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IMPACTOS GENÉTICOS E EPIGENÉTICOS DO AMBIENTE PRÉ E PÓS-NATAL NO EXCESSO DE PESO INFANTIL

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Tese submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

Orientador: Profª Drª Vanessa Suñé Mattevi

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Resumo

Introdução: Atualmente, a prevalência mundial de obesidade atingiu níveis alarmantes, inclusive em crianças. Os mecanismos epigenéticos como a metilação do DNA podem ser responsáveis pela interação entre fatores genéticos, ambientais e comportamentais responsáveis pelo desenvolvimento da obesidade e comorbidades associadas.

Objetivos: Determinar o impacto de uma intervenção nutricional e de um polimorfismo do gene *DNMT3B* (rs2424913) no perfil de metilação e seu impacto no ganho de peso corporal em uma coorte de crianças acompanhada desde o nascimento até os doze anos de idade.

Material e Métodos: Este é um estudo de coorte prospectivo realizado com 500 crianças nascidas entre 2001 e 2002 na cidade de São Leopoldo – RS. Foram analisados o impacto de orientação nutricional durante o primeiro ano de vida, os genótipos da variante rs2424913 (-149 C>T) e o padrão de metilação global aos 4 anos de idade da criança. Além disso, foram avaliados dados antropométricos, comportamentais e sociodemográficos maternos e infantis.

Resultados: Encontramos uma diferença significativa entre as médias da metilação global aos 4 anos de idade entre as crianças do grupo controle e intervenção. Ao analisar o rs2424913, encontramos diferenças significativas entre os níveis da metilação global e variáveis antropométricas para os indivíduos com genótipo TT em relação aos demais genótipos, nos diversos pontos temporais da coleta de dados.

Conclusão: A associação do genótipo TT com os padrões de metilação global indica a possível funcionalidade do SNP rs2424913, enquanto que a associação da metilação global com a intervenção nutricional realizada no primeiro ano de vida indica

que a metilação pode ser impactada por mudanças ambientais e comportamentais. Nossos resultados também demonstram que a variante genética analisada pode alterar características antropométricas que estão envolvidas no desenvolvimento de obesidade, reforçando evidências de que intervenções precoces são de suma importância na prevenção desta patologia.

Palavras-chave: Epigenética, metilação de DNA, rs2424913, obesidade, orientação nutricional.

Abstract

Introduction: Currently, the worldwide prevalence of obesity among adults, adolescents and children has reached alarming levels. Epigenetic mechanisms may be responsible for genetic, environmental and behavioral interactions responsible for the development of obesity and associated comorbidities. DNA methylation is the epigenetic modification that can regulate gene expression and has shown to be sensitive to environmental stimuli, including *in utero* and post-natal environmental conditions.

Objectives: To determine the impact of nutritional counseling and a DNMT3B gene polymorphism (rs2424913) on methylation profile and body weight gain in a cohort of children followed from birth to twelve years of age.

Materials and Methods: This is a prospective cohort study conducted with 500 children born between 2001 and 2002 in the city of São Leopoldo - RS. The impact of nutritional guidance during the first year of life, the genotypes of the rs2424913 (-149 C> T) variant and the overall methylation pattern at 4 years of age were analyzed. In addition, maternal and child anthropometric, behavioral and sociodemographic data were evaluated.

Results: We found a significant difference between the means of global methylation at 4 years of age between the children in the control and intervention group. When analyzing rs2424913, we found significant differences between the levels of global methylation and anthropometric variables for individuals with the TT genotype in relation to the other genotypes, at the different time points of data collection.

Conclusion: The association of the TT genotype with global methylation patterns indicates the possible functionality of the rs2424913SNP, while the association of global methylation with the nutritional counseling carried out in the first year of life indicates that methylation may be impacted by environmental and behavioral changes. Our results demonstrate that environmental factors can change DNA methylation, altering anthropometric characteristics that are involved in the development of obesity, reinforcing evidence that early interventions are of paramount importance in preventing this pathology.

Keywords: Epigenetics, DNA methylation, rs2424913, obesity, nutritional counseling.

Lista de abreviaturas

ADIPOQ – Gene da adiponectina

BMI ou IMC – Índice de Massa Corporal

BMI zscore – Índice de Massa Corporal ajustado pra sexo e idade

CH_3 – Grupamento metila

CID – Código Internacional de Doenças

DNA – Ácido desoxirronucléico

dmC – Deoxymetil citosina

DNMT – DNA Metiltransferase

DMNT1 – DNA Metiltransferase 1

DMNT2 – DNA Metiltransferase 2

DMNT3 – DNA Metiltransferase 3

DMNT3a – DNA Metiltransferase 3 classe a

DMNT3b – DNA Metiltransferase 3 classe b

DMNT3l – DNA Metiltransferase 3 classe l

DOHaD – Origens do desenvolvimento da saúde e doença

FTO – Fat mass and obesity associated gene

HEI – Índice de alimentação saudável

LEP – Gene da leptina

LEPR – Gene do receptor da leptina

OMS – Organização Mundial da Saúde

PPARG – Gene do receptor ativado por proliferadores de peroxissoma gama

RNA – Ácido ribonucléico

SNP – Polimorfismo de nucleotídeo único

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1.1 Obesidade

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1. REFERENCIAL TEÓRICO

1.1. Obesidade

A obesidade foi classificada como uma patologia pela Organização Mundial da Saúde (OMS) em 1948, contudo, já havia sido incorporada ao Código Internacional das Doenças (CID) em 1900 devido à ansiedade dos patologistas por estabelecer critérios a seu respeito. Em 1979, no CID 9, a obesidade foi categorizada de fato e em 1995 reconheceu-se neste sumário a morbidade relacionada a esta patologia. Portanto, a obesidade somente foi reconhecida de fato pelos patologistas e pela OMS como uma doença na última metade século XX. Atualmente, a obesidade é reconhecida como um problema se saúde pública por estar associada a complicações de saúde de curto e longo prazo (1, 2).

A obesidade é definida como um acúmulo anormal ou excessivo de gordura que contribui para o desenvolvimento de várias comorbidades associadas, como diabetes tipo II, doenças cardiovasculares, dislipidemias, hipertensão, esteatose hepática, síndrome metabólica, entre outras. A medida mais comumente utilizada para definir o excesso de peso é o índice de massa corporal (IMC), definido como peso(kg)/altura(m)^2. Para adultos com 20 anos ou mais, a OMS utiliza os seguintes pontos de corte do IMC para classificação do status de peso: <18,5 kg/m^2 (baixo peso), de 18,5 – 24,9 kg/m^2 (peso normal), 25,0 – 29,9 kg/m^2 (excesso de peso), de 30,0-39,9 kg/m^2 (obeso) e ≥ 40,0 kg/m^2 (obesidade extrema) (3, 4). Já para crianças, a OMS, adota critérios que levam em conta a curva de crescimento das mesmas, como a relação peso/altura do nascimento aos 2 anos de idade, e o IMC Z-score dos 2 aos 18 anos de idade, conforme apresentamos na tabela abaixo. (5)

Tabela 1. Critérios para a classificação de excesso de peso para menores de 18 anos.

0-2 anos		**2-5 anos**		**5-18 anos**	
Relação peso/altura z-Score		**IMC z-Score**		**IMC z-Score**	
+1	Risco de sobrepeso	+1	Risco de sobrepeso	+1	Sobrepeso
+2	Sobrepeso	+2	Sobrepeso	+2	Obesidade
+3	Obesidade	+3	Obesidade	+3	Obesidade severa

Adaptado de Valerio et al. 2018.

A prevalência mundial de obesidade triplicou entre 1975 e 2016. Em 2016, mais de 1,9 bilhões de adultos tinham sobrepeso e 650 milhões eram obesos. O aumento da prevalência de sobrepeso e obesidade em crianças e adolescentes pode ser verificado a partir dos anos 80 e vem aumentando significativamente nos últimos anos na maioria dos países. O estado de desenvolvimento do país está diretamente associado com a prevalência de obesidade, refletindo a variabilidade de status nutricionais e atividade física em muitos países (3, 6-8).

O Brasil tem passado por um período de transição política, econômica, nutricional e epidemiológica, com redução da desnutrição e aumento da prevalência de excesso de peso e obesidade. A prevalência de excesso de peso em pré-escolares no Brasil era de 3% em 1989, mantendo-se em 3,4% em 1996, aumentando 129% (ficando em 7,8%) em 2006, segundo descrito por Silveira *et al* em 2014. Já em 2019, Camargos *et al* relataram que a prevalência foi de 14,1% entre crianças e adolescentes, sendo que entre crianças menores de 2 anos de idade, a prevalência de excesso de peso e/ou obesidade infantil no país foi de 6,5% (9, 10).

O aumento destes índices está associado à mudança de hábitos da população, com aumento do consumo de alimentos ultraprocessados, e diminuição da ingesta de alimentos *in natura*. Além disso, o acesso globalizado às tecnologias que proporcionam comodidade física ao indivíduo (controle remoto, escada rolante, elevador, automóvel etc.) fez com que o nível de atividade física com tarefas simples como subir escadas e caminhar diminuísse. As crianças já não são tão estimuladas a desenvolver tantas atividades ao ar livre, tendo assim menor esforço físico. Desta forma, atividades que preveniam naturalmente a obesidade e problemas a ela associados deixaram de ser praticadas (11-13).

Nas últimas décadas, a epidemia de obesidade cresceu rapidamente transformando-se em um desafio para a saúde pública, especialmente por estar associada a comorbidades que crescem vertiginosamente, especialmente entre crianças e adolescentes, com diagnóstico aumentado em idades precoces de patologias como o diabetes tipo II, hipertensão, dislipidemias e apnéia obstrutiva do sono cada vez mais cedo e com origem ambiental. Diante disto, busca-se cada vez mais entender como os fatores ambientais influenciam no desenvolvimento de obesidade, desde a pré-concepção, infância, adolescência e vida adulta (7, 14).

Dados epidemiológicos mostram que condições nutricionais, sociais e ambientais desfavoráveis em momentos críticos do desenvolvimento humano podem afetar o metabolismo e aumentar a susceptibilidade a doenças crônicas na vida adulta. Crianças com IMC mais alto têm grandes chances de desenvolverem hiperlipidemia e resistência insulínica, assim como obesidade e doenças cardiovasculares na vida adulta (15).

Desta forma, os estudos são unânimes em reconhecer que a obesidade representa um importante problema de saúde pública que precisa ser reconhecido e manejado no início da vida. Os primeiros anos de vida são períodos críticos para o desenvolvimento de excesso de peso e obesidade ao longo da vida, assim como para o desenvolvimento de doenças cardiovasculares e diabetes mellitus tipo II na vida adulta. Por isso, o aumento da prevalência de excesso de peso e/ou obesidade infantil, especialmente em idades precoces, vem se tornando uma preocupação não somente dos profissionais de saúde, mas também das entidades governamentais de todo o mundo, ressaltando a importância e necessidade das intervenções precoces no estilo de vida, incorporando mudanças nutricionais como componente chave para o tratamento da obesidade infantil (1, 9, 16).

1.2. Genética e obesidade

A gênese da obesidade é complexa e sua prevenção não é simples, não estando restrita apenas à restrição calórica ou aumento do nível de atividade física. Menos de 1% dos casos de obesidade infantil são diretamente causadas por desordens genéticas monogênicas. Na grande maioria, os casos de obesidade são poligênicos, associados a efeito cumulativo de variações comuns em um grande número de genes interagindo com fatores ambientais (16-18).

A herdabilidade da obesidade está estimada entre 6 e 85%, dependendo da característica avaliada. Por exemplo, a herdabilidade estimada do IMC varia de 16 a 85%. Para a circunferência da cintura, a estimativa fica entre 37-81%. Para a razão entre cintura/quadril, fica entre 6-30% e para o percentual de gordura corporal varia de 35-63%. No entanto, as variantes genéticas identificadas até o momento explicam

menos de 2% da herdabilidade da obesidade. A questão desta herdabilidade “perdida” ou “desaparecida” representa uma grande lacuna no conhecimento científico atual. Sabe-se que, embora a genética tenha papel importante na gênese da obesidade, ela faz parte de uma complexa interação de com fatores ambientais e comportamentais (4, 17).

