000 vezes, $6{,}1x10^{7}$ em superfície corpórea e $6x10^{12}$ em peso (BROCK; FALCÃO, 2008).

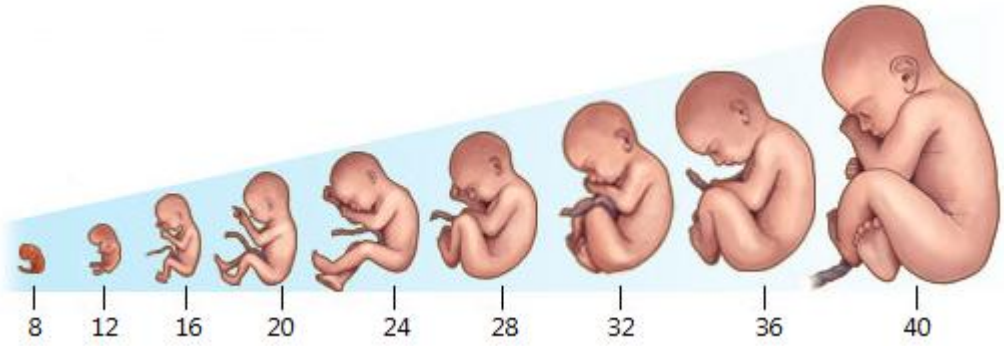

Figura 1: Crescimento fetal da 8ª a 40ª semana. Neste período, o ser humano atinge a maior velocidade de crescimento de toda sua vida (adaptado de Yale School of Medicine, 2015).

A placenta é um tecido endócrino dinâmico que exibe respostas robustas às alterações no meio maternal. Como um sistema de trocas metabólicas e de sustento fetal, a integridade placentária é fundamental para o crescimento e desenvolvimento do feto. Além disso, a placenta atua na interface entre o estado nutricional materno e perturbações ambientais, sendo sua principal a transferência de nutrientes e gases à prole (Figura 2) (NUGENT; BALE,

2015). Sabe-se que o tamanho placentário está intimamente relacionado à sua capacidade de transferência de nutrientes. No entanto, o tamanho, peso e a forma da placenta estão sujeitos a grandes variações. Assim, a morfometria placentária inadequada pode promover alteração do crescimento fetal (SALAVATI et al., 2015).

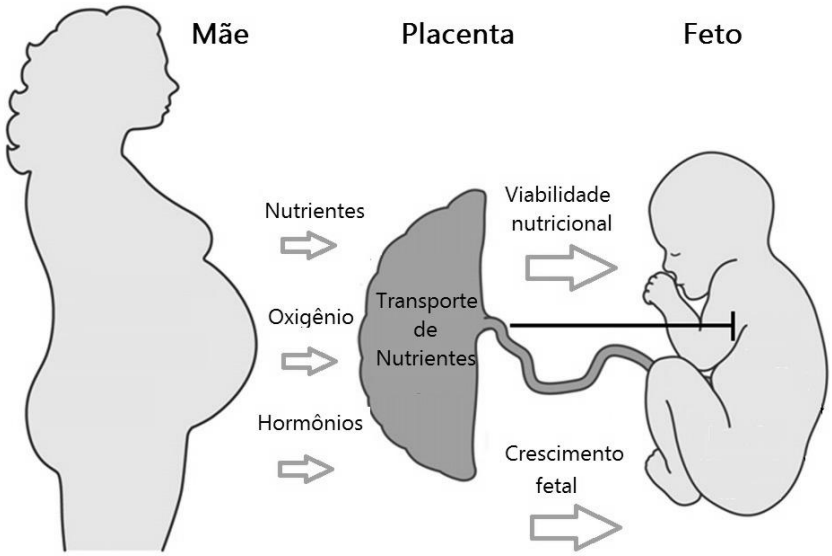

Figura 2: A placenta desempenha papel fundamental na troca de substâncias (nutrientes, hormônios e gases) entre mãe e feto, permitindo o crescimento no ambiente intrauterino (adaptado de AYE; POWELL; JANSSON, 2013).

O crescimento intrauterino também está intimamente determinado por fatores genéticos do embrião, influenciados pelo ambiente materno e pela placenta (BROCK; FALCÃO, 2008). A expressão desregulada de genes críticos ao crescimento fetal e placentário é um mecanismo plausível para a ligação de fatores ambientais modificáveis, como a nutrição materna, ao crescimento intrauterino inadequado e restrição de crescimento fetal (MUKHOPADHYAY et al., 2015).

Desde a concepção até o nascimento, o feto é exposto a um fluxo contínuo de sinais químicos transportados pelo sangue materno (DEL GIUDICE, 2012). O fator nutricional leva em consideração o estado nutricional materno e a capacidade placentária de transferir nutrientes ao feto. Tanto a subnutrição como a supernutrição podem influenciar o estado epigenético do genoma fetal e alterar a nutrição e o estado endócrino da prole (Figura 3) (JI et al., 2015).

Portanto, o fornecimento adequado de macro e micronutrientes, oxigênio e de determinados hormônios são fundamentais nesta fase precoce da vida. As perturbações no fornecimento destes componentes impactam não só o crescimento do feto, uma vez que

podem promover consequências negativas à saúde do ser humano futuramente, como susceptibilidade às doenças metabólicas na vida adulta (WARNER; OZANNE, 2010).

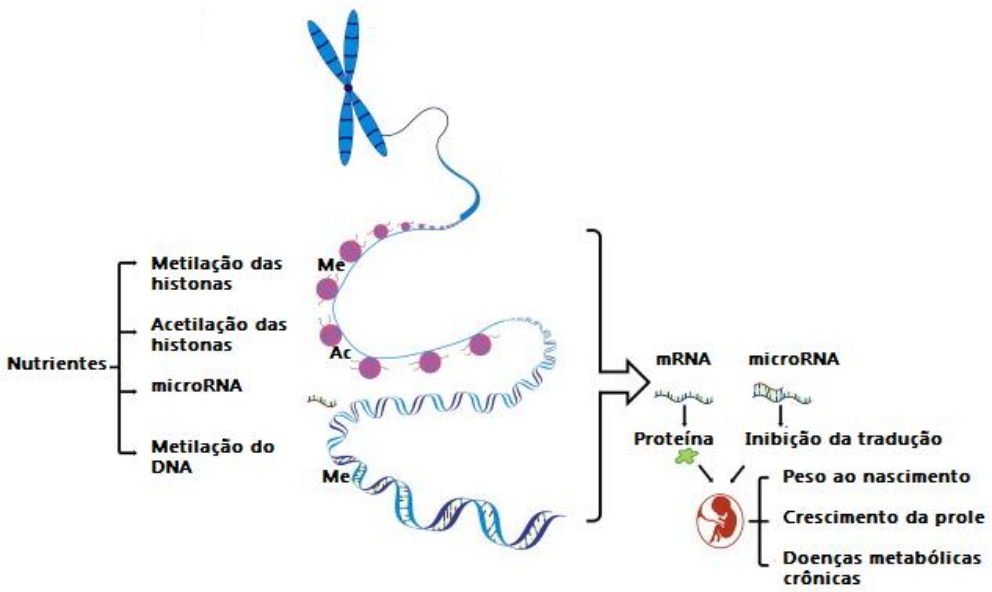

Figura 3: Regulação epigenética da expressão gênica e de programação fetal. Desnutrição materna afeta o crescimento, desenvolvimento e saúde da prole por meio de modificações epigenéticas do genoma. Os mecanismos subjacentes incluem metilação do DNA, metilação e acetilação das histonas, e síntese de microRNA. Me, metilação; Ac, acetilação (adaptado de JI et al., 2015).

Os fatores hormonais desempenham importante papel na regulação do crescimento e desenvolvimento fetal. Eles agem como mensageiros e controlam o desenvolvimento e a diferenciação dos tecidos, de acordo com as condições ambientais existentes para o feto (BROCK; FALCÃO, 2008).

Alteração do eixo hipotálamo-pituitária-adrenal (HPA) fetal no ambiente intrauterino pode ocorrer secundárias a estresses nutricionais (sub ou supernutrição) e maternos. Correlações entre alteração do peso ao nascer, concentrações plasmáticas de cortisol e o desenvolvimento de hipertensão e DT2 já foram identificados (STIRRAT; REYNOLDS, 2015). Uma quantidade crescente de evidências mostra que o estresse materno durante a gravidez exerce efeitos duradouros sobre o desenvolvimento do sistema nervoso fetal e, em última instância, sobre a fisiologia e comportamento da prole (Figura 4) (DEL GIUDICE, 2012).

Além disso, o crescimento dos vertebrados é principalmente controlado pelo hormônio polipeptídico somatotropina (hormônio de crescimento – GH). No entanto, a maior parte da regulação do crescimento e efeitos metabólicos no meio intrauterino é mediada pelo hormônio *insuline-like growth factor I* (IGF-I) ou somatomedina (WANG et al., 2015). IGF-I é amplamente expresso na maioria dos tecidos, circulam em concentrações nanomolares no

soro humano, possuindo ações endócrinas, autócrinas e parácrinas que permitem adequado crescimento e desenvolvimento fetal (BACH, 2015).

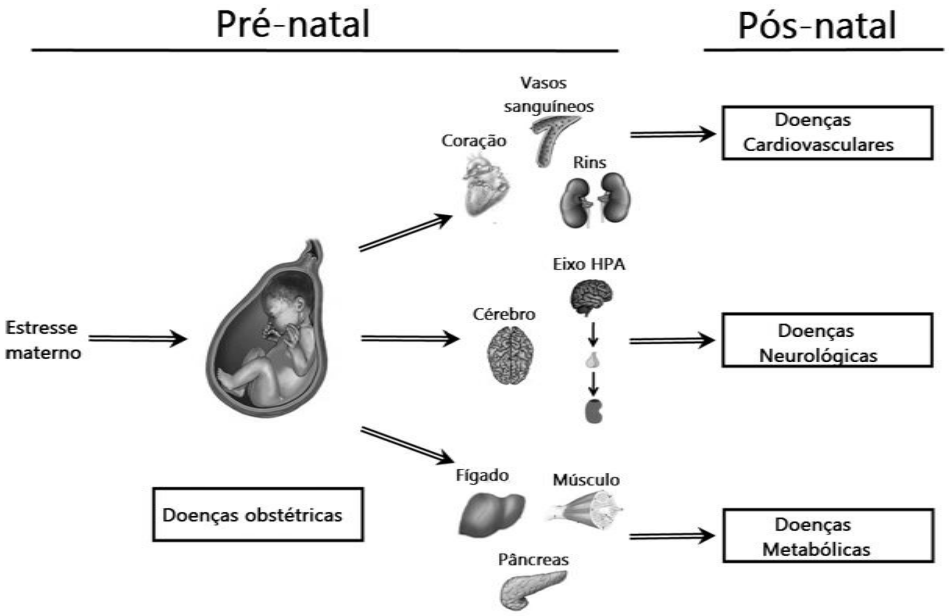

Figura 4: Impactos do estresse materno no desenvolvimento dos tecidos/ órgãos fetais. Notar aumento do risco de desenvolvimento das doenças cardiovasculares, doenças metabólicas e doenças neurológicas. HPA, hipotálamo-pituitária-adrenal (adaptado de LI; GONZALEZ; ZHANG, 2012).

2.2. Alteração do crescimento e programação fetal

Alteração do crescimento fetal é reconhecida como uma importante predição a resultados perinatais adversos. Fetos com crescimento comprometido apresentam taxas mais altas de mortalidade fetal e infantil (BOULET et al., 2006). Além disso, o ambiente intrauterino possui a capacidade de influenciar a saúde, as capacidades de aprendizagem e função social dos RNs ao longo de suas vidas. Dessa forma, torna-se extremamente importante identificar os mecanismos pelos quais a exposição a diversos fatores ambientais influencie a saúde em longo prazo (CONSTANTINOF; MOISIADIS; MATTHEWS, 2015).

A hipótese da "origem fetal das doenças" propõe que o ambiente intrauterino pode modificar a trajetória de desenvolvimento de um indivíduo (BARKER, 1995; DONZELLI et al., 2015). Os seres humanos apresentam elevada plasticidade durante seu desenvolvimento fetal e influências adversas podem permanentemente mudar a estrutura e função do seu corpo; um fenômeno conhecido como programação. Essa plasticidade pode produzir mudanças duradouras na fisiologia e metabolismo do RN (BARKER, 2012).

Programação fetal, portanto, é a resposta a um desafio específico para o organismo dos mamíferos, durante determinados momentos do crescimento intrauterino, crítico à alteração do desenvolvimento fetal (DU et al., 2015). As condições genéticas maternas e paternas (Figura 5), e os fatores ambientais adversos afetam a plasticidade do desenvolvimento, levando a alterações epigenéticas que pode dar origem a diferentes fenótipos. Esta abordagem tem-se mostrado válida particularmente para a suscetibilidade às doenças cardiovasculares e metabólicas na vida adulta (DONZELLI et al., 2015; HOCHER, 2014).

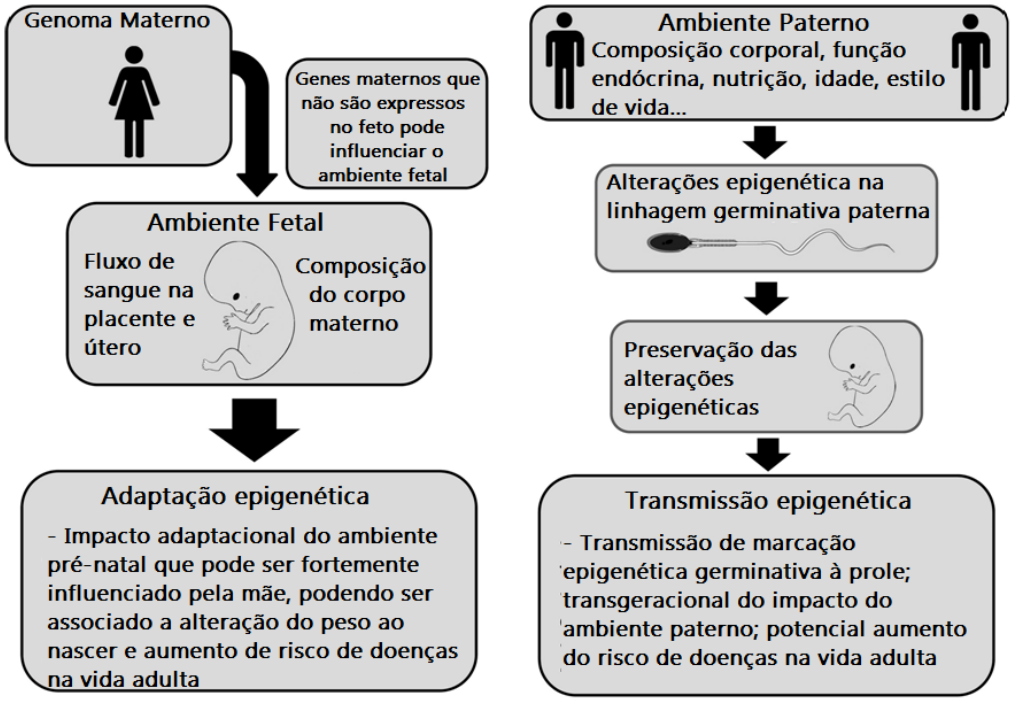

Figura 5: A hipótese de programação fetal propõe que indução de programação fetal durante os eventos vida intrauterina pode promover o desenvolvimento de doenças comuns à vida adulta e pode ser originário de eventos de programação relacionado a genes maternos e paternos que afetam o fenótipo fetal (adaptado de HOCHER, 2014).

