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Validação de *loci* de susceptibilidade à obesidade infantil identificados em estudos de varredura genômica e expressão diferencial de microRNAs circulantes.

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Dedico este trabalho aos meus pais,

Geni e Ivaldo.

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“É preciso força para sonhar e perceber que a estrada vai além do que se vê”.

(Los Hermanos)

ABSTRACT

Obesity is a multifactorial condition that presents a strong hereditary component. The prevalence in children is increasing indiscriminately and childhood obesity is rapidly emerging as a global epidemic. The identification of susceptibility molecular markers of overweight in early life could provide interventions in lifestyle to avoid obese children to become obese adults. This study aimed to investigate the genetic influence on childhood obesity using genetic and epigenetic markers through the evaluation of polymorphisms in susceptibility genes identified by genome wide association studies and circulating levels of microRNAs (miRNAs) in children followed up from birth. Molecular analyses were performed using real time polymerase chain reaction with hydrolysis probes. The genetic association analysis was performed in 745 children who were examined at birth, 12 months and 3.5 years of age. Ten single nucleotide polymorphisms were genotyped and nutritional and anthropometric parameters were compared between the genotypes. Nine circulating miRNAs were assessed in 38 children aged 6.2 years and their expressions were compared between lean and obese children and correlated with anthropometric and biochemical variables. Significant associations were identified for the polymorphisms *TMEM18* rs6548238, *NEGR1* rs2815752, *BDNF* rs6265 and rs10767664 with anthropometric phenotypes; and *SEC16B* rs10913469 with nutritional parameters. Differential circulating concentrations of miR-19b and miR-16-5p were identified with an expression of 1.96 and 1.41 times, respectively, higher in lean children in relation to the obese group. Plasma concentrations of these two miRNAs were inversely correlated with body mass index, and miR-16-5p was also inversely correlated with plasma levels of insulin. The results showed association of four of the ten studied polymorphisms with phenotypes related to obesity, showing that it is possible to detect the influence of genetic variants on anthropometric and dietary characteristics in early childhood. Furthermore, this study suggests a potential role of circulating miR-16-5p and miR-19b in childhood obesity as new biomarkers for early prevention and improvement of power of diagnosis of metabolic complications associated with obesity.

Keywords: children, overweight, obesity, polymorphisms, genetic susceptibility, microRNAs, plasma.

RESUMO

A obesidade é uma condição multifatorial que apresenta um forte componente hereditário. A sua prevalência em crianças está crescendo indiscriminadamente por todo o mundo e a obesidade infantil está rapidamente emergindo como uma epidemia global. A identificação, no início da infância, de marcadores moleculares de susceptibilidade ao ganho excessivo de peso corporal pode proporcionar intervenções precoces no estilo de vida a fim de evitar que uma criança obesa se torne um adulto obeso. Os objetivos deste estudo consistiram em investigar a influência genética na obesidade infantil utilizando marcadores genéticos e epigenéticos, através da avaliação de polimorfismos em genes de susceptibilidade identificados por *genome wide association studies* e dos níveis circulantes de microRNAs (miRNAs) em crianças acompanhadas a partir do nascimento. As análises moleculares foram feitas através da reação em cadeia da polimerase em tempo real utilizando sondas de hidrólise. A análise de associação genética foi realizada em 745 crianças que foram examinadas ao nascimento, aos 12 meses e aos 3,5 anos de idade. Dez polimorfismos de nucleotídeo único foram genotipados e parâmetros nutricionais e antropométricos foram comparados entre os genótipos. Nove miRNAs circulantes foram avaliados em 38 crianças com idade de 6,2 anos e sua expressão foi comparada entre crianças magras e obesas e correlacionada com medidas antropométricas e variáveis bioquímicas. Associações significativas foram identificadas para os polimorfismos *TMEM18* rs6548238, *NEGR1* rs2815752, *BDNF* rs6265 e rs10767664 com fenótipos antropométricos, e para *SEC16B* rs10913469 com parâmetros nutricionais. Concentrações circulantes diferenciais de miR-16-5p e miR-19b foram identificadas, com expressão de 1,96 e 1,41 vezes, respectivamente, mais elevada em crianças magras em relação ao grupo obeso. Além disso, as concentrações plasmáticas destes dois miRNAs foram correlacionadas com índice de massa corporal e os níveis de miR-16-5p foram correlacionados aos níveis plasmáticos de insulina. Os resultados encontrados revelaram associação de quatro dos dez polimorfismos estudados com fenótipos relacionados à obesidade, demonstrando que é possível detectar a influência das variantes genéticas sobre características antropométricas e dietéticas no início da infância. Além disso, o presente estudo sugere um papel potencial de miR-16-5p e miR-19b circulantes como novos biomarcadores na obesidade infantil para prevenção precoce e para melhora do poder de diagnóstico das complicações metabólicas associadas à obesidade.

Palavras-chave: crianças, excesso de peso, obesidade, polimorfismos, susceptibilidade genética, microRNAs, plasma.

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LISTA DE ABREVIATURAS

AP-2	Proteína ativadora 2
BDNF	Fator neurotrófico derivado do cérebro
DP	Desvio padrão
E/I	Relação estatura por idade
FTO	*Fat mass and obesity associated*
GWAS	*Genome wide association studies*
HOXB5	Homeobox B5
IMC	Índice de massa corporal
KCTD15	*Potassium Channel Tetramerization Domain Containing 15*
MC4R	Receptor de melanocortina 4
mRNA	RNA mensageiro
miRNAs	MicroRNAS
NEGR1	Regulador de crescimento neuronal 1
RISC	*RNA induced silencing complex*
SEC16B	*Homolog B of Saccharomyces cerevisiae 16*
SH2B1	*Src-homology-2 B adaptor protein 1*
SNC	Sistema nervoso central
SNP	Polimorfismo de nucleotídeo único
OLFM4	Olfactomedina 4
OMS	Organização Mundial da Saúde
P/E	Relação antropométrica peso por estatura
P/I	Relação antropométrica peso por idade
TMEM18	Proteína transmembrana 18
3'-UTR	Região 3' não traduzida
α-MSH	Hormônio estimulante de melanócitos alfa

SUMÁRIO

1 INTRODUÇÃO

1.1 Epidemiologia da Obesidade

A obesidade humana é uma condição multifatorial e heterogênea causada por múltiplos genes de pequeno efeito, que interagem entre si e com inúmeros fatores ambientais (1). É definida como um estado de aumento do peso corporal, mais especificamente do tecido adiposo, de magnitude suficiente para produzir consequências adversas à saúde. O seu padrão de herança é complexo e os fatores ambientais, tais como composição da dieta e nível de atividade física, desempenham um papel importante na promoção ou retardando o seu aparecimento (2). Além de impor ao indivíduo um forte estigma social, a obesidade e suas comorbidades, dentre elas diabetes tipo 2, doenças cardiovasculares, dislipidemias, hipertensão, doenças respiratórias e certos tipos de câncer, afetam a saúde de um número crescente de pessoas em todo o mundo, impactando a qualidade de vida e a longevidade da população (3).

De acordo com os resultados da Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico, 52,5% da população brasileira acima de 18 anos apresenta excesso de peso e 17,9% está obesa (4). A pesquisa salienta que excesso de peso e obesidade são fatores de risco para doenças crônicas, como hipertensão, diabetes, doenças cardiovasculares e câncer, sendo essas responsáveis por 72% dos óbitos no país.

O acúmulo de gordura é causado, invariavelmente, por um desequilíbrio entre a ingestão alimentar e o gasto de energia, um problema de solução aparentemente simples. No entanto, até hoje poucos são os avanços no sentido de conter a progressão deste problema. O balanço entre a ingestão e o gasto de energia é controlado por um complexo e poderoso sistema biológico, comandado pelo sistema nervoso central (5). O controle homeostático do balanço energético corporal é exercido por populações específicas de neurônios situados majoritariamente no hipotálamo, onde se localizam os chamados centros da fome e da saciedade (6).

Por outro lado, intimamente envolvido na patogênese da obesidade, destaca-se o tecido adiposo, que não é apenas o principal local de armazenamento de energia, mas também é considerado um órgão endócrino de alta relevância (7), constituindo um regulador fundamental do balanço energético e da homeostase da glicose nos mamíferos (8). O entendimento do mecanismo regulatório das vias responsáveis pela proliferação e diferenciação dos adipócitos é essencial para a elucidação dos mecanismos que levam à obesidade.

A prevalência mundial da obesidade em crianças vem aumentando em um ritmo alarmante, sendo apontada pela Organização Mundial de Saúde (OMS) como um dos desafios mais graves para a saúde pública do século XXI, afetando de maneira global países desenvolvidos e em desenvolvimento, especialmente em ambientes urbanos (9). Doenças de início típico na idade adulta como diabetes tipo 2, dislipidemias e doenças cardiovasculares estão sendo cada vez mais frequentemente observadas em crianças e adolescentes com excesso de peso ou obesos (10, 11). Além disso, a alta prevalência de excesso de peso em crianças está associada a um grande número de complicações que afetam os sistemas neurológico, cardiovascular, endócrino, músculo-esquelético, renal, gastrointestinal e pulmonar, além dos problemas psicossociais (12).

De acordo com estudos epidemiológicos, 70 a 80% das crianças e adolescentes obesos crescem e permanecem obesos na idade adulta (13, 14). A epidemia recente de obesidade infantil despertou grande interesse de estudo em função das suas consequências sobre a saúde clínica e pública. O que se pode presumir é que estas terão um impacto considerável no futuro sobre custos e serviços de saúde, tornando-os bastante onerosos (15).

Em 2013, pelo menos 42 milhões de crianças no mundo com menos de cinco anos de idade apresentavam excesso de peso, sendo que aproximadamente 31 milhões delas viviam em países em desenvolvimento (9). No Brasil, de acordo com o último levantamento feito pelo Instituto Brasileiro de Geografia e Estatística referente aos anos de 2008-2009, 33,4% das crianças entre 5 e 9 anos apresentavam excesso de peso e 14,2% eram obesas. Em adultos, 49% apresentavam excesso de peso e 14,6% eram obesos (16). Pesquisas populacionais brasileiras mostraram que a prevalência de excesso de peso em crianças em idade escolar triplicou entre 1974 e 1997 (17).

A infância é um importante período da vida em que intervenções em saúde eficazes podem ser realizadas, uma vez que os comportamentos associados à saúde estão em formação (18). A literatura recente sugere que a gênese do problema ocorre nos primeiros anos de vida, durante a idade pré-escolar, onde os padrões de alimentação, hábitos alimentares e práticas de alimentação orientada pelos pais são estabelecidos (19, 20).

1.2 Avaliação Nutricional na Infância

1.2.1 Antropometria

Excesso de peso e obesidade, em adultos, são definidos através do índice de massa corporal (IMC), assim calculado: peso em kilogramas/(altura em metros)². Este índice baseia-se na premissa de que a maior parte da variação no peso para pessoas de mesma altura é devida à massa de gordura corporal destes indivíduos. O IMC é um critério geral capaz de avaliar excesso de peso e obesidade e, em adultos, os valores são idade-independentes e os mesmos para ambos os sexos. De acordo com a classificação internacional utilizada pela OMS, valores de IMC $\geq$ 25 kg/m^2 caracterizam excesso de peso e valores de IMC $\geq$ 30 kg/m^2 obesidade (21).

Em crianças, este índice altera-se substancialmente com a idade, aumentando rapidamente no início da infância, diminuindo durante os anos pré-escolares e voltando a aumentar na idade adulta. Por esta razão, o IMC em crianças precisa ser avaliado usando uma curva de referência associada à idade quando a intenção for o diagnóstico individual com objetivo clínico (22). A Figura 1 apresenta as curvas de crescimento infantil baseadas no IMC por idade de acordo com o sexo.

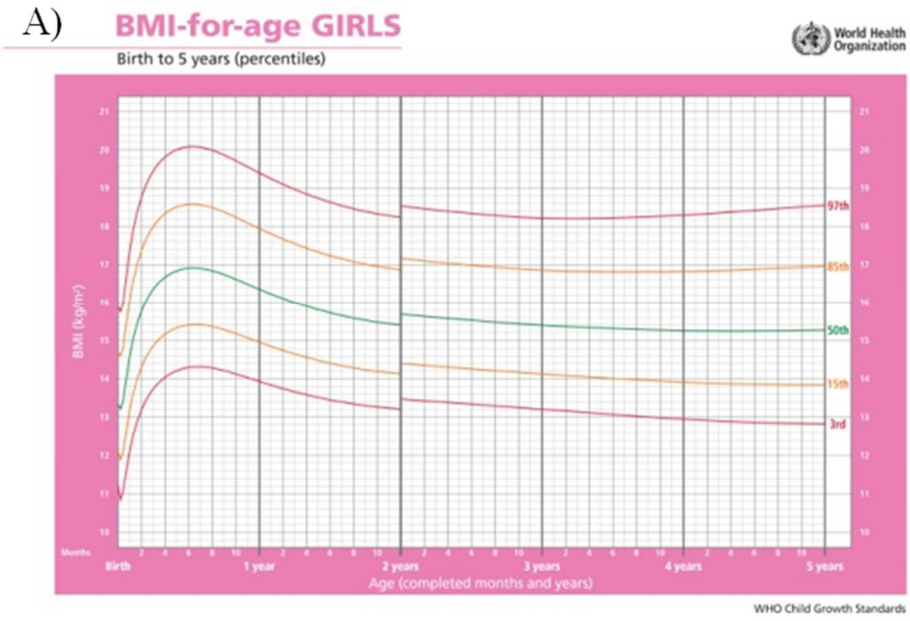
Contudo, existe outro indicador de excesso de peso ou obesidade em crianças que é mais indicado quando se deseja apresentar os dados antropométricos de grupos populacionais, o escore Z, calculado a partir da seguinte fórmula: [(valor observado – valor da mediana)/desvio padrão da população de referência] (23). Tal escore consiste na medida de quanto o indivíduo se afasta ou se aproxima da mediana em desvios-padrão (DP). Para calculá-lo, podem-se utilizar as seguintes relações: peso por estatura (P/E), peso por idade (P/I) e estatura por idade (E/I). O escore Z é mais aceito na literatura científica e é um excelente método para estudos de grupos populacionais. Um valor do escore Z igual a zero (0) significa que o valor da medida obtida da criança é exatamente igual ao valor do referencial, no caso o valor do percentil 50 das curvas de crescimento. Na Figura 2 estão ilustradas as curvas de crescimento infantil baseadas no escore Z do IMC por idade de acordo com o sexo.

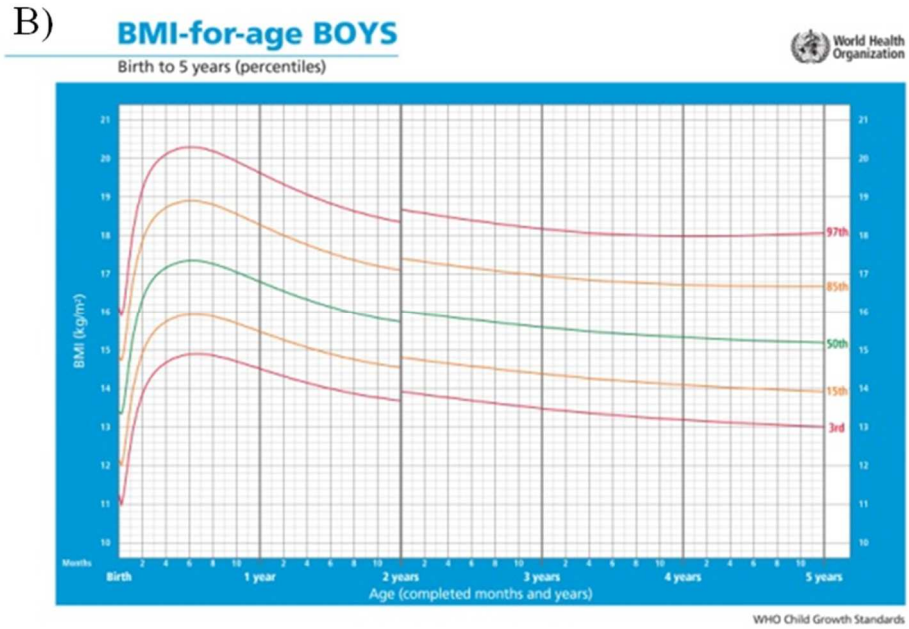
A fim de padronizar mundialmente a avaliação do diagnóstico nutricional na infância e de definir a prevalência de deficiência de peso, excesso de peso e obesidade em crianças e adolescentes, a OMS estabeleceu valores de pontos de corte para o escore Z do IMC de acordo com as curvas de crescimento para idade e sexo: a) deficiência de peso: IMCZ < -2.0 DP; b) excesso de peso: IMCZ > +1.0 DP; c) obesidade: IMCZ > +2.0 DP (9).

Além do excesso de gordura corporal, o local onde essa gordura se deposita é um fator importante na determinação das complicações associadas à obesidade (24). Existem parâmetros que utilizam critérios mais específicos para avaliar o padrão de distribuição da gordura corporal, que são a circunferência da cintura e as pregas cutâneas subescapular e tricipital.

A circunferência da cintura é uma das medidas antropométricas mais utilizadas para diagnosticar obesidade abdominal (25). Esse parâmetro auxilia na classificação quanto ao risco de desenvolvimento de alterações metabólicas, tais como dislipidemias, hipertensão arterial e hiperinsulinemia, as quais caracterizam a síndrome metabólica (26). Diversos estudos com crianças e adolescentes têm observado associação significante entre fatores de risco cardiovasculares e circunferência da cintura (27). Já a medida das pregas cutâneas é amplamente utilizada em estudos populacionais para a mensuração do tecido adiposo subcutâneo, principalmente para identificar excesso de gordura. Essas são os primeiros índices antropométricos a serem alterados quando existe excesso de peso, podendo ser avaliadas isoladamente ou em conjunto (28). As pregas cutâneas são uma medida de adiposidade que permite avaliar a composição corporal, uma vez que a gordura subcutânea constitui grande parte da gordura corporal total do indivíduo (29).