Conhecimentos obtidos nas últimas décadas demonstram que a expressão gênica pode ser afetada por fatores ambientais através de mecanismos epigenéticos (4). Atualmente, a epigenética tem oferecido novas explicações sobre os mecanismos envolvidos na patogênese da obesidade, admitindo que fatores ambientais modificam a expressão dos genes envolvidos na obesidade e suas comorbidades (3) e contribuindo na elucidação da herdabilidade perdida.

1.3. Epigenética

A epigenética refere-se a mecanismos moleculares que regulam a expressão gênica sem afetar a sequência de DNA. As alterações epigenéticas persistem ao longo da vida e contribuem para a patogênese da obesidade e complicações metabólicas associadas (19-22).

A metilação do DNA e a modificação das histonas são as duas principais marcas moleculares que compõem a informação epigenética e regulam a estrutura da cromatina e a acessibilidade ao DNA. Estudos com modelos animais demonstram que fatores ambientais influenciam e podem produzir marcas epigenéticas com consequências fenotípicas que persistem ao longo da vida (23).

Avanços no estudo das origens das doenças crônicas sugerem que fatores ambientais estão ligados a variações normais no desenvolvimento fetoplacentário. O

mecanismo desta plasticidade envolve processos epigenéticos que alteram a expressão gênica e estão permanentemente ligados ao curso destas patologias (24).

As condições ambientais durante o período pré-natal e pós-natal contribuem para o risco de desenvolvimento de doenças ao longo da vida, incluindo doenças cardiovasculares e metabólicas. Fatores de risco, como as dislipidemias em crianças, predispõem o adulto ao desenvolvimento de doenças cardiovasculares. Os genes adaptam-se aos fatores ambientais através de modificações epigenéticas, além de outros mecanismos.

1.4. Metilaçãodo DNA

A metilação do DNA é o mecanismo epigenético melhor compreendido até o momento presente. É essencial para o desenvolvimento normal, contribuindo para a regulação da expressão gênica. Ocorre após a replicação do DNA, primariamente em citosinas que estão ligadas a guaninas (dinucleotídeosCpG), sendo catalisada pelas enzimas da família das DNA metiltransferases (DNMTs). A metilação do DNA consiste na adição de um grupo metila (CH_3) ao quinto carbono de citosinas que formam dinucleotídeos CpG. Os padrões de metilação são estados dinâmicos, balanceados pelos processos de metilação e demetilação. Esta modificação epigenética regula a expressão gênica silenciando tanto genes codificantes como não-codificantes e mostra-se sensível a estímulos ambientais, incluindo condições ambientais *in utero* e pós-natais (19, 25-27).

A metilação do DNA é geralmente associada com a repressão da transcrição gênica e, por isso, baixos níveis de metilação, normalmente nas regiões promotoras dos genes, ocasionam um aumento da expressão das proteínas (20).

Há poucos anos, compreendeu-se que modificações no ambiente intrauterino alteram a metilação do DNA do recém-nascido. Modelos experimentais de programação fetal vêm demonstrando que modificações no período gestacional e neonatal resultam em mudanças na metilação, que afetam direta ou indiretamente a expressão gênica e estão associadas com processos envolvidos no balanço energético (20-22).

1.5. DNA Metiltransferases

As DNA metiltransferases (DNMTs) são enzimas responsáveis por catalisar a transferência de grupamentos metila (CH_3) para as citosinas durante o processo de metilação (28).

O controle transcricional das DNMTs pode ser responsável por mudanças nos níveis de proteínas ou atividade metabólica tanto em condições fisiológicas como em patológicas. Estudos indicam que a idade afeta a expressão das DNMTs, sugerindo que este pode ser um dos mecanismos envolvidos na desregulação dos padrões de metilação observados no envelhecimento (27).

Em humanos, são conhecidas três classes de DNMTs: DNMT1, DNMT2 e DNMT3, sendo que esta última, formada por 3 enzimas diferentes, DNMT3a, DNMT3b e DNMT3l. A DNMT1 é expressa em células proliferativas, sendo responsável pela manutenção dos padrões de metilação durante a divisão celular, garantindo que a célula-filha preserve o padrão de metilação da célula-mãe. DNMT2 parece estar envolvida na catálise das reações de metilação de RNAs. DNMT3a e b estão fortemente expressas nas células embrionárias e pouco expressas nas células somáticas, tendo como função estabelecer e manter os padrões de metilação, inclusive durante o desenvolvimento fetal, portanto são essenciais para a herança

estável dos padrões de metilação. DMNT3l é conhecida por regular a metilação, estimulando as metilações *de novo* (29-31).

Diversos estudos têm demonstrado que variantes nos genes das DNMTs, principalmente polimorfismos de nucleotídeo único (*single nucleotide polymorphisms* ou SNPs) podem afetar a expressão gênica e consequentemente a metilação do DNA. Vários estudos sugerem associações destes SNPs com diferentes patologias em humanos (28), como susceptibilidade ao câncer de mama e de pulmão e progressão do câncer de próstata (32).

1.6. DNMT3b

O gene que codifica a DNA metiltransferase 3B (DNMT3b) está localizado no cromossomo 20q11.2, sendo formado por 23 éxons e 22 íntrons. Este gene é necessário para estabelecimento e manutenção dos padrões de metilação genômico, codificando uma proteína nuclear que é responsável pelo processo de metilação *de novo* (31, 33, 34).

Vários estudos mostram que alguns dos SNPs localizados no gene *DNMT3b* podem influenciar a atividade de DNMT3b na metilação do DNA. Dentre estes polimorfismos, a variante rs2424913 tem se destacado pela sua associação com vários fenótipos complexos, como descrito a seguir (27, 29, 33, 35, 36).

O polimorfismo DNMT3b rs2424913 está localizado na região promotora do gene, a -149pb do local do sítio de inicialização da transcrição. Esta variante caracteriza-se pela substituição de uma citosina por uma timina, sendo a variante T já associada com o aumento da atividade do promotor e com maior expressão da proteína em portadores do genótipo heterozigoto (31, 34). Foi relatado que a troca de

C>T confere um aumento de 30% na atividade da região promotora do gene em ensaios *in vitro* (28, 31, 33).

A distribuição das frequências alélicas para este SNP varia de acordo com o *background* genético da população investigada. A frequência mais alta do alelo variante foi descrita na China (T=0,96-0,99), enquanto que a menor frequência foi observada na população grega (T=0,26). Frequências entre 0,42 e 0,48 foram observadas na Austrália, Estados Unidos, Polônia, Holanda e Grã-Bretanha. No Brasil, já foram reportadas diversas frequências para o alelo variante (T=0,25 em grupo controle de estudo sobre líquen plano oral em MG, por Fonseca-Silva et al em 2012; T= 0,51em grupo controle de estudo sobre câncer de cabeça e pescoço em SP, por Succi et al em 2014; T= 0,41 em grupo controle de um estudo sobre doença de Parkinson no RS por Pezzi et al em 2017). Um trabalho realizado pelo nosso grupo na população de doadores de sangue em Porto Alegre em 2019 encontrou uma frequência do alelo T= 0,43 (Veber e cols., resultados não publicados) (34, 36-38).

Diferentes estudos relataram associações do polimorfismo rs2424913 com diferentes patologias, como alguns tipos de câncer e doença de Parkinson, mas com resultados controversos. O alelo T do polimorfismo já foi associado com doença de Parkinson (29, 36) e com a diminuição do risco de desenvolver câncer de cólon (33). No entanto, estudos epidemiológicos, sugerem a correlação inversa entre doenças neurodegenerativas e risco de câncer (35).

No que diz respeito à obesidade e outras alterações do tecido adiposo, como a resistência insulínica, Ciccarone *et al*, em 2016, relataram a ligação entre obesidade, expressão alterada de DNMT3b e defeitos na metilação do DNA, propondo que o

aumento da expressão de DNMT3b contribui para a desregulação da polarização dos macrófagos do tecido adiposo, inflamação e resistência insulínica na obesidade (27).

1.7. Eventos pré-natais e pós-natais

A incidência de obesidade e desordens relacionadas (dislipidemias, hipertensão, diabetes tipo 2 e doenças cardiovasculares) vêm crescendo nas últimas décadas, ocasionando uma epidemia global de obesidade. As interações entre variações genéticas e fatores ambientais obesogênicos contribuem marcantemente para o aumento dos índices de obesidade (19).

O risco do desenvolvimento de obesidade e desordens metabólicas relacionadas após a exposição a um ambiente intrauterino desfavorável foi proposto pela primeira vez por David J. Barker *et al.* (1990) e corroborado por diversos estudos epidemiológicos, revelando a importância do ambiente uterino para a programação metabólica do recém-nascido. Atualmente, com a emergência das hipóteses envolvendo mecanismos epigenéticos, surgem explicações que ligam a exposição ao ambiente fetal adverso com desenvolvimento de obesidade ao longo da vida (20, 21, 39).

Os estudos com a coorte histórica da “Fome Holandesa” examinaram os efeitos da má nutrição no início da vida no desenvolvimento de distúrbios metabólicos e cardiovasculares. Indivíduos expostos a restrições alimentares no início da vida intra-uterina apresentaram aumento da adiposidade, que pode variar de acordo com o consumo energético, atividade física e eficiência metabólica. Este efeito foi dependente do tempo e período da vida em que estes indivíduos foram expostos à

restrição alimentar (21), sendo mais intenso quando os indivíduos foram expostos à fome no primeiro trimestre da gestação.

Sabe-se que o metabolismo lipídico materno está associado ao crescimento, acúmulo de gordura e metabolismo lipídico fetal. Estudos demonstram que estímulos nutricionais ocorridos em períodos críticos do desenvolvimento, como a gestação e o período pós-natal, alteram permanentemente o processo fisiológico, aumentando o risco de doenças crônicas no adulto (40-42).

A metilação das citosinas nas ilhas CpG têm se mostrado sensível a estímulos ambientais, incluindo condições ambientais *in utero* e no período pós-natal. As modificações epigenéticas contribuem para a patogênese da obesidade e complicações relacionadas. Estudos que os níveis de metilação de DNA em genes candidatos relacionados com desordens metabólicas e obesidade estão diminuídos no sangue e tecido adiposo de pacientes obesos e respondem a intervenções que envolvem dieta hipocalórica e exercícios. Destes genes, aqueles que vêm recebendo maior atenção são os genes da leptina (LEP) e da adiponectina (ADIPOQ) (19).

Estudos recentes demonstram que os mecanismos epigenéticos têm papel chave no controle da adipogênese, alimentação e homeostase energética, indicando que o papel da epigenética na nutrição humana e obesidade é uma relevante área de estudo para determinar os mecanismos moleculares envolvidos nas desordens metabólicas (26).

Atualmente, foca-se muito nos “Primeiros 1000 dias do bebê”. Esta ideia provém do fato dos primeiros 1000 dias de desenvolvimento serem a melhor janela para a programação da saúde e da doença no indivíduo. As evidências sugerem que as intervenções realizadas durante este período podem surtir efeitos ao longo de toda

a vida do indivíduo. Acredita-se que estes processos estejam intimamente ligados às modificações epigenéticas. A Fig. 1 esquematiza a hipótese que propõe que o risco de doenças relacionadas à obesidade/adiposidade sejam profundamente influenciadas por modificações epigenéticas durante o período intrauterino e pós-natal (43).

Três metanálises, incluindo a de Yan *et al*. 2014, demonstraram que a amamentação é um fator protetivo para obesidade. Crianças amamentadas por um perídio inferior atrês meses apresentam um pequeno efeito protetivo para obesidade, enquanto aquelas que mamaram por mais de sete meses apresentam uma proteção significativamente maior (44-46).