A avaliação do crescimento intrauterino, portanto, é um indicador sensível da saúde pós-natal. As medidas clínicas comumente utilizadas para avaliar o crescimento fetal são a comparação entre o peso, comprimento e perímetro cefálico com a idade pós-natal do RN. Outras medidas de crescimento incluem perímetro braquial, medidas de dobras cutâneas, avaliação da composição corporal, além de marcadores bioquímicos (BHATIA, 2013). Estas medidas e sua relação com a idade gestacional classificam as crianças em prematuras, a termo ou pós-termo, além de verificar se nasceram com peso adequado (AIG – adequado para idade gestacional), elevado (GIG – grande para idade gestacional) ou diminuído (PIG – pequeno para idade gestacional) (CARRASCOSA et al., 2004).

A avaliação do crescimento fetal tem sido representada através das curvas de crescimento intrauterino. Cada uma das curvas foi construída com base em dados antropométricos de amostras populacionais (estudos transversais), a partir dos quais foram calculados a média, o desvio padrão ou os diversos percentis para cada idade gestacional (BROCK; FALCÃO, 2008).

Há muitas curvas diferentes em percentil relativas ao sexo, crescimento e peso fetal. No entanto, diversos estudos apresentam como definição de GIG os RNs com peso > percentil 90, PIG < percentil 10 e AIG aqueles com um peso entre o percentil 10 e 90 (Figura 6) (GONZÁLEZ GONZÁLEZ et al., 2014). Abordagens alternativas visam classificar os RNs com alteração do crescimento fetal se baseando em análises customizadas. Essas curvas levam em consideração a interpretação de variáveis que podem afetar o peso ao nascer, como etnia, paridade, sexo do feto, ganho de peso materno durante a gestação e altura (GARDOSI; FRANCIS, 2009).

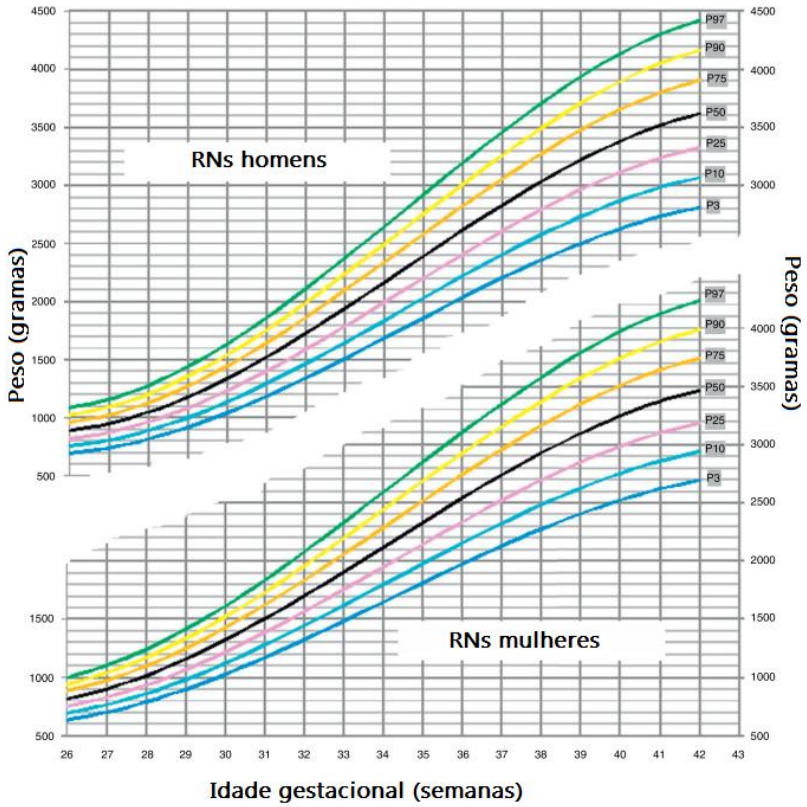

Figura 6: Curvas de percentis dos valores de peso fetal e neonatal por sexo e idade gestacional. RNs: recém-nascidos; P: percentil. Adaptado de GONZÁLEZ GONZÁLEZ et al., 2014.

A característica PIG é o resultado de um amplo espectro de adaptações fetais aos eventos que comprometeram o útero durante a gestação, como o estresse oxidativo, síndromes inflamatórias e pré-eclâmpsia (HOOIJSCHUUR et al., 2015). Esses RNs possuem um risco

aumentado ao desenvolvimento de doenças crônicas na idade adulta, tais como hipertensão, DT2 e doenças cardiovasculares, representando um grave problema de saúde para a sociedade moderna (AKAHOSHI et al., 2016).

GIG é uma indicação de alta taxa de crescimento pré-natal e está associada com vários fatores de risco durante a gravidez. DT2 pré-existente e diabetes *mellitus* gestacional (DMG), hiperglicemia, ganho de peso e hiperlipidemia materna durante a gravidez parecem ser alguns dos fatores envolvidos nessa patogênese (RÓŻDŻYŃSKA-ŚWIĄTKOWSKA et al., 2015; XIE et al., 2015). Os resultados em longo prazo dos RNs GIG são associados ao risco de desenvolvimento de doenças metabólicas na vida adulta, como obesidade e DT2 (CHIAVAROLI et al., 2015).

Portanto, mudanças no peso ou composição corporal no momento ao nascer podem ocorrer tanto na faixa superior do intervalo normal para a idade gestacional (GIG) como em significativas reduções no tamanho e peso ao nascer (PIG). Adicionalmente, ambas as condições apresentam uma maior susceptibilidade ao desenvolvimento de sequelas metabólicas na vida adulta (Figura 7) (RAMÍREZ-VÉLEZ, 2012).

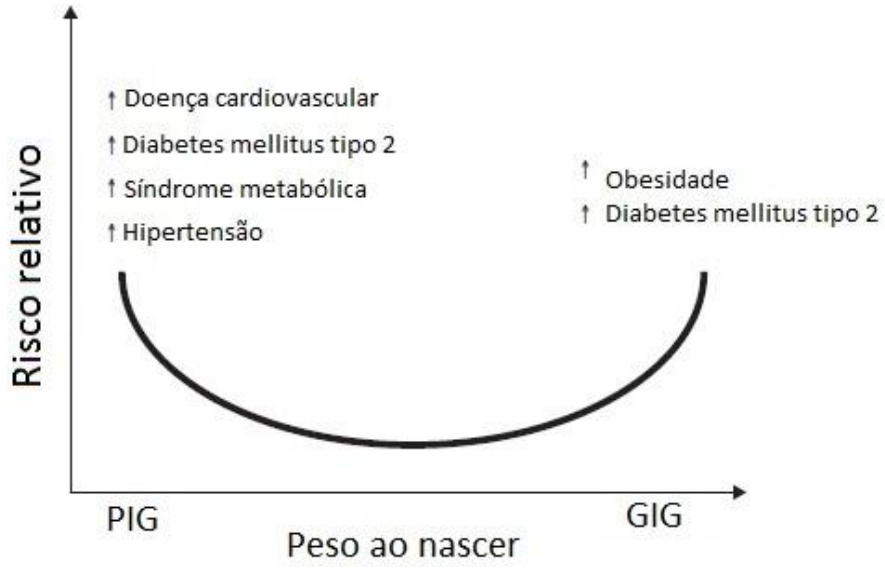

Figura 7: Gráfico representativo do risco relativo entre o peso ao nascer com a prevalência de enfermidades crônicas não transmissíveis na vida adulta, sustentada em base das observações epidemiológicas e experimentais da hipótese de Barker (adaptado de RAMÍREZ-VÉLEZ, 2012).

2.3. Gravidez, estresse oxidativo e resposta inflamatória

O desenvolvimento embrionário pode ser influenciado pela exposição às toxinas ambientais e por uma variedade de patologias, incluindo privação de oxigênio, inflamação e

doença metabólica (DENNERY, 2010). O estresse oxidativo está subjacente a muitas dessas condições, podendo causar danos às células e diretamente comprometer a saúde da prole sobrevivente. No entanto, existe pouca compreensão de como a perturbação redox no embrião influencia seu fenótipo ao longo de sua vida (NEWMAN et al., 2015)

O nascimento é um evento extremamente complexo, associado a riscos de comprometimento à integridade da saúde fetal mesmo na gravidez de mulheres saudáveis, sem histórico clínico significativo. Transição do intra para a vida extrauterina é um evento dramático e estressante. No final da gestação, muitas alterações fisiológicas em vários órgãos ocorrem de modo a permitir a sobrevivência neonatal após o nascimento (GIUFFRÈ et al., 2015)

O acúmulo de gordura e hiperlipidemia são as duas principais alterações no metabolismo lipídico associadas com a gestação. Anormalidades no metabolismo lipídico durante a gravidez podem aumentar o estresse oxidativo (EO) (HERRERA; ORTEGA-SENOVILLA, 2010). EO surge quando espécies reativas de oxigênio excessivos (EROs) não são contrabalançada pelos mecanismos de defesa antioxidante (LOY; SIRAJUDEEN; MOHAMED, 2013). Essas moléculas atuam como segundo mensageiro na cascata de sinalização celular, ativando muitas vias de sinalização redox-sensíveis, incluindo secreção de citocinas inflamatórias, o que resulta em lesão endotelial (DING et al., 2015).

Gravidez normal está associada com EO; no entanto, quando excessivo é prejudicial para a saúde materna e fetal (LOY; SIRAJUDEEN; MOHAMED, 2013). A má adaptação funcional do sistema antioxidante pode aumentar a lesão celular oxidativa e exacerbar o desenvolvimento fisiopatológico das doenças crônicas não transmissíveis ao RN, como DT2 e outras doenças metabólicas, comprometendo a saúde fetal por toda sua vida (HENRIKSEN; DIAMOND-STANIC; MARCHIONNE, 2011).

2.4. Epidemiologia e fisiopatologia da obesidade e síndrome metabólica

A prevalência da obesidade ao redor do mundo aumentou em proporções epidêmicas ao longo das últimas décadas, praticamente dobrando entre 1980 e 2008, segundo a Organização Mundial de Saúde (OMS) (BASTIEN et al., 2014). Atualmente, cerca de 70% dos adultos são classificados como obesos ou com sobrepeso (LAVIE et al., 2014). No Brasil, vem se observando um aumento da incidência da obesidade (LINHARES et al., 2012). Estudo recente analisando a prevalência dessa doença entre adultos em Pernambuco, Estado do

nordeste brasileiro, registrou que 51,9% da população apresentam obesidade abdominal (PINHO, CLAUDIA PORTO SABINO DINIZ et al., 2013).

Ainda de acordo com a OMS, obesidade é o acúmulo anormal ou excessivo de gordura, geralmente estimada pelo índice de massa corporal (IMC) (LINHARES et al., 2012), sendo uma doença crônica não transmissível (PINHO, CLAUDIA PORTO SABINO DINIZ et al., 2013). A obesidade representa uma séria ameaça à saúde humana, estando fortemente associadas a um risco aumentado de doenças crônicas degenerativas, particularmente doenças cardiovasculares (DCV), DT2 e outros distúrbios metabólicos (Figura 8) (CORREIA et al., 2011). Estimativas para 2020 apontam cerca de cinco milhões de óbitos atribuídos ao excesso de peso (MALTA et al., 2014).

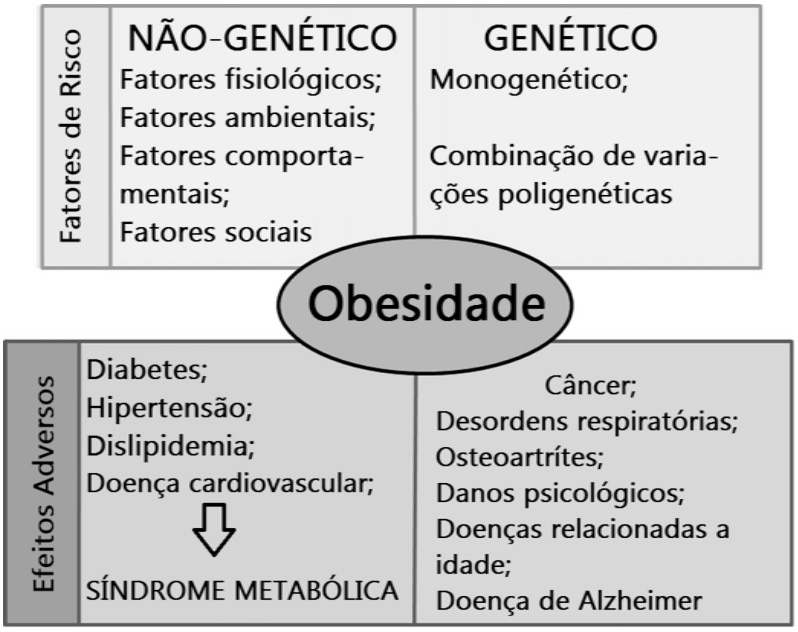

Figura 8: Consequências da obesidade. Os efeitos da obesidade incluem o desenvolvimento de diversas patologias, inclusive a síndrome metabólica. Fatores genéticos, ambientais e outros interagem para influenciar o peso do corpo de uma forma complexa (adaptado de GONZÁLEZ-CASTEJÓN; RODRIGUEZ-CASADO, 2011).

O tecido adiposo, presente em quantidade significativa na obesidade, promove a secreção de adipocitocinas pró-inflamatórias (HABIB et al., 2015). A inflamação, portanto, é uma marca característica em obesos, promovendo a liberação de citocinas que recrutam e ativam macrófagos, o que aumenta os níveis de TNF-α, IL-6 e outras citocinas inflamatórias (HOTAMISLIGIL, 2006).

A patogenicidade consequente da atividade pró-inflamatória é diretamente relacionada ao desenvolvimento de aterosclerose, que por sua vez promove ou agrava doenças

metabólicas como DT2, hipertensão arterial, resistência à insulina e dislipidemia, fatores de risco às DCV (BAYS et al., 2013). O conjunto dessas anormalidades sistêmicas contribui para o desenvolvimento da Síndrome Metabólica (SMet). Essa síndrome está fortemente relacionada a um estilo de vida ocidentalizado, caracterizado pela inatividade física e uma oferta ilimitada de alimentos ricos em gordura (HAN; LEAN, 2006).

A SMet é definida como uma desordem complexa que consiste em um conjunto de fatores de risco de origem metabólica (Figura 9). Estima-se que 20% a 25% da população mundial adulta podem ter essa síndrome atualmente, o que representa um desafio de saúde pública ao redor do mundo (MARTINS et al., 2015). No entanto, as taxas de incidência da SMet ao redor do mundo variam muito. Fatores relacionados às características da população estudada podem influenciar essas taxas, como fatores genéticos, étnicos, culturais, demográficas, socioeconômicos e clínicos (OLIVEIRA et al., 2015)

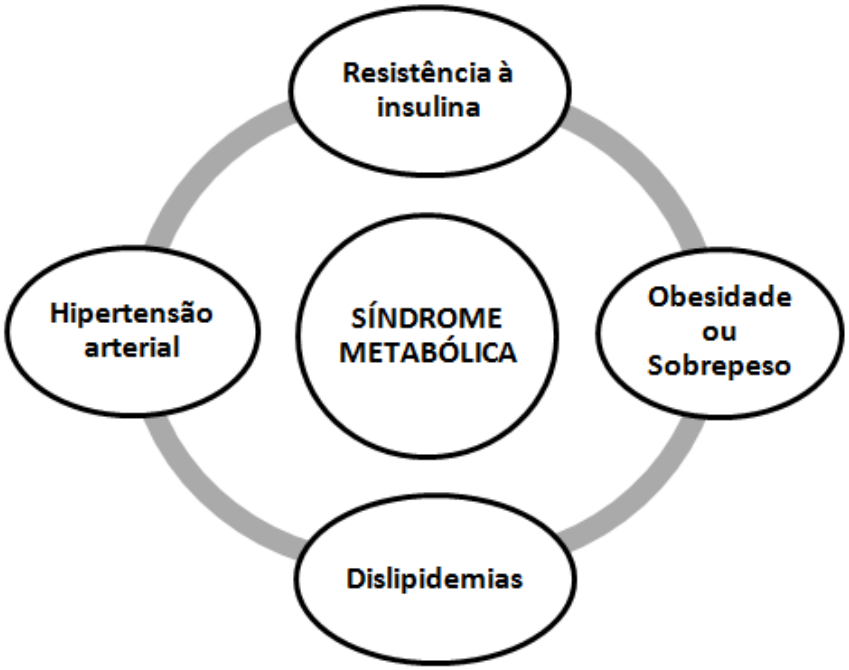

Figura 9: Os fatores de risco que definem a síndrome metabólica são produtos de uma combinação de predisposição genética, nutrição, estilo de vida e inatividade física.