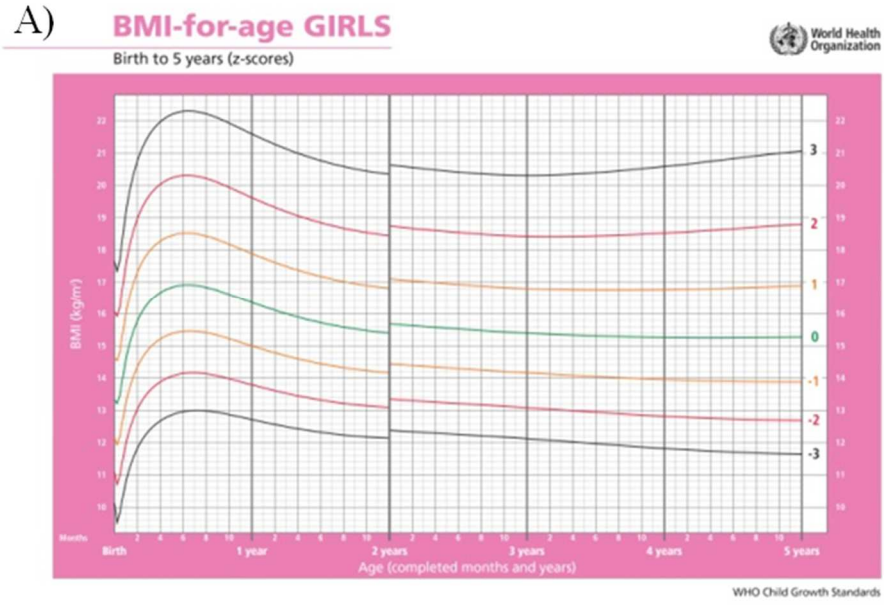
Figura 1. Curvas de crescimento infantil baseadas no índice de massa corporal por idade de acordo com o sexo: A) Meninas, B) Meninos.

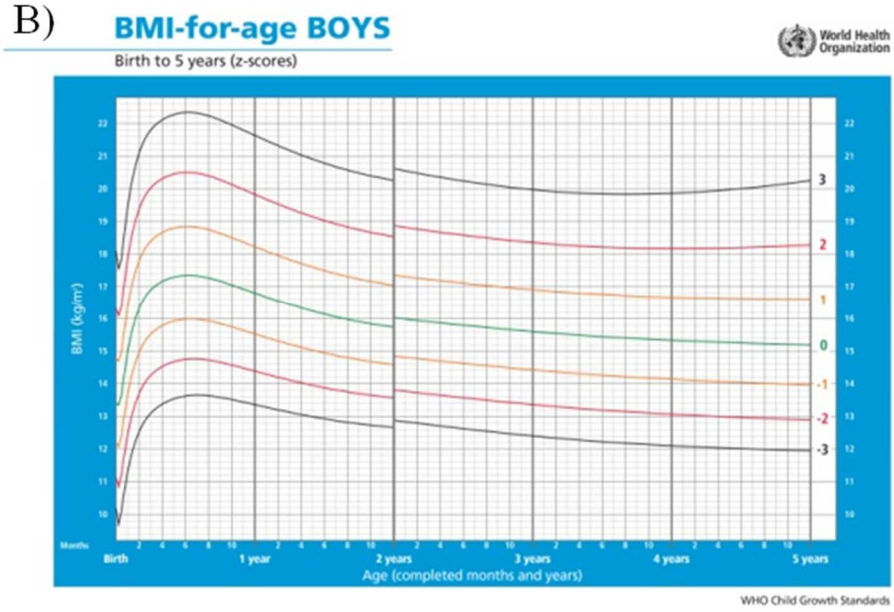




Fonte: Organização Mundial de Saúde
http://www.who.int/childgrowth/standards/bmi_for_age/en/

Figura 2. Curvas de crescimento infantil baseadas no escore Z do índice de massa corporal por idade de acordo com o sexo: A) Meninas, B) Meninos.





Fonte: Organização Mundial de Saúde
http://www.who.int/childgrowth/standards/bmi_for_age/en/

1.2.2 Avaliação Dietética

O consumo alimentar e o *status* nutricional de crianças podem ser avaliados através de inquéritos alimentares, como o inquérito recordatório de 24 horas, em que as mães ou os cuidadores são solicitados a descreverem todos os alimentos e bebidas ingeridos nas últimas 24 horas, assim como as quantidades e o modo de preparo desses alimentos (30). Os inquéritos recordatórios devem ser realizados em dias alternados e utilizadas as médias de dois inquéritos para o cálculo das variáveis dietéticas relacionadas ao consumo de frutas, vegetais e alimentos ricos em açúcar e gordura. Os alimentos presentes em cada um dos inquéritos recordatórios são classificados de acordo com o método proposto por Monteiro *et al*., que considera a natureza, a extensão e a finalidade do processamento de alimentos (31).

O cálculo nutricional da ingestão alimentar é realizado através de softwares específicos, tais como o DietWin® (Porto Alegre, Brasil) e o NutWin® (São Paulo, Brasil), que são baseados na Tabela Brasileira de Composição de Alimentos (32). Esses softwares contabilizam a ingestão calórica e de micro e macronutrientes presentes nos inquéritos recordatórios.

Nos últimos anos, os pesquisadores vêm utilizando uma nova ferramenta dietética com o propósito de estimar a qualidade global da dieta através da avaliação dos inquéritos recordatórios de 24 horas de cada criança, o Índice de Alimentação Saudável. Esse índice é utilizado tanto para avaliar o sucesso de intervenções nutricionais quanto o consumo alimentar de indivíduos de todas as faixas etárias (33). Consiste em um instrumento que atribui pontuações para a dieta, levando em consideração o desequilíbrio entre os componentes da dieta ao invés dos nutrientes isolados, tendo sido ligado a doenças crônicas e às concentrações plasmáticas dos nutrientes.

1.3 Aspectos Genéticos e Ambientais na Obesidade

O estilo de vida da sociedade contemporânea é apontado como o principal responsável pelo rápido crescimento da prevalência de obesidade (34). Se a eficiência em acumular energia favoreceu a sobrevivência em períodos de escassez de alimentos, nos dias atuais ocorre o inverso. A facilidade de acesso e o baixo custo de alimentos altamente palatáveis e de grande densidade energética, normalmente ricos em gorduras e açúcares, mas pobres em vitaminas, minerais e outros micronutrientes, aliados ao menor requerimento de atividade física na vida diária, favorecem o acúmulo de gordura em indivíduos com predisposição genética (35).

Excesso de peso e obesidade resultam de uma combinação de influência genética, comportamental e ambiental sobre metabolismo, dieta e atividade física. De maneira simplificada, são o resultado de um desequilíbrio entre consumo alimentar e gasto energético, que pode ocorrer devido a alterações genéticas em três principais vias: a) ingestão alimentar; b) gasto energético; e c) armazenamento de energia (adipogênese) (36). Quando a ingestão energética ultrapassa o consumo, o excesso resultante é armazenado principalmente sob a forma de gordura no tecido adiposo e este depósito de armazenamento é utilizado como fonte primária de energia durante os períodos de déficit energético (37). Numerosos fatores neurais, hormonais, ambientais e genéticos interagem de forma complexa na regulação da homeostase energética (37). Quando existe predisposição genética para a obesidade, o ambiente e o estilo de vida do indivíduo potencializam o seu desenvolvimento. Portanto, as diferenças genéticas podem esclarecer algumas das discrepâncias encontradas no ganho de peso entre populações.

Diversos estudos com gêmeos, famílias e filhos adotivos foram realizados a fim de avaliar o grau de herdabilidade da obesidade humana. A maioria deles sugere um forte componente genético, estimando que a herdabilidade do índice de massa corporal varia entre 40 e 70% (38-40). Isso significa que a maior parte da variação interindividual da adiposidade é devida a fatores genéticos. Indivíduos com alto risco genético para obesidade são mais susceptíveis aos efeitos de um ambiente não saudável (41).

Já é amplamente aceito que intervenções no estilo de vida com modificações dos hábitos alimentares associadas à atividade física constituem a mais importante maneira de prevenção do o excesso de peso na infância e na adolescência (42). Sabe-se que fatores genéticos apresentam um importante papel também na obesidade infantil, mas seus efeitos

podem ser diferentes, ou ainda, podem resultar de outras variantes genéticas que tenham efeitos apenas no início da infância (43).

Skidmore *et al*. relataram que o ambiente obesogênico pós-natal foi mais importante do que o ambiente fetal no desenvolvimento de adiposidade em mulheres gêmeas de 18 a 80 anos, e que um maior peso ao nascer está associado à maior proporção de massa corporal magra em relação ao tecido adiposo na idade adulta (44). Além disso, cada vez mais os fatores epigenéticos estão ganhando atenção como um importante fator associado à obesidade infantil, já que consistem em mudanças genéticas herdáveis possivelmente modificadas pelos hábitos de vida e pelo ambiente social em que o indivíduo está inserido (20).

1.4 Genes Investigados

Nas últimas décadas, a identificação dos componentes genéticos que contribuem para a variação interindividual na obesidade e fenótipos relacionados vem sendo investigada intensivamente por inúmeros pesquisadores. Essa busca tem sido concentrada principalmente em populações adultas, que constituem um alvo mais acessível para estes estudos. No entanto, as associações observadas entre variantes genéticas e medidas antropométricas nessas populações provavelmente sejam o reflexo da interação entre genes e fatores ambientais aos quais o indivíduo é exposto desde a sua infância (45).

Os avanços tecnológicos e o sequenciamento do genoma humano têm permitido a varredura de todo o genoma a fim de identificar *loci* de susceptibilidade associados a diversas doenças, numa abordagem *hypothesis-free*. Esses estudos de varredura genômica, os GWAS (*genome wide association studies)*, representam uma ferramenta bastante útil na identificação de marcadores moleculares para doenças humanas complexas, embora na maioria deles a busca seja apenas por polimorfismos de nucleotídeo único (SNPs) com frequência do alelo raro (MAF) superior a 5%, desconsiderando-se as variantes raras. A descoberta de rotas potenciais para novas terapias, melhora do diagnóstico e prevenção de doenças são os principais objetivos desses estudos (46).

Diversos GWAS têm sido realizados na busca de genes e variantes genéticas envolvidos na obesidade humana, a maioria deles em adultos e alguns em crianças. Esses estudos rastreiam o genoma todo de milhares de indivíduos na busca por centenas de SNPs. Contudo, a maioria das variantes genéticas identificadas parece conferir um efeito pequeno e poucos alelos causais têm sido identificados. Uma importante metanálise recentemente

publicada envolvendo mais de 300.000 indivíduos revelou que 97 *loci* genéticos estão associados ao IMC e a fenótipos relacionados à obesidade, sendo que a maioria deles parece conferir risco de obesidade através de seus efeitos no sistema nervoso central (47).

Os critérios de inclusão dos genes analisados no presente estudo levaram em consideração a necessidade de investigar e/ou confirmar, em crianças, associações que vêm sendo propostas em adultos e também identificar possíveis variantes genéticas que estejam influenciando fenótipos relacionados à obesidade no início da infância.

Para a análise de associação, após busca na literatura realizada nos meses de agosto e setembro de 2012, foram selecionadas as variantes genéticas de susceptibilidade à obesidade previamente identificadas pelos GWAS que foram replicadas em um maior número de estudos. Além disso, foram levados em consideração os resultados mais fortemente associados à obesidade ($P<5x10^{-8}$) nos GWAS de adultos e alguns deles em amostras pediátricas. Os polimorfismos selecionados foram verificados na base de dados do *The International HapMap Project* e também na literatura, a fim de constatar se já haviam sido descritos em populações de ancestralidade europeia. As frequências dos polimorfismos foram verificadas, dando-se preferência àqueles cujos alelos raros apresentavam frequência superior a 5%. A maioria dos genes selecionados é expresso ou conhecido por atuar no sistema nervoso central e está envolvido no controle da ingestão de alimentos e no gasto energético, embora também desempenhem funções periféricas relacionadas ao tecido adiposo (48, 49).

É importante salientar que, até o momento, as variantes genéticas mais fortemente associadas à obesidade identificadas pelos GWAS estão localizadas no gene *fat mass and obesity associated* (*FTO*). Frayling et al., na busca por genes de susceptibilidade ao diabetes tipo 2, identificaram uma variante genética comum no gene *FTO*, que conferia risco ao diabetes através do seu efeito sobre o IMC (50). Estudos subsequentes confirmaram sua associação com IMC e predisposição à obesidade na infância e na vida adulta (51-53). A importância do *FTO* na obesidade infantil no Brasil foi previamente confirmada pelo nosso grupo (54). Por este motivo, o *FTO* não foi incluído no presente trabalho.

A seguir, a Tabela 1 apresenta genes e variantes genéticas associadas à obesidade e fenótipos relacionados identificadas através de GWAS, conforme busca na literatura realizada no momento da seleção dos genes a serem estudados.

Tabela 1. Genes e variantes genéticas associadas ao índice de massa corporal identificadas nos GWAS.

Gene mais próximo	Cromossomo	SNP	Referências
NEGR1*: regulador do fator de crescimento neuronal 1	1	rs2815752 rs2568958	(52, 55, 56)
SEC16B*: *homolog B of S. cerevisiae 16*	1	rs10913469	(53, 55-57)
TNNI3K*: *serine/threonine protein kinase	1	rs1514175	(53, 56)
TMEM18*: proteína transmembrana 18	2	rs6548238 rs7561317	(52, 53, 55)
INSIG2: *insulin induced gene* 2	2	rs7566605	(58)
POMC: pró-opio melanocortina	2	rs1042571	(53)
SFRS10: fator de *splicing* arginina/serina 10	3	rs7647305	(57)
ETV5: *ets variant* 5	3	rs7647305	(57)
DGKG: diacilglicerol quinase gama	3	rs7647305	(57)
GNPDA2: glicosamina-6-fosfato desaminase 2	4	rs10938397	(52)
PRL: prolactina	6	rs4712652	(59)
PTER: fosfotriesterase associado	10	rs10508503	(59)
BDNF*: fator neurotrófico derivado do cérebro	11	rs6265 rs10767664	(56, 57, 60)
MTCH2: carreador mitocondrial homólogo 2	11	rs10838738	(52)
BCDIN3D: *BCDIN3 domain containing*	12	rs7138803	(57)
FAIM2: molécula inibidora de apoptose Faz	12	rs7138803	(53, 57)
OLFM4: olfactomedina 4	13	rs9568856	(53, 56)
NRXN3: neurexina 3	14	rs10150332	(56)
SH2B1*: *Src-homology-2 B adaptor protein 1*	16	rs7498665	(52, 55-57)
ATP2A1: ATPase	16	rs7498665	(57)
FTO**⁺**: *fat mass and obesity associated*	16	rs9939609 rs8050136 rs1421085	(50-53, 55, 57, 59, 61)
MAF: fator de transcrição *v-maf musculoaponeurotic fibrosarcoma*	16	rs1424233	(59)
HOXB5: homeobox B5	17	rs9299	(53, 56)
ACE: enzima conversora de angiotensina 1	17	rs4351	(57)
NPC1: proteína de membrana Niemann-Pick C1	18	rs1805081	(59)
MC4R**⁺**: receptor de melanocortina 4	18	rs17782313 rs12970134 rs52820871 rs2229616	(52, 53, 55, 59, 62-65)
CHST8: sulfotransferase carboidrato 8	19	rs29941	(57)
KCTD15*: *potassium channel tetramerization domain containing 15*	19	rs29941 rs11084753	(52, 55-57)

Fonte: http://www.ncbi.nlm.nih.gov/pubmed/

⁺maior número de GWAS
*segundo maior número de GWAS

Desta forma, no presente trabalho foram selecionadas dez variantes genéticas associadas à obesidade identificadas pelos GWAS e localizadas em nove genes ou próximas a eles, descritos a seguir.

1.4.1 Receptor de Melanocortina 4

O gene que codifica o receptor de melanocortina 4 (MC4R) foi o primeiro lócus no qual mutações foram associadas à obesidade mórbida (IMC $\geq$ 40 kg/m^2) em humanos e representa a segunda causa genética mais fortemente associada à obesidade comum, precedida apenas pelo gene *FTO* (65-67). O MC4R é um receptor transmembrana acoplado à proteína G expresso em diversas regiões cerebrais, incluindo o hipotálamo (68). É regulador chave da homeostase energética através da via de sinalização das melanocortinas, tendo o hormônio estimulante de melanócito alfa (α-MSH) como ligante, que leva à diminuição na ingestão de alimentos e ao aumento do gasto energético (69, 70).

Pelo menos 72 polimorfismos no gene *MC4R* foram descritos até o momento, poucos deles levando à formação de uma proteína mutada ou defeituosa (71). Dentre essas mutações, algumas parecem causar retenção intracelular da proteína deformada, enquanto outras levam à ligação anormal do seu agonista ou a um defeito na interação ligante-receptor (71). O polimorfismo rs17782313, aqui investigado, é uma transição C>T localizada no cromossomo 18:60183864 a 188 kb *downstream* do gene *MC4R* e tem sido associado ao aumento do IMC, afetando a ingestão e o gasto energético na infância, adolescência e idade adulta (52, 56, 65, 72-75). Essa variante genética parece modular o comportamento relacionado ao apetite, exercendo um papel regulatório na expressão ou na tradução do *MC4R* (76). Estudos recentes em humanos e em modelos animais detectaram os genótipos desse polimorfismo associados a diversas alterações alimentares, tais como hiperfagia, preferência por alimentos com alto teor de gordura, resposta à saciedade e sensação de prazer ao comer, sugerindo que o seu efeito sobre o IMC seja através de comportamentos alimentares aumentados mediados por mecanismos centrais (72, 77, 78).

1.4.2 Proteína Transmembrana 18

A proteína transmembrana 18 (TMEM18) localiza-se na membrana nuclear e apresenta um domínio C-terminal específico, carregado positivamente, que se liga ao DNA. Em animais é abundantemente expressa no cérebro e em outras regiões centrais, incluindo aquelas envolvidas na ingestão alimentar (79). Já em humanos, uma ampla distribuição foi observada, com níveis máximos de expressão no cérebro e tecido adiposo, além do fígado e músculo esquelético (48). A proteína ligada ao DNA causa repressão da transcrição de

diversos genes, possivelmente por impedir a ligação dos fatores de transcrição às suas regiões promotoras (48).

O mecanismo pelo qual polimorfismos no gene *TMEM18* estão envolvidos na regulação do IMC e no balanço energético ainda não está bem estabelecido, embora as associações com obesidade sejam replicadas em diversos estudos. Um dos mecanismos moleculares propostos seria através da repressão da transcrição de genes ligados à ingestão de alimentos e gasto energético, uma vez que foi observado que uma mutação que levou à perda dos aminoácidos da porção C-terminal impediu a ligação da proteína ao DNA, mostrando que a extensão C-terminal é essencial para essa ligação (80). Outro mecanismo recentemente sugerido seria através do seu papel regulatório no tecido adiposo, onde a supressão incompleta dos níveis de mRNA levou à inibição da diferenciação dos adipócitos, sugerindo que a proteína TMEM18 deve atuar no silenciamento de genes também durante a adipogênese (48,79).

Variantes no gene *TMEM18* ou próximas a ele têm mostrado fortes associações com obesidade em GWAS de crianças e adultos. O polimorfismo que tem apresentado o maior tamanho de efeito é o rs6548238, uma transição C>T localizada no cromossomo 2:634905 a 2 kb *upstream* do gene *TMEM18,* o qual foi associado ao aumento de IMC e dobras cutâneas (53, 56, 81-83). Evidências demonstram que é pequena a probabilidade de que essa variante genética esteja ligada a SNPs causais dentro do gene *TMEM18*, no entanto ela poderia modular a transcrição do *TMEM18* afetando a ligação dos fatores de transcrição ou de co-reguladores da transcrição (83).