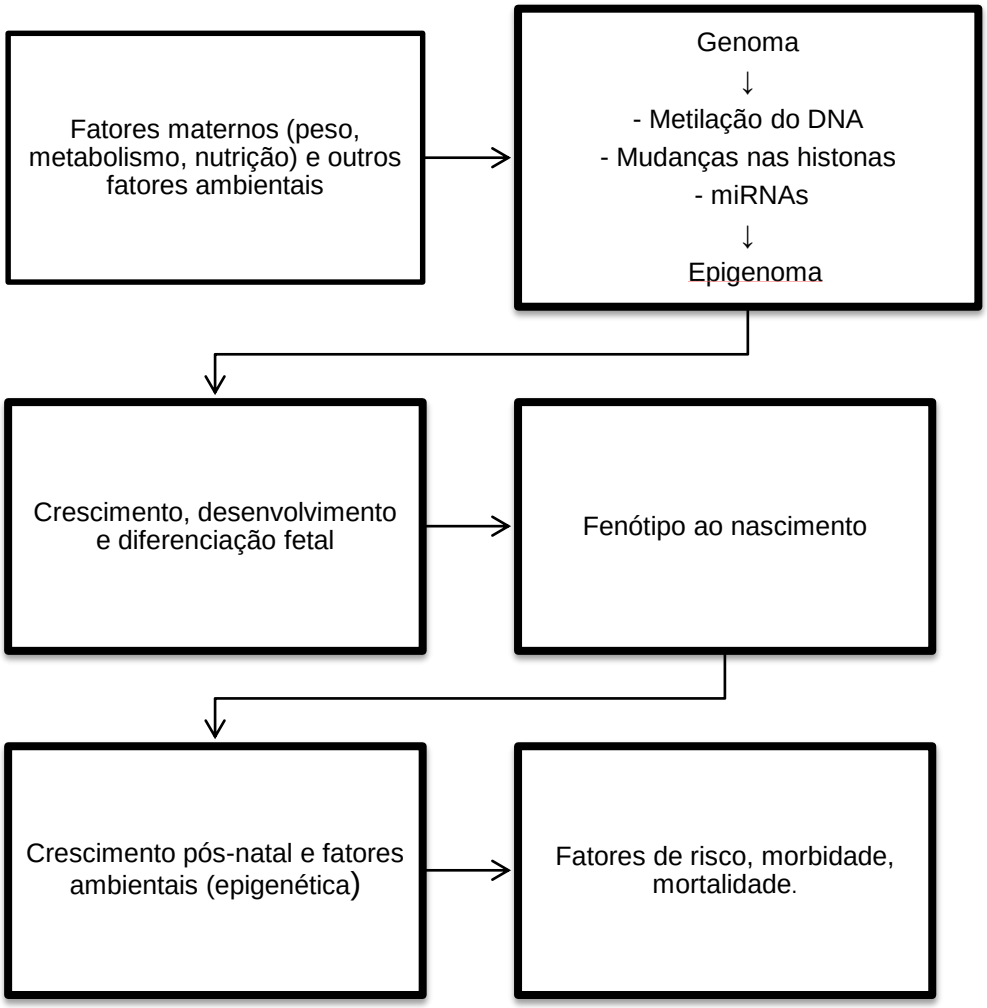

Fig 1. Influência do epigenoma no fenótipo dos indivíduos. Adaptado de Yajnik, 2014

Diversos estudos já foram realizados com a coorte estudada na presente Tese, demonstrando o impacto positivo de uma intervenção nutricional realizada no primeiro ano de vida sobre o tempo de amamentação exclusivo, diminuindo a ocorrência de morbidades nas crianças do estudo. Além disso, já foi relatado que a diminuição do número de horas de sono nas crianças está associada com aumento do IMC. O hábito de assistir televisão foi associado ao aumento da circunferência da cintura e o aumento do consumo de alimentos ultraprocessados foi associado com aumento da obesidade central. Foram ainda encontradas associações entre variantes em genes envolvidos na fisiopatologia da obesidade (como *FTO*, *LEPR*, *PPARG, 5HTTLPR* entre outros) com alterações fenotípicas associadas a obesidade (8, 11, 12, 47-52).

Dadas as evidências apresentadas, pode-se perceber que eventos adversos intrauterinos têm sido associados o desenvolvimento de obesidade e doenças cardiometabólicas na vida adulta. Entretanto, os mecanismos subjacentes a esta “programação fetal” ainda não estão totalmente esclarecidos. Igualmente, é sabido que fatores ambientais pós-natais, tais como a amamentação e a introdução adequada de alimentação complementar, são fatores protetivos para o excesso de peso e obesidade. Os mecanismos através dos quais se dá esta proteção não são completamente conhecidos. Estudos realizados na área da genética permitem a formulação da hipótese de que a desregulação epigenética possa ser o resultado da interação destes fatores ambientais sobre o genoma humano. O mecanismo que vem sendo mais explorado atualmente é a metilação de DNA, que já apresentou relação com a “programação fetal” em diversos estudos com modelos animais (53). O papel da metilação do DNA na “programação fetal” e pós-natal associada ao desenvolvimento de excesso de peso e obesidade infantil necessita ser melhor

elucidado, através de medidas de metilação do DNA comparadas com medidas associadas ao desenvolvimento de obesidade. Da mesma forma cabe avaliar o papel das DNMTs neste processo, visto que elas são responsáveis pela catálise das reações de metilação do DNA. Neste contexto, a DNMT3b, que está envolvida com o processo de metilação *de novo*, merece atenção especial.

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3. OBJETIVOS

3.1. Objetivo geral

Determinar o impacto de uma intervenção nutricional e de um polimorfismo do gene *DNMT3B* (rs2424913) no perfil de metilação e seu impacto no ganho de peso corporal em uma coorte de crianças acompanhada desde o nascimento até os doze anos de idade.

3.2. Objetivos específicos

- Determinar o perfil de metilação global aos 4 anos de idade das crianças da coorte em estudo.

- Comparar os perfis de metilação entre crianças submetidas à intervenção nutricional no primeiro ano de vida e controles, segundo os “Dez passos para alimentação saudável para crianças até 2 anos de idade”, do Ministério da Saúde.

- Relacionar a variação destes perfis de metilação com fatores pré-natais, como ganho de peso da mãe e tabagismo durante a gestação e pós-natais, como os padrões nutricionais da criança, especialmente no que se refere ao período de amamentação.

- Relacionar os níveis de metilação com os genótipos para o polimorfismo do gene DNMT3B (rs2424913) apresentados pelas crianças da coorte em estudo.

- Relacionar estes genótipos com características antropométricas associadas ao desenvolvimento de obesidade e alterações metabólicas nas crianças desta coorte.

4. ARTIGO 1

Impact of maternal dietary counselling in the first year of life on DNA methylation in a cohort of children.

Artigo publicado no periódico Genetics and Molecular Biology

Impact of maternal dietary counselling in the first year of life on DNA methylation in a cohort of children.

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Abstract

Epigenetic changes established during prenatal and early life, including DNA methylation, have been suggested as potential mediators of environmental exposures on later life outcomes, especially cardiometabolic complications and overweight. The effect of a dietary intervention performed in the first year of life on global methylation profile in leukocytes samples from a cohort of children born from 2001 to 2002 in southern Brazil was examined. Overall methylation measurements were performed on DNA samples from 237 children at 4 years old. A highly significant difference in the mean values of methylation was found between the control and intervention groups (intervention group mean: 2.199 ± 1.306%, control group mean: 1.649 ± 1.114%, *T*-test = 3.24, P = 0.001). The nutritional counseling in the first year of life has increased breastfeeding time and stimulated the development of healthier eating habits. Therefore, it is possible that these factors, together, had contributed to increase global DNA methylation. These alterations in the early stages of life can have a positive impact, influencing the remodeling of epigenetic mechanisms that can prevent complex diseases such as obesity and cardiovascular diseases in adult life.

Keywords: epigenetics, DNA methylation, dietary intervention, metabolic diseases.

Introduction

The world has witnessed, in the last decades, a dramatic increase in the incidence of childhood overweight, obesity, hypertension, dyslipidemia, insulin resistance, and diabetes, which have been considered serious public health problems [1, 2]. An emerging hypothesis for this increase is that higher exposure to an “obesogenic” environment in early life may contribute to this epidemic in childhood. Such metabolic alterations have a multifactorial etiology that involves complex interactions among genetic background, hormones and different environmental factors [3, 4].

Epigenetic processes are involved in regulation of gene expression and exhibit plasticity to environment during development. Animal models and human data show that epigenetic alterations can underlie these interactions and their impact on metabolic changes [5-7], as exemplified by the Dutch famine of 1944-45 studies [8]. DNA methylation is the most investigated epigenetic mark. It is essential for normal development and contributes to the gene regulation. DNA methylation is usually associated with repression of gene transcription, and therefore, low levels of methylation, usually in the promoter regions of the genes, lead to increased protein expression [2, 9-11].

Meta-analyses showed that breastfeeding is a protective factor for obesity and global DNA methylation patterns are known to be altered by early nutrition [12, 13]. One of the most important moments to prevent diseases of adulthood is the early life. Understanding as maternal diet and early nutrition modulate the embryonic, fetal and perinatal environment through epigenetic changes is important in reducing the risk of obesity and metabolic disturbances risk. Yajnik (2014) recently have focused on the

“first 1000 days” of life as the most important window in the programming of health and disease and offer hope that intervention in early life could help prevent these disorders [1, 6, 14, 15].

Intervention studies are essential in the elucidation of the impact of the early life environment on the epigenetic markers. Longitudinal cohort studies are costly to perform and sustain, because they should include extensive, prospectively collected data and biological samples at multiple time points across the life course. However, these studies can provide an opportunity to increase the understanding of the dynamic nature of epigenetic patterns and how changes occur in response to environmental, lifestyle and behavioral factors [16]. Intervention studies regarding the relationship between epigenetic alterations, early life exposures and metabolic diseases development, as overweight, obesity, hypertension, dyslipidemia, insulin resistance, diabetes in adult life in humans are still scarce.

The present group has performed, since 2001, a randomized trial designed with the objective to assess the impact of dietary counseling given to mothers during the first year of infants’ lives on food consumption, nutritional status, and lipid profiles of 500 children from a low-income population setting from the south of Brazil. These children have been under follow-up since birth until 12 years old. Our studies have shown that the intervention has positively influenced breastfeeding and reduced the occurrence of morbidity, as diarrhea, respiratory problems, dental caries and medication use [17]. Similarly, dietary counseling during the first year of life was found to improve the healthy eating index measured in these children at preschool age [18]. Genetic variants associated with overweight and increase in body mass index at 4 years of age were also found in this study population [19-21].

While the effect of the fetal and early childhood environment on health outcomes in adult life has been widely discussed, the empirical assessment of this hypothesis remains a challenge [22]. Based on this information and knowing that the early stages of life are considered critical windows for genetic and epigenetic modifications and for the establishing habits that will influence lifelong health patterns, we hypothesized that the effect of dietary counseling could be due to changes on methylation levels in this cohort. Therefore, the present study evaluated DNA samples collected at 4 years old from children enrolled in an intervention study to promote healthy feeding practices during the first year of their lives in order to evaluate the effect of this intervention on global DNA methylation levels.

Materials and Methods

Patients

This research is part of a cohort study that included 500 children born from 2001 to 2002 in a public Hospital of Southern Brazil. Inclusion criteria were healthy, singleton, full-term (>37 weeks) and normal birth weight (>2500 g). Exclusion criteria were HIV-positive mothers, infants with congenital malformations or infants who were admitted to neonatal intensive care units, and individuals with breastfeeding impediments. The children were randomly assigned in control and intervention groups at birth. The intervention group followed a diet based on the “Ten Steps to Healthy Feeding”, as advised by Brazilian Ministry of Health, during the first year of life [17]. For children of the intervention group, ten home visits were performed, the first in 10 days after birth, monthly up to 6 months of life, then at 8, 10 and 12 months. The home visits for children of the control group occurred at 6 and 12 months of life. During home

visits anthropometric, dietary, socioeconomic, demographic and health data were collected. These data and biological samples for biochemical evaluation were collected from all children at the ages of 1, 4, 8 and 12 years. Randomization data are summarized in Figure 1. More detailed information on randomization and data collection of this cohort trial are available in Costa, Campagnolo *et al.* 2017 [23]. The study was approved by the Research Ethics Committees of the institutions involved.

Nutritional variables analysis

The Healthy Eating Index (HEI) is an indicator used to measure diet quality. It evaluates through 10 components different aspects of healthy nutrition, based on the individual's nutritional need. For this study, the consumption of 6700 KJ (1600 Kcal) was recommended, a criterion adopted for children between 3 and 4 years. Further information on the calculation of this indicator in this sample can be found in Vitolo, Rauber *et al.* 2010 [18].

Were considered as high sugar density food when they had 50% or more sugar in 100g of composition (e.g. candies, soft drink, sugar and honey), and as high lipid density food when they had 30% or more fat content in 100g of composition (e.g. salty snacks, filled cookies and chocolate).