Obesidade e SMet formam um agrupamento de múltiplos fatores de risco para aterosclerose, hipertensão arterial, glicemia elevada e dislipidemia aterogênica, promovendo o aumento do risco de mortalidade e morbidade cardiovascular (SATOH-ASAHARA et al., 2015). A elevada prevalência dessas doenças não pode ser apenas atribuída ao estilo de vida e dieta isolada, uma vez que o elemento genético à sua susceptibilidade é indiscutível (DEARDEN; OZANNE, 2015).

Fundamentalmente, a síndrome metabólica também está associada com aumento ou distribuição do tecido adiposo. A gordura visceral abdominal produz quantidades aumentadas de citocinas pró-inflamatórias, tais como IL-6 e TNF-α, hormônios e biomarcadores de inflamação sistêmica comuns à obesidade (KOWALSKA; OLEJNIK, 2016). Tudo isso contribui para o desenvolvimento de doenças metabólicas, como resistência à insulina e DT2 (GONZÁLEZ-CASTEJÓN; RODRIGUEZ-CASADO, 2011).

2.5. Via de Sinalização Wnt e *TCF7L2*

Os níveis circulantes de citocinas inflamatórias são preditores potentes ao desfecho do desenvolvimento das DCV em pacientes obesos (SPOTO et al., 2014). Além de promoverem a elevação dessas citocinas, os adipócitos também contribuem ao aumento da produção de espécies reativas de oxigênio, estresse oxidativo e diminuição das respostas anti-inflamatórias (BAYS et al., 2013). A resposta inflamatória promove a ativação da via de sinalização Wnt canônica, resultando na supressão de genes críticos da adipogênese através de translocação nuclear de β-catenina (LI et al., 2011).

A via de sinalização Wnt participa de numerosos processos fundamentais ao desenvolvimento embrionário e manutenção da homeostase na vida adulta, incluindo proliferação, sobrevivência, auto-renovação e diferenciação celular (DUCHARTRE; KIM; KAHN, 2015). Múltiplas disfunções nessa via tem sido relacionadas a diversas doenças metabólicas, incluindo DT2 (WELTERS; KULKARNI, 2008).

Essa via de sinalização pode ser ativada por três vias: canônica, polaridade celular planar não-canônica e via não canônica Wnt/cálcio (DUCHARTRE; KIM; KAHN, 2015). A via canônica é a mais bem caracterizada na literatura, sendo responsável pela regulação da transcrição de genes controlados a partir dos níveis e localização da proteína multifuncional β-catenina (WELTERS; KULKARNI, 2008).

Quando a via de sinalização Wnt não está ativada, há a formação do complexo formado por glicogênio sintase-quinase 3β (GSK3β), polipose adenomatosa coli (APC), proteína axin (AXIN) e caseína quinase 1α (CK1α) no citoplasma. Esse complexo permite a GSK3β fosforilar resíduos de serina/treonina da β-catenina, encaminhando essa molécula para ubiquitinação e posterior degradação no proteassomo, impedindo a transcrição dos genes-alvo dessa molécula (Figura 10) (PIERZYNSKI et al., 2015; SMITH, 2007).

No entanto, a via Wnt canônica pode ser ativada após a ligação de uma molécula WNT ao receptor *Frizzled* (FZD) associado a uma lipoproteína co-receptora (LRP). A formação do

complexo receptor permite a ativação da proteína *dishevelled* (DSH), que conduz a uma cascata de sinalização intracelular, promovendo fosforizações de resíduos de serina/treonina e consequente inibição de GSK3β. Isso resulta na desmontagem do complexo constituído por GSK3β/APC/AXIN/CK1α, permitindo o aumento citoplasmático de β-catenina e sua consequente translocação nuclear (PIERZYNSKI et al., 2015; SMITH, 2007), podendo ativar diversos genes, como o *transcription factor 7-like 2* (*TCF7L2*) (Figura 10B) (KUZMICKI et al., 2011).

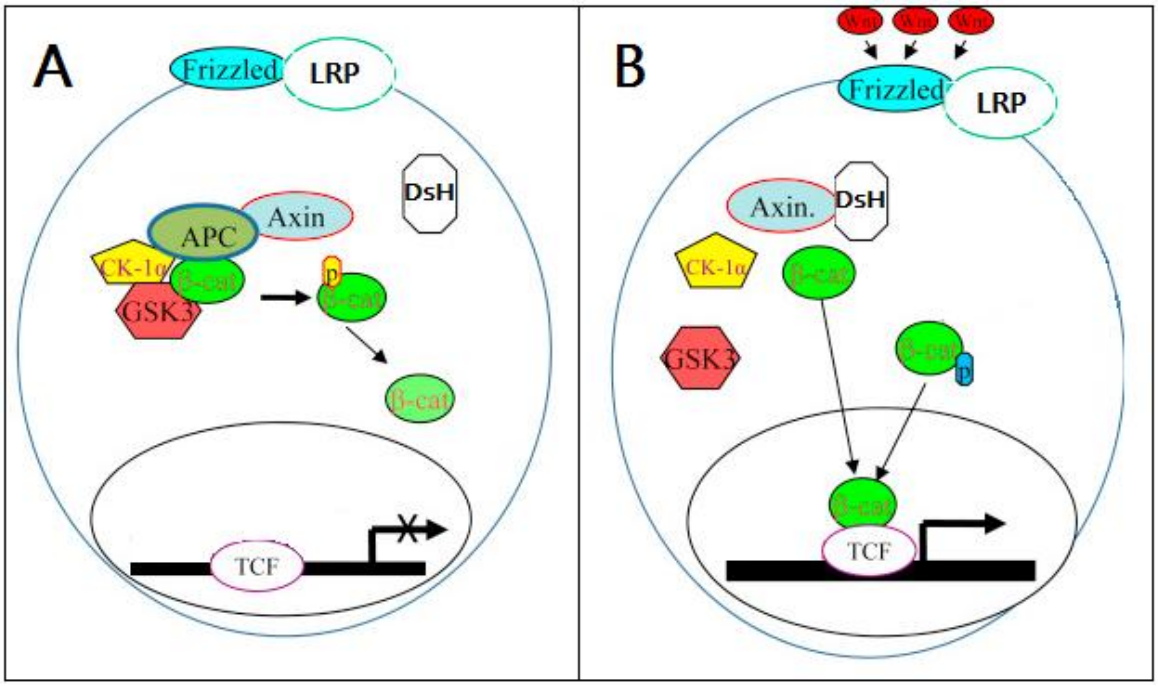

Figura 10: Via de sinalização Wnt canônica. A) Na ausência da molécula WNT, o complexo GSK3β/APC/AXIN/CK1α conduz a ubiquitinação e degradação de β-catenina. B) Quando a molécula WNT se liga aos receptores Frizzled e LRP, a proteína Dsh promover a inibição da GSK3β. Isto resulta na desmontagem do complexo GSK3β/APC/AXIN/CK1α, elevando β-catenina citoplasmática, promovendo a transcrição gênica. GSK3β: complexo de glicogênio sintase-quinase 3β (GSK3); APC: polipose adenomatosa coli; AXIN: proteína axina; CK1α: caseína-quinase 1α; LRP: lipoproteína co-receptora (adaptado de JIN, 2015).

O gene *TCF7L2* é um fator de transcrição localizado no braço longo do cromossomo 10, na região 25.3 (10q25.3) (DE MELO et al., 2015), abrangendo 215,9 kb e composto por 17 éxons. O gene possui dois domínios principais: um de ligação à β-catenina (éxon 1) e outro de ligação ao DNA (éxons 10 e 11). Pelo menos cinco éxons podem ser emendados por *splicing* alternativo (Figura 11), e a maioria dos tecidos humanos expressam níveis detectáveis desse fator de transcrição (PANG; SMITH; HUMPHRIES, 2013).

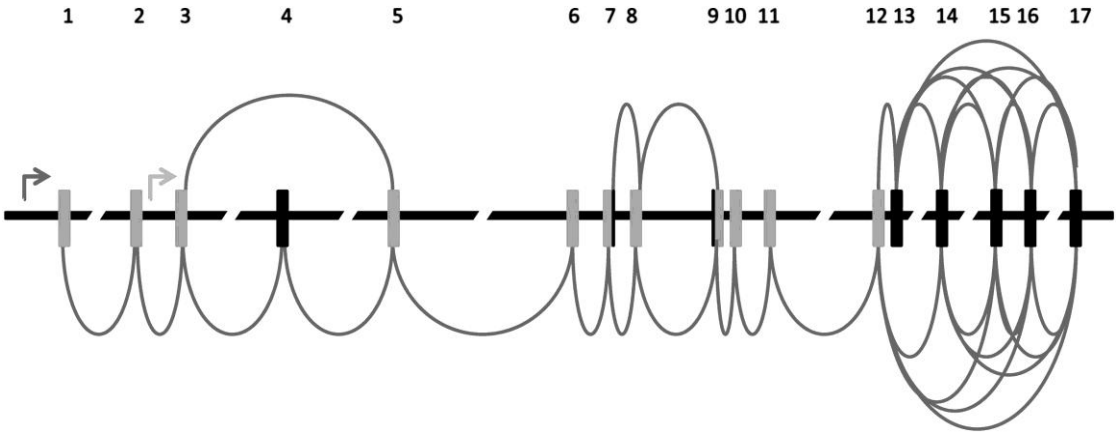

Figura 11: Estrutura do *TCF7L2*. As setas indicam os locais de início da transcrição. Barras cinzas indicam os éxons. Barras pretas indicam os éxons de processamento alternativo. As linhas redondas entre os éxons indicam os produtos por *splicing* alternativo (fonte PANG; SMITH; HUMPHRIES, 2013).

Embora os SNPs associados a DT2 estejam localizados nos íntrons (regiões não codificantes), eles desempenham um importante papel na manifestação de diversas patogenias metabólicas, como fenótipo ao desenvolvimento de DT2 (GRANT et al., 2006) e resistência à insulina (LIU et al., 2009). No entanto, não está claro como estes SNPs desempenham suas influências no *splicing* alternativo, na expressão ou na estrutura da proteína desse gene (PANG; SMITH; HUMPHRIES, 2013).

TCF7L2 codifica uma proteína de 596 aminoácidos que interage com a β-catenina da via Wnt, induzindo a expressão de vários genes, como o peptídeo semelhante à glucagon-1 (GLP-1) (DE MELO et al., 2015). A maioria dos genes de risco à diabetes foi associada principalmente por prejudicarem a função normal das células beta. Polimorfismos de nucleotídeo único (SNPs) de *TCF7L2* foram relatados por promoverem a desregulação dessas células, contribuindo assim à tolerância prejudicada de glicose e DT2 (SHEN; FANG; GE, 2015). Além disso, SNPs mais bem estudados desse gene, tais como 49080T>C (rs7901695), 103894G>T (rs12255372) e 53341C>T (rs7903146) (GRANT et al., 2006) também foram correlacionados à susceptibilidade de elevar a taxa de IMC (DE MELO et al., 2015).

3. OBJETIVOS

3.1. Geral

Identificar a frequência dos principais polimorfismos e analisar a expressão do gene *TCF7L2* em amostras de recém-nascidos com alteração do crescimento fetal e de pacientes adultos obesos.

3.2. Específicos

3.2.1. Identificar a frequência dos polimorfismos 49080T>C (rs7901695), 103894G>T (rs12255372) e 53341C>T (rs7903146) do gene *TCF7L2* em amostras de recém-nascidos com alteração do crescimento fetal (pequenos para idade gestacional, PIG; e grandes para idade gestacional, GIG) e de recém-nascidos apropriados para idade gestacional (AIG).

3.2.2. Identificar a frequência dos polimorfismos 49080T>C (rs7901695), 103894G>T (rs12255372) e 53341C>T (rs7903146) do gene *TCF7L2* em amostras de sujeitos adultos obesos diabéticos, obesos não diabéticos e de indivíduos não obesos.

3.2.3. Avaliar a expressão do gene *TCF7L2* em amostras de recém-nascidos (PIG, GIG e AIG), correlacionado aos achados referente à expressão dos indivíduos adultos (obesos diabéticos, obesos não diabéticos e não obesos).

3.2.4. Avaliar a susceptibilidade do gene *TCF7L2* ao desenvolvimento de síndrome metabólica, diabetes tipo 2, doenças cardiovasculares e obesidade.

3.2.4. Correlacionar os dados clínicos e moleculares.

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5. MANUSCRITO 1

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Functional analysis of transcription factor 7-like 2 (*TCF7L2*) variants associated to metabolic syndrome development in newborns and adults

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Abstract

Introduction: Fetal growth depends on interactions between mother, placenta, and fetus. Metabolic exchanges *in utero* establish physiological patterns that influence the health all lifelong. *TCF7L2* is one important gene that influences the fetal growth due to its role as deregulating insulin secretion. The aim of this study was to determine the influence of *TCF7L2* polymorphisms (49080T>C; 103894G>T; 53341C>T) and expression in birth weight as imprint for metabolic syndrome in adults.

Methods: This study was performed in two cohorts: 98 newborns and 51 adults. Were enrolled 11 newborns with small for gestational age (SGA); 41 large for gestational age (LGA); and 46 appropriate for gestational age (AGA). In adult cohort, were included 12 obese with type 2 diabetes (T2D); 17 non-T2D obese; and 22 healthy adults.

Results: *TCF7L2* 49080T>C the most prevalent in the study population (38.9%) compared to 103894G>T (27.7%) and 53341C>T (31.9%). mRNA levels showing statistical difference between newborns and adults cohorts (p=0.001). SGA neonates presented a *TCF7L2* expression higher than LGA (1.751 and 1.229, respectively) (p=0.017). No statistical significance was observed in adult cohort (p=0.115). LGA and obese adult groups reveled strong similarity (p=0.922).

Discussion: 53341C>T, 103894G>T, and 49080T>C allelic frequencies were similar to the findings in other studies. SGA showed higher relative expression than LGA, being similar to T2D obese adults. *TCF7L2* may influence the birth weight, therefore contributing for MetS and obesity in adult life. However, the mechanism of these metabolic disorders development seems to show different pathways through lifetime.

Keywords:

Fetal programming; *TCF7L2 gene*; Metabolic syndrome; Abnormal fetal growth; Obesity.