1.4.3 Fator Neurotrófico Derivado do Cérebro

O fator neurotrófico derivado do cérebro (BDNF) é um fator de crescimento que está envolvido no desenvolvimento e sobrevivência de neurônios no sistema nervoso central (84). Nos últimos anos, evidências crescentes têm demonstrado que o BDNF desempenha um papel importante na regulação central da ingestão de alimentos e no controle do peso corporal (85). Contudo, pouco se sabe sobre o seu mecanismo na patofisiologia da obesidade infantil. Uma das hipóteses se relaciona à regulação do peso corporal através do seu envolvimento na via de sinalização do MC4R, modulando a ingestão de alimentos (86). O gene *BDNF* é altamente expresso no hipotálamo, onde desempenha um papel chave no metabolismo da energia, regulação da ingestão de alimentos e IMC, através de mecanismos centrais e periféricos (49).

Um estudo recente detectou expressão de *BDNF* no tecido adiposo subcutâneo e visceral e destacou seu possível papel regulatório na diferenciação dos adipócitos humanos (48).

Dentro do gene *BDNF*, a variante que vem mostrando o maior número de associações com fenótipos relacionados à obesidade é o rs6265, uma transição G>A localizada no cromossomo 11: 27658369 que resulta na substituição do aminoácido valina por metionina no códon 66 (val66met), afetando a produção da proteína madura e levando à diminuição da produção de BDNF (87, 88). Este SNP inicialmente foi associado a distúrbios neurológicos e psiquiátricos, mas tanto os GWAS quanto os estudos com genes candidatos têm sugerido o seu envolvimento no comportamento alimentar e no desenvolvimento da obesidade (57, 89). Resultados de estudos de associação envolvendo crianças e/ou adolescentes encontraram a variante rs6265 conferindo risco aumentado de obesidade (49, 90).

Outro polimorfismo no gene *BDNF* que mostrou forte associação com IMC em uma grande metanálise de GWAS de obesidade em adultos foi o rs10767664 (56), uma transversão A>T intrônica localizada no cromossomo 11:27704439. Em crianças também foi demonstrada associação com risco de obesidade e fenótipos relacionados (90) e, recentemente em adultos, foi sugerido o seu envolvimento na modulação do apetite e no risco de obesidade (91). O mecanismo pelo qual essa variante genética influencia a adiposidade e, possivelmente, o comportamento alimentar ainda é incerto.

Os dois SNPS no gene *BDNF*, rs6265 e rs10767664, estão em forte desequilíbrio de ligação, de acordo com os blocos haplotípicos catalogados pelo *The International HapMap Project* para a população CEU de ancestralidade Europeia (*Utah residents with northern and western European ancestry*). Apesar da sugerida associação não aleatória entre alelos desses dois diferentes *loci*, ambos os SNPs foram analisados no presente trabalho, já que os sinais de associação identificados pelos GWAS para cada um deles mostraram fortes associações com obesidade, além de considerarmos que a alta heterogeneidade da população Brasileira pode afetar os padrões de desequilíbrio de ligação em relação a outras populações (92).

1.4.4 *Potassium Channel Tetramerization Domain Containing 15*

A proteína *potassium channel tetramerization domain containing 15* (KCTD15) inibe a atividade transcricional da proteína ativadora-2 (AP-2) através da interação com o seu domínio de ativação, afetando a formação da crista neural durante o desenvolvimento embrionário (93). A crista neural é uma população de células multipotentes que contribui para

a formação de ossos, cartilagens, tecido conectivo da face, neurônios e células da glia do sistema nervoso periférico, além de células de pigmentação da pele (94).

Diversos GWAS têm identificado variantes no gene *KCTD15* ou próximas a ele associadas com risco de desenvolver obesidade, porém o mecanismo molecular destas associações ainda é pouco conhecido. Dentre as variantes genéticas descritas, o polimorfismo rs11084753 é o que tem mostrado fortes evidências de associação com obesidade. É uma transição A>G localizada no cromossomo 19:33831232 a 17kb *downstream* do gene *KCTD15*. Estudos prévios encontraram associação dos genótipos deste SNP com obesidade e fenótipos relacionados em crianças, adolescentes e adultos (52, 75, 82). A hipótese proposta sobre o mecanismo molecular desse gene na etiologia da obesidade pode resultar de um efeito neuronal do *KCTD15* sobre o balanço energético, uma vez que o gene é expresso em níveis elevados no hipotálamo (82). Estudos *in vitro* e *in vivo* demonstraram que a proteína KCTD15 está envolvida na modulação do comportamento alimentar e de recompensa por localizar-se em regiões hipotalâmicas (95). Além disso, Zarelli et al. demonstraram que a AP-2 regula importantes fatores na adipogênese, sugerindo um possível papel regulatório da *KCTD15* na patofisiologia da obesidade (93).

1.4.5 Regulador do Crescimento Neuronal 1

O regulador do crescimento neuronal 1 (NEGR1) é uma proteína que desempenha um papel chave no desenvolvimento neuronal e é altamente expressa em diversas regiões cerebrais, incluindo o hipotálamo (96). A proteína parece exercer seus efeitos sobre a ingestão de alimentos e a regulação do gasto energético através da modulação da nesfatina-1, uma molécula anorexígena identificada em 2006 (79). Em modelos animais, mutações de perda de função do *NEGR1* levaram à diminuição da massa corporal (96). O gene parece ser diferentemente regulado no tecido adiposo de indivíduos magros e obesos (97), o que levou Bernhard et al. a sugerirem uma possível função regulatória do *NEGR1* no tecido adiposo humano durante a diferenciação dos adipócitos, uma vez que a expressão do gene foi menor no tecido adiposo subcutâneo de indivíduos obesos (48).

Na busca por variantes genéticas de susceptibilidade à obesidade, diversos estudos têm identificado *loci* no gene *NEGR1* ou próximos a ele que estão associadas à obesidade comum. Um dos polimorfismos que vem mostrando fortes evidências de associação é o rs2815752, uma transição C>T localizada no cromossomo 1:72346757 a 64 kb do gene *NEGR1*. Diversos

GWAS e estudos de replicação encontraram associação com IMC e risco de obesidade em crianças, adolescentes e adultos (52, 56, 74, 98, 99). As bases moleculares que explicam a influência dessa variante genética no controle da homeostase energética ainda são investigadas. Evidências iniciais apontam que esse SNP englobaria sítios de ligação de repressores transcricionais do *NEGR1*, afetando a sua expressão (100).

1.4.6 *Homolog B of Saccharomyces cerevisiae 16*

A proteína *homolog B of Saccharomyces cerevisiae 16* (SEC16B) é uma isoforma do Sec16, que se localiza na membrana do retículo endoplasmático e medeia o transporte de proteínas para o complexo de Golgi (101). Diversos estudos têm associado o gene *SEC16B* com risco de obesidade (57, 98, 102, 103). No entanto, pouco se sabe acerca do seu mecanismo no acúmulo de gordura corporal. O gene é expresso no hipotálamo e no tecido adiposo subcutâneo, o que indica um possível envolvimento na regulação central e periférica da obesidade (104). O mecanismo central proposto relacionado à ingestão de alimentos seria através da regulação da secreção de neupeptídeos orexígenos e anorexígenos, tais como o neuropeptídeo Y e a proopiomelanocortina (105). Já a expressão periférica do *SEC16B* poderia afetar a síntese e o transporte da lipase, inibindo a decomposição de gordura (75).

O polimorfismo identificado pelos GWAS que tem mostrado significantes associações com obesidade é o rs10913469, uma transição C>T intrônica no gene *SEC16B* localizada no cromossomo 1:177944384. As associações encontradas são entre os genótipos desse SNP e baixo peso ao nascer para a idade gestacional, IMC aumentado e risco de obesidade em amostras adultas e pediátricas (53, 56, 82, 106-108). Até o momento, pouco se sabe acerca das funções moleculares dessa variante genética sobre a regulação central e periférica da obesidade. Uma das hipóteses seria através de alterações no controle da distribuição da energia corporal entre os tecidos magro e adiposo ou através de alterações na eficiência metabólica durante a termogênese (108).

1.4.7 *Src-homology-2 B adaptor protein 1*

O gene que codifica a *Src-homology-2 B adaptor protein 1* (SH2B1) é expresso no músculo esquelético, linfócitos, rins, ovários, pâncreas, tecido adiposo além do hipotálamo e hipófise (48, 109). O seu envolvimento nas vias de regulação dos sistemas relacionados à homeostase energética e obesidade ainda não é bem estabelecido. A função que vem sendo

proposta para a proteína SH2B1 é a de modular a via de sinalização JAK-STAT (*Janus Kinase - Signal Transducer and Activator of Transcription*) (79). Esse sistema de transdução de sinal e ativação da transcrição é descrito como a principal via de sinalização intracelular, que liga os receptores da superfície celular aos seus alvos transcricionais no núcleo da célula. A SH2B1 é capaz de ligar a leptina ao seu receptor no hipotálamo e, portanto, aumentar a atividade das proteínas JAK através da sua função como proteína adaptadora, uma vez que contribui para recrutar proteínas alvo para os receptores, alterando a via de sinalização intracelular da leptina e insulina no cérebro (79, 110). Além disso, estudos em animais demonstraram que a inativação do gene *SH2B1* leva à hiperfagia, resistência à leptina e insulina e obesidade (109). Tendo em vista o mecanismo proposto, a SH2B1 parece atuar sobre o comportamento alimentar contribuindo para o desequilíbrio energético e o desenvolvimento de obesidade.

Variantes genéticas no *SH2B1* associadas à obesidade foram identificadas em diversos GWAS. Dentre elas, a mais replicada é polimorfismo rs7498665, uma transição G>A que resulta na substituição do aminoácido alanina por treonina no códon 434 e localiza-se no cromossomo 16:28871920. Diversos autores encontraram associação entre os genótipos dessa variante com IMC e outros parâmetros de adiposidade, além de risco de obesidade em crianças, adolescentes e adultos (52, 56, 110, 111). As consequências moleculares funcionais desse polimorfismo ainda estão sendo investigadas e os resultados iniciais apontam para uma possível influência na expressão do *SH2B1* e na atividade da proteína (111). Análises funcionais *in vitro* revelaram que esse polimorfismo não alterou a via de sinalização da leptina mediada pelo STAT (112).

1.4.8 Olfactomedina 4

Olfactomedina 4 (OLFM4) é uma glicoproteína de matriz extracelular, membro da família das olfactomedinas, que facilita a adesão celular através da interação com lectinas e caderinas na superfície celular endógena (113). Inicialmente foi descrita como um fator anti-apoptótico que promove o crescimento tumoral, estando relacionada a diversos tipos de câncer (114). O gene *OLFM4* é expresso na medula óssea, próstata, intestino, estômago e pâncreas e sua expressão frequentemente está aumentada em alguns tipos de tumores (115). Embora a função desta glicoproteína não seja bem estabelecida, existem evidências que a relacionam com a microbiota intestinal e apontam para uma relação entre esta microbiota e

risco de obesidade (53). Um importante GWAS identificou um novo *lócus* próximo ao gene *OLFM4* que mostrou fortes sinais de associação com obesidade em adultos (56). O polimorfismo identificado foi o rs9568856, uma transição A>G localizada a 500 kb do gene no cromossomo 13:53490846. Consistente com esse achado, Bradfield et al. confirmaram a associação dessa variante genética com obesidade em crianças (53). Posteriormente a este estudo, não foram encontrados outros que replicassem a associação. Além disso, não foi encontrada na literatura nenhuma evidência funcional desta variante na etiologia da obesidade. Warrington et al. encontraram associação do polimorfismo rs12429545 próximo ao gene *OLFM4* com IMC aos 8 anos de idade (116). Esse SNP localiza-se no cromossomo 13:53528071 e pode estar em desequilíbrio de ligação com o rs9568856.

1.4.9 Homeobox B5

Os genes homeobox (*HOX)* são uma família de fatores de transcrição altamente conservados que medeiam a regulação transcricional de genes alvo durante a diferenciação e o desenvolvimento embrionário (117, 118). Speliotes et al. identificaram, em adultos, um novo *lócus* de susceptibilidade à obesidade localizado no gene *HOXB5* (56). Essa variante genética é o rs9299, uma transição A>G que se localiza na região 3’ não traduzida (3’- UTR) do gene, no cromossomo 17:48592068. Em uma metanálise de GWAS para obesidade infantil, a associação desse lócus com obesidade no início da vida foi confirmada (53). Fu et al. revelaram que o *HOXB5* é expresso durante o desenvolvimento do intestino, porém pouco se sabe sobre o seu envolvimento na patofisiologia da obesidade (119). Um dos possíveis mecanismos seria através da *up-regulation* de fatores de transcrição homeobox observada após a perda de gordura corporal, afetando processos essenciais para a função do tecido adiposo, tais como a adipogênese (53, 118, 120).

Na literatura, foi encontrado apenas o estudo de Albuquerque et al. investigando os polimorfismos rs9568856 e rs9299 com obesidade e fenótipos relacionados em crianças Portuguesas, mas os autores falharam em encontrar associações estatisticamente significativas (67). Os autores que identificaram esses dois sinais de associação na obesidade infantil sugerem que esses *loci* podem influenciar a adiposidade através de diferentes aspectos da função intestinal, já que a composição da microbiota intestinal tem sido descrita como um dos mecanismos causadores de obesidade (53).

1.5 MicroRNAs

1.5.1 Definição e Mecanismo de Regulação

Os microRNAs (miRNAs) são uma classe de moléculas de RNA endógeno não codificantes, com 18 a 25 nucleotídeos, que regulam a expressão gênica dos eucariotos em nível pós transcricional. São evolutivamente bem conservados e se ligam ao seu transcrito alvo nas regiões codificantes e 3'- UTR, podendo inibir a tradução e desestabilizar seus mRNAs alvos. Estima-se que eles controlem a expressão de, no mínimo, um terço do genoma humano (121).

Os genes de miRNAs são transcritos no núcleo das células pela enzima RNA polimerase II resultando em um pri-miRNA, que é então processado para formar o pré-miRNA. Esse é transportado para o citoplasma da célula, onde é clivado por endonucleases originado o miRNA, o qual se associa ao complexo RISC (*RNA induced silencing complex*), que localiza o sítio de ligação do miRNA em ambas as regiões 3'-UTR ou codificante do mRNA alvo (122).

Desde a sua descoberta em humanos, no ano 2000 (123), inúmeros miRNAs e seus genes alvo têm sido associados à obesidade, tanto no mecanismo de adipogênese quanto na função dos adipócitos maduros, ação da insulina (homeostase da glicose) e metabolismo de lipídios (124). Padrões de expressão alterados de miRNAs foram encontrados no tecido adiposo (125, 126). Ortega et al. avaliaram a expressão de 723 miRNAs em adipócitos humanos e constataram que a expressão de 70 miRNAs estava aumentada ou diminuída durante a diferenciação (127). Além disso, encontraram 11 miRNAs desregulados nos adipócitos de indivíduos obesos e 17 correlacionados com IMC, glicose em jejum e triglicerídeos. No tecido cerebral também foi observada expressão diferencial de miRNAs, onde estão ligados à regulação de fatores neuronais específicos da obesidade, em particular o controle do apetite e a sinalização neural ao fígado, músculo, pâncreas e trato gastrointestinal, influenciando o metabolismo (128).

Esses pequenos RNAs têm sido reconhecidos como uma classe de reguladores epigenéticos do metabolismo e da homeostase da energia, principalmente devido ao fato de que a regulação simultânea de um grande número de genes alvo pode ser exercida por um único miRNA (128, 129). No genoma humano já foram identificados mais de 950 miRNAs, sendo que um único miRNA pode regular centenas de genes (130) e muitos genes sofrem ação de múltiplos miRNAs (131, 132). Diferentes abordagens computacionais têm sido

desenvolvidas com o intuito de identificar os sítios alvo dos miRNAs em todo o genoma, como os algoritmos TargetScan, miRBase, miRanda, Pictar, mi-Records, Tarbase, Diana-Path, miRTarBase, entre outros.

Um melhor entendimento da regulação da adipogênese e da homeostase energética é essencial na identificação de novos biomarcadores e alvos ou estratégias terapêuticas para o tratamento da obesidade e de suas co-morbidades. A investigação dessas pequenas moléculas e de seus alvos genéticos pode elucidar novas vias envolvidas nos processos metabólicos, aprofundando o entendimento acerca dos seus mecanismos, assim como identificando novos possíveis marcadores e novas abordagens terapêuticas, incluindo o diagnóstico precoce.

1.5.2 MicroRNAs Circulantes

Nos últimos anos, os miRNAs têm sido encontrados em níveis estáveis e abundantes nos fluidos corporais como soro e plasma, dentro de exossomas ou outras microvesículas circulantes, e esses níveis circulantes parecem ser doença-específicos (133,134). Perfis de expressão alterados de miRNAs circulantes foram encontrados em diversas doenças, tais como câncer, diabetes e obesidade, mostrando seu potencial como novos biomarcadores minimamente invasivos para doenças humanas e fazendo deles alvos promissores para o diagnóstico, prognóstico e tratamento de doenças complexas e de suas comorbidades (19, 135).

Apesar dos miRNAs circulantes estarem rapidamente ganhando destaque e emergindo como potenciais marcadores de doenças, poucos estudos têm investigado os seus níveis de expressão circulantes na obesidade. Ortega et al. conduziram o primeiro estudo a fim de verificar o perfil circulante dos miRNAs e constataram expressão desregulada no plasma de indivíduos obesos mórbidos (135). Os autores sugerem cinco miRNAs (miR-142-3p, miR-140-5p, miR-15a, miR-520c-3c e miR-423-5p) como potenciais biomarcadores para estimar risco e classificação de pacientes com obesidade mórbida. Pescador et al. conduziram outro importante estudo a fim de determinar o painel dos miRNAs circulantes associados à obesidade comum em adultos e apontaram quatro miRNAs (miR-138, miR-15b, miR-376a e miR-503) como potenciais marcadores preditivos de obesidade e diabetes tipo 2 (134).

Na literatura, foi encontrado apenas o estudo de Prats-Puig et al. investigando a associação entre os níveis de miRNAs plasmáticos e obesidade infantil. Os autores avaliaram o perfil de expressão de miRNAs circulantes utilizando a metodologia *TaqMan miRNA low-*

density arrays (TLDAs), que abrange 754 miRNAs, a fim de identificar quais miRNAs circulantes estariam desregulados em crianças obesas (11). Os resultados revelaram pelo menos 15 miRNAs desregulados, incluindo miR-221, miR-28-3p, miR-486-5p, miR-486-3p, miR-142-3p, miR-130b e miR-423-5p. A detecção precoce de um perfil alterado de miRNAs circulantes pode ser uma estratégia promissora para identificar crianças obesas que possam vir a sofrer de alterações metabólicas.