Global Methylation Analysis

DNA samples were obtained from peripheral leukocytes using a standard salting-out technique from biological samples collected at 4 years old. DNA methylation was quantified by an enzyme-linked immunosorbent assay. The degree of DNA methylation was expressed in terms of percent methylation. The Methylflash™ Methylated DNA Quantification Kit (Colorimetric, Base Catalog # P-1034; Epigentek Group INC., Farmindgale, NY, USA) was used for the analysis of global methylation.

Experiments were performed in accordance with the manufacturer´s instructions using appropriate controls and input of DNA was 80ng per sample.

Statistical Analysis

The homogeneity of the sample related to anthropometric, dietary, demographic and socioeconomic variables distribution among the control and intervention groups subsequent to randomization was verified using the chi-square test. Methylation data were asymmetrically distributed and therefore logarithmically transformed prior to statistical analyses. Other variables evaluated presented normal distribution. *T test* was used to compare the means of methylation between the intervention and control groups. Statistical analyses were performed in SPSS version 22.0 for Windows software (IBM, Armonk, NY) and differences were considered significant when $p<0.05$.

Results

Of the 500 children initially allocated to control (n=300) and intervention (n=200) groups in 2001-2002, 354 were found for 4th-year interviews in 2005-2006. From these, 345 children with complete data were obtained [23]. For this study, global methylation analyses were performed in 250 samples of these children aged 3-4 years old whose DNA was available, being 151 samples from the control and 99 from the intervention group (Fig. 1). After the evaluation of the distribution of the methylation levels, presented as percentage of deoxy-methyl cytosine (% dmC) in the whole sample, 13 samples were considered as outliers, because they presented levels outside the range of (1.5 x interquartilic interval) from the first or third quartile. These samples were excluded from further analyses.

From the 237 children analyzed in study, 132 (55.69%) were boys. The proportion of boys and girls was not different between groups (Table 1). Both groups were also similar regarding pre and postnatal variables evaluated, such as total breastfeeding duration, gestational weight gain, maternal smoking during gestation. However, exclusive breastfeeding time was significantly higher in the intervention group. A highly significant difference in mean global methylation values was found between control and intervention groups (intervention group mean: 2.199 ± 1.306%; control group mean: 1.649 ± 1.114%; *T test* = 3.24; *P* = 0.001) (Fig2).

The influence of the other available categorical variables considered possibly relevant for this outcome (%dmC) was also evaluated (Table 2). We analyzed pre-gestational (body mass index of the mother), gestational (smoking, mother weight gain, sex of the child), post-natal (type of delivery, exclusive breastfeeding up to 4 months, total breastfeeding time, group in which the child was allocated, consumption of high lipid and high sugar density foods in the first year of life), and smoking of the mother and of someone else in the house until 4 years old. None of these variables were associated with methylation status. For the continuous variables (pre-gestational BMI, total breastfeeding duration, exclusive breastfeeding duration, amount of ingested folate and B12 vitamin in the first year and healthy eating index in the first year; suplementar Table S1) univariate regressions with %dmC were also evaluated, but none of them was significantly associated with the outcome.

Discussion

DNA methylation is an epigenetic alteration that has impact on DNA transcription, potentially modifying the phenotypic patterns. In this work, our main goal

was to verify the possibility of a nutritional counseling performed in the first year of life to have impact on global methylation of children's DNA. Furthermore, we aimed to understand how some environmental factors present in the early stages of life, such as nutritional status of mother during pregnancy, smoking, breastfeeding and gender, are related to DNA methylation. It is believed that these environmental changes may impact childhood obesity and collaborate to chronic disease development in adult life through changes in DNA methylation patterns.

As demonstrated in a previous publication by our group, the present intervention in the first year of life has positively influenced the duration of exclusive breastfeeding and improved early feeding practices [17]. A lower proportion of children with diarrhea, respiratory problems, use of medication, and dental caries in the 12-16 months period was also observed. Therefore, through our current findings, we can observe that children in the intervention group, who were exposed to better nutritional practices, such as adequate introduction of complementary feeding, reduced exposure to high lipid- and sugar-dense foods and duration of exclusive breastfeeding than controls, had higher methylation levels. In this context, we may suggest that these better practices together would impact on the methylation profile and would be protective in the long term for the development of complex diseases such as obesity.

In 2017, Hartwig, *et al*., in a systematic review, found five studies conducted in humans about the effects of breastfeeding on DNA methylation. These investigations were highly heterogeneous, but all of the papers analyzed by the authors showed interactions between breastfeeding and DNA methylation. In an article published by *Nutrients* in 2014, Verduci *et al.* hypothesize several ways how breast milk can have a beneficial effect on child's health and decrease the development of complex diseases such as obesity in adulthood. The involvement of epigenetic mechanisms in this

beneficial impact of breastfeeding, especially DNA methylation, is possible. However, it is difficult to state clearly how breastfeeding modifies the methylation profile of DNA. One of the possibilities would be the supply of nutrients that would interfere in the function of one-carbon metabolism that would regulate the availability of methyl groups for biological methylation reactions [24, 25].

To our knowledge, this is the first study to evaluate the methylation profile in a human population that underwent a dietary counseling. It is worth mentioning that the individuals were allocated to the groups through randomization and that there were no significant differences between these groups with regard to demographic and socio-economic characteristics. However, we are aware that the global methylation analysis performed here is limited and more detailed methylation analyses in specific locations of the genome are necessary. The main limitation found in the reviewed articles is the deficiency of studies on the theme, which makes the mechanisms of interaction between breastfeeding and DNA methylation still poorly understood, as well as making it difficult to evaluate confounding variables, which was also present in our study [12, 26]. On the other hand, this is a longitudinal study which used multiple time points to data collection, increasing the power to recognize the real impacts of environmental factors on epigenetic mechanisms and their influence on the pathophysiology of complex diseases of adult life [16].

Many studies have demonstrated that the nutritional status of the mother and metabolic dysregulation during gestation are crucial factors for development and health of the next generation. The Barker theory or fetal programming is the best known that proposes the influence of environmental factors on the epigenetic mechanisms involved in the obesity development [27-30]. Recently, derived from Barker theory and others, the Developmental Origins of Health and Disease (DOHaD) theory has

emerged, which describes a strong association between life events (pre and postnatal) and biological/epigenetic responses that define the risk of development diseases. The periods of greater epigenetic plasticity are the prenatal, neonatal and pubertal stages when epigenetic markers are associated with disease risk and can be modified through lifestyle changes [31, 32]. Based on these premises, we can deduce that the nutritional counseling performed in the first year of life of the children in this study impacted markedly in DNA methylation mechanism, altering the epigenetic profile of the individuals and may have been influenced by environmental factors such as exclusive breastfeeding. Given the above, we believe that the beneficial effect of breastfeeding and an adequate intrauterine environment verified in different studies can be, at least in part, mediated through changes in DNA methylation occurring within the first 1000 days of life, that persist throughout the development of the individual until adulthood. Longitudinal studies and follow-up of our cohort may be important to respond to this question.

In a previous study with this same cohort of children, it was observed that those with excessive weight gain in early life had a negative impact, increasing the susceptibility of children to have insulin resistance at 8 years old [23]. In addition, it was observed that nutritional counseling in the first year of life improved early feeding practices, also improving the lipid profile of children at 8 years old [33]. It is possible that methylation profile change may be involved in these metabolic changes.

In summary, we report the association between a nutritional intervention with global DNA methylation. Although far from conclusive, these findings reinforce that interventions in the early stages of life can have a positive impact, and proposes a mechanism for these effects through DNA methylation. Further investigations are needed to understand the complex interactions between environmental factors such

as nutritional patterns in epigenetic mechanisms, in order to predict the effect and validity of an intervention in early life as a therapeutic strategy in preventing adult diseases.

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Table 1 Descriptive characteristics of the cohort groups

		Control		Intervention		*p*-value
		n	**%**	**n**	**%**	
Sex	Boys	78	53.8	54	58.7	0.459
	Girls	67	46.2	38	41.3	
Total breastfeeding duration	<12 months	83	58.0	45	49.5	0.198
	≥12 months	60	42.0	46	50.5	
Exclusive breastfeeding duration	<4 months	106	74.1	53	58.2	0.011
	≥4 months	37	25.9	38	41.8	
Gestational weight gain	Low	40	30.1	33	40.2	0.214
	Adequate	44	33.1	27	32.9	
	Excessive	49	36.8	22	26.8	
Maternal smoking during gestation	Yes	23	16.8	8	9.3	0.116
	No	114	83.2	78	90.7	

p-values from chi-squared-tests

Table 2. Comparison of methylation levels between biodemographic variables of the sample

	Category	N	Mean	SD	*p*
Pre-gestational BMI	Eutrophic	143	1.87	1.28	0.742
	Overweight	56	1.84	1.05	
	Obese	21	1.88	1.15	
Gestational smoking	Yes	31	1.86	1.26	0.686
	No	192	1.88	1.22	
Gestational weight gain	< 9Kg	69	2.11	1.30	0.191
	9.1 to 15.9 Kg	92	1.80	1.18	
	>16 Kg	63	1.66	1.28	
Sex of the child	Boys	132	1.89	1.23	0.416
	Girls	105	1.83	1.21	
Type of delivery	Natural	123	1.79	1.23	0.148
	Caesarean	85	1.94	1.16	
High lipid density food consumption	Yes	107	1.73	1.19	0.065
	No	127	1.95	1.20	
High sugar density food consumption	Yes	62	2.02	1.38	0.628
	No	172	1.78	1.12	
HEI (4 years)	0 - 50.99	24	1.57	0.97	0.394
	51 - 80	178	1.92	1.27	
	80.01 - 100	23	1.68	1.02	
Mother smoking (4 years)	Yes	49	1.92	1.31	0.976
	No	173	1.83	1.18	
Another smoking family member (4 years)	Yes	81	1.71	1.17	0.109
	No	150	1.93	1.21	

Independent samples *t*-test with *ln*-transformed methylation levels, SD: standard deviation, HEI: Healthy eating index.

Table S1 Linear regressions analysis of global methylation

	Univariate model			
	B	**95% CI**	**R**	***p***
Global Methylation				
Pre-gestational BMI (kg/m^2)	0.013	-0.006, 0.033	0.089	0.188
Exclusive breastfeeding (months)	0.027	-0.015, 0.069	0.083	0.208
Total breastfeeding (months)	0.002	-0.007, 0.011	0.049	0.613
Folate ingest amount (µg/24 hours)	-0.034	-0.002, 0.001	0.034	0.617
B12 vitamin ingest amount (µg/24 hours)	0.002	-0.010, 0.013	0.018	0.793
HEI (score)	-0.001	-0.009, 0.008	0.012	0.855

Global methylation levels were *ln*-transformed; HEI: healthy eating index.