5.1. Introduction

Obesity has been increasing in epidemic proportions over last decades, and is considered a risk factor for cardiovascular disease (CVD), dyslipidemia and type 2 diabetes (T2D) [1,2]. The occurrence of two or more systemic diseases, such hypertension, abdominal obesity, dyslipidemia, and altered glucose tolerance characterizes Metabolic Syndrome (MetS), a condition that affects more than 20% of the adults worldwide [3–5].

Obesity, T2D and also MetS may be influenced by the intrauterine environment [6]. The fetal programming [7] and developmental origins of disease models [8] indicates that during the prenatal period the organism is susceptible to genetic and environmental factors, with a persistent influence over metabolic disease development in adulthood [9].

The exact role of genes in birth weight variation is still poorly understood. However, changes in body composition and weight at birth, such as occurs in newborns large and small for gestational age (LGA and SGA, respectively), can lead to metabolic sequel in the adulthood [10]. Genes that promote diabetes or fasting glucose alteration, as transcription factor 7-like 2 (*TCF7L2*), are good candidates for genes that influence birth weight [11,12].

Belonging to transcription factor 4 family (TCF4), *TCF7L2* is activated by Wnt signaling pathway, which virtually controls all aspects of embryonic development [13]. Activation of this pathway results in β-catenin increment in the cytoplasm, which is translocated to the nucleus and activates TCF7L2 and other transcription factors, like Sox family members, FOXO, PPARγ, and nuclear receptors [14]. Some *TCF7L2* polymorphisms were described as promoting T2D and insulin resistance, despite they are located in non-coding regions, such 49080T>C (rs7901695), 103894G>T (rs12255372), and 53341C>T (rs7903146) [12]. Then, it is still unclear if these SNPs play a role in alternative splicing or post-transcriptional regulation [15].

The aim of this work was to determine the *TCF7L2* expression level, as well polymorphisms frequency, in newborns and adults in order to evaluate its role in abnormal birth weight and adult obesity.

5.2. Methods

5.2.1. Study population

A total of 149 patients were investigated in two independent cohorts. Ninety-eight newborns were included in the first cohort: 11 SGA, 41 LGA, and 46 appropriate for gestational age (AGA). Fifty-one adults were included in the second cohort: 12 T2D obese, 17 non-T2D obese, and 22 healthy adults.

Samples from umbilical cord were collected at the moment of the birth in Candida Vargas Maternal Institute, João Pessoa/PB - Brazil in March 2015. Full-term newborns from vaginal delivery or non-elective cesarean section, without perinatal asphyxia, or acute fetal suffering signs were included in this study. Newborns from mothers with preeclampsia, gestational diabetes, congenital syndromes, connective tissue diseases, chronic infection, alcoholism, or tobacco addiction during the current pregnancy were not included. Siblings were also excluded from this study. Newborns were classified according to their birth weight in small for gestational age (SGA, lowest 10th percentile), appropriate for gestational age (AGA, between 10th and 90th percentile), and large for gestational age (LGA, higher 90th percentile) based on birth weight and gestational age.

Peripheral blood was collected from morbid obese patients undergoing bariatric procedures at Hospital das Clínicas da UFPE - Recife/PE, Brazil. These patients were morbidly obese as defined by the 1991 National Institutes of Health (NIH) consensus meeting for obesity: body mass index (BMI) greater than 35 kg/m^2, showing at least one comorbidity. Comorbidities included diseases such as: T2D, hyperlipidemia, hypertension, obstructive sleep apnea, heart disease, stroke, asthma, and back and lower-extremity weight-bearing degenerative problems. Adults with obesity due to psychiatric disturbances, pregnancy, chemistry dependence, or cancer treatment history were excluded from this study. Healthy adults with BMI less than 25 kg/m^2 were recruited at the same public service institution.

Ethics Committee approval was obtained for both cohorts studied in this research, in accordance with The Code of Ethics of the World Medical Association. All participants signed a consent forms (mothers signed the consent form when were admitted to the hospital) and privacy rights were assured.

5.2.2. Sample collection and nucleic acids extraction

Blood from umbilical cord was quickly collected and maintained in K3 EDTA tube (BD Vacuette®) for DNA analysis and also in EDTA tubes containing RNAlater® Solution (Life Technologies™) for RNA analysis. Peripheral blood from adults were collected and maintained in PAXgene® tubes to perform DNA and RNA analysis. The samples were stored at -80ºC until nucleic acids extraction through automated system QIAsymphony® (Qiagen, USA). QIAsymphony® DNA Mini Kit and PAXgene® Blood *RNA Kit* were used for extraction of DNA and RNA, respectively. All nucleic acid samples were evaluated in NanoDrop® – 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) prior to be used to genotyping or qPCR analysis.

5.2.3. TCF7L2 Genotyping

Three *TCF7L2* polymorphisms were genotyped using fluorescent probes (TaqMan®, Life Technologies): 49080T>C (rs7901695, Life ID - C_384583_10; 103894G>T (rs12255372, Life ID: C_291484_20); 53341C>T (rs7903146, Life ID: C_29347861_10). Genotyping reactions were performed twice in 25 μL reaction, using TaqMan® Universal PCR Master Mix (Life Technologies), according to manufacturer's guidelines. 20 ηg/μL DNA was used as input for genotyping assay trough PCR cycles as follow: 10 min at 95ºC, 40 cycles of 15 seconds at 92ºC and 60 seconds at 60ºC. DNA amplification and allelic discrimination plot were performed in StepOnePlus™ System (Life Technologies).

5.2.4. Reverse transcription and quantitative PCR (qPCR)

For functional analysis of *TCF7L2*, the complementary DNA (cDNA) was obtained from mRNA using QuantiTect Reverse Transcription Kit® (Qiagen, USA), following the manufacturer's protocol. cDNA purity and concentration was quantified in NanoDrop® – 2000 Spectrophotometer. All samples were tested twice for 25 μL final volume reaction: 100 ηg/μL cDNA; 10 μM TCF7L2 primers (Fw: 5'-CAC ACT TAC CAG CCG ACG TA-3' and Rv: 5'-TCC TGT CGT GAT TGG GTA CA-3') and 12.5 μL Rotor-Gene SYBR® Green PCR Kit (Qiagen, USA), according to manufacturer's guidelines. β-Actin (ACTB) gene was used as housekeeping gene. ACTB primers were purchased from Qiagen (Hs_ACTB_1_SG QuantiTect Primer Assay) and used according to manufacturer's protocol. Quantitative PCR

was performed at real-time PCR Cycler RotorGene Q® (Qiagen, USA) with the follow setup: 40 cycles of 5 seconds at 95ºC and 10 seconds at 60ºC. Melting curve (Tm) was analyzed to determine the quality of the reaction.

5.2.5. Statistical analysis

All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, CA). Comparisons of parameters with normal distribution were performed using the Student t-test for independent samples, and nonparametric test Mann–Whitney. ANOVA One-Way was performed for comparison between adults and newborn groups. Hardy–Weinberg equilibrium was tested among controls using Haploview v.4.2. Hypothesis tests were performed considering a *p*-value of <0.05 as statistical significance.

5.3. Results

5.3.1. TCF7L2 polymorphisms genotyping

A total of 149 individuals were investigated for *TCF7L2* 49080T>C (rs7901695), 103894G>T (rs12255372), and 53341C>T (rs7903146). Table 1 summarizes the genotypic and allelic frequency for all individual. *TCF7L2* 49080T>C polymorphism was more prevalent in the population (38.9%) compared to103894G>T (27.7%) and 53341C>T (31.9%). No significant difference between genotype groups was found. The genotypic and allelic distributions are in Hardy-Weinberg equilibrium.

5.3.2. TCF7L2 expression

TCF7L2 mRNA levels were analyzed in newborns and adults cohorts, showing statistical difference (p=0.001) (Fig. 1A). In newborn group, *TCF7L2* mRNA levels showed to be lower in SGA (8.230) compared to LGA (9.150) and AGA (9.130) (Fig. 1B). Thus, significant difference was observed in AGA *vs*. SGA (p=0.029) and LGA *vs.* SGA (p=0.017). In adult group, no significant difference was observed in mRNA levels between healthy and obese, 8.170 and 8.180 (p=0.810), respectively (Fig. 1C).

TCF7L2 relative expression showed no difference between newborns with abnormal birth weight (non-AGA) (1.153) and obese adults (0.959) (p=0.549) (Fig. 2A). However, in newborn cohort a difference can be observed for SGA *vs.* LGA (1.751 and 1.229,

respectively), with statistical significance (p=0.017) (Fig. 2B). In adult cohort, TCF7L2 relative expression showed great difference between non-T2D obese (0.664) and T2D obese (1.285), but without statistical significance (p=0.115) (Fig. 2C).

Newborns non-AGA revealed an expression profile similar to T2D obese (p=0.421). Although SGA and non-T2D obese showed a discrepancy in TCF7L2 relative expression, the statistical difference was slightly above the borderline (p=0.064). Otherwise, the expression profile is quite similar between SGA and T2D obese (p=0.422). The analysis of LGA and obese adult groups revealed strong similarity (p=0.922), as well no difference can be observed to T2D and non-T2D obese groups.

5.4. Discussion

The hypothesis about "fetal programming" has been analyzed in molecular field. This hypothesis proposes that adult metabolic disorders like hypertension, insulin resistance, and dyslipidemia can be a result of the fetal period adaptation [16]. Some studies demonstrate that newborns with abnormal birth weight has increased susceptibility for metabolic disorders in adulthood [17–20]; while other studies show no association [21]. This disagreement can be even more controversial when LGA and SGA newborns are evaluated for adult MetS development [20].

Insulin is one of the major growth factors in fetal life, so disorders that affect its secretion or promote resistance can lead to fetal growth changes [22]. An extensive study with 24.053 subjects demonstrated that *TCF7L2* was the first T2D gene to be associated with abnormal birth weight [11], demonstrating that *TCF7L2* can be a good candidate for "fetal programming".

Our study, the *TCF7L2* 53341C>T (rs7903146) polymorphism showed 28.6% frequency for T allele in population from João Pessoa, being similar to 31.4% frequency in obese individuals from Brasilia [23] and 29% frequency in pregnant with gestational diabetes in Curitiba [24]. Otherwise, higher level of expression was observed in our study regarding to Pernambuco (36.9%), being comparable to the TCF7L2 expression level of 33.5% observed in Espírito Santo for obese and diabetic population [25].

Phillips et al. reported a case–control study that demonstrates the association between 53341C>T and MetS risk in French population [26]; that could be due to the association of T allele with the decrease of *TCF7L2* expression in fat tissues [27]. However, the report from Bodhini et al. [28] showed no correlation between this polymorphism and MetS in India.

The T allelic frequency of *TCF7L2* 103894G>T polymorphism was 23.2% in João Pessoa population, being similar to 25% frequency reported for T2D population from Piauí [29]; while for Pernambuco, the frequency of 34.6% was higher than 30% obtained for Curitiba populations [24]. There are studies reporting the correlation between 103894G>T and MetS [30,31], that could be related to SGA condition.

The analyzing for *TCF7L2* 49080T>C polymorphism showed 37.5% C allele frequency in João Pessoa individuals and 40.5% frequency for individual from Pernambuco. The C allele was more frequent than all polymorphisms tested, but no other data was found in Brazil for comparison. The frequency of C allele was 48.8% in African population [32] and 45.5% in African-Americans healthy individuals [33]; in China population, was 1.3% [32].

It is well-established that LGA condition is a risk factor for obesity [34]. Our results showed the same profile for *TCF7L2* expression in LGA and obese adults, corroborating that this gene can influence in obesity. However, our results also show higher *TCF7L2* relative expression in SGA than LGA, being similar to T2D obese adults. These results are not in agreement with data reported for newborns from New Zealand [35], France [36] and Netherlands [37]. Despite the controversial data, it was already observed that SGA neonates shows high incidence of MetS development, including insulin resistance [38] and obesity [39]. In our population of obese and non-obese adults no correlation was observed with *TCF7L2*, corroborating with other studies [40,41]. However, *TCF7L2* seems to have a role in T2D development [12] *and* in adipogenesis deregulation [42].

TCF7L2 may influence the birth weight, therefore contributing for obesity and T2D in adult life. However, the mechanism of development of these metabolic disorders seems to show different pathways through lifetime. Then, further analysis is necessary to understand the role of *TCF7L2* in MetS development in adulthood, including follow-up studies.

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5.5 References

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Table 1: Genotype and allele frequencies of the *TCF7L2* polymorphisms (49080T>C, 103894G>T, and 53341C>T) for neonates and adult patients.

SNPs	Genotype allele	All subjects (n=149)	SGA (n=11)	LGA (n=41)	AGA (n=46)	Newborns p value	Obese (n=29)	Non-obese (n=22)	Adults p value
49080T>C	T/T	49 (32.89%)	5 (45.45%)	11 (26.83%)	17 (39.96%)	0.747	9 (31.04%)	7 (31.82%)	0.694
	T/C	81 (54.36%)	5 (45.45%)	25 (60.98%)	23 (50.00%)		15 (51.72%)	13 (59.09%)	
	C/C	19 (12.75%)	1 (9.10%)	5 (12.19%)	6 (13.04%)		5 (17.24%)	2 (9.09%)	
	T	61.20%	68.20%	57.30%	62.00%		56.90%	61.40%	
	C	38.90%	31.80%	42.70%	38.00%		43.10%	38.60%	
103894G>T	G/G	74 (49.66%)	7 (63.64%)	24 (58.54%)	22 (47.83%)	0.748	13 (44.83%)	8 (36.36)	0.787
	G/T	66 (44.30%)	4 (36.36%)	15 (36.58%)	22 (47.83%)		13 (44.83%)	12 (54.55%)	
	T/T	9 (6.04%)	0 (0.00%)	2 (4.88%)	2 (4.34%)		3 (10.34%)	2 (9.09%)	
	G	72.30%	81.80%	76.80%	71.70%		67.20%	63.60%	
	T	27.70%	18.20%	23.20%	28.30%		32.80%	36.40%	
53341C>T	C/C	64 (42.95%)	6 (54.55%)	18 (43.90%)	22 (47.83%)	0.765	9 (31.04%)	9 (40.91%)	0.765
	C/T	72 (48.32%)	5 (45.45%)	20 (48.78%)	19 (41.30%)		17 (58.62%)	11 (50.00%)	
	T/T	13 (8.73%)	0 (0.00%)	3 (7.32%)	5 (10.87%)		3 (10.34%)	2 (9.09%)	
	C	68.80%	77.30%	68.30%	68.50%		60.30%	65.90%	
	T	31.90%	22.70%	31.70%	31.50%		39.70%	34.10%	

SGA: small for gestational age; LGA: large for gestational age; AGA: adequate for gestational age.