O presente trabalho selecionou nove miRNAs cuja expressão foi avaliada em amostras plasmáticas de crianças magras e obesas. Essa seleção foi baseada no padrão de miRNAs circulantes na obesidade infantil previamente descrito por Prats-Puig *et al.* (11). Foram escolhidos os sete miRNAs mais diferentemente expressos no plasma de crianças magras e obesas (P<0,001): miR-486-5p, miR-486-3p, miR-221-3p, miR-28-3p, miR-142-3p, miR-130b-3p, miR-423-5p. O oitavo miRNA selecionado (miR-16-5p) foi baseado nos resultados de expressão aumentada em 150 vezes no tecido adiposo subcutâneo de mulheres não obesas em relação às obesas em uma amostra da cidade de Porto Alegre, RS (Gasparotto et al., em preparação). Inicialmente, foram testados quatro controles endógenos como fatores normalizadores, baseados na sua estabilidade no plasma de crianças, de acordo com Prats-Puig et al. (11): miR-223-3p, miR-106a-5p, miR-146a-5p e miR-19b. No entanto, entre os 12 miRNAs aqui analisados, três foram selecionados e utilizados como controles endógenos devido à sua maior estabilidade entre todos os ensaios avaliados com o software ExpressionSuite versão 1.0.3: miR-223-3p, miR-106a-5p e miR-142-3p. Portanto, nove foram os miRNAs candidatos investigados neste estudo.

2 JUSTIFICATIVAS

O número de crianças que está acima do peso ou são obesas vem aumentando dramaticamente ao longo dos últimos anos, como consequência da predisposição genética associada ao aumento da ingestão de alimentos altamente calóricos e à falta de atividade física diária. Essa situação é bastante preocupante, já que as coloca em um risco crescente de doenças crônicas, como doenças cardíacas, hipertensão arterial, dislipidemias, diabetes e problemas emocionais na adolescência e na idade adulta. Estudos revelam que o excesso de peso na infância é fator determinante de obesidade futura e que uma criança obesa tem mais de 50% de chances de tornar-se um adulto obeso (13, 14, 136).

A falta de orientação nutricional criada pelo descaso e pela ausência de profissionais das áreas de nutrição e endocrinologia, tanto em escolas públicas quanto privadas é, em parte, causadora de distúrbios sociais relacionados à nutrição da nossa população. A interação entre genes e ambiente é importante no desenvolvimento da obesidade e pode apresentar importantes implicações em nível de saúde pública, já que é possível afetar a expressão dos genes através de mudanças nos fatores ambientais, tais como atividade física e dieta. Essas evidências podem motivar as autoridades para que intervenções em saúde sejam realizadas nas crianças com susceptibilidade genética à obesidade. Intervenções com o intuito de modificar os fatores de risco associados à dieta precisam ser realizadas em fases precoces da vida, a fim de evitar a formação de preferências por alimentos altamente calóricos e pobres em nutrientes (137).

Os tratamentos atuais da obesidade incluem intervenções no estilo de vida, dieta e agentes farmacológicos. As pesquisas visam o melhor entendimento dos mecanismos moleculares da obesidade associado ao desenvolvimento de novas estratégias terapêuticas e de marcadores precoce para as doenças decorrentes dessa condição.

A maioria das associações de variantes genéticas de susceptibilidade à obesidade foi descrita principalmente em populações europeias e asiáticas, embora a epidemia de obesidade infantil esteja atingindo de forma importante também os países em desenvolvimento, como o Brasil. Segundo os últimos dados publicados pelo Instituto Brasileiro de Geografia e Estatística, uma em cada três crianças e um em cada cinco adolescentes brasileiros apresentam excesso de peso (16). Pesquisas populacionais brasileiras mostram que a prevalência de excesso de peso em crianças em idade escolar triplicou entre 1974 e 1997 (138), sendo observada uma prevalência de 33,5% no ano de 2008-2009 (16). Os desafios atuais no tratamento da obesidade e suas comorbidades incluem a busca por biomarcadores que sejam preditivos de saúde ou doença metabólica.

Os miRNAs têm sido vistos como potenciais novos biomarcadores para muitas patologias, como consequência de sua expressão tecido-específica e associação com variáveis clínico-patológicas. Sua descoberta na circulação sanguínea tem levado à investigação de seu uso como uma nova classe de biomarcadores minimamente invasivos de diversas doenças (139,140). Devido ao seu importante papel como integrante-chave da regulação das complexas vias metabólicas no organismo humano, os miRNAs associados à obesidade têm sido alvo de diversos estudos nos últimos anos, porém poucos são os estudos que avaliam o seu perfil na circulação sanguínea em indivíduos obesos e ainda mais escassas são as investigações em crianças.

É importante salientar que nem todos os marcadores genéticos de susceptibilidade ao aumento do IMC em adultos identificados pelos GWAS foram replicados na obesidade infantil. Por outro lado, outros *loci* foram identificados apenas em crianças com formas extremas de obesidade ou em grandes estudos de coortes compilados em metanálises (19). Além disso, os resultados encontrados em crianças pelo presente grupo (54, 141-143) evidenciam a necessidade de explorar a investigação de genes relacionados à obesidade visando um melhor entendimento dos mecanismos moleculares da obesidade nas crianças Sul Riograndenses.

3 OBJETIVOS

O presente trabalho teve como objetivos:

3.1. Objetivo geral

Investigar a influência genética na obesidade infantil utilizando marcadores genéticos e epigenéticos, através da avaliação de polimorfismos em genes de susceptibilidade identificados pelos estudos de varredura genômica e do perfil de expressão de miRNAs circulantes em crianças acompanhadas desde o nascimento.

3.2. Objetivos específicos

3.2.1 Determinar os genótipos de dez polimorfismos representando nove *loci* de susceptibilidade nos genes *MC4R* (rs17782313), *TMEM18* (rs6548238), *BDNF* (rs6265 e rs10767664), *KCTD15* (rs11084753), *NEGR1* (rs2815752), *SEC16B* (rs10913469), *SH2B1* (rs7498665), *OLFM* (rs9568856) e *HOXB5* (rs9299) em uma amostra de crianças.

3.2.2 Analisar a associação das variantes nos genes acima descritos com medidas antropométricas e de ingestão alimentar ao nascimento, 12 meses e 3,5 anos de idade.

3.2.3. Avaliar a expressão de microRNAs circulantes em amostras de plasma de crianças magras e obesas e a sua correlação com parâmetros antropométricos e bioquímicos.

4 ARTIGOS CIENTÍFICOS ELABORADOS

Os resultados obtidos a partir deste trabalho permitiram a elaboração de dois artigos científicos.

Artigo 1

Validation of obesity susceptibility loci identified by genome-wide association studies in early childhood in South Brazilian children.

Aceito para publicação no periódico *Pediatric Obesity* em 04 de janeiro de 2016, Fator de Impacto 4.86

Artigo 2

Differential plasma concentrations of miR-16-5p and miR-19b in Brazilian lean and obese children.

Submetido ao periódico *International Journal of Obesity* em 17 de fevereiro de 2016, Fator de Impacto 5.004

4.1 Artigo 1

ORIGINAL ARTICLE

Validation of obesity susceptibility loci identified by genome-wide association studies in early childhood in South Brazilian children.

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Keywords: children, overweight, genetic susceptibility, energy intake

Running title: Validation of genes involved in childhood obesity.

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What is already known about this subject:

- Obesity is a heritable complex trait.

- Genome-wide association studies have identified several loci in the human genome containing genetic variants conferring an increased risk of developing overweight and obesity.

- Nine from the most accepted susceptibility genes to obesity-related phenotypes herein investigated have previously shown significant associations in adults.

What this study adds:

- Gene variants in/near *TMEM18, BDNF, NEGR1,* and *SEC16B* influence body mass index, subcutaneous fat and food intake in children in a very young age.

- Due to its longitudinal design, the present study provides some evidence about the age at which the action of the analyzed gene variants begins to be expressed on the obesity-related phenotypes.

- Influence of genetic components on this complex phenotype may be detected in young children, where environmental exposure and its impact have occurred for a relatively short period of their lifetime.

ABSTRACT

Background: The prevalence of childhood obesity has been dramatically increasing in developing countries as it has been reported for developed nations. Identifying susceptibility genes in early life could provide the foundations for interventions in lifestyle to prevent obese children to become obese adults.

Objectives: To evaluate the influence of genetic variants related to obesity identified by genome-wide association studies (*MC4R*, *TMEM18*, *KCTD15*, *SH2B1*, *SEC16B*, *BDNF*, *NEGR1*, *OLFM4* and *HOXB5* genes) on anthropometric and dietary phenotypes in two Brazilian cohorts followed-up since birth.

Methods: 745 children were examined at birth, after 1 year and after 3.5 years of follow-up. Ten single nucleotide polymorphisms were genotyped. Anthropometric and dietary parameters were compared among genotypes. Children were classified as overweight when BMI-Z score was > +1.

Results: Overweight prevalence was 30.7% at 3.5 years old. Significant associations were identified at 3.5 years for *TMEM18* rs6548238, *NEGR1* rs2815752, *BDNF* rs10767664 and rs6265 (1y and 3.5y) with anthropometric phenotypes, and at 3.5 years for *SEC16B* rs10913469 with dietary parameters.

Conclusions: Our results indicate that genetic variants in/near these genes contribute to obesity susceptibility in childhood and highlight the age at which they begin to affect obesity-related phenotypes.

INTRODUCTION

Childhood obesity prevalence has increased very fast over recent decades and now it represents a serious public health concern not restricted to developed nations, but in the developing countries as well. Preventing overweight from early childhood is of great importance in terms of public health since most of times overweight tracks through adulthood (1). This complex phenotype results from the interaction of environmental and multiple genetic factors influencing body mass index (BMI), with heritability estimations varying from 40 to 70% (2).

Genome-wide association studies (GWAS) have identified several loci in the human genome containing genetic variants associated with common diseases, such as obesity. More than 97 genetic loci have been robustly associated with obesity-related traits (3), but, in general, a little overlap in the results of these studies has been observed. A review of GWAS for obesity-related traits published until September 2012 (during the conception of this study) revealed that until that moment, nine GWAS had been published (4-12) and their results were compared. The ten variants which displayed significant signals in a higher number of GWAS were selected for this study.

Although associations of many common genetic variants with obesity have been replicated mainly in European and Asian populations, until now, few studies reporting the association of genetic variants with the risk of common obesity have been performed in South American children. In this study, we evaluated the influence of the most replicated genetic variants related to obesity previously identified through GWAS: melanocortin 4 receptor (*MC4R*), transmembrane protein 18 (*TMEM18*), brain-derived neurotrophic factor (*BDNF*), potassium channel tetramerization domain containing 15 (*KCTD15*), neuronal growth regulator 1 (*NEGR1*), Src-homology-2 B adaptor protein 1 (*SH2B1*), homolog of S. cerevisiae Sec16 (*SEC16B*), olfactomedin 4 (*OLFM4*), and homeobox B5 (*HOXB5*) on anthropometric

and food intake parameters in two cohorts totaling 745 Brazilian children followed-up since birth. Our aim was to validate the role of these obesity loci in Brazilian children and to identify at which age the genetic effect starts to manifest on the phenotype.

METHODS

The study included children from two birth cohorts from different cities in southern Brazil:

São Leopoldo Cohort

This was a cohort study nested in a randomized field trial in which 500 mother-child pairs were recruited at birth between October 2001 and July 2002 in São Leopoldo city, state of Rio Grande do Sul, Brazil (13). This city is located on the metropolitan region of the state and has currently about 200,000 inhabitants. Mothers were recruited at the maternity wards from the only city hospital that assists mainly the low-income populations. The children were randomized into intervention and control groups. The intervention consisted of dietary advice about breastfeeding and complementary feeding based on the “Ten steps for healthy feeding for Brazilian children from birth to 2 years of age” (13, 14). HIV-positive mothers and infants with congenital malformation were not eligible for the study. The children were reevaluated at the ages of 1 year and 3.9 years for the collection of dietary and anthropometric data.

Porto Alegre Cohort

This was also a cohort study nested in a randomized field trial performed between April of 2008 and May of 2012. Seven hundred and fifteen women were recruited during the third trimester of pregnancy at health centers in the eight district areas of the city of Porto Alegre, capital of the state of Rio Grande do Sul, Brazil. This city has currently about 1,400,000 inhabitants and is about 40 km apart from São Leopoldo. The intervention

consisted of an update to the ‘‘Ten steps to healthy eating for children younger than two years” (14) guide for all professionals working in the selected health centers, in addition to providing educational materials based on the food guide, to be delivered to all mothers undergoing prenatal and child care. HIV-positive women were excluded from the study. Subsequent phases of data collection happened at the ages of 1 year and 3.2 years.

The intervention was not the primary objective of the present research and the participation in intervention/control group was used as confounding factor in statistical analyses.

Dietary and anthropometric data collection

Fruits, vegetables, and lipid- and sugar dense foods consumption was assessed through dietary recalls for both cohorts. Structured questionnaires and two 24-hour recalls were applied at each stage with the mother or primary caregiver. The 24-hour recalls were performed on two nonconsecutive days, irrespective of the day of the week, and the mean values were used in the analyses.

At 1 year children were weighed naked using a portable digital scale (Techline, São Paulo, Brazil), and length was measured using an infant stadiometer (Serwital Inc, Porto Alegre, Brazil). At 3.2 (Porto Alegre) and 3.9 (São Leopoldo) years, children were directly weighted barefoot and wearing light clothes using a digital scale (Techline), and height was measured with children standing straight using a stadiometer (SECA, Hamburg, Germany). Tricipital and subscapular skinfold thicknesses and waist circumference were also measured. Body mass index (BMI) was calculated as [weight (kg)/height $(m)^2$], and BMI-for-age Z-scores (BMI-Z) were estimated based on the World Health Organization standards (15). Children were classified as overweight when BMI-Z was > +1. We used the term overweight referring to overweight and obese children combined.

Ethnicity was self-defined by parents through skin color, as whites and nonwhites (black and brown), as officially used in demographic censuses in the country. Previous studies performed in this geographic region show the Amerindian influence is very low (16). The children's mothers provided informed written consent and the studies were approved by the Ethics Committee of the Federal University of Health Sciences of Porto Alegre. Further details for both cohorts can be found elsewhere (13, 17).

Molecular analyses

Ten single nucleotide polymorphisms (SNPs) were genotyped: *MC4R* rs17782313, *TMEM18* rs6548238, *BDNF* rs6265 and rs10767664, *KCTD15* rs11084753, *NEGR1* rs2815752, *SH2B1* rs7498665, *SEC16B* rs10913469, *OLFM4* rs9568856 and *HOXB5* rs9299. For the São Leopoldo cohort DNA was extracted from whole blood, and in the Porto Alegre cohort DNA was extracted from epithelial buccal cells, both using methods of precipitation with a high salt concentration. The ten gene variants were genotyped through real-time polymerase chain reaction using TaqMan® SNP genotyping assays (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

Categorical variables are presented as relative frequencies (%) and continuous variables as means and standard deviations. Genotype and allele frequencies were calculated by gene counting. The chi-square test was used to verify if these frequencies were in agreement with those expected under Hardy-Weinberg equilibrium and to compare allele frequencies between the two localities and genotype/allele frequencies within ethnic groups.

Normality for each continuous variable was assessed by visual inspection using histograms of the respective distributions. Mean variables with normal distribution were compared among genotypes for each SNP using analysis of variance (ANOVA). When

significant results were found, the post-hoc Tukey test was used to identify which genotypes had different means from the others. All continuous variables were adjusted for the effects of gender, cohort and intervention group using multiple linear regressions without genotypes and then adding the respective residues to the mean of the variable before the comparisons between genotypes by ANOVA. For those genotypes with fewer than 20 individuals, the mean values for the rare genotype were combined with heterozygotes for analyses and mean variables were compared with T-tests for independent variables.

Sum of skinfolds were transformed into their natural logarithms prior to analysis in order to achieve a normal distribution. When the continuous variables were not normally distributed even after logarithmic transformation (sugar dense foods, SDF; and lipid dense foods, LDF), after visual inspection at histograms, we ran Kruskal-Wallis test when there were three genotypes or Mann-Whitney test when two genotypes were present.

For longitudinal analyses, we calculated the percentage change in BMI between 1 and 3.5 years of age as $[(BMI_3 - BMI_1)/BMI_1]x100$ for each individual and compared their means among genotypes using ANOVA or T-tests. This variable was also adjusted for the effects of gender, cohort and intervention group using multiple linear regressions before the comparisons between genotypes, as described before for continuous variables.

Control for multiple testing was performed by controlling the false discovery rate (FDR) at a 0.10 level as described by Benjamini and Hochberg in 1995 (18). *P*-values were corrected by the number of phenotypes analyzed for each gene variant, as there was a correlation between the phenotypes measured. Analyses for each gene were considered as independent hypotheses. Therefore, all corrected *P*-values in Table 3 and Supporting Information Tables S2, S3 and S4 should be evaluated using 0.10 as critical value. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, for windows version 20.0).

RESULTS

Seven hundred and forty five children were followed up since birth until 3.2 years old (Porto Alegre) and 3.9 years old (São Leopoldo), and were genotyped for the ten gene polymorphisms. The average age of children at the second evaluation was 1 year old, and at the last one was 3.5 years old. At 1 year, 41.1% of children were overweight and 13.7% were obese. These figures diminished a little at 3.5 years old, when 30.7% of children were overweight and 10.5% were obese. Descriptive data are presented in Table 1.

Minor allele frequencies (MAF) for the polymorphisms studied are shown in Table 2, together with National Center for Biotechnology Information dbSNP Database for European population (HapMap CEU: Utah residents with ancestry from northern and western Europe), Sub-Saharan African population (Hapmap YRI: Yoruba in Ibadan, Nigeria), and the Asian populations frequencies (HapMap CHB: Han Chinese in Beijing, China; and HapMap JPT: Japanese in Tokyo, Japan). There were only minor differences between allele frequencies in the two cohorts (Table 2). The frequencies for the majority of variants were closer to the CEU data (*NEGR1* rs2815752, *TMEM18* rs6548238, *BDNF* rs6265 and rs10767664, *SH2B1* rs7498665, *KCTD15* rs11084753) or intermediate between CEU and YRI (*OLFM4* rs9568856, *HOXB5* rs9299). For *SEC16B* rs10913469 and *MC4R* rs17782313, MAF was lower than in both populations. Genotype and allele frequencies were not different between ethnic groups (whites and non-whites) in our sample (Supporting Information Table S1). No differences were observed regarding either anthropometric or dietary measurements among ethnicities, also. Therefore, the individuals from both ethnicities were grouped in further analyses.