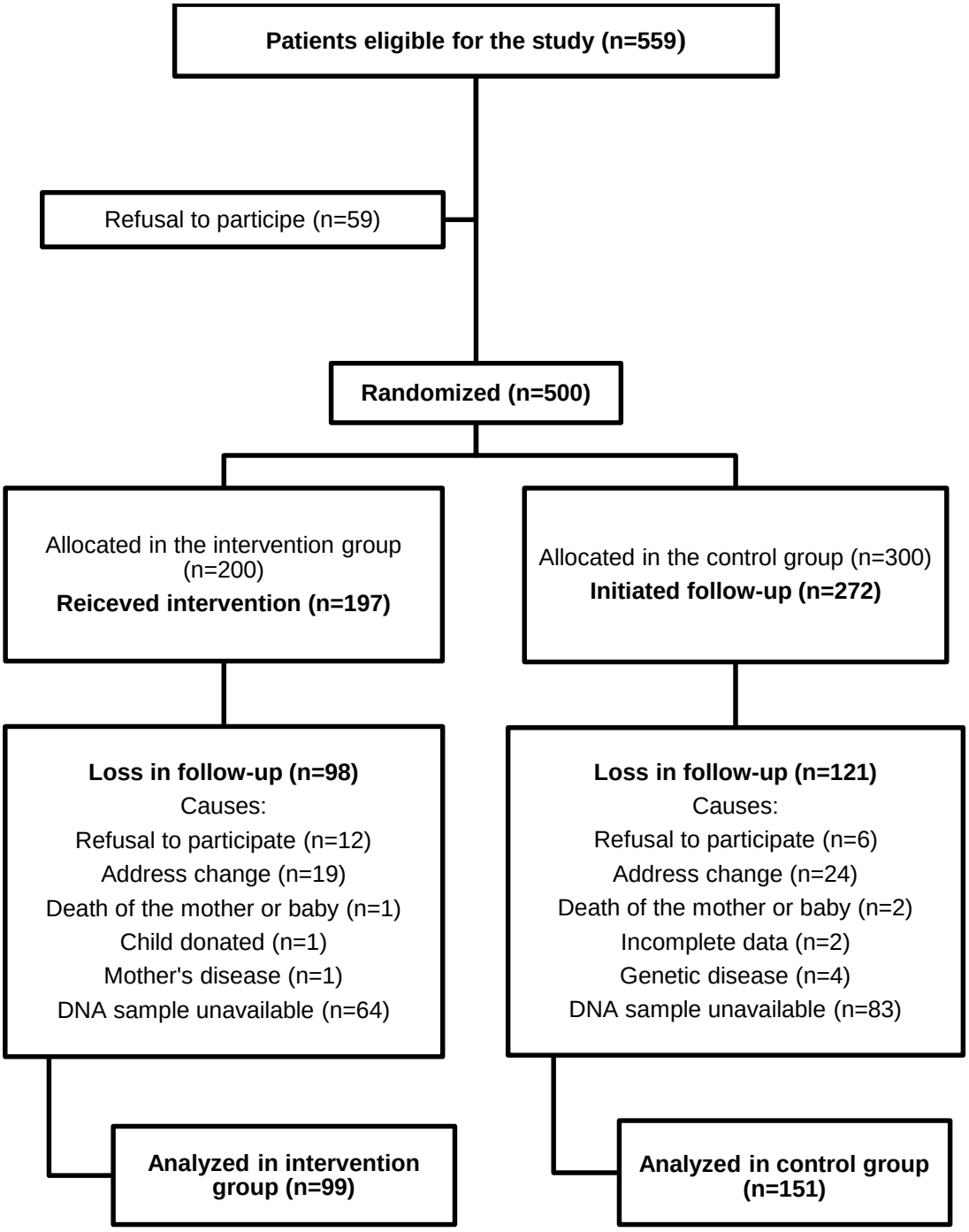

Figure 1. Study overview.

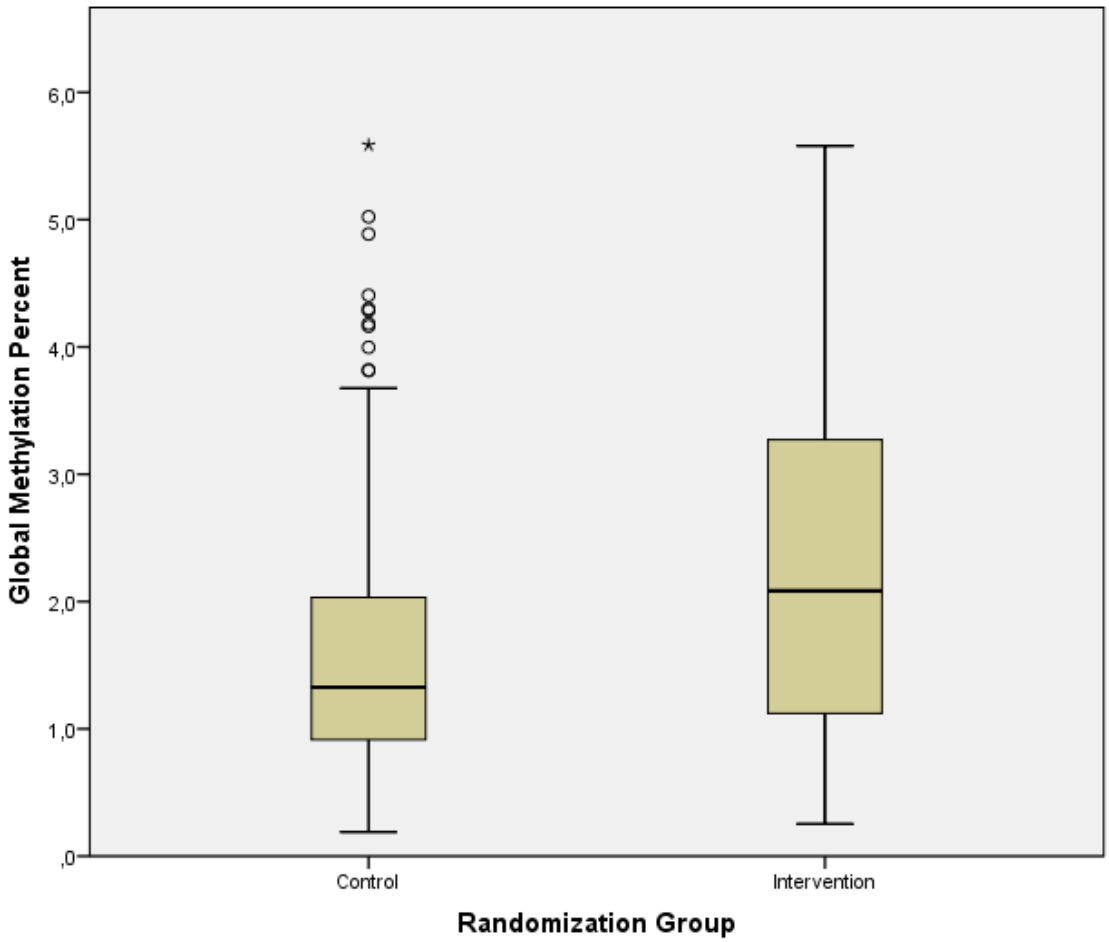

Figure 2. Methylation profile of the intervention and control groups. Data are presented as medians and range of variation. Boxes represent the interval between the first and third quartiles. Comparison was made through T-test for independent samples with ln-transformed values, *P*<0.001.

5. ARTIGO 2

DNMT3B rs2424913 gene variant is associated with DNA methylation and anthropometrics in children from 4 to 12 years old

Em preparação para submissão ao periódico *Journal of Pediatrics*

DNMT3B rs2424913 gene variant is associated with DNA methylation and anthropometrics in children from 4 to 12 years old

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Abstract

The global increase in prevalence of obesity among adults, adolescents and children has reached alarming levels, making this disease a serious public health problem. The etiology of obesity is complex and multifactorial. Currently, epigenetic alterations are being considered an important approach in order to understand the mechanisms of interaction among genes and environmental and behavioral factors involved in the genesis of obesity. In this study, we examined the association of the DNA methyltransferase 3 (*DNMT3*) gene -149 C>T variant (rs2424913) genotypes with global DNA methylation and the changes in anthropometric parameters in a cohort of 171 children followed since birth to 12 years old. Genotypes were obtained using real-time polymerase chain reaction and global DNA methylation was measured on blood samples collected at 4 years old through enzyme-linked immunosorbent assays. Our results showed that the TT genotype is associated with increase in global methylation levels at 4 years old and higher changes in body mass index, waist circumference, subscapular subcutaneous fat, body fat mass, body lean mass and basal metabolic rate from 4 to 12 years. These results suggest that this promoter *DNMT3* gene variant can be predictive of the increased risk of development of obesity in children.

Introduction

Obesity is defined as an excessive accumulation of body fat that is associated with the development of several short and long-term comorbidities, such as hypertension, diabetes, cardiovascular diseases, metabolic syndrome, among others (1, 2).

In the last decades, the global prevalence of obesity and its complications has been growing rapidly and constantly. The global prevalence of obesity increased three times between 1975 and 2016, with more than 1.9 billion overweight and 650 million obese adults in 2016. The situation is as alarming among children and adolescents as it is among adults. As a result, obesity was recognized as an important public health issue, and has come to be considered a global epidemic (2-5).

The pathogenesis of obesity is complex, depending on the interaction of genetic, environmental, and behavioral factors. It is estimated that genetic factors account for 6-85% of the heritability of excessive body weight, depending on the trait evaluated (e.g., for body mass index, the heritability was estimated among 16-85%; for waist circumference, 37-81%; for percentage body fat, 35-63%, and for waist/hip ratio, 6-30%). However, the already identified genetic variants explain less than 2% of this heritability. In most cases, obesity is polygenic, resulting from the cumulative effect of common variations in several genes in interaction with environmental factors. In this context, attention has shifted to the assessment of the interactions between genetic variants and the obesogenic environment (3, 6-8).

Epigenetics refers to the molecular mechanisms that regulate gene expression without changing the DNA sequence. Epigenetic changes have been proposed to underlie, at least in part, the mechanisms through which genes interact with the

environment, possibly being involved in the pathogenesis of adiposity and its comorbidities. DNA methylation is an important epigenetic mechanism responsible for regulation of gene expression and chromatin organization (2, 3, 9).

We have previously reported that a nutritional intervention during the first year of life in a cohort of Brazilian children that has been followed since birth is associated with methylation patterns of the study population (10). This association suggests that changes in eating habits in the early stage of life, as higher exclusive breastfeeding duration and lower sugar intake, among others, may change the epigenetic mechanisms involved in the pathogenesis of obesity in later life.

The establishment and maintenance of DNA methylation is mediated through enzymes called DNA methyltransferases (DNMTs). Currently 3 DNMTs are known in humans: DNMT1, DNMT2 and DNMT3. DNMT1 is responsible for the maintenance and stability of methylation patterns during cell division, ensuring that the daughter cell preserves the parental methylation pattern. DNMT2 appears to be involved in the methylation of small RNAs. Finally, the DNMT3 family is composed by 3 classes: DNMT3a andDNMT3b, that are involved in the establishment and maintenance of *de novo* methylation patterns during fetal development, and DNMT3l, a methylation regulator that stimulates *de novo* methylation (9, 11-13).

DNA methyltransferase 3b (DNMT3b) gene is located on the long arm of chromosome 20 (20q11.2), including 23 exons and 22 introns. A single nucleotide polymorphism (SNP), rs2424913 (-149 C>T), has been associated with increased activity in the promoter region and increased transcription of the gene (12-16).

Given that changes in DNA methylation have been associated with the risk of developing cardiometabolic disorders in adulthood and that there is evidence that

variation on the DNMT3B gene may be associated with methylation levels, the purpose of this article is to investigate the possible association between the DNMT3B -149 C> T (rs2424913) variant and the levels of overall methylation and the increased risk of excessive weight gain in children.

Materials and Methods

Study population

The children cohort analyzed in the present study was drawn from a randomized trial performed in their first year of life, as follows. The study design, phases and sample sizes are presented in Figure 1. Population and randomization procedures of this study have been extensively described in previous studies (17, 18). Briefly, 500 full-term children born from 2001 to 2002 in a public Hospital of Southern Brazilwere included. The children were randomized into intervention and control groups at birth. The mothers of the children allocated in the intervention group were instructed to provide their children a diet based on the Health Ministry of Brazil booklet called “Ten Steps to Healthy Feeding” during the first year of life the child (18). Home visits were made during the first year of life to children of both groups. Children of the intervention group received ten home visits, the first in 10 days after birth, monthly up to 6 months of life, then at 8, 10 and 12 months. For the children of the control group two visits at 6 and 12 months of life were performed. In these home visits, anthropometric, dietary, socioeconomic, demographic and health data were collected. These data and biological samples for biochemical evaluation were collected from all children found at the ages of 1, 4, 8 and 12 years.

Anthropometric data were collected during four phases of the study. The

weights of children were measured using a portable digital scale (Techline®; São Paulo, Brazil) and their heights were measured using a portable stadiometer (Seca®; Hamburg, Germany) with the children dressed in light clothes and no shoes. From the data collection in the 3-4 years of children, triciptal and subscapular skinfold thicknesses and waist circumference were measured. The body mass index (BMI) was calculated [weight(kg)/height(m)^2], and transformed in BMI Z-scores using the World Health Organization Growth Standards charts specific for sex and age. Children were classified as overweight when BMI Z-score was >+1.

In the subsequent data collections (8-9 and 12-13 years old), children's bioimpedanciometry was performed (Byodinamics 450®, Shoreline, USA), obtaining body fat weight (kg), lean mass weight (kg) and basal metabolic rate (kcal/day). The bioimpedanciometry data collected from children who were not fasting for 4 hours, did physical exercise in the last 24 hours, drank alcohol in the last 48 hours, took diuretics in the last week, girls who were menstruating and those who did not urinate for at least 30 minutes before the exam were disregarded because they could interfere in the analyzes.