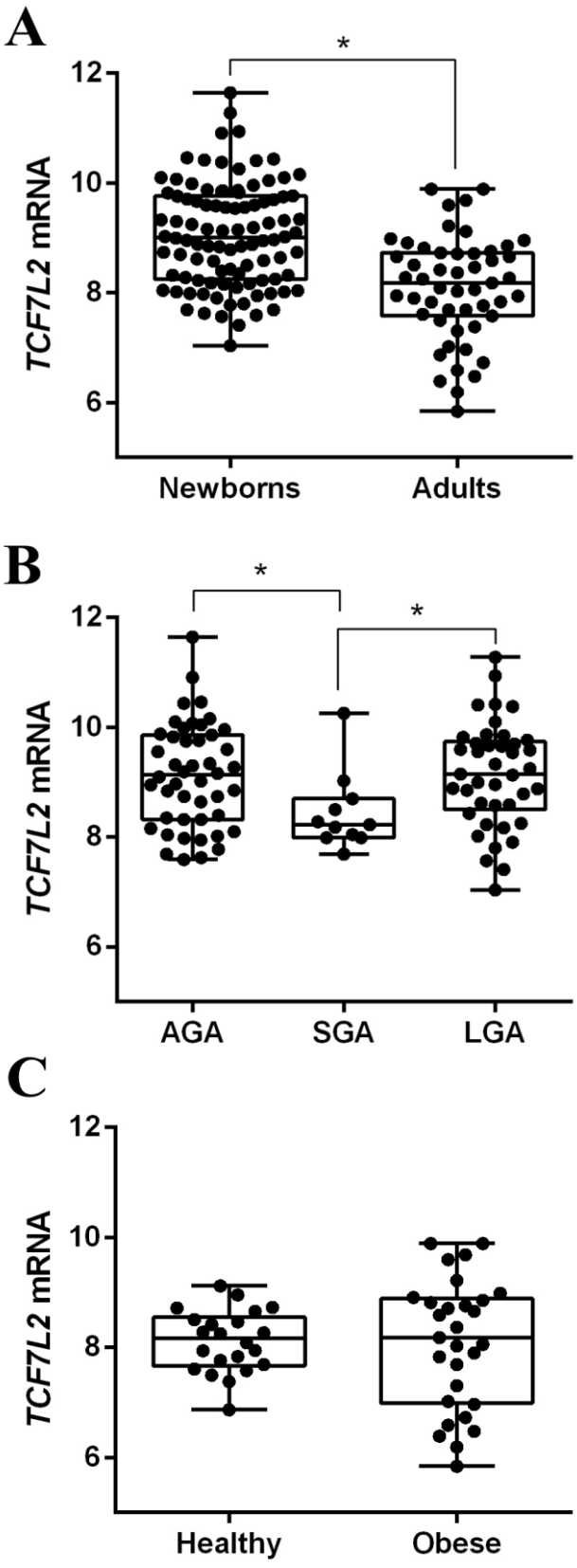

Figure 1: A, B and C. Levels of *TCF7L2* mRNA. A) Comparison between the newborns and adults cohorts (p=0.001) B) Analysis for newborns with abnormal fetal growth (SGA and LGA) and AGA. Statistical significance between SGA compared to AGA (p=0.029) and LGA (p=0.017). C) Comparison between adults healthy and obese, without statistical significance (p=0.810).

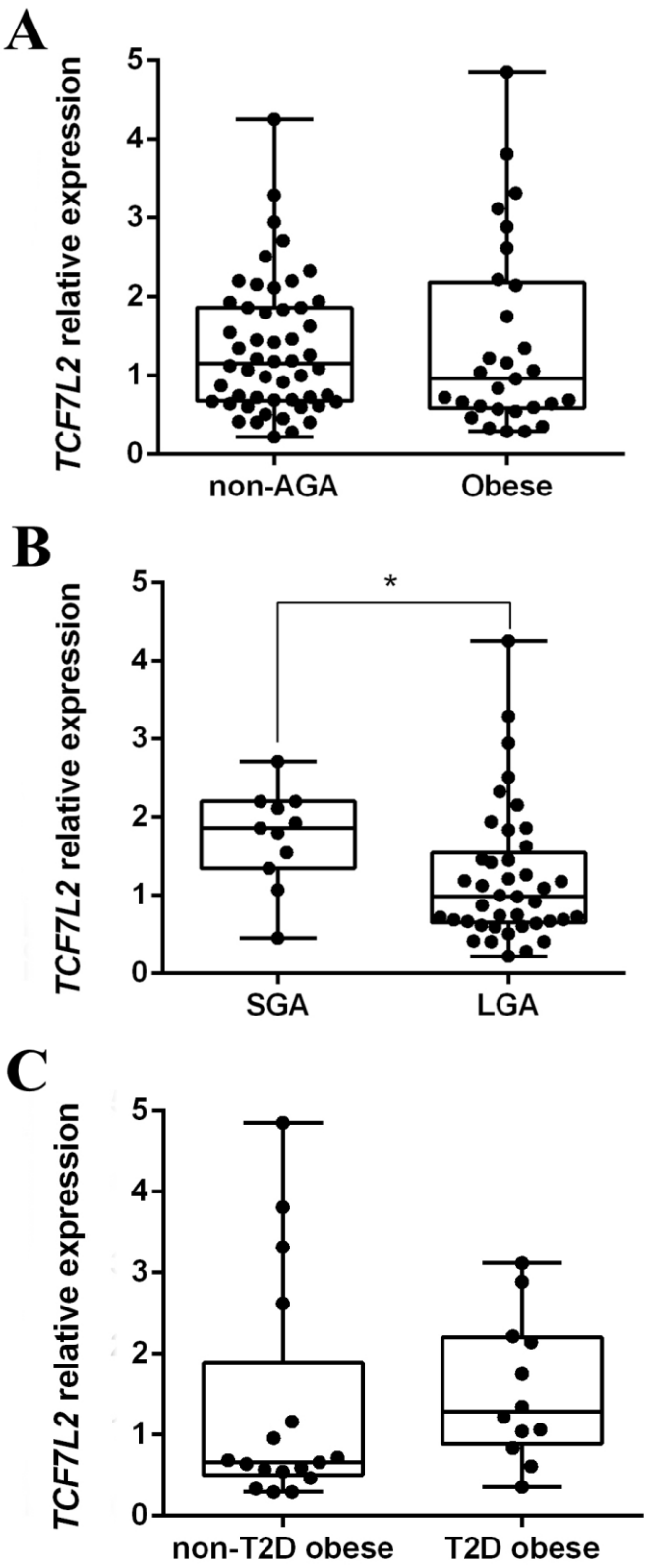

Figure 2: A, B and C. *TCF7L2* relative expression. A) Comparison between the newborns with abnormal weight (non-AGA) and obese adults (p=0.549) B) Analysis for newborns cohort with a difference observed between SGA and LGA with statistical significance (p=0.017). C) A great difference between non-T2D obese and T2D obese, without statistical significance (p=0.115).

6. MANUSCRITO 2

The role of *TCF7L2* to metabolic syndrome development in adulthood due intrauterine imprinting affected to oxidative stress

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The role of *TCF7L2* to metabolic syndrome development in adulthood due intrauterine imprinting affected to oxidative stress

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Abstract

Fetal growth and development are dynamic processes that depend on sophisticated interactions between mother, placenta, and fetus. Several stimuli can modulate the fetal growth and susceptibility to the development of common diseases in adulthood such as obesity, cardiovascular disease, metabolic syndrome, and diabetes. This observation has been referred as "fetal programming", but the molecular interactions that lead to this biochemical behavior is still unclear. Bioinformatics tools can be applied to the prediction of metabolic networks, helping to improve the knowledge of the biological response in metabolic disorders. The network analysis was performed to evaluate the interaction between Wnt signaling pathway and oxidative stress, during pregnancy. Inflammatory events contribute to increase the level of cytokines, such TNFα and IL-6, and consequent *TCF7L2*. Furthermore, this gene interacts with other genes and products related to Wnt signaling pathway, like *GCC*, *DKK1*, *CTNNB1*, *APOE*, *APOC1*, and *FTO*. It was observed that Wnt pathway and *TCF7L2* transcription has a role in the development MetS, also related to SGA and LGA birth weight phenotype. Further studies including TCF7L2 and a set of related genes could indicate how the early life is connected to the adulthood, highlighting new biomarkers for prevent the development of metabolic diseases.

Abbreviations:

MetS, Metabolic Syndrome; SGA, Small for Gestational Age; LGA, Large for Gestational Age.

Keywords:

TCF7L2; Biomolecular Interactions; Fetal Growth; Metabolic Syndrome.

6.1. Introduction

Fetal growth and development are dynamic processes that depends on sophisticated interactions among the mother, placenta and fetus [1,2]. So, in 1995 David J. P. Barker suggested an explanatory model for the predisposition of the development of metabolic disorders in adult life due the intrauterine environment conditions, mainly for newborns with abnormal birth weight [3–5].

About 85% of term newborns show normal birth weight of 2500-4000 g (Appropriate for Gestational Age – AGA), but 8% newborns show abnormal birth weight. Newborns weighting less than 2500 g and 10th percentile are classified as Small for Gestational Age – SGA), while newborns with more than 4000 g and 90th percentile are classified as Large for Gestational age – LGA) [6]. SGA and LGA infants show increased susceptibility to obesity, cardiovascular diseases (CVD), type 2 diabetes (T2D) and metabolic syndrome (MetS) [6–8], suggesting that molecular interactions during fetal growth can explain the development of metabolic disorders in adult life [9,10].

Oxidative stress in pregnancy can modulate the expression of genes involved in cell growth and response, as *TCF7L2*. This gene is related to the deregulation of insulin levels, therefore is also associated to MetS development [9,11,12]. TCF7L2, a transcription factor that plays a role in Wnt signaling pathway, virtually controls all aspects of embryonic development [13].

Thus, the aim of this study was to perform a bioinformatics analysis focused in the molecular interactions of *TCF7L2* that can lead to the development of abnormal birth weight and metabolic disorders in adult life.

6.2. Methods

6.2.1. Data and text mining

The data and text mining was performed in three different databases: PubMed (http://www.ncbi.nlm.nih.gov/pubmed), ScienceDirect (http://www.sciencedirect.com/) and LILACS (http://lilacs.bvsalud.org/). The crossings descriptors (keywords) used were: "*TCF7L2"*, "Barker hypothesis", "intrauterine growth biology", "small for gestational age", "large for gestational age", "insulin growth factor", "diabetes", "metabolic syndrome", "bioinformatics", "Wnt pathway", "Wnt pathway signaling", "glucose homeostasis", "*TCF7L2* pregnancy", "Fetal growth and TCF7L2", "Fetal programming and metabolic

syndrome", "TCF7L2 and SGA and LGA", "TCF7L2 and abnormal birth weight", "TCF7L2 and metabolic syndrome" and "Wnt pathaway and TCF7L2".

6.2.2. Bioinformatics tools

The analysis of molecular interactions of TCF7L2 was performed through bioinformatics tools available in online databases: NCBI databases (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD) (http://www.ncbi.nlm.nih.gov/); GeneMANIA© (University of Toronto) (http://www.genemania.org/); KEEG (Kyoto Encyclopedia of Genes and Genomes, Kanehisa Laboratories) (http://www.kegg.jp/); The Wnt Homepage (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). MetaCore™ (Thomson Reuters) (version 6.17 build 64668) a systems of biological analysis, performed a manually-curated interaction of genes and protein related to Wnt pathway in relation to TCF7L2.

6.3. Results and discussion

The pregnancy is marked for high production of reactive oxidizing species generated by oxygen (ROS) and nitrogen (RNS); molecules mainly synthesized in the mitochondria of placental tissue [14,15]. This tissue releases high levels of the antiangiogenic factors and *debris* in maternal circulation, leading to the systemic activation of leukocytes and stimulating major platelet aggregation, vasoconstriction, and widespread inflammatory response.

The reactive oxidizing species, such as •O2−, can react with nitric oxide (NO), the molecule synthesized by NO synthase through L-arginine amino acid. This enzyme is fundamental in pregnancy regulation, allowing placental vasodilatation. Due this oxidative stress state, the NO oxidation promotes the production of ONOO– (peroxynitrite) [16], leading to inflammatory response and cytokines synthesis.

6.3.1. Molecular interaction of Wnt signaling pathway

The canonical Wnt signaling pathway occurs in the presence of WNT molecule. Normally, this molecule is absent. Thus, in the cytoplasm occurs the recruitment of the protein complex formed by glycogen synthase kinase-3β (GSK3β), APC, casein-kinase-1 (CK1), and Axin. This complex allows the phosphorylation of β-catenin, leading to

ubiquitination and degradation of the molecule through Proteasome (Fig. 1A). Otherwise, when WNT ligand binding of a canonical Frizzled receptor and LRP5/6 co-receptor, the protein Dishevelled (DsH) is phosphorylated and promotes the GSK3β/APC/Axin/CK1 complex disassembly. Lastly, β-catenin is accumulated in the cytoplasm for posterior migration to the nucleus, contributing for gene transcription (Fig. 1B).

The Wnt signaling pathway is regulated by oxidative stress during pregnancy (Figure 2). This phenomenon occurs due their predisposition to inflammatory events, leading to an increase in cytokines concentration. The molecules involved in Wnt pathway can be stimulated by several factors, such as endothelial nitric oxide synthase (NOS). Oxidative stress stimulates the expression of inducible nitric oxide synthase (*iNOS*) expression, inhibiting dickkopf WNT signaling pathway inhibitor 1 (DKK1), responsible for the competitive inhibition of the complex LRP-Frizzled receptor and also necessary to the activation of Wnt pathways in the presence of WNT molecule. Thus, *iNOS* induces β-catenin stabilization and pathway activation is facilitating *TCF7L2* transcription [17]. This explains the fact already described in the literature about the relation between increased *TCF7L2* expression and increased *iNOS* expression [17,18]

By another hand, some of the most common pro-inflammatory cytokines, TNFα e IL-6, are involved in Wnt pathway, contributing to the inhibition of APC/GSK3β/Axin/CK1 complex, which results in increment of cytoplasmic β-catenin and subsequent activation of *TCF7L2* transcription. Furthermore, Wnt signaling promotes the phosphorylation of DsH, which also results in high levels of β-catenin. [14], This phenomenon can occur due these cytokines contribute to Dsh phosphorylate, which binds to CK1. However, Axin molecule levels remained low in the presence of IL-6 and even further reduced in the presence of TNFα [19–21]. Without CK1 or Axin there is no formation of APC/GSK3β/Axin/CK1 complex, avoiding β-catenin ubiquitination and degradation.

6.3.2. TCF7L2 interaction network

A total of 20 molecules were found to be involved in the TCF7L2 network, according to 7 categories: co-expression; physical interactions; genetics interactions; shared protein domains; co-localization; pathway; and prediction (Figure 3).

These molecules are related to different metabolisms: 2 molecules in carbohydrate metabolism – GCGR and GLP2R; 3 molecules in lipid metabolism – FFAR4, LRP5, and LRP6; 4 molecules in nucleic acid regulation – CHD8, SALL1, NR5A1, and SOX1. BCL9

and TAX1BP3 were also linked to TCF7L2 metabolism and play a role in cancer development. Additionally, other 10 molecules are involved in different reaction showing participation in regulatory events – GRP, CDX2, KREMEN1, KREMEN2, DKK2, DKK3, BCL9, CTNNBIP1, LEF1, and CTNNA1.

Three molecules GCG, CTNNB1 and DKK1 showed to be more related to *TCF7L2* interactions, involved in gene modulation through Wnt signaling pathway. The protein encoded by this *GCG* is a preproprotein that is cleaved into mature peptides, such glucagon, and important carbohydrate regulator. *CTNNB1* encodes a protein necessary for cell growth and adhesion between cells. Lastly, *DKK1* is involved in embryonic development through its inhibition of the WNT signaling pathway.

The increment of maternal *TCF7L2* expression is related to weight gain or gestational diabetes development. The increase in glucose offering to the fetus results in LGA newborn [22]. *GCG* and *GLP-1* genes, involved in glucose metabolism, can deregulate the events of insulin secretion and glucose absorption that can impact the fetal growth. The interaction of *TCF7L2* and *GCG* promotes β-catenin increment and *TCF7L2* transcription, also inducing *GLP-1* expression in enteroendocrine cells [23,24]. This mechanism affects the intrauterine fetal growth and may be involved in the susceptibility to metabolic disorders in adulthood [25]. Additionally, DKK1 can promote adipogenesis in cells with a low degree of differentiation, showing a fundamental role of Wnt signaling pathway in repressing adipogenesis [26].