Mean weight and length were compared among different genotypes at birth, and other anthropometric and dietary variables were compared at 1 year and 3.5 years old (Table 3 and

Supporting Information Tables S2, S3 and S4). Significant associations of individual SNPs located at three of the nine genes investigated with anthropometric measurements were found and they are summarized in Table 3. For *TMEM18* rs6548238 we could observe that C/C homozygotes presented higher BMI-Z compared to T-allele carriers (*P*=0.007; *Pcorr*=0.049) at the age of 3.5. Carriers of the minor allele (A) of *BDNF* rs6265 presented lower BMI-Z at 1 year (*P*=0.026; *Pcorr*=0.091) and sum of skinfolds at 3.5 years old (*P*=0.011; *Pcorr*=0.077) than G/G homozygotes. At this same age, the minor T-allele of *BDNF* rs10767664 was associated with a reduction in the sum of skinfolds (*P*=0.009; *Pcorr*=0.063) when compared to the A/A genotype. For the *NEGR1* rs2815752, the A/A homozygotes presented higher BMI-Z (*P*=0.042; *Pcorr*=0.098) and sum of skinfolds at 3.5 years old (*P*=0.023; *Pcorr*=0.098). The post-hoc Tukey test revealed that these differences were due to a higher BMI and amount of subcutaneous fat in the A/A homozygotes than in the A/G heterozygotes. Longitudinal analyses demonstrated that individuals bearing the A/A genotype had also a lower variation in BMI from 1 to 3.5 years (*P*= 0.030; *Pcorr*=0.098). All results showed above remained significant when corrected for multiple comparisons using a FDR = 0.10.

Only one of the investigated genes presented associations with parameters related to dietary patterns: *SEC16B* rs10913469 (Supporting Information Table S2). The T/T genotype was associated with higher intake of total energy, carbohydrates, and lipids compared to C-carriers at 3.5 years (*P*=0.006, *Pcorr*=0.048; *P*=0.016, *Pcorr*=0.064; *P*=0.036, *Pcorr*=0.096; respectively).

We did not find any significant associations between *SH2B1*, *HOXB5*, *KCTD15*, *OLFM4*, *SEC16B*, *MC4R* and anthropometric parameters (Supporting Information Table S3), neither for *NEGR1*, *SH2B1*, *HOXB5*, *KCTD15*, *BDNF*, *TMEM18*, *OLFM4* and *MC4R* gene variants with dietary phenotypes (Supporting Information Table S4).

DISCUSSION

The advent of GWAS has brought several advances to the identification of new susceptibility loci for several complex characteristics, such as obesity. However, these studies have been focused in populations from North America and Europe, while the South American populations remained unexplored in this context.

In the present study, we were able to validate the associations of five of the 10 analyzed gene variants in a cohort of children from the South of Brazil followed from birth until 3.5 years of age. The *TMEM18, BDNF* and *NEGR1* gene variants investigated were associated with BMI-Z and subcutaneous fat in our setting, and the *SEC16B* SNP, although not associated with these phenotypes, was shown to be associated with higher daily intake of energy provided from carbohydrates and lipids at the age of 3.5. Our results also show that the *BDNF* variants effect begins earlier, at the age of 1, than the effect of the *NEGR1* and *TMEM18* gene variants, which seem to begin a little later, being detected after the age of 3.5.

A more recent GWAS, published in 2013 by Wheeler et al. (19), performed analyses with a high number of severely obese children and controls, identified nine genome-wide significant signals in eight loci, named: *FTO, MC4R, TMEM18, NEGR1, PRKCH, LEPR, PACS1*, and *RMST*, the last four being detected in GWAS for the first time. Although their sample is not totally comparable with ours, because their study focused on children in the extreme tail of the BMI distribution (BMI-Z more than 3 SD from the mean), we were able to replicate half of these findings in the present (*TMEM18, NEGR1*) and in previous studies performed with the same sample of children (*FTO* (20) and *LEPR* (21)). Of course, we must take in consideration that the specific gene variants were not the same, although located in or near the same genes, and that three of them (*PRKCH, PACS1,* and *RMST*) were not evaluated by us.

From the ten polymorphisms included in the present study, we identified significant associations for *NEGR1* rs2815752, *TMEM18* rs6548238, *BDNF* rs6265 and rs10767664 with anthropometric phenotypes (Table 3) and for *SEC16B* rs10913469 with dietary parameters (Supporting Information Table S2). A common feature between these genes is that they are expressed or known to act in the central nervous system, highlighting the importance of the neuronal component to the obesity susceptibility, although they have also peripheral functions related to adipose tissue (6, 22).

The associations reported herein were directionally consistent with results from GWAS: *TMEM18* rs6548238 C-allele (23, 24), *BDNF* rs6265 G-allele (25), rs10767664 A-allele (25), *NEGR1* rs2815752 A-allele (26, 27). Regarding *BDNF*, Zegers *et al*. screened the coding region of the gene and did not find mutations associated with overweight or obesity in children and adolescents (28). On the other hand, Gardner *et al*. revealed *BDNF* promoter methylation levels associated with satiety responsiveness in female children (29).

In our study, we found *SEC16B* rs10913469 T/T genotype associated with increased energy, carbohydrates, and lipids intake. Other studies also in pediatric samples observed the C-allele associated with BMI and risk of obesity. The minor allele for this SNP was found in our sample with a lower frequency than in European-derived (CEU) or African (YRI) population samples (Table 2). Therefore, these discrepancies may be due to population heterogeneity and/or different linkage disequilibrium patterns. It is important to notice that the individuals studied herein are all from Rio Grande do Sul, the Brazilian southernmost state, where the Amerindian and African influence is reduced in comparison with all other geographic regions from Brazil. One striking difference between Brazil and other countries that congregate European and African descents, like the United States of America, is the high degree of admixture that can be found between these ethnicities, making very difficult to determine ancestrality from phenotypic characteristics (30). Therefore, the finding that most

gene variants presented intermediate frequencies between Euro and Afro descendants was expected.

A limitation to our study is that our moderated sample size may not have been large enough to detect an influence of some of the studied polymorphisms on obesity-related traits. Furthermore, although we have not detected any difference in genotype frequencies or anthropometric and dietary measurements between ethnicities, our results may be interpreted with caution due to the bi-ethnic nature of our population. It is also noteworthy that this is a longitudinal study and our two cohorts are very well characterized in terms of anthropometry and food intake, which represents an important differential factor from many other studies. Our results suggest that the follow-up of our two cohorts may reveal other associations as the age of the children increase and thus provide interesting information.

In conclusion, we found that among the more than 97 *loci* that have been reported to associate with susceptibility to obesity, those located in or near *TMEM18, BDNF, NEGR1,* and *SEC16B* contribute to obesity susceptibility in childhood, as demonstrated by their associations in our two Brazilian pediatric cohorts. We reasoned that it is very relevant to detect the influence of the genetic component in this complex phenotype in childhood, where environmental exposure and its interaction with genotypes have occurred for a relatively short period of their lifetime. The continuity of the follow-up of the two cohorts may, in the future, bring more important findings in relation to these questions.

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared.

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MRZ carried out the experiments, the statistical analyses and wrote the manuscript. CS performed nutritional evaluation and database construction. PDBC and MRV were

responsible for the cohort selection, nutritional evaluation and sample collection. SA participated in the statistical analyses and manuscript writing. VSM conceived the experiments, analyzed and interpreted data and wrote the manuscript. All authors approved the final version. This work has been financially supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil).

REFERENCES

1. Whitaker RC, Wright JA, Pepe MS, Seidel KD, Dietz WH. Predicting obesity in young adulthood from childhood and parental obesity. *N Engl J Med* 1997; **337:** 869-873.

2. Maes HH, Neale MC, Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. *Behav Genet* 1997; **27:** 325-351.

3. Locke AE, Kahali B, Berndt SI, *et al.* Genetic studies of body mass index yield new insights for obesity biology. *Nature* 2015; **518:** 197-206.

4. Loos RJ, Lindgren CM, Li S, *et al.* Common variants near MC4R are associated with fat mass, weight and risk of obesity. *Nat Genet* 2008; **40:** 768-775.

5. Thorleifsson G, Walters GB, Gudbjartsson DF, *et al.* Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat Genet* 2009; **41:** 18-24.

6. Willer CJ, Speliotes EK, Loos RJ, *et al.* Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009; **41:** 25-34.

7. Speliotes EK, Willer CJ, Berndt SI, *et al.* Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet* 2010; **42:** 937-948.

8. Bradfield JP, Taal HR, Timpson NJ, *et al.* A genome-wide association meta-analysis identifies new childhood obesity loci. *Nat Genet* 2012; **44:** 526-531.

9. Wang K, Li WD, Zhang CK, *et al.* A genome-wide association study on obesity and obesity-related traits. *PloS One* 2011; **6:** e18939.

10. Zhao J, Bradfield JP, Li M, *et al.* The role of obesity-associated loci identified in genome-wide association studies in the determination of pediatric BMI. *Obesity* 2009; **17:** 2254-2257.

11. Fall T, Ingelsson E. Genome-wide association studies of obesity and metabolic syndrome. *Mol Cell Endocrinol* 2014; **382:** 740-757.

12. Xi B, Chandak GR, Shen Y, Wang Q, Zhou D. Association between common polymorphism near the MC4R gene and obesity risk: a systematic review and meta-analysis. *PloS One* 2012; **7:** e45731.

13. Vitolo MR, Rauber F, Campagnolo PD, Feldens CA, Hoffman DJ. Maternal dietary counseling in the first year of life is associated with a higher healthy eating index in childhood. *J Nutr* 2010; **140:** 2002-2007.

14. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. Dez passos para uma alimentação saudável: guia alimentar para menores de 2 anos. Um guia para o profissional da saúde na atenção básica [in Portuguese] Ministério da Saúde: Brasília, Brazil, 2010.

15. WHO Child Growth Standards: Lenght/height-for-age, weight-for-age, weight-for-lenght, wight-for-height and body mass index-for-age: Methods and development. World Health Organization: Geneva, 2006.

16. Zembrzuski VM, Callegari-Jacques SM, Hutz MH. Application of an African Ancestry Index as a genomic control approach in a Brazilian population. *Ann Hum Genet* 2006; **70:** 822-828.

17. Valmorbida JL, Vitolo MR. Factors associated with low consumption of fruits and vegetables by preschoolers of low socio-economic level. *Jornal de Pediatria* 2014; **90:** 464-471.

18. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *J R Statist Soc B* 1995; **57:** 289-300.

19. Wheeler E, Huang N, Bochukova EG, *et al.* Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity. *Nat Genet* 2013; **45:** 513-517.

20. da Silva CF, Zandona MR, Vitolo MR, *et al.* Association between a frequent variant of the FTO gene and anthropometric phenotypes in Brazilian children. *BMC Med Genet* 2013; **14:** 34.

21. Zandona MR, Rodrigues RO, Albiero G, *et al.* Polymorphisms in LEPR, PPARG and APM1 genes: associations with energy intake and metabolic traits in young children. *Arq Bras Endocr Metab* 2013; **57:** 603-611.

22. Bernhard F, Landgraf K, Kloting N, *et al.* Functional relevance of genes implicated by obesity genome-wide association study signals for human adipocyte biology. *Diabetologia* 2013; **56:** 311-322.

23. Rask-Andersen M, Jacobsson JA, Moschonis G, *et al.* Association of TMEM18 variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats. *Eur J Hum Genet : EJHG* 2012; **20:** 192-197.

24. den Hoed M, Ekelund U, Brage S, *et al.* Genetic susceptibility to obesity and related traits in childhood and adolescence: influence of loci identified by genome-wide association studies. *Diabetes* 2010; **59:** 2980-2988.

25. Mei H, Chen W, Jiang F, *et al.* Longitudinal replication studies of GWAS risk SNPs influencing body mass index over the course of childhood and adulthood. *PloS One* 2012; **7:** e31470.

26. Mejia-Benitez A, Klunder-Klunder M, Yengo L, *et al.* Analysis of the contribution of FTO, NPC1, ENPP1, NEGR1, GNPDA2 and MC4R genes to obesity in Mexican children. *BMC Med Genet* 2013; **14:** 21.

27. Ntalla I, Panoutsopoulou K, Vlachou P, *et al.* Replication of established common genetic variants for adult BMI and childhood obesity in Greek adolescents: the TEENAGE study. *Ann Hum Genet* 2013; **77:** 268-274.

28. Zegers D, Hendrickx R, Verrijken A, *et al.* Screening for genetic variants in BDNF that contribute to childhood obesity. *Pediatr Obes* 2014; **9:** 36-42.

29. Gardner KR, Sapienza C, Fisher JO. Genetic and epigenetic associations to obesity-related appetite phenotypes among African-American children. *Pediatr Obes* 2015.

30. Durso DF, Bydlowski SP, Hutz MH, Suarez-Kurtz G, Magalhaes TR, Pena SD. Association of genetic variants with self-assessed color categories in Brazilians. *PloS One*; **9:** e83926.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Polymorphisms frequencies and anthropometric and dietary measurements according to ethnic groups.

Table S2 Association of the *SEC16B* rs10913469 polymorphism with dietary parameters.

Table S3 Measures of anthropometric parameters according to *SH2B1*, *HOXB5*, *KCTD15*, *OLFM4*, *SEC16B* and *MC4R* gene variants.

Table S4 Measures of dietary parameters according to *NEGR1*, *SH2B1*, *HOXB5*, *KCTD15*, *BDNF*, *TMEM18*, *OLFM4* and *MC4R* gene variants.

Table 1 Descriptive characteristics of the two cohorts at birth and over the years

Characteristic	Porto Alegre (n=423)	São Leopoldo (n=322)	*P*-value
Boy (%)	50.7	57.6	0.059[a]
Ethnicity (whites) %	55.9	43.0	0.001[a]
Maternal age at child's birth < 20 years (%)	29.8	25.5	0.199 [a]
Monthly household income < $500 (%)	45.7	96.3	<0.001[a]
Maternal schooling > 8 years (%)	70.2	28.3	<0.001[a]
Employed mother (%)	33.6	35.4	0.612[a]
Exclusive breastfeeding < 4 months (%)	74.7	66.4	0.016[a]
At birth			
Weight (kg)	3.29±0.52	3.36±0.46	0.053[b]
Length (cm)	49.21±3.19	48.79±2.00	0.031[c]
Length/age Z-score	-0.26±1.29	-0.40±1.03	0.057[b]
1 year			
Weight (Kg)	10.12±1.29	9.92±1.19	0.033 [b]
Length (cm)	74.8±3.0	75.3±3.1	0.037 [b]
BMI Z-score	0.99±1.03	0.61±1.09	<0.001[b]
Length/age Z-score	-0.03±0.93	-0.32±1.09	<0.001[b]
Overweight (%)	47.0	35.2	0.003[a]
Obesity (%)	18.0	9.4	0.001[a]
3.2 years*/3.9 years**			
Weight (Kg)	15.80±2.32	16.82±2.58	<0.001[b]
Height (cm)	93.8±4.4	103.1±4.6	<0.001[b]
BMI Z-score	0.87±1.25	0.22±0.99	<0.001[c]
Length/age Z-score	-0.15±1.04	0.06±1.04	<0.006[b]
Overweight (%)	42.3	19.1	<0.001[a]
Obesity (%)	16.6	4.4	<0.001[a]

Data are presented as mean ± standard deviation or percentage. *na*, data not available. [a]Chi square test; [b]Student's T test; [c]Mann-Whitney U test. *Porto Alegre children were followed-up until 3.2 years old. **São Leopoldo children were followed-up until 3.9 years old. Children were classified as overweight when BMI-Z was > +1 and obese when BMI-Z was > +2.

Table 2 Minor allele frequencies for the polymorphisms analyzed

Nearest Gene	Chr	SNP	MA	Hapmap CEU[a]	Hapmap YRI[b]	Hapmap CHB/JPT[c]	São Leopoldo	Porto Alegre	*P*-value[d]
NEGR1	1	rs2815752	G	0.36	0.47	0.08/0.05	0.34	0.33	0.811
SEC16B	1	rs10913469	C	0.25	0.34	0.24/0.26	0.17	0.18	0.755
TMEM18	2	rs6548238	T	0.15	0.10	*n.a.*/0.18	0.15	0.12	0.276
BDNF	11	rs6265	A	0.19	0.004	0.37/0.42	0.15	0.14	0.748
BDNF	11	rs10767664	T	0.20	0.04	*n.a.*/0.35	0.17	0.16	0.834
OLFM4	13	rs9568856	A	0.13	0.31	0.20/0.30	0.22	0.25	0.397
SH2B1	16	rs7498665	G	0.38	0.21	0.13/0.18	0.39	0.39	0.958
HOXB5	17	rs9299	C	0.37	0.90	0.39/0.49	0.43	0.47	0.397
MC4R	18	rs17782313	C	0.26	0.31	0.24/0.23	0.17	0.20	0.348
KCTD15	19	rs11084753	A	0.31	0.31	0.67/0.62	0.33	0.36	0.394

Chr, chromosome; MA, minor allele; *n.a.*, data not available; [a]European population; [b]Sub-Saharan African population; [c]Asian populations; [d]Chi square test between Porto Alegre and São Leopoldo localities.

Table 3 Associations of *NEGR1*, *BDNF* and *TMEM18* polymorphisms with anthropometric parameters.