Ethical approval to undertake this study was obtained from the Research Ethics Committees of the institutions involved. All mothers of the children included in the cohort signed an informed consent form when they were invited to participate of this study.

Genetics and Epigenetics analysis

DNA samples were obtained from peripheral leukocytes using a standard salting-out technique from blood samples collected at 4 years old.

One single nucleotide polymorphism (SNP) in *DMNT3B* (rs2424913) with the potential to be associated with *de novo* methylation was selected (12, 19). This SNP was analyzed by real-time polymerase chain reaction using hydrolysis probes to discriminate genotypes (Taqman; Applied Biosystem, Foster City, CA).

Global DNA methylation quantification was performed by enzyme-linked immunosorbent assay using Methylflash™ Methylated DNA Quantification Kit (Colorimetric, Base Catalog # P-1034; Epigentek Group INC., Farmindgale, NY, USA). Experiments were performed in accordance with the manufacturer´s instructions using appropriate controls and DNA input was 80ng per sample.

Data analysis

The chi-square test was used to verify the homogeneity of the sample regarding anthropometric, dietary, demographic and socioeconomic categorical variables distribution among the control and intervention groups subsequent to randomization.

Accordance of genotype frequencies distribution to Hardy-Weinberg equilibrium expectations was also calculated using the chi-square test. This same test was used to examine differences in genotype and allele frequencies between relevant subgroups according to categorical variables.

Global methylation data were asymmetrically distributed and were logarithmically transformed to attain normal distribution before the statistical analyses were performed. Other continuous variables evaluated presented normal distribution.

Analysis of variance for repeated measures were performed to evaluate the longitudinal effects of genotypes over anthropometric variables.

Independent samples Student's *T-test* was used to compare the means of

continuous variables of interest, such as DNA methylation and anthropometric data between genotypes. Genotypes were grouped into carriers of the C allele (CC+CT) and homozygous for the T allele (TT) for these analyses.

Correlation analyses between DNA methylation levels and BMI were performed using the non-parametric Spearman's correlation coefficient.

Statistical analyses were performed in SPSS version 22.0 for Windows software (IBM, Armonk, NY) and differences were considered significant when $p<0.05$.

Results

At baseline, in 2001-2001, 500 children were allocated into the study: 200 children in the intervention group and 300 in the control group. From these, only 344 were found and presented complete data were available after the fourth year interviews in 2005-2006 (Fig.1). For the global methylation analysis, 237 samples of these children at 3-4 years of age were analyzed. Subsequently, 171 DNA samples were available for analysis of the rs2424913 polymorphism, with 104 samples from the control group and 67 from the intervention group.

The main sociodemographic characteristics of the samples analyzed herein according to the group where children were initially randomized are presented in Table 1. Sex proportions, prevalence of mother's smoking during pregnancy, mother's schooling and family income were not different between the intervention and control groups. The proportion of women who breastfed their children for 4 months or more was higher in the intervention group than in controls (40.6 vs. 28.6%, $p = 0.024$).

The anthropometric variables analyzed during the various stages of the study (at birth, 1, 4, 8 and 12 years) are presented in Table 2. None of these variables presented significant differences between groups.

Frequency of the minor C allele for the genotyped SNP (rs2424913) was 0.45. Genotype frequencies were distributed according to those expected under Hardy-Weinberg equilibrium (X^2= 0.47; p=0.49). Genotypic frequencies were not different between the intervention and control groups, as shown in Table 3.

The longitudinal effects of genotypes over anthropometric measures in the different steps of children evaluation were evaluated through analysis of variance for repeated measures and are shown in Fig.2. BMI Z-scores, waist circumference and subscapular skinfold were measured at 4, 8 and 12 years. For waist circumference and skinfolds, there was a significant difference among genotypes at the three time points (p_{SNPs}:0.017 and 0.024, respectively). For BMI Z-scores, the means were marginally different (P=0.059). As can be seen in the graphs and post-hoc tests, these differences were among the children with the TT genotype and the carriers of the C-allele. Body fat weight, lean mass weight and basal metabolic rate were measured through bioimpedanciometry at 8 and 12 years. All these three measures were also significantly different among genotypes. These differences were observed in the TT genotype, as well.

Interaction analyses between time collection and genotypes were performed and demonstrated that genotype effect is independent of the time of data collection for most of characteristics evaluated (p_i> 0.05). As can be seen in the graphs presented in Fig. 2, the difference in the behavior of genotypes remained at different times of data collection.

To further explore these results, we also compared the means of these measures transversally between C-carriers and TT homozygotes at the different ages through T-tests for independent samples (Table 4). Children aged 3-4 and 8-9 years old with the TT genotype presented higher BMI than the mean expected for their age and sex than carriers of the C allele. However, at 12-13 years the difference between BMI Z-scores means was not significant (p=0.146). For mean waist circumference and subscapular skin fold significant differences were obtained in all stages of the study (respectively, 3-4 years old, p=0.013 and p=0.075; 8-9 years old, p=0.035 and p=0.017; 12-13 years old, p=0.007 and p=0.014). Other significant differences were observed for body fat weight at 8-9 and 12-13 years old, lean mass weight and basal metabolic rate at 12-13 years old.

The global methylation levels evaluated at 3-4 years old of children presented significant difference between genotypic groups (p= 0.030) being the mean of global methylation for TT genotype (n=50) 2.13 (SD±1.27) and for genotypes carrying the C allele (n=121) was 1.78 (SD± 1.17).

Correlation analyses between global methylation levels at 3-4 years and BMI Z-scores at different ages were also performed. BMI Z-score at ages 3-4 exhibited a small but significant correlation with methylation ($r_{Spearman}$ = 0.152, p = 0.018). However, this correlation disappeared at 8 ($r_{Spearman}$ = 0.057, p = 0.401) and 12 years ($r_{Spearman}$ = -0.055, p = 0.497).

Discussion

The factors that determine global methylation levels and their impact on human health have been the focus of considerable interest to researchers due to their possible

role in the normal development of individuals as well as in the emergence of diseases. We investigated the hypothesis that a functional promoter polymorphism in the DNA methyltransferase 3 gene was related to weight gain and body fat measurements in children accompanied from birth to 12 years of age. The main result found was that individuals with the homozygous TT genotype had higher measures of total body mass, measures of body fat and basal metabolic rate than individuals with the C allele, as well as higher levels of global methylation. The observed associations were quite consistent considering that they remained over time and several obesity-related phenotypes were carefully evaluated.

At the beginning of the study, children were randomized into intervention and control groups. As can be seen from the results presented in Tables 1, 2 and 3, the two groups of children evaluated were homogeneous in relation to anthropometric and sociodemographic variables, thus not representing confounding variables in our analysis. The allelic and genotypic frequencies of the analyzed polymorphism were also similar in both groups. Therefore, analyzes of the association between the investigated genotypes and anthropometric measurements were performed in the cohort as a whole. Previous studies by our group have demonstrated that the intervention in the first year of life has positively influenced the duration of exclusive breastfeeding and improved early feeding practices (10, 18, 20), but it did not have any effect over anthropometric measurements of the children.

The longitudinal analyzes carried out in the present study showed that individuals homozygous for the T allele from 3-4 years of age have a higher BMI, waist circumference and subcutaneous fat than heterozygous individuals for the C allele. In addition, after 8 years of age, TT individuals also had higher lean mass, fat mass and basal metabolic rate compared to individuals homozygous for the C allele and

heterozygous, whose anthropometric variables were quite similar for BMI. The effect of the TT genotype on BMI seems to be reduced at 12 years of age, when it lost its significance. However, it is clear that the effect of the rs2424913 polymorphism is significant for all the variables analyzed and that the difference between the effects of the genotypes remains over time in the different phases of the study. To clarify whether this effect remains along the lifetime, it is of paramount importance to follow up on this sample and to verify the impact of this gene variant in other samples of adolescents and adults.

Potter et al. (2013) evaluated 2 polymorphisms located in the DNMT1 and 8 variants located in the DNMT3 gene of a sample of 333 newborns from UK and found that the rs2424913 SNP was the one with the greatest association with global methylation levels (21). The association reported by them was in the same direction of that reported herein, with individuals with the T-allele presenting higher methylation levels. These authors also found an association between the maternal genotype for this polymorphism and the levels of overall methylation of children at birth.

Shen et al. (2002) have demonstrated that the rs2424913 T-allele is functional, causing the increase of the activity of the DNMT3 gene, which is also in line with our findings. In 2016, another study suggested that increased expression of DNMT3b contributes to the dysregulation of adipose tissue, inflammation and insulin resistance in obesity and that this fact is associated with increased methylation again, reaffirming the link between the altered expression of DNMT3b, methylation defects and obesity predisposition (16, 19, 22).

We found a significant association between the T allele and the increase in global methylation levels at 4 years of age in the present sample, which corroborates

the evidence of functionality of this SNP. These methylation levels were significantly correlated with BMI at this age. However, there was no correlation between the levels of global methylation and the increase in anthropometric measures described here at 8 and 12 years of age. However, methylation levels were only assessed at age 4 and we are aware that global methylation is a non-specific measure. Therefore, the present study does not allow to infer which genes are the target of DNA methyltransferase 3 and whether there is hyper or hypomethylation of them in obesity. The in-depth investigation of the methylome in the present and other cohorts may bring clarification about the mechanism of the association between the studied variant and the risk of weight gain in children.

Given the above, we conclude that the associations found in this study, as well as the reports from previous studies, corroborate that the TT genotype of the rs2424913 polymorphism increases the activity of the promoter region of the DNMT3b gene, leading to increases in the level of global methylation, changing anthropometric characteristics associated with increased risk of developing obesity in children.

Further studies are needed to understand the mechanisms by which methylation changes are involved in the development of obesity, but our findings reinforce the evidence that the best time for any intervention to alter these epigenetic mechanisms and prevent the development of obesity is at the beginning of life.

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Table 1. Sociodemographical characteristics of children according to trial group

Characteristics	Categories	Randomization Group				$p^{\#}$
		Intervention		Control		
		n	%	n	%	
Sex	Boys	98	56.3	136	55.3	0.833
	Girls	76	43.7	110	44.7	
Exclusive breastfeeding	<4 months	79	59.4	135	71.4	0.02
	≥4 months	54	40.6	54	28.6	4
Mother's schooling	≤ 8 years	99	73.3	133	70.0	0.51
	> 8 years	36	26.7	57	30.0	2
Maternal smoking during pregnancy	Yes	19	13.2	31	16.9	0.488
	No	125	86.8	164	84.1	
Family income at 12 months	≤ $300	115	85.8	152	81.3	0.284
	> $300	19	14.2	35	18.7	

[#]Chi-squared tests

Table 2. Anthropometric characteristics of children according to trial group at birth and at 1, 4, 8, and 12 years old

Characteristics	Categories	Randomization Group: Intervention n	Intervention % or Mean ± SD	Control n	Control % or Mean ± SD	*p*
At 12 months						
Weight (kg)		163	9.9 ± 1.2	233	9.9 ± 1.2	0.942[&]
Height (cm)		163	75.0 ± 2.9	234	75.5 ± 3.3	0.141[&]
BMI z-score		134	0.66 ± 1.07	187	0.57 ± 1.09	0.506[&]
Overweight	**Yes**	58	35.6	85	36.5	0.855[#]
	No	105	64.4	148	63.5	
At 3-4 years						
Weight (kg)		144	17.0 ± 2.8	200	16.8 ± 2.5	0.358[&]
Height (cm)		144	103.1 ± 4.4	200	103.2 ± 4.7	0.772[&]
BMI z-score		144	0.40 ± 1.26	200	0.20 ± 1.04	0.118[&]
Waist circumference (cm)		139	51.0 ± 3.7	197	50.7 ± 3.6	0.474[&]
Subscapular skin fold (mm)		139	6.05 ± 2.55	196	5.74 ± 2.29	0.255[&]
Overweight	**Yes**	31	21.5	40	19.9	0.712[#]
	No	113	78.5	161	80.1	
At 8-9 years						
Weight (kg)		128	27.51 ± 6.2	181	26.8 ± 5.6	0.306[&]
Height (cm)		128	127.1± 6.9	181	127.0 ± 7.0	0.945[&]
BMI z-score		128	0.51 ± 1.45	176	0.29 ± 1.31	0.166[&]
Waist circumference (cm)		128	57.9 ± 7.3	181	56.4 ± 6.2	0.062[&]
Subscapular skin fold (mm)		128	8.25 ± 5.25	181	7.70 ± 4.66	0.339[&]
Body fat weight (kg)		126	5.9 ± 3.5	178	5.8 ± 2.9	0.759[&]
Lean mass weight (kg)		125	21.7 ±3.6	178	21.1 ± 3.5	0.130[&]
Basal metabolic rate (kcal/day)		126	655.4 ± 115.0	178	640.5 ± 105.7	0.251[&]
Overweight	**Yes**	38	29.7	47	26.1	0.489[#]
	No	90	70.3	133	73.9	
At 12 years						
Weight (kg)		90	49.8 ± 13.2	124	49.2± 12.6	0.703[&]
Height (cm)		90	153.6± 7.8	124	154.5 ± 7.0	0.352[&]
BMI z-score		90	0.66 ± 1.57	120	0.64 ± 1.25	0.912[&]
Waist circumference (cm)		89	69.3 ± 9.8	123	68.2 ± 9.3	0.400[&]
Subscapular skin fold (mm)		90	13.57 ± 7.89	124	12.80 ± 8.17	0.490[&]
Body fat weight (kg)		77	13.5 ± 6.3	107	13.0 ± 6.7	0.592[&]
Lean mass weight (kg)		77	35.9 ± 7.5	107	36.2 ± 7.3	0.763[&]
Basal metabolic rate (kcal/day)		77	1092.3 ± 228.4	107	1102.2 ± 222.4	0.769[&]
Overweight	**Yes**	41	45.6	46	37.7	0.251[#]
	No	49	54.4	76	62.3	

[&]*p* of T-test; [#]*p* of chi-square. n is less than the total when there are samples with incomplete data.