In the fetal development period occurs protein-protein physical interactions, beyond pathways interaction and gene-protein prediction between *TCF7L2* and CTNNB1. These data complement studies that have suggested the essential involvement of β-catenin/*TCF7L2* in the Wnt signaling pathway for pancreatic development [27] and its possible tissue disorders [28].

Although the role of β-catenin in pancreatic β-cell development remains unclear and controversial [29], *TCF7L2* has been associated to T2D through β-cell dysfunction. This molecules could modulate fasting lipid levels in familial hyperlipidemia, suggesting this may also regulate adipokines secretion and lipid metabolism [30]. Moreover, FFAR4 may interact directly with *TCF7L2* since those genes have co-expression; and LRP5 and LRP6 share the same pathway with CTNNB1. Since *TCF7L2* had interconnections with *APOE, APOC1*, and *FTO* [31], it has been assumed that *TCF7L2* is related to MetS and atherogenic dyslipidemia development [32].

TCF7L2 seems to contribute to susceptibility for MetS development, but more than one pathway could be responsible for the development of T2D and obesity. Therefore, it is

necessary to perform new computational and experimental analysis to improve the knowledge about TCF7L2 network and its relation with the “fetal programming” as the trigger for the development of metabolic disorders in adult life.

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6.4. References

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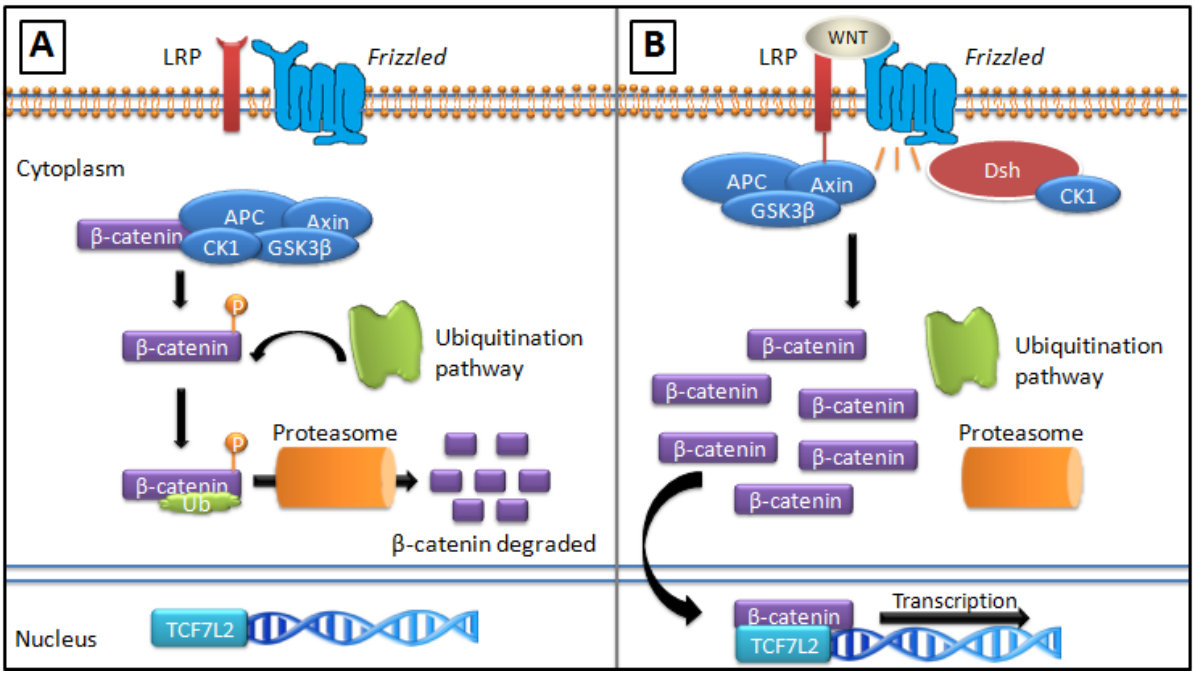

Figure 1: Canonical Wnt signaling pathway. A) In the absence of WNT molecule. B) The Wnt signaling pathway activated.

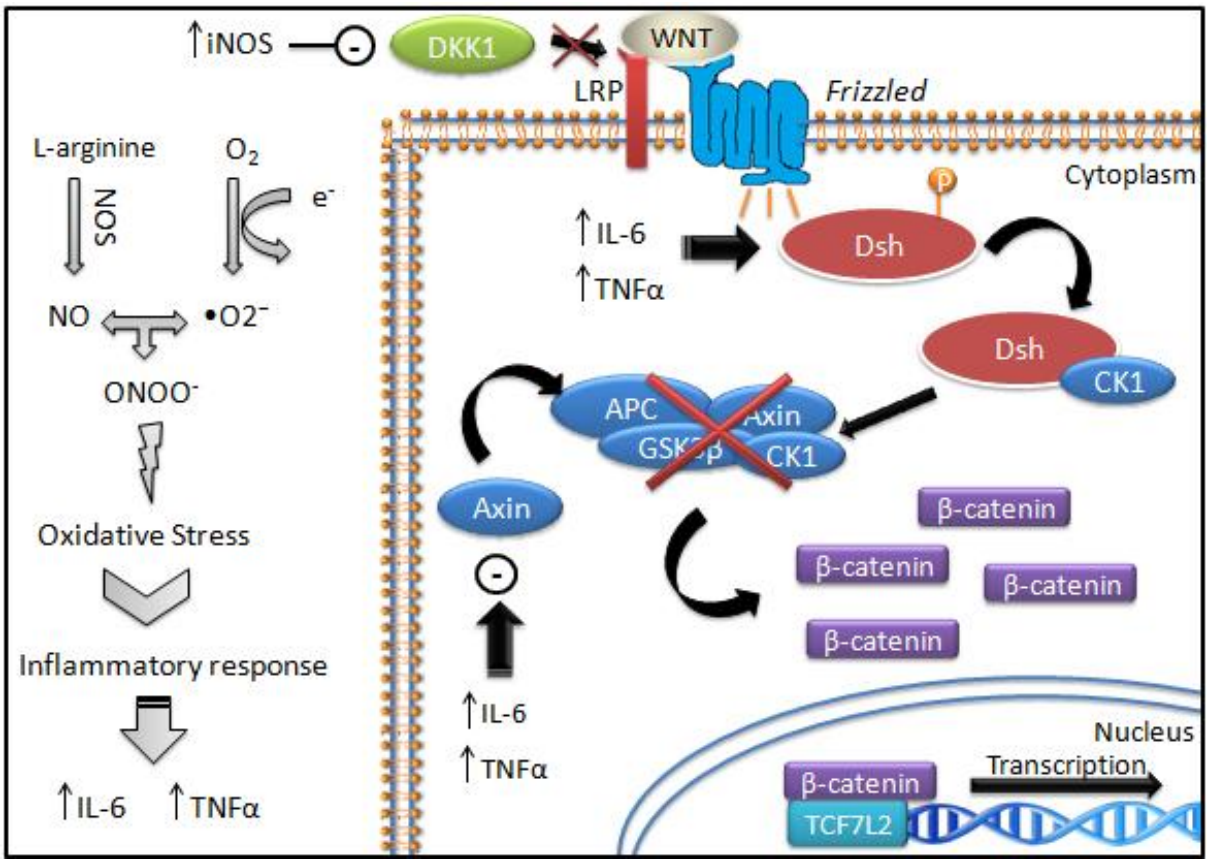

Figure 2: Oxidative stress during pregnancy promotes an intense inflammatory response in intrauterine ambient through IL-6 and TNFα. These cytokines interact with molecules from Wnt signaling, and promotes the stabilization of β-catenin levels, inducing gene transcription.

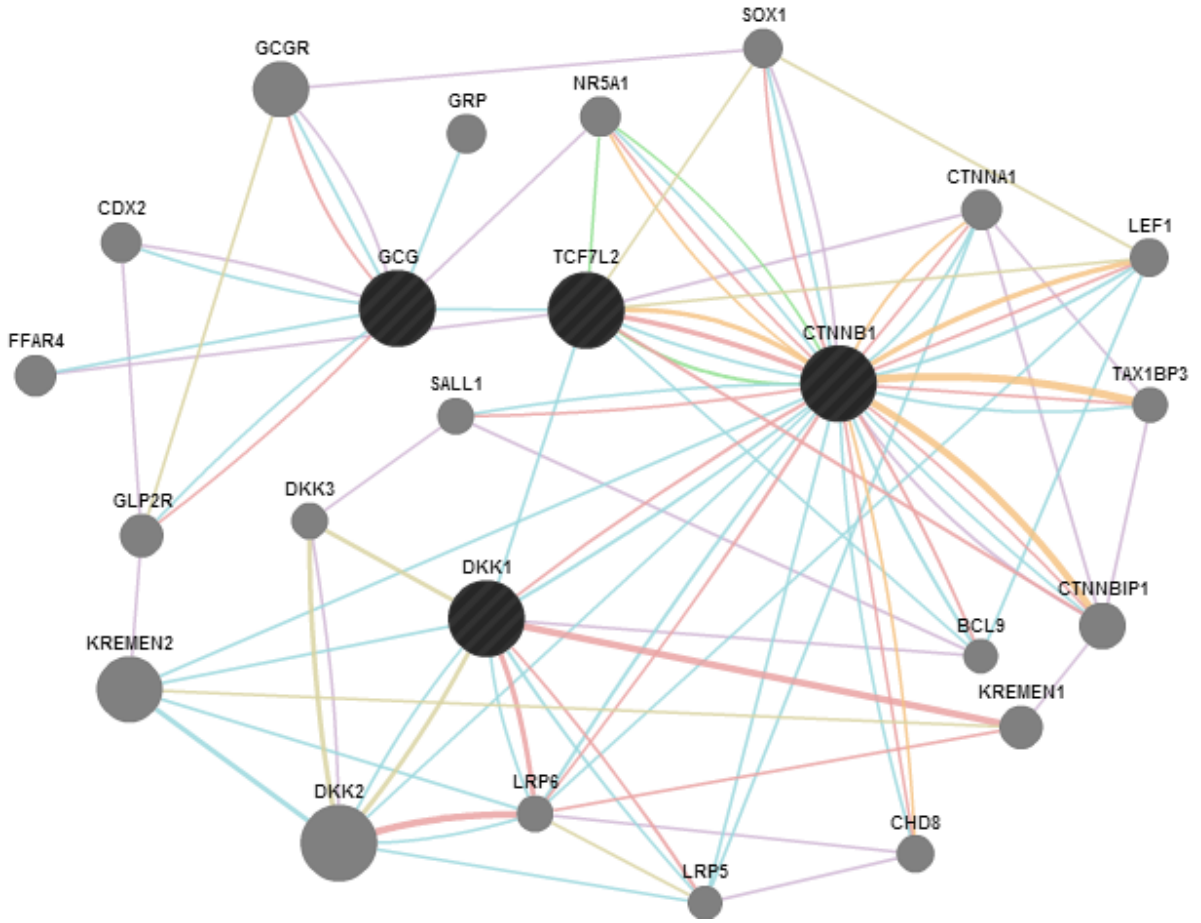

Figure 3: Molecular interactions network related to Wnt signaling pathway through *TCF7L2*, *GCG*, *DKK1* and *CCND1* genes (dashed gray circles) and their products (light gray circles). Lines: green – genetics interaction; light blue – pathway; red – physics interaction; orange – prediction; beige – shared protein domains; purple – co-expression; dark blue – co-localization.

Table 1: Molecules that interacting to *TCF7L2* and Wnt signaling pathway.

Symbol	Molecule name
GCGR	Glucagon receptor
GRP	Gastrin-releasing peptide
CDX2	Caudal type homeobox 2
FFAR4	Free fatty acid receptor 4
GLP2R	Glucagon-like peptide 2 receptor
KREMEN2	Kringle containing transmembrane protein 2
KREMEN1	Kringle containing transmembrane protein 1
LRP5	Low density lipoprotein receptor-related protein 5
LRP6	Low density lipoprotein receptor-related protein 6
DKK2	Dickkopf WNT signaling pathway inhibitor 2
DKK3	Dickkopf WNT signaling pathway inhibitor 3
BCL9	B-cell CLL/lymphoma 9
CTNNBIP1	Catenin, beta interacting protein 1
TAX1BP3	Tax1 (human T-cell leukemia virus type I) binding protein 3
CHD8	Chromodomain helicase DNA binding protein 8
SALL1	Spalt-like transcription factor 1
LEF1	Lymphoid enhancer-binding factor 1
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kda
SOX1	SRY (sex determining region Y)-box 1
NR5A1	Nuclear receptor subfamily 5, group A, member 1

7. CONCLUSÕES

A frequência alélica dos principais polimorfismos do gene *TCF7L2* (53341C>T, 103894G>T e 49080T>C) na população do nordeste brasileiro foi semelhante a outros estudos brasileiros e globais, sendo 49080T>C o mais prevalente. Os recém-nascidos PIG apresentaram os maiores níveis de expressão relativa de TCF7L2 em comparação com os neonatos GIG. Curiosamente, o perfil de expressão de TCF7L2 em neonatos GIG foi similar aos observados em pacientes obesos. Análises *in silico* demonstraram que a TCF7L2, juntamente com GCC, DKK1, CTNNB1, pode influenciar no crescimento fetal e suscetibilidade ao desenvolvimento de síndrome metabólica através de diferentes vias metabólicas.

8. PERSPECTIVAS

Estudos adicionais utilizando ferramentas de bioinformática em associação com dados clínicos, bioquímicos e moleculares deverão permitir a compreensão do gatilho de ativação do gene *TCF7L2* para o crescimento fetal inadequada e susceptibilidade às doenças metabólicas. Além disso, estudos que permitem o acompanhamento de indivíduos desde o nascimento até a vida adulta podem esclarecer sobre o papel da TCF7L2 e de outros genes no desenvolvimento da síndrome metabólica na idade adulta.

APÊNDICE A – Manuscrito técnico realizado durante o Mestrado.

Comparison of the performance of two commercial nucleic acids extraction kits from human samples

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Comparison of the performance of two commercial nucleic acids extraction kits from human samples

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Abstract

The development of new protocols for nucleic acids extraction is very important in the molecular biology field, contributing to many applications to improve the human health in society. Two commercial kits were tested for DNA and RNA extraction, analyzing their yield and purity. Omega Bio-tek© kits showed to be a simple, rapid and effective method for the isolation of DNA in whole blood, tissue biopsy, and formalin-fixed paraffin-embedded (FFPE) tissue. Good results were also found for recovering RNA from tissue biopsy samples. Comparing with Qiagen© kits, a well established commercial kit for scientific experiments, Omega Bio-tek© showed similar values of DNA yield and purity for human fresh and frozen whole blood and tissue samples. Also, Omega Bio-tek© showed better DNA purity for FFPE breast tissue. None of the kits showed DNA integrity for FFPE pancreas tissue, despite Omega Bio-tek© had better DNA yield. Omega Bio-tek© kit has a slight advantage over Qiagen© kit regarding RNA yield, but both kits showed excellent real-time PCR efficiency through standard curves analysis. Thus, Omega Bio-tek© kits appear to be recommendable for utilization in the molecular biology field.

Keywords

DNA extraction; RNA extraction; PCR; Whole blood; Tissue biopsy; FFPE.

1. Introduction

The molecular biology field has advanced greatly in the past five years. Currently, it is an important moment of experimental knowledge expansion, using new technologies that contribute to the improvement in human diagnosis, treatment, and clinical follow-up [1]. It is very common in researches involving molecular biology that the effectiveness results analysis and protocols only can be checked after long and tiresome procedures, many times indirectly, like to nucleic acids isolation and purification protocols. Furthermore, the quality of the nucleic acid extraction is extremely important for this subsequent handling success [2].