Phenotype	Genotype		Genotype		Genotype		*P* value	*P* corrected
	mean±SD		mean±SD		mean±SD			
NEGR1 rs2815752	A/A		A/G		G/G			
At birth								
Length (cm)	48.9±2.8	305	49.0±2.8	306	49.2±2.9	75	0.944[a]	0.944
Weight (g)	3320±525	305	3314±541	306	3282±597	75	0.848[a]	0.944
1 year								
BMI Z-score	0.84±1.14	291	0.83±1.09	295	0.63±1.21	73	0.355[a]	0.497
3.5 years								
BMI Z-score[c]	0.68±1.21	300	0.44±1.13	306	0.57±1.19	77	0.042[a]	0.098*
Sum of skinfolds (mm)[d]	15.7±5.9	301	14.5±4.1	304	14.3±4.4	76	0.023[a]	0.098*
Waist circumference (cm)	52.1±5.9	302	51.2±3.4	306	51.3±4.0	76	0.072[a]	0.126
BMI variation 1y - 3.5y (%)[e]	-7.2±10.3	287	-9.2±8.5	292	-7.3±8.4	73	0.030[a]	0.098*
BDNF rs6265	A-carriers		G/G					
At birth								
Length (cm)	49.1±2.8	200	49.0±2.7	542			0.469[b]	0.656
Weight (g)	3309±55	200	3301±530	542			0.909[b]	0.909
1 year								
BMI Z-score	0.64±1.17	192	0.85±1.11	521			0.026[b]	0.091*
3.5 years								
BMI Z-score	0.48±1.14	199	0.59±1.18	538			0.330[b]	0.588
Sum of skinfolds (mm)	14.3±4.3	196	15.4±5.3	541			0.011[b]	0.077*
Waist circumference (cm)	51.3±5.0	197	51.7±4.7	544			0.336[b]	0.588
BMI variation 1y - 3.5y (%)	-7.6±9.7	190	-7.9±9.3	514			0.580[b]	0.676
BDNF rs10767664	T-carriers		A/A					
At birth								
Length (cm)	49.1±2.8	213	49.0±2.7	522			0.692[b]	0.884
Weight (g)	3304±538	213	3303±536	522			0.884[b]	0.884
1 year								
BMI Z-score	0.68±1.17	205	0.84±1.10	501			0.082[b]	0.287
3.5 years								
BMI Z-score	0.44±1.10	212	0.61±1.20	519			0.167[b]	0.389
Sum of skinfolds (mm)	14.2±4.0	208	15.4±5.4	522			0.009[b]	0.063*
Waist circumference (cm)	51.3±4.9	209	51.8±4.8	524			0.262[b]	0.458
BMI variation 1y - 3.5y (%)	-8.1±9.6	203	-7.6±9.5	495			0.770[b]	0.884

TMEM18 rs6548238	T-carriers		C/C			
At birth						
Length (cm)	49.1±2.9	189	49.0±2.7	556	0.477[b]	0.552
Weight k(g)	3286±540	189	3311±531	556	0.552[b]	0.552
1 year						
BMI Z-score	0.67±1.03	180	0.83±1.15	534	0.099[b]	0.259
3.5 years						
BMI Z-score	0.33±1.16	188	0.65±1.18	552	0.007[b]	0.049*
Sum of skinfolds (mm)	14.7±4.6	187	15.2±5.2	551	0.363[b]	0.508
Waist circumference (cm)	51.1±5.1	187	51.8±4.6	555	0.111[b]	0.259
BMI variation 1y - 3.5y (%)	-8.7±8.5	177	-7.4±9.8	528	0.153[b]	0.268

Data are presented as mean ± standard deviation. BMI, body mass index. Variables were adjusted by gender, cohort and intervention group. [a]Oneway-ANOVA; [b]Student's T test; [c]Tukey test A/A x A/G *P*=0.052, A/A x G/G *P*=0.723, A/G x G/G *P*=0.753; [d]Tukey test A/A x A/G *P*=0.060, A/A x G/G *P*=0.071, A/G x G/G *P*=0.726; [e]Tukey test A/A x A/G *P*=0.031, A/A x G/G *P*=0.999, A/G x G/G *P*=0.262. Statistical test performed with sum of skinfolds variable ln-transformed. *Significant corrected *P*-values according to Benjamini-Hochberg method with the false discovery rate of 0.10.

Validation of obesity susceptibility loci identified by genome-wide association studies in early childhood in South Brazilian children.

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Table S1 Polymorphisms frequencies and anthropometric and dietary measurements according to ethnic groups.

	Whites			Non whites			$P_{genotype}$[a]	P_{allele}[a]
Genotypes	n (%)	Allele	Frequency	n (%)	Allele	Frequency		
NEGR1 rs2815752								
A/A	157 (45.9)	A	0.68	142 (42.5)	A	0.65	0.220	0.264
A/G	154 (45.0)	G	0.32	148 (44.3)	G	0.35		
G/G	31 (9.1)			44 (13.2)				
SEC16B 10913469								
T/T	263 (68.8)	T	0.82	246 (69.7)	T	0.83	0.835	0.677
C/T	104 (27.2)	C	0.18	96 (27.2)	C	0.17		
C/C	15 (3.9)			11 (3.1)				
TMEM18 rs6548238								
C/C	282 (73.4)	C	0.85	267 (75.6)	C	0.87	0.188	0.318
C/T	92 (24.0)	T	0.15	83 (23.5)	T	0.13		
T/T	10 (2.6)			3 (0.8)				
BDNF rs6265								
G/G	277 (72.9)	G	0.84	262 (74.0)	G	0.86	0.092	0.300
G/A	87 (22.9)	A	0.16	87 (24.6)	A	0.14		
A/A	16 (4.2)			5 (1.4)				
BDNF rs10767664								
A/A	265 (70.3)	A	0.83	255 (72.9)	A	0.85	0.584	0.339
A/T	94 (24.9)	T	0.17	83 (23.7)	T	0.15		
T/T	18 (4.8)			12 (3.4)				
OLFM4 rs9568856								
A/A	16 (4.3)	A	0.22	20 (5.8)	A	0.25	0.336	0.204
A/G	130 (35.3)	G	0.78	134 (39.0)	G	0.75		
G/G	222 (60.3)			190 (55.2)				
SH2B1 rs7498665								

A/A	147 (38.7)	A	0.60	137 (39.1)	A	0.62	0.226	0.466
A/G	159 (41.8)	G	0.40	161 (46.0)	G	0.38		
G/G	74 (19.5)			52 (14.9)				
HOXB5 rs9299								
C/C	72 (19.3)	C	0.43	79 (23.2)	C	0.47	0.074	0.139
C/T	176 (47.3)	T	0.57	162 (47.5)	T	0.53		
T/T	123 (33.0)			101 (29.6)				
MC4R rs17782313								
T/T	263 (69.6)	T	0.82	234 (66.9)	T	0.81	0.190	0.668
C/T	92 (24.3)	C	0.18	102 (29.1)	C	0.19		
C/C	23 (6.1)			14 (4.0)				
KCTD15 rs11084753								
A/A	55 (14.5)	A	0.35	45 (12.7)	A	0.34	0.785	0.740
A/G	160 (42.1)	G	0.65	152 (42.9)	G	0.66		
G/G	165 (43.4)			157 (44.4)				
Anthropometric measurements	**mean±SD**	**n**		**mean±SD**	**n**			***P*[b]**
At birth								
Length (cm)	49.1±2.8	485		49.0±2.7	433			0.774
Weight (g)	3279±495	485		3320±553	433			0.230
1 year								
BMI Z-score	0.75±1.11	439		0.83±1.17	394			0.333
3.5 years								
BMI Z-score[c]	0.67±1.12	393		0.55±1.28	362			0.207
Sum of skinfolds (mm)	15.6±4.9	390		14.8±5.5	363			0.077
Waist circumference (cm)	51.9±5.2	396		51.5±4.5	361			0.168
Dietary measurements								
1 year								
Energy intake (kcal/day)	1008±434	421		966±445	380			0.173
Carbohydrates intake(kcal/day)	575±256	424		562±273	377			0.490
Lipids intake (kcal/day)	273±137	424		257±136	377			0.103
3.5 years								
Energy intake (kcal/day)	1508±427	393		1514±419	353			0.850
Carbohydrates intake (kcal/day)	838±253	393		847±257	348			0.641
Lipids intake (kcal/day)	445±162	393		432±152	348			0.294
SDF intake (kcal/day)	116±108	393		114±100	353			0.822
LDF intake (kcal/day)	132±176	393		136±173	353			0.774

SDF, sugar dense foods (50% or more in 100 grams); LDF, lipid dense foods (30% or more in 100 grams). [a]Chi square test; [b]Student's T test. All statistic tests were run between whites and non-whites.

Table S2 Association of the *SEC16B* rs10913469 polymorphism with dietary parameters.

Phenotype	T/T		C-carriers		*P* value	*P* corrected
1 year	mean±SD	n	mean±SD	n		
Energy intake (kcal/day)	982±429	471	982±434	211	0.961[a]	0.985
Carbohydrates intake(kcal/day)	560±260	485	561±256	218	0.885[a]	0.985
Lipids intake (kcal/day)	265±137	485	262±136	218	0.985[a]	0.985
3.5 years						
Energy intake (kcal/day)	1541±423	504	1445±418	227	0.006[a]	0.048*
Carbohydrates intake (kcal/day)	859±252	511	808±261	232	0.016[a]	0.064*
Lipids intake (kcal/day)	447±160	511	420±154	232	0.036[a]	0.096*
SDF intake (kcal/day)	113±98	504	118±117	227	0.812[b]	0.985
LDF intake (kcal/day)	141±177	504	129±176	227	0.215[b]	0.430

Data are presented as mean ± standard deviation. SDF, sugar dense foods (50% or more in 100 grams); LDF, lipid dense foods (30% or more in 100 grams). [a]Student's T test; [b]Mann-Whitney test. Variables were adjusted by gender, cohort and intervention group.*Significant corrected *P*-values according to Benjamini-Hochberg method with the false discovery rate of 0.10.

Table S3 Measures of anthropometric parameters according to *SH2B1, HOXB5, KCTD15, OLFM4, SEC16B* and *MC4R* gene variants.

Phenotype	Genotype		Genotype		Genotype		*P*-value	*P* corrected
	mean±SD	n	mean±SD	n	mean±SD	n		
***SH2B1* rs7498665**	A/A		A/G		G/G			
At birth								
Length (cm)	49.1±2.7	283	48.9±2.9	324	49.2±2.5	131	0.654[a]	0.916
Weight (g)	3346±517	283	3272±559	324	3306±515	131	0.263[a]	0.916
1 year								
BMI Z-score	0.78±1.10	275	0.79±1.16	307	0.82±1.09	126	0.950[a]	0.951
3.5 years								
BMI Z-score	0.54±1.10	282	0.53±1.20	323	0.71±1.28	128	0.495[a]	0.916
Sum of skinfolds (mm)	14.9±4.8	277	15.1±5.1	327	15.3±5.7	128	0.951[a]	0.951
Waist circumference (cm)	51.6±5.7	281	51.3±3.9	326	52.4±4.6	129	0.110[a]	0.770
BMI variation 1y - 3.5 y (%)	-8.0±8.8	271	-8.1±9.7	305	-6.8±9.8	123	0.574[a]	0.916
***HOXB5* rs9299**	C/C		C/T		T/T			
At birth								
Length (cm)	49.1±3.4	151	49.0±2.6	340	48.9±2.5	230	0.971[a]	0.971
Weight (g)	3245±597	151	3329±520	340	3295±511	230	0.272[a]	0.574
1 year								
BMI Z-score	0.82±1.15	142	0.79±1.15	332	0.74±1.07	218	0.773[a]	0.901
3.5 years								
BMI Z-score	0.54±1.28	151	0.58±1.16	339	0.51±1.09	227	0.586[a]	0.820
Sum of skinfolds (mm)	14.7±4.6	151	15.3±5.5	340	14.8±4.4	226	0.328[a]	0.574
Waist circumference (cm)	51.7±5.4	153	51.8±5.1	341	51.2±3.6	226	0.272[a]	0.574
BMI variation 1y - 3.5 y (%)	-8.6±10.2	140	-7.3±9.4	329	-8.2±8.5	214	0.165[a]	0.574
***KCTD15* rs11084753**	A/A		A/G		G/G			
At birth								
Length (cm)	48.9±2.0	101	49.0±2.8	315	49.1±2.9	326	0.783[a]	0.785
Weight (g)	3350±468	101	3297±505	315	3301±582	326	0.681[a]	0.785
1 year								
BMI Z-score	0.84±1.09	97	0.73±1.10	302	0.84±1.16	313	0.451[a]	0.785
3.5 years								
BMI Z-score	0.53±1.12	98	0.52±1.11	316	0.63±1.27	324	0.391[a]	0.785
Sum of skinfolds (mm)	15.2±5.2	99	14.7±4.3	312	15.4±5.6	325	0.263[a]	0.785
Waist circumference (cm)	51.6±3.9	100	51.5±5.3	315	51.8±4.3	325	0.785[a]	0.785
BMI variation 1y - 3.5 y (%)	-8.8±9.6	94	-7.9±8.5	300	-7.5±10.2	309	0.493[a]	0.785

***OLFM4* rs9568856**	G/G		A-carriers			
At birth						
Length (cm)	49.0±2.6	418	49.0±2.9	303	0.961[b]	0.998
Weight (g)	3303±507	418	3307±574	303	0.998[b]	0.998
1 year						
BMI Z-score	0.80±1.11	403	0.78±1.16	289	0.091[b]	0.637
3.5 years						
BMI Z-score	0.54±1.18	415	0.55±1.16	301	0.956[b]	0.998
Sum of skinfolds (mm)	15.1±5.1	415	14.8±4.8	300	0.493[b]	0.998
Waist circumference (cm)	51.7±4.8	418	51.5±4.6	300	0.616[b]	0.998
BMI variation 1y - 3.5 y (%)	-8.1±9.8	399	-7.6±8.9	284	0.569[b]	0.998
***SEC16B* rs10913469**	T/T		C-carriers			
At birth						
Length (cm)	49.1±2.9	512	48.9±2.5	231	0.351[b]	0.828
Weight (g)	3302±536	512	3314±533	231	0.828[b]	0.828
1 year						
BMI Z-score	0.83±1.07	493	0.82±1.10	220	0.824[b]	0.828
3.5 years						
BMI Z-score	0.58±1.16	512	0.54±1.23	226	0.543[b]	0.828
Sum of skinfolds (mm)	15.0±4.7	507	15.2±5.8	229	0.818[b]	0.828
Waist circumference (cm)	51.6±4.9	510	51.8±4.5	230	0.602[b]	0.828
BMI variation 1y - 3.5 y (%)	-8.0±9.1	487	-7.5±10.2	217	0.686[b]	0.828
***MC4R* rs17782313**	C-carriers		T/T			
At birth						
Length (cm)	48.9±2.7	233	49.0±2.8	504	0.831[b]	0.831
Weight (g)	3335±513	233	3295±544	504	0.302[b]	0.513
1 year						
BMI Z-score	0.76±1.20	224	0.82±1.09	483	0.554[b]	0.646
3.5 years						
BMI Z-score	0.45±1.14	231	0.63±1.20	500	0.032[b]	0.224
Sum of skinfolds (mm)	14.8±5.0	233	15.2±5.2	496	0.152[b]	0.513
Waist circumference (cm)	51.5±4.1	232	51.8±5.1	501	0.367[b]	0.513
BMI variation 1y - 3.5y (%)	-8.2±9.5	223	-7.6±9.5	475	0.319[b]	0.513

Data are presented as mean ± standard deviation. Variables were adjusted by gender, cohort and intervention group. [a]Oneway-ANOVA; [b]Student's T test. Statistical test performed with sum of skinfolds variable ln-transformed. Corrected *P*-values according to Benjamini-Hochberg methods with the false discovery rate of 0.10.

Table S4 Measures of dietary parameters according to *NEGR1*, *SH2B1*, *HOXB5*, *KCTD15*, *BDNF*, *TMEM18*, *OLFM4* and *MC4R* gene variants.

Phenotype	Genotype		Genotype		Genotype		*P*-value	*P* corrected
	mean±SD	n	mean±SD	n	mean±SD	n		
***NEGR1* rs2815752**	A/A		A/G		G/G			
1 year								
Total energy intake (kcal/day)	981±423	271	962±421	287	1028±421	72	0.028[a]	0.224
Carbohydrates (kcal/day)	557±248	281	546±247	295	588±273	77	0.456[a]	0.812
Lipids (kcal/day)	271±145	281	255±132	295	266±127	77	0.644[a]	0.812
3.5 years								
Total energy intake (kcal/day)	1487±416	300	1521±402	299	1551±444	74	0.570[a]	0.812
Carbohydrates (kcal/day)	835±253	306	843±246	304	868±268	75	0.765[a]	0.812
Lipids (kcal/day)	429±156	306	447±160	304	441±148	75	0.558[a]	0.812
SDF (kcal/day)	111±97	300	115±108	299	107±96	74	0.812[b]	0.812
LDF (kcal/day)	130±170	300	151±189	299	154±184	74	0.754[b]	0.812
***SH2B1* rs7498665**	A/A		A/G		G/G			
1 year								
Total energy intake (kcal/day)	950±425	268	1020±440	293	931±393	117	0.033[a]	0.160
Carbohydrates (kcal/day)	542±252	276	579±270	306	537±224	119	0.046[a]	0.160
Lipids (kcal/day)	256±143	276	272±133	306	251±131	119	0.060[a]	0.160
3.5 years								
Total energy intake (kcal/day)	1499±425	281	1510±426	317	1530±416	128	0.602[a]	0.688
Carbohydrates (kcal/day)	828±244	283	850±271	326	851±240	129	0.367[a]	0.489
Lipids (kcal/day)	437±156	283	436±162	326	439±155	129	0.816[a]	0.816
SDF (kcal/day)	107±90	281	122±115	317	112±105	128	0.263[b]	0.420
LDF (kcal/day)	151±181	281	133±175	317	116±167	128	0.112[b]	0.224
***HOXB5* rs9299**	C/C		C/T		T/T			
1 year								
Total energy intake (kcal/day)	1034±457	140	964±409	314	975±437	205	0.451[a]	0.613
Carbohydrates (kcal/day)	583±279	147	551±239	321	555±268	214	0.402[a]	0.613
Lipids (kcal/day)	272±144	147	259±138	321	266±131	214	0.532[a]	0.613
3.5 years								
Total energy intake (kcal/day)	1575±436	150	1472±398	338	1530±442	221	0.045[a]	0.122
Carbohydrates (kcal/day)	886±283	155	821±227	340	840±268	227	0.046[a]	0.122
Lipids (kcal/day)	457±160	155	422±148	340	451±167	227	0.025[a]	0.122
SDF (kcal/day)	120±118	150	115±101	339	110±98	221	0.849[b]	0.849
LDF (kcal/day)	129±166	150	134±174	339	149±188	221	0.537[b]	0.613

***KCTD15* rs11084753**	A/A		A/G		G/G			
1 year								
Total energy intake (kcal/day)	1010±427	93	968±425	292	979±434	297	0.816[a]	0.963
Carbohydrates (kcal/day)	586±245	94	554±253	299	553±267	312	0.677[a]	0.963
Lipids (kcal/day)	273±142	94	260±139	299	262±132	312	0.612[a]	0.963
3.5 years								
Total energy intake (kcal/day)	1526±449	96	1512±417	313	1507±423	321	0.880[a]	0.963
Carbohydrates (kcal/day)	850±264	97	842±245	314	842±264	331	0.948[a]	0.963
Lipids (kcal/day)	440±171	97	441±162	314	435±153	331	0.963[a]	0.963
SDF (kcal/day)	101±82	96	112±99	313	122±114	321	0.355[b]	0.963
LDF (kcal/day)	99±139	96	144±195	313	142±166	321	0.182[b]	0.963
***BDNF* rs6265**	A-carriers		G/G					
1 year								
Total energy intake (kcal/day)	969±443	184	989±434	497			0.696[c]	0.867
Carbohydrates (kcal/day)	553±272	192	564±259	512			0.867[c]	0.867
Lipids (kcal/day)	255±129	192	267±141	512			0.466[c]	0.867
3.5 years								
Total energy intake (kcal/day)	1497±401	194	1516±424	536			0.581[c]	0.867
Carbohydrates (kcal/day)	836±234	194	845±260	543			0.659[c]	0.867
Lipids (kcal/day)	436±148	199	438±160	543			0.856[c]	0.867
SDF (kcal/day)	113±106	194	115±103	536			0.566[d]	0.867
LDF (kcal/day)	144±174	194	135±178	536			0.747[d]	0.867
***BDNF* rs10767664**	T-carriers		A/A					
1 year								
Total energy intake (kcal/day)	971±438	197	984±425	478			0.759[c]	0.962
Carbohydrates (kcal/day)	553±270	205	560±253	494			0.962[c]	0.962
Lipids (kcal/day)	258±129	205	266±140	494			0.604[c]	0.962
3.5 years								
Total energy intake (kcal/day)	1513±406	207	1506±422	517			0.928[c]	0.962
Carbohydrates (kcal/day)	845±238	212	839±260	525			0.872[c]	0.962
Lipids (kcal/day)	442±151	212	436±161	525			0.422[c]	0.962
SDF (kcal/day)	110±104	207	115±105	517			0.397[d]	0.962
LDF (kcal/day)	144±172	207	135±179	517			0.785[d]	0.962
***TMEM18* rs6548238**	T-carriers		C/C					
1 year								
Total energy intake (kcal/day)	958±412	175	988±443	508			0.627[c]	0.877
Carbohydrates (kcal/day)	546±241	181	563±262	524			0.768[c]	0.877
Lipids (kcal/day)	253±134	181	268±137	524			0.359[c]	0.877