Table 3. Genotype and allele frequencies of *DNMT3b* gene SNP rs2424913

		Randomization group		
	Frequencies	**Intervention**	**Control**	
Genotype	**n (%)**	**n (%)**	**n (%)**	***p****
CC	34 (17.8)	11 (16.2)	23 (18.9)	
CT	99 (51.8)	34 (50.0)	64 (52.5)	0.740
TT	58 (30.4)	23 (33.8)	35 (28.6)	
C Allele Frequency	0.44	0.41	0.45	0.530

*Chi-square

Table 4. Comparison of anthropometric measures among genotypes at different ages

Characteristics	Genotypes	3-4 years old			8-9 years old			12-13 years-old		
		n	Mean ± SD	*P*	n	Mean ± SD	*p*	n	Mean ± SD	*p*
BMI Zscore	CC + CT	125	0.07 ± 0.87	0.014	115	0.11 ± 1.05	0.002	84	0.33 ± 1.26	0.146
	TT	57	0.60 ± 1.38		47	0.77 ± 1.66		30	0.79 ± 1.91	
Waist circumference (cm)	CC + CT	122	50.19 ± 2.73	0.013	115	55.57 ± 4.83	0.035	83	66.23 ± 8.02	0.007
	TT	57	51.56 ± 4.60		48	57.90 ± 8.42		31	71.56 ± 11.84	
Subscapular skin fold (mm)	CC + CT	121	5.59 ± 1.65	0.075	115	6.86 ± 3.36	0.017	84	11.08 ± 7.51	0.014
	TT	57	6.30 ± 3.63		48	8.63 ± 5.97		31	15.45 ± 10.19	
Body fat weight (kg)	CC + CT		N.A.		113	5.30 ± 2.18	0.011	74	11.76 ± 5.94	0.029
	TT		N.A.		47	6.53 ± 3.77		25	15.09 ± 7.92	
Lean mass weight (kg)	CC + CT		N.A.		112	20.72 ± 3.01	0.232	74	34.35 ± 6.19	0.003
	TT		N.A.		47	21.42 ± 3.50		25	39.09 ± 8.22	
Basal metabolic rate (kcal/day)	CC + CT		N.A.		113	630.94 ± 91.94	0.260	74	1038.99 ± 177.26	0.005
	TT		N.A.		47	651.11 ± 106.57		25	1157.23 ± 238.70	
Global Methylation (%)	CC + CT	121	1.78 ± 1.17	0.030		N.A.			N.A.	
	TT	50	2.13 ± 1.27			N.A.			N.A.	

p-values obtained by Student's T-test for independent samples. *p* for global methylation was calculated with Ln-transformed variable.

N.A.= not available for this age.

Fig.1. Design of the study and number of children evaluated at 1, 4, 8, and 12 years.

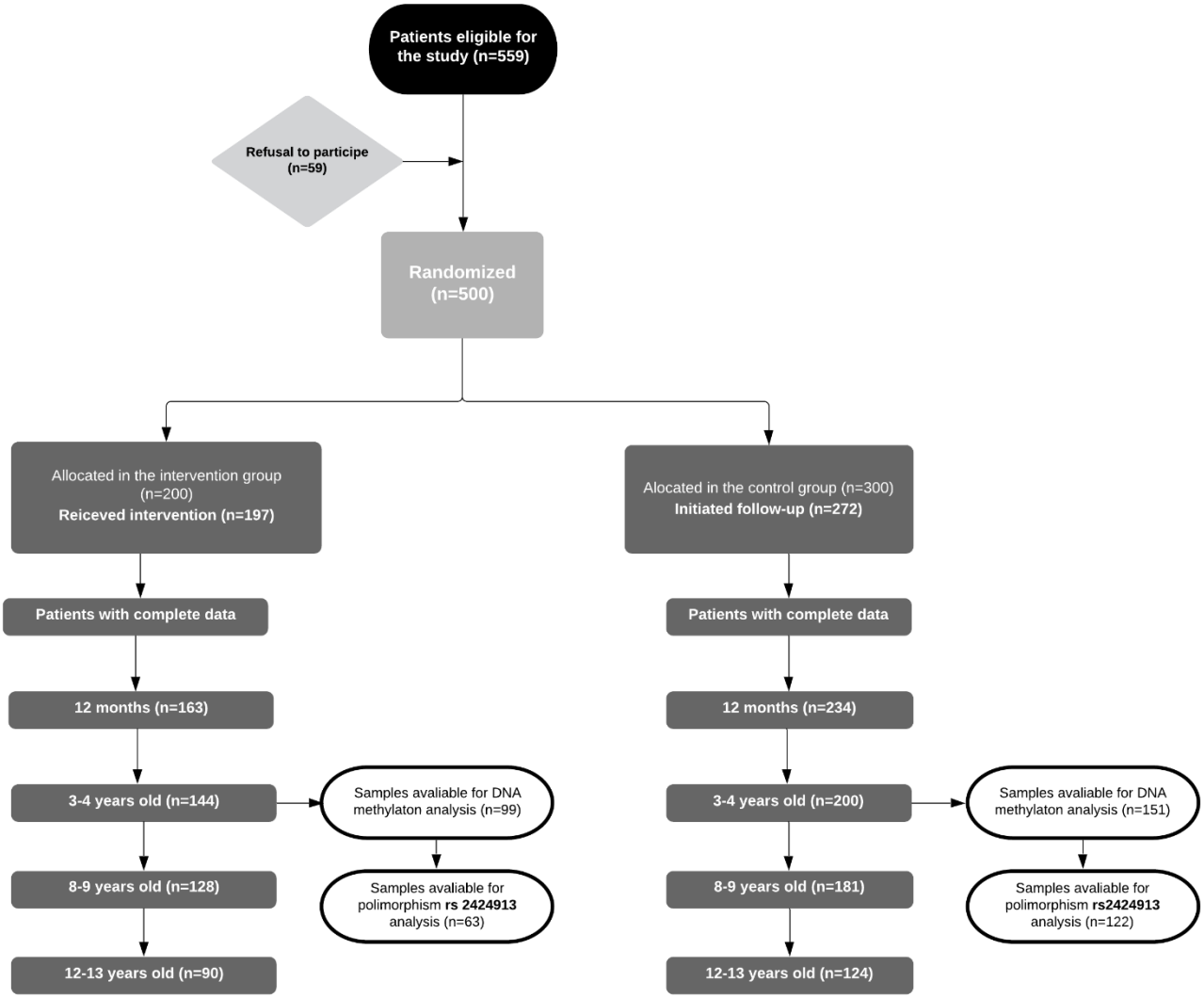
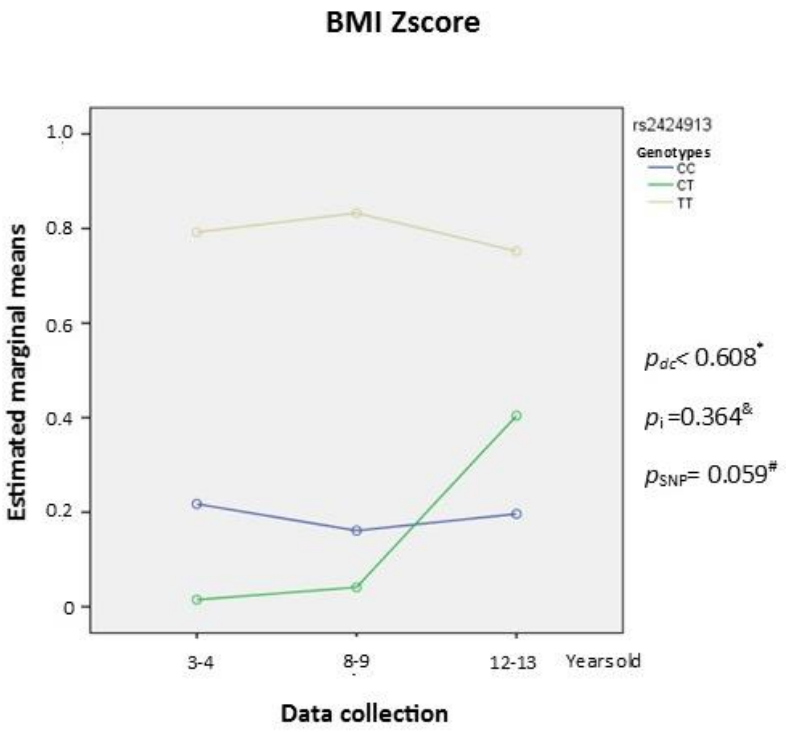
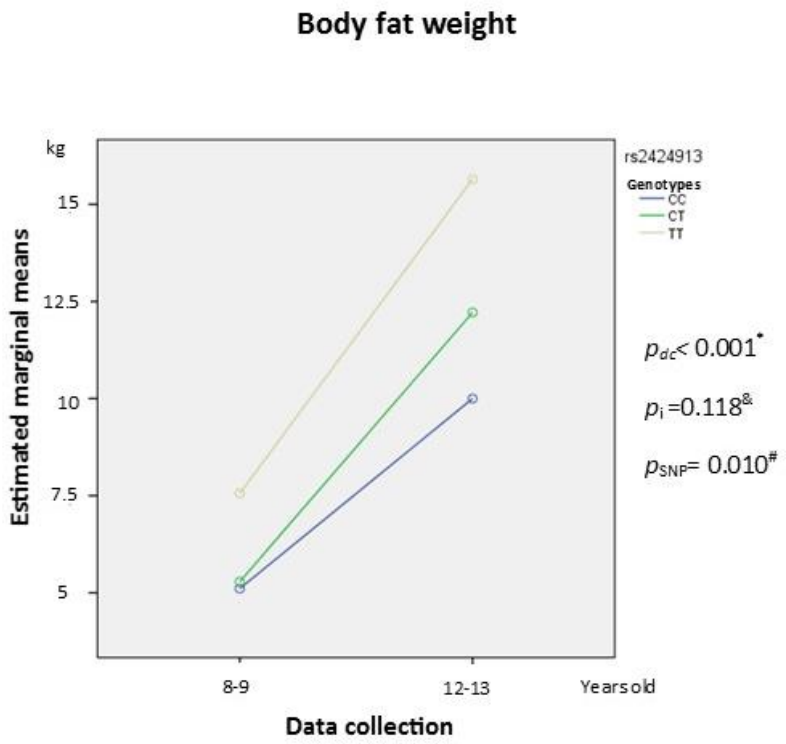


Fig.2. Comparison of anthropometric variables at different ages of children according to rs2424913 genotypes with analysis of variance for repeated measures (Part 1)