Nucleic acids serve as templates for a wide array of analysis techniques [3], like DNA and RNA sequencing [4] [5], polymerase chain reaction (PCR) [6], real-time PCR (qPCR), and microarray tests [5] [7]. Through efficient nucleic acid isolation, it can be possible to realize many important applications for the society, such as in aid of forensic science [8], paternity tests [9], safety and quality in food industry[10] [11], diagnosis of infectious diseases [12], and molecular diagnostics of genetic abnormalities [13].

In the last two decades, a large number of extraction methods were developed, since in-house manual methods until commercial high-end DNA extraction kits by automated system [14]. The best method is assumed based on several factors, such as the DNA quality and purity required [15], and the removal of any potential inhibitor that will interfere with subsequent downstream processing [14].

Ever since the proposal of the central dogma of molecular biology [16], RNA was considered simply as an intermediate between DNA and protein [17]. However, this molecule is now known to play much more functional roles, as a result of the improvement of the techniques for RNA isolation and purification [18]. Thus, RNA is now employed for gene expression and transcriptomic analysis [5]. Furthermore, in partnership with next generation sequencing technologies, RNA sequencing can be used to investigate the development of many diseases, like cancer [19].

Despite major advances in the isolation and purification of DNA and RNA, all analysis depend on the availability and quality of pure nucleic acids [3] found in a wide range of human sample sources, as formalin-fixed paraffin-embedded (FFPE) tissues [20], peripheral blood mononuclear cells (PBMCs) [21] and tissues from biopsies [22]. A variety of extraction methods assists to promote increasing scientific information, allowing scientists to develop the nucleic acid-based diagnosis [3] through gene expression profiles, genome

sequences, and methylation patterns, contributing to accurately identification of genetic diseases [23].

Increasing demand for clinical diagnosis requires reliable methods for efficient recovery of nucleic acids from clinical samples [24]. Thus, the development of new protocols for DNA and RNA extraction and purification is required for improvement in diagnosis, and various commercial kits should be tested by comparing them with well-established kits used in laboratory routine.

The goal of this work was to analyze Omega Bio-tek© kits that offer a simple, rapid and effective method for the isolation of nucleic acids, being comparable to Qiagen© kits, a well established kit used in molecular biology field.

2. Material and methods

2.1. Commercial kits

E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek©, Norcross, GA) (OmegaDk) was tested for DNA extraction and compared to Qiagen DNeasy Blood and Tissue kit (Qiagen©, Germantown, MD) (QiagenDk). RNA extraction was tested using E.Z.N.A.® Tissue RNA Kit (Omega Bio-tek©, Norcross, GA) (OmegaRk) and compared to Qiagen RNeasy® Mini Kit (Qiagen©, Germantown, MD) (QiagenRk). All kits were based on selective binding of the nucleic acids to a membrane mini-column and performed according to manufacturer's directions. To ensure reproducibility, kits were tested by two different operators, following the same protocols, equipment, and laboratory structure.

2.2. Sample preparation for DNA extraction

2.2.1. Whole blood

Two different samples were tested for whole blood DNA extraction. Fresh samples were collected by venipuncture in ethylenediamine tetraacetic acid tube (K3 EDTA tube, BD Vacuette®) and immediately processed for DNA extraction. Frozen whole blood samples, stored at -20ºC, were tested after thawing at room temperature for 30 min. OmegaDk required 200 µL of anticoagulated blood, but no more reagents besides those provided by the kit. QiagenDk required 100 µL of anticoagulated blood and 100 µL PBS pH 7.4 (Gibco®, Life Technologies™), not provided in the kit. For both sample types tested by both kits, five steps were performed: cell lysis and adjusted binding conditions, binding to mini-column, washing,

drying, and elution. Despite the difference to sample impute, in final step was obtained the same elution volume of 200 µL, following manufacturer's directions.

2.2.2. Tissue biopsy

Tissue DNA extraction was performed on hyperplasic breast biopsy sample, stored in TRIzol® Reagent (Life Technologies™) at -80ºC. The sample amount input for OmegaDk was 30 mg whereas QiagenDk was 25 mg, following manufacturer's directions. In both assays, tissue was sliced into small pieces prior to the first step of tissue digestion, and the cell lyses. Samples were incubated in a shaking water bath for 2.5 hours, mixing by inversion and vortex every 30 minutes. It was followed by binding step with mini-column, washing, drying and elution. Despite the different sample quantities used for each kit, the same elution volume of 200 µL was obtained at the final step.

2.2.3. Formalin-fixed paraffin-embedded tissues (FFPE)

Formalin-fixed paraffin-embedded tissue DNA extraction was realized in samples of pancreas and breast tissues, stored under temperature room. The sample amount was 25 mg for OmegaDk and QiagenDk, being sliced into small pieces of 0.3 mm. Xylene (Merck KGaA©, Darmstadt, Germany) was used for removing paraffin. The first step was the tissue digestion and cell lysis, being incubated in a shaking water bath for 2.5 hours, mixed by inversion and vortex every 30 minutes. The following steps were binding to mini-column, washing, and drying. The final elution volume was 100 µL to OmegaDk and 200 µL to QiagenDk. All procedures were performed according to manufacturer's recommendations.

2.3. DNA quantification

Qubit® dsDNA BR Assay Kit (Invitrogen™) was used in Qubit® 2.0 Fluorometer (Invitrogen™, Paisley, UK), a quantitation system fluorescence-based dyes that bind specifically to the nucleic acid. It was used to determine the DNA yield extracted from samples of whole blood, tissue biopsy, and FFPE.

2.4 PCR amplification

Conventional PCR was realized to determine DNA quality as a way of comparing the two kits in their different extractions types. We performed PCR reaction with GoTaq® Green Master Mix (Promega©, Madison, WI), 10 ρM β-globin forward primer (5'-CAA CTT CAT CCA CGT TCA CC-3'), 10 ρM β-globin reverse primer (5'-GAA GAG CCA AGG ACA GGT AC-3'), and Ultra Pure Water (Gibco®), according to manufacturer's protocol. PCR amplification was tested using 1 and 2 μL of each DNA sample, for each kit. PCR cycling was performed in Veriti® 96-Well Thermal Cycler (Life Technologies™), with the following settings: 95º C for 2 min; 95º C for 1 min, 62º C for 1 min and 72º C for 30 sec by 35 cycles; 72º C for 5 min. Results were observed in 1% agarose gel electrophoresis containing 0.4 μg/μL of ethidium bromide ($C_{21}H_{20}BrN_3$), in 1× Tris-Borate-EDTA buffer pH 8.3, at 100 V for 45 min.

2.5. Sample preparation for RNA extraction

Tissue RNA extraction was performed on hyperplasic breast biopsy sample, stored in RNAlater® Tissue Solution (Life Technologies™) at -80ºC. The proper amount of starting material was defined to 30 mg for both kits, OmegaRk and QiagenRk. Tissue was disrupted using a mortar and pestle, and liquid nitrogen was added to improve the assay yield. After the liquid nitrogen evaporated and before the tissue thawing, the sample was macerated and homogenized using a needle (20-gauge) and syringe. Cell lysis, mini-column binding, washing, drying, and elution were performed according to manufacturer's guidelines. Eluate volumes for OmegaRk and QiagenRk were 40 μL and 30 μL, respectively. In both kits, 2-Mercaptoethanol ($HOCH_2CH_2SH$) (Sigma-Aldrich©, St. Louis, MO), provided by the user, was added to prepare the buffers for the tissue cell lysis.

2.6. RNA quantification

All RNA samples were quantified and analyzed for purity, by NanoDrop® – 2000 Spectrophotometer (Thermo Scientific©, Wilmington, DE). Ratio analysis was performed at 260 nm/280 nm (mean ± standard deviation [SDs], 1.90 ± 0.05) to access the RNA purity.

2.7. Real-time PCR

QuantiTect Reverse Transcription Kit® (Qiagen©) was used to obtain cDNA, following the manufacturer's directions. cDNA was stored at -20ºC until quantification by NanoDrop® – 2000 Spectrophotometer. A standard curve was performed in real-time PCR (qPCR) for the RPLP0 gene (Ribosomal Protein, Large, P0 gene), a common stable endogenous gene (primer sequence: forward 5'-TCT ACA ACC CTG AAG TGC TTG ATA TC-3' and reverse 5'-GCA GAC AGA CAC TGG CAA CAT T-3'). The qPCR test was performed in RotorGene Q® (Qiagen©) using Rotor-Gene SYBR® Green PCR Kit (Qiagen©), according to manufacturer's guidelines. 100 ηg cDNA template and 5 μM RPLP0 primers were used to 25 μL final volume reaction.

The qPCR standard curve reflects the reaction efficiency, estimating the concentration of the known samples in serial dilutions and their variation between replicates [25]. Therefore efficiency calculated from the slope of the perfect standard curve should be 1.00 (100%), but values ranging from 90% and 110% (which correspond to slopes between -3.58 and -3.10) are acceptable. R^2 coefficient, correlation obtained for the standard curve that represents how well the experimental data fit the regression line, should be > 0.99 [26].

2.8. Statistical analysis

All statistical analysis were performed using GraphPad Prism version 6.0 (GraphPad Software©, CA). Once sample input or elution volume were different in all tests, the final values were submitted to normalization, allowing the analysis of the parity between the results of different kits. Pairwise comparisons were conducted, as appropriate, using non-parametric data in the Unpaired t-test, considering standard deviations (SDs). The coefficient of variation (CV) was calculated by the ratio between SDs and median variation from replicate samples. A p-value less than 0.05 were considered as statistically significant.

3. Results

3.1. Evaluation of genomic DNA extracted

3.1.1. DNA yield and purity from different sample types

Fresh whole blood extraction through PBMCs showed final DNA yield of 9.30 μg/mL for OmegaDk and 6.78 μg/mL for QiagenDk. Regarding frozen whole blood samples, the

values were 7.65 μg/mL and 6.66 μg/mL, respectively. In agarose gel electrophoresis, it was observed a strong amplicon for all samples in both kits, using either 1 or 2 μL of a sample extracted (Fig. 1A).

Hyperplasic breast biopsy tissue showed very similar DNA yield, 4.12 μg/mL for OmegaDk and 4.16 μg/mL for QiagenDk. β-globin amplification also showed a strong amplicon for both samples, either using 1 or 2 μL of the sample extracted (Fig. 1B). For FFPE breast tissue, OmegaDk showed only 1.00 μg/mL DNA yield, but it was possible to visualize amplification using either 1 or 2 μL of the sample extracted (Fig. 1C). For QiagenDk, the DNA concentration was under the cut-off for quantification, resulting in discrete amplification for 1 μL sample volume, but no amplification occurred with 2 μL sample volume. FFPE pancreas tissue was allowed high DNA recovery of 15.00 μg/mL for OmegaDk and 4.98 μg/mL for QiagenDk. However, no amplification was observed in agarose gel.

3.1.2. Extraction reproducibility and comparative performance analysis

Two operators, with different levels of experience in molecular biology, performed the DNA extraction procedures. The results were reproducible for all kits tested, exhibiting an efficient performance for DNA extraction (Table 1). The coefficient variation (CV) at final DNA yield showed values between 0.04 and 0.36. The CV for the operators was quite similar in both kits using fresh whole blood, but breast biopsy tissue showed a high discrepancy between CV values.

A comparative analysis for the kits was assessed using the mean of DNA yield. For fresh whole blood and frozen whole blood samples, no statistical significance was observed, with a p-value of 0.6721 and 0.8353, respectively (Fig. 2A and 2B). Fresh tissue sample extraction also showed no statistical significance, p-value = 0.5046 (Fig. 2C). For FFPE breast tissue, as QiagenDk elution showed undetectable DNA concentration, it was not possible to obtain the statistical data. However, FFPE pancreas tissue OmegaDk showed threefold DNA yield higher than QiagenDk, being statistical significance (p-value= 0.0486) (Fig. 2D).

3.2. Evaluation RNA extracted

3.2.1. RNA yield, purity and real-time PCR efficiency for biopsy tissue

Only hyperplasic breast biopsy tissue was submitted to RNA extraction, showing a yield of 89.07 ηg/μL for OmegaRk and 69.50 ηg/μL for QiagenRk. Sample purity was considered adequate, respecting the mean and standard deviation of 1.90 ± 0.05.

cDNA was used to evaluate the RNA quality by qPCR, using threshold cycle (Ct) for data analysis. Ct value is a relative measure of the target concentration in the reaction, defined as the number of cycles required for the fluorescent signal to surpass the threshold. Standard curves were performed using a ten-fold serial dilution, ranging from 1:1 to 1:10,000. OmegaRk' sample showed Ct values between 15.08 and 28.05, while QiagenRk' sample showed values from 16.31 to 29.30, with minimal variation within replicates (Table 2). The difference of Ct means in each of the five points of the serial dilution for both kits was 1.23, 2.11, 1.75, 1.72, and 1.65. The overall Ct means was 1.69.

The Ct values obtained in the qPCR demonstrate the efficiency of each sample extracted with each kit. Through the standard curve, we collected the data of slope, R^2 coefficient and efficiency for OmegaRk that showed to be -3.300, 0.99547 and 1.01 (101%), respectively (Fig. 3A). Regarding to QiagenRk, the slope was -3.353, R^2 = 0.99973, and efficiency was 0.99 (99%) (Fig. 3B).

3.2.2. Extraction performance for biopsy tissue

Reproducibility and comparative efficiency for the kits were performed using the mean of RNA yield, only from hyperplasic breast biopsy tissue, obtained from two operators. OmegaRk showed RNA yield of 63.60 ηg/μL (SD 36.02 and CV 0.57), while QiagenRk was 59.65 ηg/μL (SDs 13.93 and CV 0.23). The result of RNA yield for the second operator was twice smaller than for the first operator using OmegaRk, but was quite similar using QiagenRk. Despite the high level of OmegaRk SD and CV, the variation of RNA yield between kits was low (~3.95 ηg/μL); showing efficiency for both kits, since no statistical significance was observed (*p*-value of 0.8983).

4. Discussion

Commercial extraction kits are rapid, cost-effective, and efficient as a strategy for DNA and RNA extraction [27], mainly in samples with small amounts of DNA and various levels of degradation [28]. Thus, the commercial kits comparison is an important procedure for laboratory routine in research and diagnosis of many diseases based in genetic analysis.

Many tests are available for nucleic acids extraction using whole blood [29]. We observed that, for fresh whole blood DNA extraction, both kits showed similar DNA yield. However, QiagenDk presented better performance with a small advantage over OmegaDk. In frozen whole blood samples, OmegaDk demonstrates the best performance in replication tests with minimal standard deviation, probably due to the use of heated elution buffer reagent. Besides, OmegaDk had fewer discharges throughout the process, thus avoiding waste by recycling pipe. Surprisingly, frozen whole blood showed similar DNA yield with both kits, despite the literature [30] seem to advocate for isolation of PBMCs from whole blood before freezing in a suitable cryopreservative, to prevent adventitious damage after storage.

Efficient nucleic acids extraction from tissue samples, such breast tissue, are important to molecular profiling of tumors, research and diagnostics. Breast biopsy shows a high fat content and low cell number [31], increasing the difficulty for DNA extraction. However, both kits showed a good performance for breast hyperplasia tissue preserved in TRIzol®. Similar DNA yields were obtained for both kits, but QiagenDk showed small advantage. OmegaDk has tiresome procedures, beyond the additional step for incubation in water shaking did not provide better results.