3.5 years						
Total energy intake (kcal/day)	1465±421	187	1526±423	546	0.074[c]	0.296
Carbohydrates (kcal/day)	811±236	191	853±262	554	0.070[c]	0.296
Lipids (kcal/day)	430±165	191	441±156	554	0.560[c]	0.877
SDF (kcal/day)	111±96	187	115±107	546	0.902[d]	0.902
LDF (kcal/day)	146±180	187	134±174	546	0.663[d]	0.877
***OLFM4* rs9568856**	G/G		A-carriers			
1 year						
Total energy intake (kcal/day)	966±412	383	995±453	278	0.424[c]	0.989
Carbohydrates (kcal/day)	553±250	398	563±272	286	0.504[c]	0.989
Lipids (kcal/day)	261±130	398	267±146	286	0.411[c]	0.989
3.5 years						
Total energy intake (kcal/day)	1508±435	412	1518±395	296	0.803[c]	0.989
Carbohydrates (kcal/day)	841±258	421	847±248	299	0.874[c]	0.989
Lipids (kcal/day)	440±164	421	436±150	299	0.536[c]	0.989
SDF (kcal/day)	115±107	412	114±101	296	0.989[d]	0.989
LDF (kcal/day)	144±184	412	131±166	296	0.646[d]	0.989
***MC4R* rs17782313**	T/T		C-carriers			
1 year						
Total energy intake (kcal/day)	981±428	465	979±428	212	0.912[c]	0.978
Carbohydrates (kcal/day)	563±257	476	556±253	216	0.795[c]	0.978
Lipids (kcal/day)	265±135	476	261±139	216	0.843[c]	0.978
3.5 years						
Total energy intake (kcal/day)	1516±420	504	1498±428	221	0.607[c]	0.978
Carbohydrates (kcal/day)	841±249	510	842±263	224	0.978[c]	0.978
Lipids (kcal/day)	441±157	510	429±163	224	0.386[c]	0.978
SDF (kcal/day)	111±109	504	120±92	221	0.040[d]	0.320
LDF (kcal/day)	132±170	504	148±188	221	0.519[d]	0.978

Data are presented as mean ± standard deviation. SDF, sugar dense foods (50% or more sugar in 100 grams); LDF, lipid dense foods (30% or more in 100 grams). Variables were adjusted by gender, cohort and intervention group. [a]Oneway ANOVA; [b]Kruskal-Wallis test; [c]Student's T test; [d]Mann-Whitney test. Corrected *P*-values according to Benjamini-Hochberg method with the false discovery rate of 0.10.

4.2 Artigo 2

ORIGINAL ARTICLE

Differential plasma concentrations of microRNA-16-5p and microRNA-19b in Brazilian lean and obese children.

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Running Title: Circulating microRNAs in obese and lean children

Conflict of Interest: The authors declare no conflict of interest.

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ABSTRACT

Background/Objectives: Circulating microRNAs (miRNAs) are been considered valuable biomarkers of many metabolic diseases, such as obesity, and potential promising targets for the diagnosis and clinical monitoring of adiposity and its complications. Our objective was to investigate whether circulating miRNAs differ between lean and obese children in southern Brazil. **Subjects/Methods:** Nine plasma miRNAs were assessed by quantitative polymerase chain reaction in 38 children aged 6.2 years (18 lean and 20 obese). We compared miRNA expression between lean and obese children and performed correlation analyses with anthropometric measurements and biochemical variables. **Results:** Two circulating miRNAs were differentially expressed in childhood, with decreased levels of miR-16-5p (P=0.008) and miR-19b (P=0.046) in obese children compared to the lean group. The plasma concentrations of these two miRNAs were inversely correlated with body mass index, and miR-16-5p was inversely correlated with plasma insulin levels. **Conclusions:** Our findings contribute to a better understanding of the role of miRNAs in physiopathology of obesity and suggest a potential role of circulating miR-16-5p and miR-19b in childhood obesity as novel biomarkers for the early prevention and to improve the diagnostic power of obesity-associated metabolic complications. To our knowledge, this study provides the first evidence of obesity-related circulating miRNAs in South American children.

INTRODUCTION

The prevalence of childhood obesity has increased worldwide over the recent decades. Obesity is an important risk factor for many chronic diseases including hypertension, dyslipidemia, type 2 diabetes mellitus and premature cardiovascular complications, which have been previously found only in adults[1]. One of the greatest present challenges is to find out early biomarkers for the diagnosis and clinical monitoring of adiposity and its complications that can be used still in childhood, in order to prevent the occurrence of asymptomatic clinical conditions such as atherosclerosis in adulthood[2, 3]. Many factors such as inappropriate diet and sedentary lifestyle are contributing to the obesity growing epidemics, but it is widely recognized as an heritable condition, with multiple genetic factors influencing its development.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level by pairing with the 3' untranslated region of messenger RNAs (mRNAs), leading to translation repression and/or mRNA degradation[4]. They are believed to play important regulatory roles in most biological processes, including adipocyte differentiation, insulin action and fat metabolism[5] and may be modulated by inflammatory events according to physiological and pathological states[6]. Several studies have reported evidence of miRNA dysregulation in obese adipose tissue in human and animal models[7-10]. Furthermore, miRNAs have also been found in body fluids, such as serum and plasma, in a stable and cell independent form[11]. Altered expression profiles of circulating miRNAs have been found in various diseases such as cancer, diabetes and obesity, showing their potential as new non-invasive biomarkers for human diseases, and making them promising targets for therapeutic strategies[12, 13].

Although circulating miRNAs are emerging as putative disease biomarkers, to our knowledge only one study investigated the association between plasma miRNAs expression and childhood obesity. In this cross-sectional study, Prats-Puig et al.[3] found differential plasma concentrations of 15 circulating miRNAs in lean and obese children: miR-486-5p, miR-486-3p, miR-142-3p, miR-130b, miR- 423-5p, miR-532-5p, miR-140-5p, miR-16-1, miR-222, miR-363, miR-122, miR-221, miR- 28-3p, miR-125b and miR-328.

Our group has been studying the influence of common genetic variants on the risk of obesity-related phenotypes in Brazilian children[14-17]. However, meta-analyses of genome-wide association studies concluded that, so far, only 2.7% of the variation in body mass index in the general population has been explained by gene variants [18]. Thus, the lack of explanation this so-called "missing heritability" in adiposity may rely, at least in part, on miRNAs.

These recent observations prompted us to investigate whether circulating levels of miRNAs selected among those which presented the higher significant differences between lean and obese children in previous studies[3] (miR-16-5p, miR-19b, miR-28-3p, miR-130b-3p, miR-146a-5p, miR-221-3p, miR-423-5p, miR-486-3p and miR-486-5p) in childhood differ between lean and obese children in southern Brazil. We hypothesize that the very early detection of an abnormal circulating miRNA profile may allow early prediction of metabolic comorbidities.

SUBJECTS AND METHODS

Recruitment and clinical assessments

This was a cross-sectional analysis nested in a cohort study, performed between April of 2008 and June of 2015, with children followed since birth until six years old.

The recruitment phase occurred during the third trimester of pregnancy at health centers in the eight district areas of the city of Porto Alegre, state of Rio Grande do Sul, Brazil. An intervention was carried out with primary health care professionals, which consisted in an update of the ‘‘Ten steps to healthy eating for children younger than two years’’[19] guide for all professionals working in the selected health centers, in addition to providing educational materials based on the food guide, to be delivered to all mothers undergoing prenatal and child care. This intervention was not the primary objective of the present research and the participation in intervention/control group was used as confounding factor in statistical analyses. More details about data collection can be found elsewhere[20, 21]. The children were reevaluated at the ages of 1 year, 3.2 years and 6.2 years for the collection of dietary and anthropometric data.

The present study assessed 40 children when they were 6.2 years old. Children were directly weighted barefoot and wearing light clothes using a digital scale (Líder®, Araçatuba, Brazil), and height was measured with children standing straight using a stadiometer (Alturaexata®, Belo Horizonte, Brazil). Body mass index (BMI) was calculated as [weight (kg)/height $(m)^2$], and BMI-for-age Z-scores (BMI-Z) were estimated based on the World Health Organization standards[22]. From the 206 children evaluated in this phase of follow-up of the whole cohort, those 20 children with lower BMI-Z and those 20 children with higher BMI-Z values were selected for the present study. The obese group consisted of children with BMI-Z > +2 and the lean group was constituted by children with BMI-Z < -1. Waist circumference was also measured in the supine position at the narrowest point of the trunk. Tricipital and subscapular skinfolds were measured using an adipometer (WCS, Cardiomed®, Curitiba, Brazil). Blood pressure was measured in the supine position on the right arm after a 10-min rest, using

an electronic sphygmomanometer (Omron Healthcare, Bannockburn, USA) with cuff size appropriate for arm circumference.

The research was approved by the Ethics Committee of the Federal University of Health Sciences of Porto Alegre. Informed written consent was obtained from all the parents.

Biochemical measurements

Venous blood samples were obtained after a 12-h fast. Biochemical parameters such as circulating glucose, total cholesterol, high density cholesterol (HDL), triglycerides and C-reactive protein were measured by spectrophotometry or turbidimetry (BS-120 Chemistry Analyzer, Mindray, Shenzhen, China); and low density lipoprotein (LDL) was calculated by the Friedewald formula[23]. Insulin levels were measured by chemiluminescence (IMMULITE 1000® Immunoassay System, Siemens Healthcare Diagnostics, Flanders, USA). Fasting insulin sensitivity was estimated based on fasting insulin and glucose levels using the homeostasis model assessment (HOMA IR)[24].

Analysis of circulating miRNAs

Based on the circulating pattern of miRNAs in childhood obesity previously described by Prats-Puig et al.[3], we chose the seven most differentially expressed miRNAs between obese and lean children (miR-486-5p, miR-486-3p, miR-221-3p, miR-28-3p, miR-142-3p, miR-130b-3p, miR-423-5p. The eighth miRNA (miR-16-5p) was selected based on its overexpression in the subcutaneous adipose tissue of non-obese women in a southern Brazilian sample (Gasparotto et al., personal communication).

EDTA plasma samples were obtained by standard venipuncture and immediately centrifuged with a laboratory centrifuge at 1000*g* for 15 min at 4 °C. Circulating RNA

was extracted from a plasma volume of 500 μL using the mirVana PARIS isolation kit (Life Technologies®, Carlsbad, California, USA) according to the manufacturer's instructions and stored at -80ºC. RNA integrity and quantification were measured with BioSpec-nano (Shimadzu®, Kyoto, Japan).

Fifty microliters of RNA were eluted, and a fixed volume (3 μL) was used as input into the reverse transcription (RT) reaction with the TaqMan® miRNA RT Kit (Life Technologies®). At total of eight target miRNAs and four endogenous controls were reverse transcribed using a pool of individual stem-loop primers of the selected microRNA assays (Life Technologies®). Multiplex RT products were preamplified using the TaqMan® PreAmp Master Mix (Life Technologies®). All cDNAs were stored at -20ºC for no longer than five days. Subsequently, analysis of individual miRNAs was performed in duplicate for relative miRNA expression quantification using TaqMan® hydrolysis probes (Life Technologies®) carried out on a StepOnePlus™ Real-Time PCR System (Life Technologies®) to verify the presence of the target circulating miRNAs. All experiments were performed following MIQE guidelines[25]. Moreover, samples of both groups were included in each batch, in order to discard any measurement bias.

MiRNAs expression was measured using threshold cycle (C_T) relative to the geometrical mean of the endogenous controls ($2^{-\Delta Ct}$). Initially, we tested four endogenous controls as normalizing factors based on their stability in plasma of children[3]: miR-223-3p, miR-106a-5p, miR-146a-5p and miR-19b. However, among the 12 miRNAs herein analyzed, three were selected as endogenous controls due to their highest stability among all assays evaluated with ExpressionSuite Software version 1.0.3 (Life technologies®): miR-223-3p, miR-106a-5p and miR-142-3p. Therefore, the expression of the remaining nine miRNAs was compared between the lean and obese groups.

We used the DIANA-miRPath, a free web-based computational tool that performs miRNA pathway analysis using the predicted miRNA targets or even experimentally validated miRNA interactions[26]; and the TargetScanHuman[27] and miRTarBase[28] online databases to identify the target genes and their molecular pathways.

Statistical analysis

Demographic and clinical continuous variables were compared between the two groups (lean and obese) using Student's t-test for independent samples. Chi-squared test with Yates continuity correction was used to compare categorical variables between groups. The $2^{-\Delta Ct}$ approach was used to normalize miRNA data to the endogenous controls and to calculate the differential expression between groups[29]. Differential expression values lower than 1.0 were inverted to facilitate interpretation. All data were tested for normal distribution and equivalence of variances. Student's t-test for independent samples was used to compare mean normalized miRNA levels between lean and obese children when the miRNA levels presented a normal distribution, and Mann-Whitney U test was used when their distribution was asymmetric. Multiple regression analyses adjusted for potential confounding factors and Spearman's statistic were used to study correlations between circulating levels of individual miRNAs, anthropometric measurements and biochemical variables. All statistical analyses were performed using the SPSS 20.0 program (SPSS Inc., Chicago, USA). Statistical significance was defined as *P*-values <0.05.

RESULTS

Clinical and biochemical characteristics of the children are shown in Table 1. Forty children had plasma samples analyzed for the nine candidates obesity-related

circulating miRNAs. Due to unsuccessful RNA extraction, two of the 40 children were excluded of the analyses. The mean age of children was 6.2 ± 0.2 years, and 55.3% were boys. MiRNA concentrations were not different between boys and girls.

The relative expression for each miRNA in the two groups analyzed is shown in Table 2. Two circulating miRNAs presented significantly different medians between lean and obese children. Increased circulating concentrations of miR-16-5p and miR-19b (both $P < 0.05$) were identified in lean children, showing an expression about 1.96 and 1.41 times, respectively, higher in plasma samples of lean than in the obese group ($P = 0.008$, $P = 0.046$; respectively) (Figure 1).

The relative expression of miR-16-5p and miR-19b was significantly correlated with BMI-Z (Table 3). For miR-16-5p, we found the following correlation values: BMI-Z (r = -0.443, $P = 0.008$), skinfolds (r= -0.433, $P = 0.009$), waist (r = -0.404, $P = 0.016$). However, when adjusted by BMI-Z, the correlations with skinfolds and waist did not survive ($P > 0.05$), indicating that the correlation of this miRNA with subcutaneous or central fat is secondary to the correlation with BMI-Z. The same was observed with miR-19b, significant correlations were observed with BMI-Z (r = -0.348, $P = 0.032$), skinfolds (r = -0.404, $P = 0.012$) and waist (r = -0.372, $P = 0.021$), and the last two disappeared after controlling for BMI-Z (Table 3).

The correlations between biochemical variables (fasting glucose, total cholesterol, HDL, LDL, triglycerides, C-reactive protein, insulin levels and homeostasis model assessment of insulin resistance) and miRNAs expression were also evaluated. Univariate analyses showed a significant correlation between miR-16-5p and plasma insulin levels (r = -0.351, $P = 0.039$) (Table 4).

Multivariate regression analyses were also performed for the identification of combined factors associated with changes in BMI-Z. Parameters with P values < 0.200

in the bivariate analyses were entered into a multivariate analysis model and removed stepwise. Only those variables with significant standardized regression coefficients (beta) were maintained in the final model. The combined effect of circulating concentrations of miR-16-5p, together with fasting insulin levels, contributed to explain about 41% of the variance in BMI (miR-16-5p, beta = -0.300, P= 0.042; insulin, beta = 0.487, P = 0.002; complete model, r^2 = 0.413, P < 0.001). The effect of miR-16-5p alone on BMI-Z contributed to explain about 20% of BMI variation (r^2 = 0.196, P = 0.008).

DISCUSSION

MiRNAs research has advanced and highlighted their important regulatory role in most biological processes controlling gene expression. Alterations in miRNAs expression patterns have been demonstrated in various physiological and pathological states. Considering the findings that miRNAs can be found at stable levels in serum and plasma, within circulating exosomes or other microvesicules[30], these circulating molecules may be useful as minimally invasive biomarkers for the diagnosis, prognosis, and possible therapeutics of complex diseases and their comorbidities. We identified two circulating miRNAs differently expressed in lean and obese children, miR-16-5p and miR-19b, which showed decreased concentrations in the obese group and had an inverse correlation with measures of body fat.

Our very recent findings have reported that expression of miR-16-5p in subcutaneous adipose tissue is higher in non-obese than in obese women, suggesting a low expression of its target genes in non-obese group. Since this miRNA has the vascular endothelial growth factor (VEGF) as one of its target genes[31], the authors suggested that this miRNA may be involved in the alterations of angiogenesis observed

in obesity (Gasparotto et al., unpublished results). Recent studies have demonstrated the effect of this proangiogenic gene in body fat regulation[32]. Indeed, obese children have an increased risk for early development of metabolic and cardiovascular abnormalities, including endothelial dysfunction, which is an early risk marker of cardiovascular disease, even before the onset of dyslipidemia or hypertension process[1, 33]. In our study, children with an increase in BMI-Z and insulin levels showed decreased circulating miR-16-5p. According to our analysis *in silico* performed with DIANA-mirPath, miR-16-5p regulates the expression of at least 13 genes involved in insulin signaling pathways, affecting the glucose and lipid homeostasis as well as the protein synthesis[26]. Thus, we believe that plasma level of this miRNA may be influenced by changes in homeostasis of insulin. It is known that obesity leads to an altered glucose metabolism and precedes diabetes, and adiposity is involved in the etiology of insulin resistance, where lipid moieties from the white adipose tissue disturb insulin signaling[34]. The circulating concentration of miR-16-1-3p was found to be deregulated in obese children in the study conducted by Prats-Puig et al.[3], who identified an increase of this miRNA in plasma from prepubertal obese children when compared with lean children. This is a mature miRNA originating from the 3' end of the pre-miRNA, while the miRNA investigated in the present study is the mature miRNA originating from the 5' end of the same pre-miRNA[35]. The two mature miRNAs could be functional, co-expressed differently from tissue to tissue and target on different mRNAs, suggesting tissue-dependent regulatory functions[36, 37]. Furthermore, according to our analysis *in silico* in this online database, miR-16-5p and miR-19b modify the transforming growth factor beta (TGF-β) signaling pathway targeting eight genes, modulating angiogenic properties, angiogenesis, apoptosis and cellular cycle, among others[26]. Members of the

TGF-β superfamily are known to control many aspects of adipogenesis, adiposity and energy expenditure[38].