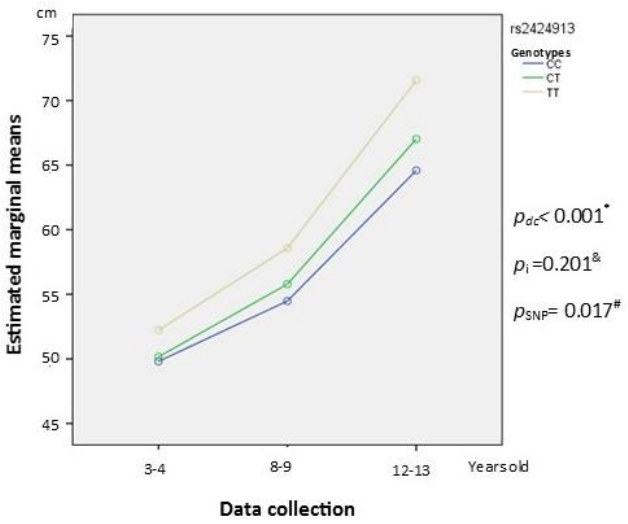
Post Hoc Bonferroni: TT x CC, p=0.280; TT x CT , p=0.062; CC x CT, p= 1.00



Post Hoc Bonferroni: TT x CC, p=0.016; TT x CT , p=0.033; CC x CT, p= 0.997

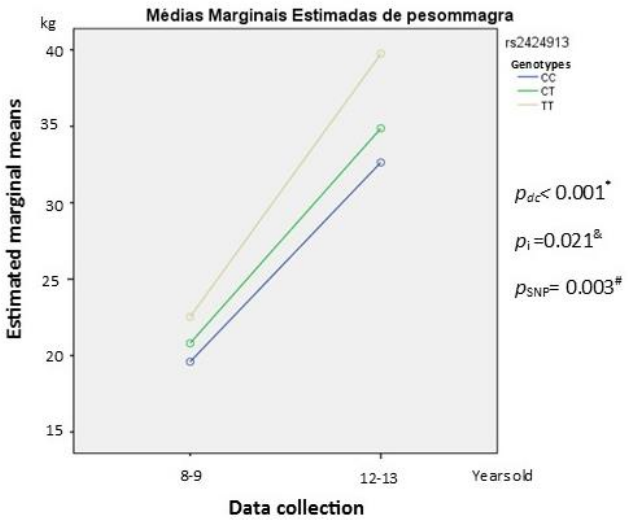
Fig.2. Comparison of anthropometric variables at different ages of children according to rs2424913 genotypes with analysis of variance for repeated measures (Part 3)

Waist circumference



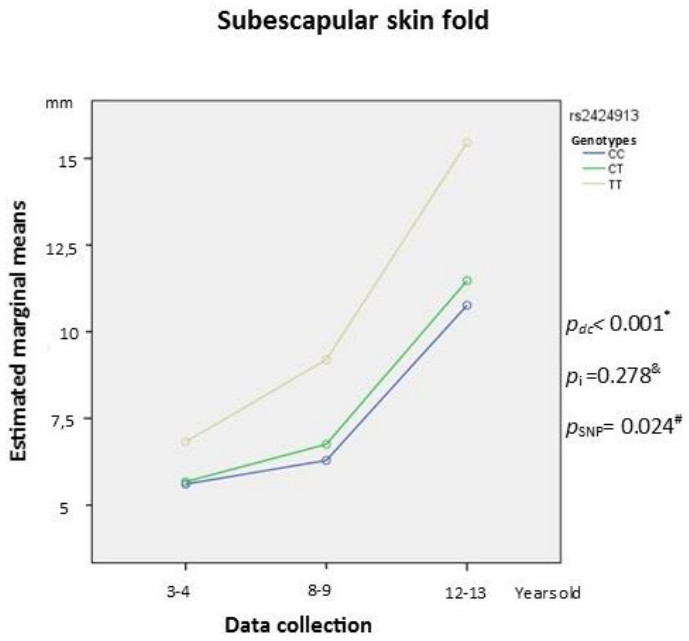
Post Hoc Bonferroni: TT x CC, p=0.032; TT x CT , p=0.049; CC x CT, p= 1.00

Lean mass weight

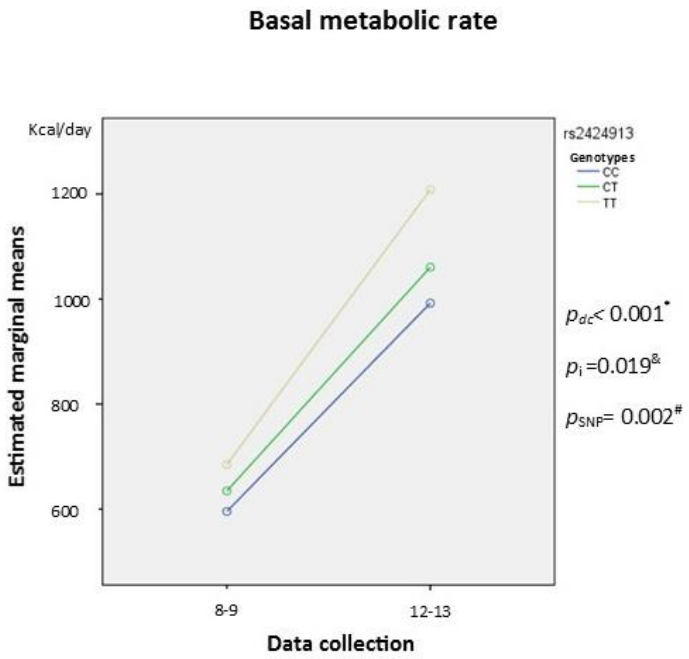


Post Hoc Bonferroni: TT x CC, p=0.003; TT x CT , p=0.016; CC x CT, p= 0.561

Fig.2. Comparison of anthropometric variables at different ages of children according to rs2424913 genotypes with analysis of variance for repeated measures (Part 3)



Post Hoc Bonferroni: TT x CC, *p*=0.081; TT x CT , *p*=0.037; CC x CT, *p*= 1.00



Post Hoc Bonferroni: TT x CC, *p*=0.003; TT x CT , *p*=0.017; CC x CT, *p*= 0.519

* *p Greenhouse*-Geisser for data collection obtained by analyzes of variance for repeated measures; & *p Greenhouse*-Geisser for data collection x DNMT3b interaction obtained by analyzes of variance for repeated measures; # *p* for DNMT3b effect obtained by analyzes of variance for repeated measures.

6. CONCLUSÕES

Os primeiros mil dias do desenvolvimento infantil, desde a concepção até os dois anos de idade, consistem em um tema de extrema relevância, pois tem sido demonstrado que esta janela temporal é crítica para a indução de distúrbios fisiopatológicos que podem levar à obesidade na infância e na vida adulta, bem como às comorbidades cardiometabólicas classicamente associadas a este fenótipo. Os padrões epigenéticos consistem em um possível mecanismo subjacente a este fenômeno de *imprinting* que ocorre neste período específico do desenvolvimento humano. Na presente Tese, buscou-se avaliar tanto o papel de influências ambientais quanto de influências genéticas nos padrões de metilação e as suas consequências para a saúde infantil.

A respeito das influências ambientais sobre os níveis de metilação global, pudemos verificar que as crianças que foram submetidas à intervenção com aconselhamento nutricional durante o primeiro ano de vida tiveram níveis de metilação global aos 4 anos de idade maiores do que aquelas que não receberam estas orientações. Esta observação nos permite inferir que as mudanças ambientais e comportamentais ocasionadas pela intervenção realizada no primeiro ano da criança modificaram o perfil de metilação global destas crianças, corroborando o fato de que o ambiente é capaz de modificar mecanismos epigenéticos como a metilação do DNA.

No que tange o comportamento do polimorfismo rs2424913 em relação à metilação global e às características antropométricas da população que estudamos, é possível afirmar que há evidências sugestivas de que este polimorfismo seja funcional. Desta forma, o genótipo TT do SNP rs2424913 aumentaria a atividade da região promotora do gene *DNMT3b*, ocasionando o aumento dos níveis de metilação global

do DNA, modificando características fenotípicas associadas ao aumento do risco de desenvolvimento de obesidade em crianças.

Através dos nossos achados, não somos capazes de inferir se a hipermetilação global do DNA é positiva ou negativa em relação ao aumento do risco de desenvolvimento de obesidade nas crianças. Acreditamos que esta seja uma medida inespecífica para ser avaliada neste aspecto. O presente estudo teve um caráter exploratório. Portanto, mais estudos neste sentido se fazem necessários, no intuito de se avaliar quais genes estão hipo ou hipermetilados nesta condição, o que permitirá uma compreensão mais aprofundada nos processos de interação entre o ambiente e a expressão gênica. Contudo, podemos sugerir que intervenções ambientais e comportamentais realizadas precocemente podem modificar características fenotípicas associadas a alterações metabólicas relacionadas à obesidade, corroborando a importância da atenção aos primeiros 1000 dias do desenvolvimento infantil, sendo este o melhor período para realizar-se uma intervenção preventiva.

Mais estudos são necessários para elucidar os mecanismos através dos quais fatores ambientais e comportamentais, modificam a expressão gênica afetando a saúde infantil e aumentando a susceptibilidade ao desenvolvimento de obesidade e comorbidades associadas. Na coorte deste estudo, poderíamos incluir ainda o estudo de expressão de DNMT3b; estudo de metilação global do DNA por ELISA nas idades de 8 e 12 anos, afim de verificar se o padrão de metilação se mantém ao longo dos anos; estudo da metilação do DNA por metodologia mais específica como pirossequenciamento para todos os momentos de coleta de dados; estudo de metilação de DNA gene específica para genes previamente associados à alterações metabólicas (LEP, ADIPOQ, FTO, PPARG, IGF, entre outros); por fim, comparar esta

coorte com a coorte da cidade de Porto Alegre, também estudada por este grupo de pesquisa.

7. ANEXOS

Anexo 1 Aprovação pelo comitê de ética

UNIVERSIDADE DO VALE DO RIO DOS SINOS - UNISINOS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Impacto da implementação do Programa dos Dez Passos para Alimentação Saudável durante o primeiro ano de vida nas condições nutricionais e de saúde na adolescência.

Pesquisador: Paula Dal Bó Campagnolo
Área Temática:
Versão: 3
CAAE: 18426813.4.0000.5344
Instituição Proponente: Universidade do Vale do Rio dos Sinos - UNISINOS
Patrocinador Principal: Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul

DADOS DO PARECER

Número do Parecer: 407.263
Data da Relatoria: 01/10/2013

Apresentação do Projeto:
O projeto refere-se à quarta fase de um estudo que iniciou em 2001, em São Leopoldo/RS, sobre o impacto de um programa nacional do Ministério da Saúde, que normatiza as diretrizes da alimentação saudável para crianças menores de dois anos. A pesquisadora principal é a professora Dra Paula Dal Bó Campagnolo juntamente com seus colaboradores. Tem como objetivo geral avaliar o impacto da implementação do Programa dos Dez passos para alimentação saudável durante o primeiro ano de vida, nas condições nutricionais e de saúde na adolescência.

Objetivo da Pesquisa:
Os objetivos da pesquisa são relevantes, abrangentes e poderão ser alcançados com a metodologia proposta. A pesquisa é de grande magnitude pois envolve uma equipe multiprofissional e faz parte de uma pesquisa maior de extrema relevância para a saúde pública.

Avaliação dos Riscos e Benefícios:
As solicitações foram atendidas.

Comentários e Considerações sobre a Pesquisa:
Além dos benefícios já citados, contribuirá na busca por respostas quanto ao efeito das exposições ambientais nas condições nutricionais e de saúde das crianças e adolescentes ao longo

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do tempo, gerando conhecimento que possa direcionar programas de promoção da saúde e prevenção de doenças. Pode servir como um método de avaliação dessa Política Pública e fornecer subsídio para tomada de decisão quanto à continuidade ou não do Programa, e podem indicar dificuldades da implementação e sugerir melhorias que contribuam para a reformulação do mesmo ao longo do tempo.

Considerações sobre os Termos de apresentação obrigatória:

As considerações foram atendidas.

Recomendações:

Foram atendidas.

Conclusões ou Pendências e Lista de Inadequações:

Foram atendidas conforme foi solicitado anteriormente.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

SAO LEOPOLDO, 26 de Setembro de 2013

Assinador por:
José Roque Junges
(Coordenador)

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