FFPE tissues are a valuable source of DNA for retrospective molecular studies and identification of specific molecular markers [32]. However, DNA extraction from this type of sample remains a challenge [33]. In FFPE breast tissue, OmegaDk showed better results than QiagenDk, allowing the DNA quantification despite the low nucleic acid amount. The excessive fat in FFPE breast tissue do not influence in automatic extraction system [34], but this effect could not be proved in the column-based extraction system, due to the low DNA yield in both kits. In FFPE pancreas tissue, it was possible to obtain the predicted DNA concentration, mainly in OmegaDk, probably related to an additional step for sample incubation. Residual chemical contamination from nucleic acids extraction can also affect the DNA quality, being related to paraffinization protocol that leads to protein–protein and protein–DNA crosslinking, inducing chain breaks [35]. It can reduce the accuracy of DNA

quantification, leading to an erroneous overestimation of the nucleic acid concentration, and also inhibiting the PCR amplification.

The qPCR employed for gene expression analysis under a variety of conditions requires RNA of high quality [7], where sample preparation method and professional training are crucial. As expected, an operator with less dexterity and laboratory time had more difficulties in acquiring material extracted. Probably due to the difficulty in the reproducibility of maceration using the jacket of liquid nitrogen, applied as a method to ensure thorough grinding and to minimize the sample loss [36]. This procedure definitely reflected on the final RNA yield, but no statistical difference was observed between kits' performance.

Although the RNA measurements were not the best in reproducibility tests, mainly for OmegaRk, the means for both kits were similar. It is noteworthy that the difference between the Ct means did not compromise the qPCR efficiency, once the standard curve showed correlation coefficient near 1.00. Further analysis for the comparative performance of the kits should be done for blood RNA, including tests genomic integrity. Furthermore, each laboratory should test the efficiency of the kits for a specific tissue sample, including intra and inter-assay.

5. Conclusion

The DNA yields for fresh and frozen whole blood, tissue biopsy and the RNA yield for tissue, obtained from both kits, showed similar results. E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek©) revealed to be the best for FFPE tissue, although it is necessary to optimize the sample input, according to the tissue type. Finally, based on our findings here, Omega Bio-tek© showed good performance for nucleic acid extraction in different samples types, showing a simple and rapid processing similar to Qiagen© kits, a commercial market leader company.

6. Acknowledgments

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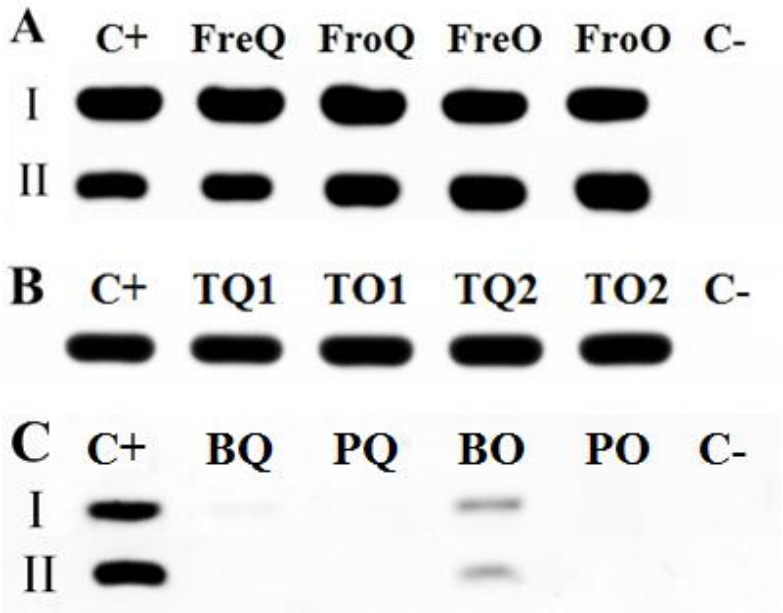

Figure 1: Agarose gel electrophoresis was realized to analyze integrity and purity of DNA extracted from human samples after conventional PCR. (A) Whole Blood sample fresh and frozen, with 1 µl (I) and 2 µl (II) of DNA – C+: Positive control; FreQ: Fresh/Qiagen; FroQ; Frozen/Qiagen; FreO: Fresh/Omega Bio-tek; FroO: Frozen/Bio-tek; and C-: Negative Control. (B) Hyperplasic breast tissue sample, where C+: Positive control; TQ1: Tissue/Qiagen 1 µl; TO1: Tissue/Omega Bio-tek 1 ul; TQ2: Tissue/Qiagen 2 µl; TO2: Tissue/Omega Bio-tek 2 µl; and C-: Negative Control. (C) FFPE breast and pancreas tissue, with 1 µl (I) and 2 µl (II) of DNA – C+: Positive Control; BQ: breast/Qiagen; PQ: pancreas/Qiagen; BO: breast/Omega Bio-tek; PO: pancreas/Omega Bio-tek; and C-: Negative Control.

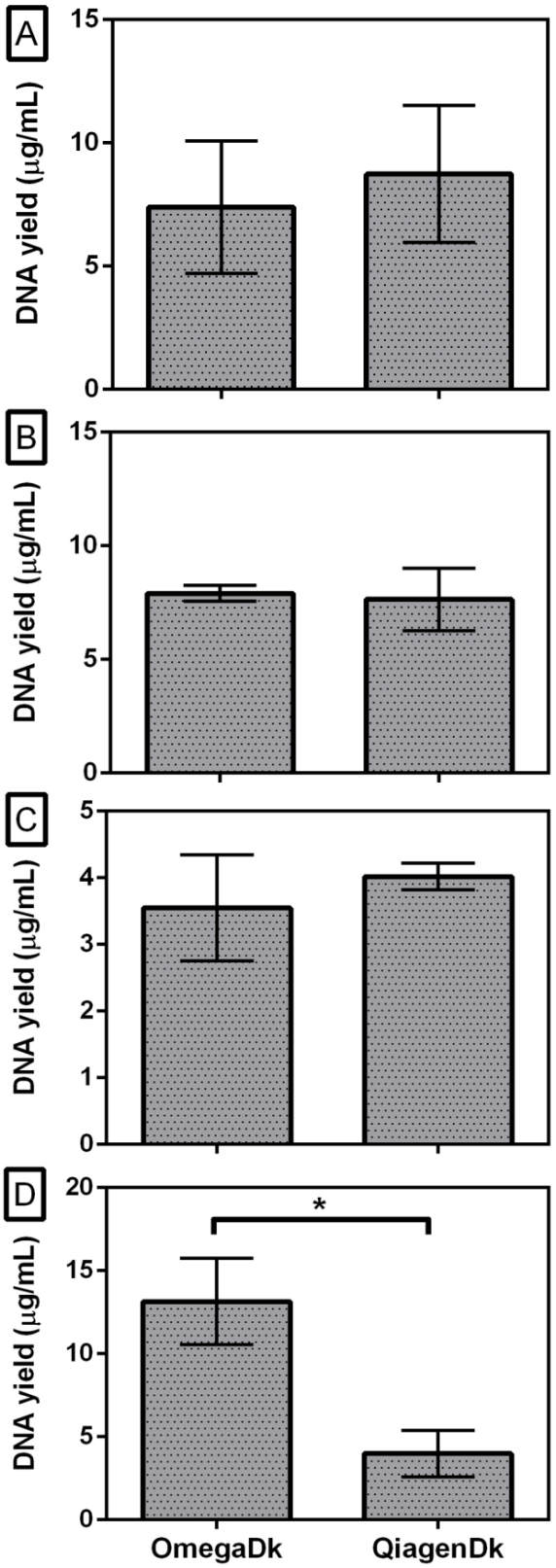

Figure 2: Graphical representation of DNA extraction reproducibility analysis from human samples, comparing the performance of both kits tested – OmegaDk and QiagenDk, with all samples properly normalized by the ratio of starting material or final elution volume following the supplier's recommendations. (A) Fresh whole blood sample. (B) Frozen whole blood sample without DNA preservative added. (C) Fresh breast tissue extraction. (D) FFPE pancreas tissue extraction. * Statistical significance ($p \leq 0.05$).

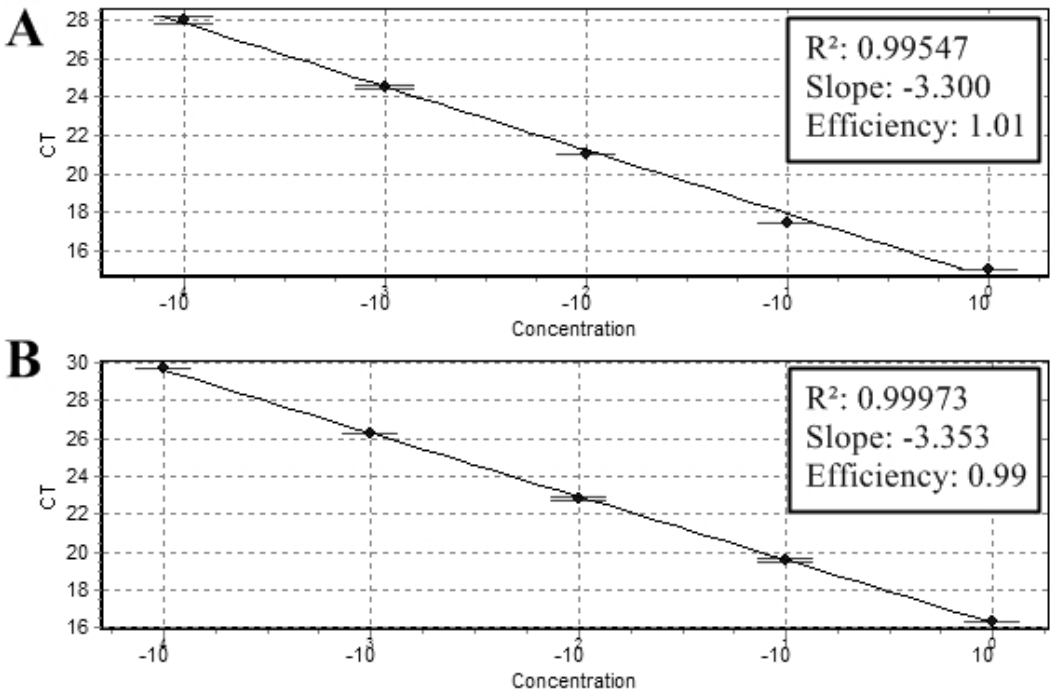

Figure 3: Standard curves built with cDNA standards of known concentrations, in serial dilution and showing the standards errors mean (double line per plotting) from RNA extracted by OmegaRk (A) and QiagenRk (B). *X-axis:* initial amount of cDNA impute, in logarithmic scale. *Y-axis*: Ct values. Standard curve images produced with Rotor Gene Q® software.

Table 1: Reproducibility analysis of DNA extraction by Omega Bio-tek© and Qiagen© kits, in µg/mL.

	OmegaDk					QiagenDk				
	Op 1	Op 2	Mean	SD	CV	Op 1	Op 2	Mean	SD	CV
Fresh Whole Blood	9.30	5.50	7.40	2.68	0.36	6.78	10.70	8.74	2.77	0.32
Frozen Whole Blood	7.65	8.15	7.90	0.35	0.04	6.66	8.62	7.64	1.38	0.18
Breast tissue biopsy	4.12	2.98	3.55	0.80	0.22	4.16	3.88	4.02	0.20	0.05
FFPE breast tissue	1.00	1.10	1.05	0.07	0.06	> 1.0	> 1.0	-	-	-
FFPE pancreas tissue	15.00	11.30	13.15	2.61	0.20	4.98	3.00	3.99	1.40	0.35

Comparing the performance between both kits tested through by two different operators. Op 1: operator 1. Op 2: operator 2. SDs: standard deviations. CV: coefficient of variation.

Table 2: Ct analysis to RPLP0 housekeeping gene in serial dilution, with 100 ηg of cDNA impute from breast biopsy tissue samples extracted by Omega Bio-tek© and Qiagen© RNA kits.

Sample concentration	OmegaRk		QiagenRk	
	Ct	SDs	Ct	SDs
1	15.08	0.05	16.31	0.00
1:10	17.49	0.03	19.60	0.08
1:100	21.08	0.01	22.83	0.10
1:1,000	24.54	0.11	26.26	0.07
1:10,000	28.05	0.22	29.70	0.03

Amplification curves built with cDNA standards of known concentrations, in serial dilution (logarithmic scale). Ct: Cycle threshold. SDs: standard deviations. Ct values provided by Rotor Gene Q® software.

APÊNDICE B – Patente desenvolvida durante o Mestrado

< Uso exclusivo do INPI >

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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: José Luiz de Lima Filho

1.2 Qualificação: Médico

1.3 CNPJ/CPF: 21638241449

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☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

PAINEL GENÉTICO NA PREDIÇÃO DE CÂNCER GINECOLÓGICO ASSOCIADO À SÍNDROME METABÓLICA

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 2/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: José Luiz de Lima Filho

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☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

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☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	6
☒	11.6	Reivindicações.	2
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, _____ por melhor representar a invenção (sujeito à avaliação do INPI).	1
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: _____ nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☐	11.13	Outros (especificar) Lista de inventores	1

12. Total de folhas anexadas: 12 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Recife, 21 de outubro de 2015
Local e Data

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Prof. José Luiz de Lima Filho
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PAINEL GENÉTICO NA PREDIÇÃO DE CÂNCER GINECOLÓGICO ASSOCIADO À SÍNDROME METABÓLICA

RELATÓRIO DESCRITIVO

Campo da Invenção

[01] A presente invenção refere-se ao campo de métodos e dispositivos baseados em um painel genético de biomarcadores para a identificação de predisposição ao desenvolvimento de neoplasias específicas da mulher (mama, ovário, endométrio e colo uterino) associadas à síndrome metabólica em pacientes recém-nascidas com crescimento fetal anormal, não excluindo outras doenças.

Antecedentes da Invenção

[02] O câncer é a segunda maior causa de morte no mundo, ultrapassado apenas pelas doenças cardiovasculares. Caracterizada por alterações morfológicas e funcionais a nível celular leva a proliferação anômala e multiplicação desordenada com capacidade invasiva para tecidos adjacentes. Existem mais de 200 tipos de cânceres, com incidência global desigual, e dependente de diversos fatores tais como idade, sexo, ambientais e comportamentais. Segundo a Organização Mundial de Saúde (OMS), estima-se que em 2030 serão cerca de 27 milhões de novos casos de câncer, com 17 milhões de mortes no mundo.

[03] Diversos tipos de cânceres são exclusivos das mulheres: colo uterino, endométrio e ovário, por exemplo. O câncer de mama, apesar de atingir ambos os sexos, apresenta maior prevalência em mulheres. Segundo o Instituto Nacional de Câncer (INCA), durante o biênio 2014/2015, estão previstos mais de 273.000 casos de câncer em mulheres, dos quais 84.290 (~30,8%) correspondem aos quatro tipos de neoplasias supracitados.

[04] A realização de diagnóstico precoce de câncer é crucial para melhor prognóstico e sobrevida das pacientes. No câncer do colo uterino, o método padrão de diagnóstico é o exame citológico das células cervicais (através do

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RESUMO

A invenção refere-se a um painel de marcadores genéticos que possuem potencial de diagnóstico e predição ao desenvolvimento de câncer ginecológico (mama, colo de útero, endométrio e ovário) em associação à síndrome metabólica, não excluindo outras doenças metabólicas, em recém-nascidas que apresentam alteração do crescimento fetal, para o diagnóstico precocemente do desenvolvimento destes fenótipos patológicos.