Initially, we had chosen miR-19b as an endogenous control, since a previous study with ten healthy children had pointed it as one of the most stable circulating miRNAs[3]. However, we found significant increased plasma levels of miR-19b in lean that in obese children and an inverse correlation of miR-19b with measures of body fat. A recent study by Xue et al. has demonstrated that miR-19b also has as one of its target genes the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a transcriptional coactivator known to be a key regulator of energy metabolism and mitochondrial biogenesis[39]. The authors concluded that this miRNA induces endothelial cell dysfunction by suppression of PGC-1α human atherosclerotic vessel samples. Literature data and results from bioinformatics analysis revealed that miR-19b might also influence the expression of the adrenergic receptor beta 1 (ADRB1)[27, 40]. Beta-adrenergic receptors are known to affect energy homeostasis through regulation of lipolysis and thermogenesis by catecholamines[41], and ADRB1 has been involved in both stimulation of lipolysis and the proliferation of brown fat cells in animal models[42]. To our knowledge miR-19b has not been previously reported to be related to either adipogenesis or obesity. Most of studies have reported it deregulated in multiple tumor types, with a tumor-promoting activity[43]. Thus, the importance of our study is that it suggests miR-19b as a new candidate in the field of obesity.

We acknowledge there are limitations in our study. We are aware that the miRNAs investigated may not represent the whole bulk of circulating miRNAs altered in our obese children. The cross-sectional design of our study does not allow us to establish causative relationships between the altered miRNAs and obesity. On the other hand, children herein studied are enrolled in a longitudinal study followed-up since

birth, and likely will be re-evaluated in 3-4 years. Therefore, the circulating concentrations of miRNAs might be analyzed again in order to verify possible changes in plasma levels. Moreover, our children are very well characterized in terms of anthropometry, which constitutes a differential characteristic from other studies.

In conclusion, our findings indicate differential plasma concentrations of miR-16-5p and miR-19b in lean and obese children, suggesting a potential role of these miRNAs in childhood obesity as novel biomarkers for the early prevention and to improve the diagnostic power of obesity-associated metabolic complications. To our knowledge, we provided the first evidence in South American children about obesity-related circulating miRNAs. These findings provide a better understanding regarding the role of miRNAs in physiopathology of obesity, pointing to new signaling pathways, and justify the need of future investigations to determine the prognostic value of these circulating miRNAs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Khalyfa A, Gozal D. Exosomal miRNAs as potential biomarkers of cardiovascular risk in children. *J Transl Med* 2014; **12:** 162.

2. Omran A, Elimam D, He F, Peng J, Yin F. Potential role of blood microRNAs as non-invasive biomarkers for early detection of asymptomatic coronary

atherosclerosis in obese children with metabolic syndrome. *Med Hypotheses* 2012; **79:** 889-893.

3. Prats-Puig A, Ortega FJ, Mercader JM, Moreno-Navarrete JM, Moreno M, Bonet N*, et al.* Changes in circulating microRNAs are associated with childhood obesity. *J Clin Endocrinol Metab* 2013; **98:** E1655-1660.

4. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010; **466:** 835-840.

5. McGregor RA, Choi MS. microRNAs in the regulation of adipogenesis and obesity. *Curr Mol Med* 2011; **11:** 304-316.

6. Perera RJ, Ray A. MicroRNAs in the search for understanding human diseases. *BioDrugs* 2007; **21:** 97-104.

7. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* 2009; **58:** 1050-1057.

8. Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A*, et al.* MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PloS one* 2010; **5:** e9022.

9. Bengestrate L, Virtue S, Campbell M, Vidal-Puig A, Hadaschik D, Hahn P*, et al.* Genome-Wide Profiling of MicroRNAs in Adipose Mesenchymal Stem Cell Differentiation and Mouse Models of Obesity. *PloS one* 2011; **6:** e21305.

10. Martinelli R, Nardelli C, Pilone V, Buonomo T, Liguori R, Castano I*, et al.* miR-519d overexpression is associated with human obesity. *Obesity* 2010; **18:** 2170-2176.

11. Pescador N, Perez-Barba M, Ibarra JM, Corbaton A, Martinez-Larrad MT, Serrano-Rios M. Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. *PloS one* 2013; **8:** e77251.

12. Ortega FJ, Mercader JM, Catalan V, Moreno-Navarrete JM, Pueyo N, Sabater M*, et al.* Targeting the circulating microRNA signature of obesity. *Clin Chem* 2013; **59:** 781-792.

13. Wang YC, Li Y, Wang XY, Zhang D, Zhang H, Wu Q*, et al.* Circulating miR-130b mediates metabolic crosstalk between fat and muscle in overweight/obesity. *Diabetologia* 2013; **56:** 2275-2285.

14. da Silva CF, Zandona MR, Vitolo MR, Campagnolo PD, Rotta LN, Almeida S*, et al.* Association between a frequent variant of the FTO gene and anthropometric phenotypes in Brazilian children. *BMC medical genetics* 2013; **14:** 34.

15. Zandona MR, Rodrigues RO, Albiero G, Campagnolo PD, Vitolo MR, Almeida S*, et al.* Polymorphisms in LEPR, PPARG and APM1 genes: associations with energy intake and metabolic traits in young children. *Arquivos brasileiros de endocrinologia e metabologia* 2013; **57:** 603-611.

16. Miranda RC, Vetter SB, Genro JP, Campagnolo PD, Mattevi VS, Vitolo MR*, et al.* SLC6A14 and 5-HTR2C polymorphisms are associated with food intake and nutritional status in children. *Clin Biochem* 2015.

17. Galvao AC, Kruger RC, Campagnolo PD, Mattevi VS, Vitolo MR, Almeida S. Association of MAOA and COMT gene polymorphisms with palatable food intake in children. *J Nutr Biochem* 2012; **23:** 272-277.

18. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR*, et al.* Genetic studies of body mass index yield new insights for obesity biology. *Nature* 2015; **518:** 197-206.

19. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. Dez passos para uma alimentação saudável: guia alimentar para menores de 2 anos. Um guia para o profissional da saúde na atenção básica [in Portuguese] Ministério da Saúde: Brasília, Brazil, 2010.

20. Chaffee BW, Feldens CA, Vitolo MR. Cluster-randomized trial of infant nutrition training for caries prevention. *J Dent Res* 2013; **92:** 29S-36S.

21. Valmorbida JL, Vitolo MR. Factors associated with low consumption of fruits and vegetables by preschoolers of low socio-economic level. *Jornal de pediatria* 2014; **90:** 464-471.

22. WHO Child Growth Standards: Lenght/height-for-age, weight-for-age, weight-for-lenght, wight-for-height and body mass index-for-age: Methods and development. World Health Organization: Geneva, 2006.

23. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18:** 499-502.

24. Madeira IR, Carvalho CN, Gazolla FM, de Matos HJ, Borges MA, Bordallo MA. [Cut-off point for Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index established from Receiver Operating Characteristic (ROC) curve in the detection of metabolic syndrome in overweight pre-pubertal children]. *Arquivos brasileiros de endocrinologia e metabologia* 2008; **52:** 1466-1473.

25. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M*, et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55:** 611-622.

26. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I*, et al.* DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic acids research* 2015; **43:** D153-159.

27. TargetScanHuman 7.0. 2006 - 2015, Whitehead Institute for Biomedical Research. http://www.targetscan.org/.

28. Hsu SD, Tseng YT, Shrestha S, Lin YL, Khaleel A, Chou CH*, et al.* miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. *Nucleic acids research* 2014; **42:** D78-85.

29. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; **3:** 1101-1108.

30. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L*, et al.* Detection of microRNA expression in human peripheral blood microvesicles. *PloS one* 2008; **3:** e3694.

31. Fiedler J, Thum T. MicroRNAs looping around angiogenesis. *Arterioscler Thromb Vasc Biol* 2011; **31:** 2367-2368.

32. Shungin D, Winkler TW, Croteau-Chonka DC, Ferreira T, Locke AE, Magi R*, et al.* New genetic loci link adipose and insulin biology to body fat distribution. *Nature* 2015; **518:** 187-196.

33. Bhattacharjee R, Alotaibi WH, Kheirandish-Gozal L, Capdevila OS, Gozal D. Endothelial dysfunction in obese non-hypertensive children without evidence of sleep disordered breathing. *BMC Pediatr* 2010; **10:** 8.

34. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S*, et al.* Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes* 2014; **63:** 4089-4099.

35. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. *Nat Rev Genet* 2012; **13:** 358-369.

36. Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafa R*, et al.* Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. *Gastroenterology* 2012; **142:** 886-896 e889.

37. Ro S, Park C, Young D, Sanders KM, Yan W. Tissue-dependent paired expression of miRNAs. *Nucleic acids research* 2007; **35:** 5944-5953.

38. Zamani N, Brown CW. Emerging roles for the transforming growth factor-{beta} superfamily in regulating adiposity and energy expenditure. *Endocr Rev* 2011; **32:** 387-403.

39. Xue Y, Wei Z, Ding H, Wang Q, Zhou Z, Zheng S*, et al.* MicroRNA-19b/221/222 induces endothelial cell dysfunction via suppression of PGC-1alpha in the progression of atherosclerosis. *Atherosclerosis* 2015; **241:** 671-681.

40. Volk N, Paul ED, Haramati S, Eitan C, Fields BK, Zwang R*, et al.* MicroRNA-19b associates with Ago2 in the amygdala following chronic stress and regulates the adrenergic receptor beta 1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2014; **34:** 15070-15082.

41. Tafel J, Branscheid I, Skwarna B, Schlimme M, Morcos M, Algenstaedt P*, et al.* Variants in the human beta 1-, beta 2-, and beta 3-adrenergic receptor genes are not associated with morbid obesity in children and adolescents. *Diabetes Obes Metab* 2004; **6:** 452-455.

42. Soloveva V, Graves RA, Rasenick MM, Spiegelman BM, Ross SR. Transgenic mice overexpressing the beta 1-adrenergic receptor in adipose tissue are resistant to obesity. *Mol Endocrinol* 1997; **11:** 27-38.

43. Huhn D, Kousholt AN, Sorensen CS, Sartori AA. miR-19, a component of the oncogenic miR-17 approximately 92 cluster, targets the DNA-end resection factor CtIP. *Oncogene* 2015; **34:** 3977-3984.

Figure Legend

Figure 1. Median normalized circulating concentration values for miR-16-5p (A) and miR-19b (B) according to two groups: lean (BMI-Z < -1) and obese (BMI-Z > +2) children. *P < 0.05 compared with lean group after Mann-Whitney U test. Boxes show interquartile ranges. Error bars represent lowest and highest values. Dots show outlier values.

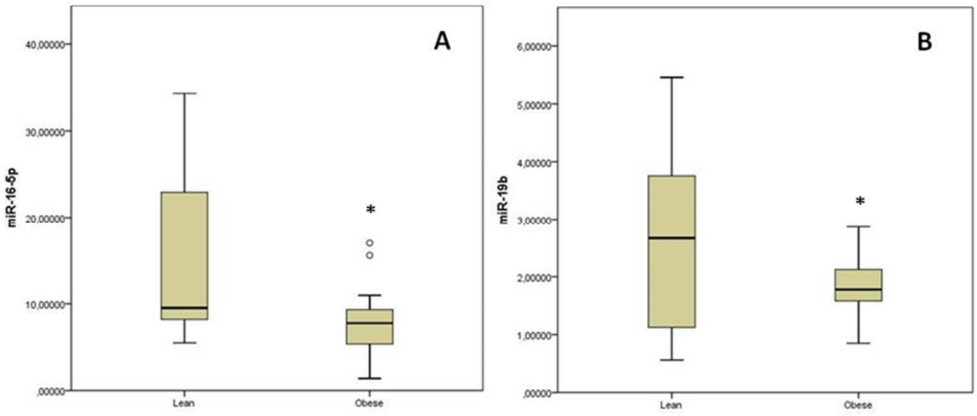
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Table 1. Biodemographic characteristics of the children.

Characteristics	Lean (n=18)	Obese (n=20)	*P* value
Boys, n	13	8	0.095[a]
Age, years	6.25 ± 0.22	6.16 ± 0.18	0.165[a]
Clinical parameters			
BMI- Z	-1.55 ± 0.36	3.23 ± 0.64	<0.001[b]
Waist circumference, cm	48.8 ± 3.1	65.9 ± 3.7	<0.001[b]
Skinfolds, mm	11.69 ± 2.32	38.8 ± 10.4	<0.001[b]
Systolic blood pressure, mm Hg	93.6 ± 12.0	101.9 ± 12.1	0.047[b]
Diastolic blood pressure, mm Hg	59.1 ± 16.3	63.7 ± 8.2	0.285[b]
Biochemical variables			
Glucose, mmol/L	4.56 ± 0.39	4.85 ± 0.42	0.034[b]
Total cholesterol, mmol/L	4.23 ± 0.56	4.00 ± 0.80	0.315[b]
HDL cholesterol, mmol/L	1.78 ± 0.42	1.59 ± 0.33	0.144[b]
LDL cholesterol, mmol/L	2.09 ± 0.55	1.96 ± 0.61	0.517[b]
Triglycerides, mmol/L[d]	0.79 ± 0.48	0.95 ± 0.48	0.182[b]
C-reactive protein, mg/L	2.98 ± 8.41	2.43 ± 4.72	0.805[b]
Insulin (μIU/mL)	2.35 ± 0.70	7.39 ± 4.81	<0.001[c]
HOMA-IR	0.48 ± 0.15	1.64 ± 1.16	<0.001[c]

Abbreviations: BMI-Z, body mass index for age Z-score; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BP, blood pressure, HOMA-IR, homeostasis model assessment for insulin resistance. Data are means ± standard deviation. [a]χ^2 with Yates continuity correction, [b]Student's *t* test, [c]Mann-Whitney U test, [d]Statistic test performed with triglycerides variable Ln-transformed.

Table 2. Normalized means for the miRNAs analyzed in lean and obese children.

miRNA	Lean	Obese	Fold change (obese/lean)	*P* value[a]
miR-16-5p	15.36 ± 9.57	7.83 ± 3.89	-1.96	**0.008[a]**
miR-486-5p	0.026 ± 0.016	0.019 ± 0.009	-1.41	0.090[a]
miR-486-3p	0.0018 ± 0.0025	0.0007 ± 0.0008	-2.55	0.086[a]
miR-221-3p	0.020 ± 0.011	0.025 ± 0.012	1.23	0.211[b]
miR-28-3p	0.0103 ± 0.0070	0.0096 ± 0.0044	-1.07	0.724[b]
miR-130b-3p	0.0049 ± 0.0038	0.0055 ± 0.0034	1.11	0.641[b]
miR-423-5p	0.0074 ± 0.0034	0.0068 ± 0.0027	-1.09	0.527[b]
miR-19b	2.59 ± 1.43	1.83 ± 0.55	-1.41	**0.046[a]**
miR-146a-5p	0.37 ± 0.16	0.32 ± 0.11	-1.13	0.368[b]
miR -106a-5p[c]	21.97 ± 1.83	21.50 ± 1.22		
miR -142a-3p[c]	24.68 ± 1.91	24.12 ± 1.27		
miR-223-5p[c]	18.66 ± 1.80	17.98 ± 1.23		

[a]Mann-Whitney U test, [b]Student's *t* test, [c]Endogenous controls. Data are $2^{-\Delta Ct}$ means ± standard deviation. For the endogenous controls, data are threshold cycle (C_T) means ± standard deviation. Significant differences are shown in bold.

Table 3. Correlation analysis between anthropometric parameters and normalized miRNAs concentrations

miRNA	BMI-Z[a]		Skinfolds[a]		Skinfolds adjusted by BMI[b]				Waist[a]		Waist adjusted by BMI[b]			
	R	*P*	R	*P*	Beta	$P_{partial}$	R_{model}	P_{model}	R	*P*	Beta	$P_{partial}$	R_{model}	P_{model}
miR-16-5p	-0.443	**0.008**	-0.433	**0.009**	-0.184	0.610	0.450	**0.027**	-0.404	**0.016**	0.183	0.725	0.446	**0.029**
miR-486-5p	-0.345	**0.034**	-0.343	**0.035**	-0.170	0.655	0.352	0.099	-0.311	0.058	0.266	0.680	0.351	0.100
miR-486-3p	-0.280	0.089	-0.306	0.062	-0.295	0.449	0.306	0.179	-0.259	0.117	0.104	0.853	0.281	0.236
miR-221-3p	0.250	0.130	0.228	0.169	0.003	0.995	0.250	0.322	0.235	0.156	-0.053	0.925	0.251	0.321
miR-28-3p	-0.077	0.646	-0.109	0.516	-0.222	0.583	0.121	0.774	-0.182	0.274	-1.276	**0.023**	0.380	0.065
miR-130b-3p	0.127	0.448	0.091	0.587	-0.138	0.733	0.139	0.710	0.123	0.463	0.017	0.976	0.127	0.753
miR-423-5p	-0.038	0.521	-0.058	0.731	-0.133	0.744	0.067	0.924	-0.053	0.753	-0.194	0.739	0.068	0.922
miR-19b	-0.348	**0.032**	-0.404	**0.012**	-0.501	0.183	0.406	**0.042**	-0.372	**0.021**	-0.463	0.395	0.373	0.072
miR-146a-5p	-0.168	0.313	-0.241	0.145	-0.504	0.203	0.270	0.266	-0.265	0.108	-1.219	**0.028**	0.393	**0.053**

Abbreviations: BMI-Z, body mass index for age Z-score; R, correlation coefficient; Beta, partial standardized regression coefficient; R_{model}, complete model regression coefficient; $P_{partial}$ refers to the Beta; P_{model} refers to the complete model. [a]Spearman statistic; [b]Multiple linear regression. Significant differences are shown in bold.

Table 4. Correlation analysis between biochemical variables and normalized miRNAs concentrations

miRNA	Insulin (μIU/mL)		HOMA-IR	
	R	*P*	R	*P*
miR-16-5p	-0.351	**0.039**	-0.254	0.147
miR-486-5p	-0.218	0.194	-0.148	0.389
miR-486-3p	-0.199	0.238	-0.151	0.379
miR-221-3p	0.036	0.832	-0.009	0.960
miR-28-3p	0.154	0.363	0.096	0.579
miR-130b-3p	-0.010	0.951	0.037	0.831
miR-423-5p	-0.045	0.793	-0.027	0.878
miR-19b	-0.140	0.408	-0.048	0.783
miR-146a-5p	0.142	0.403	0.144	0.401

Abbreviations: HOMA-IR, homeostasis model assessment for insulin resistance; R, correlation coefficient. *P*-values are by Spearman statistic. Significant difference is shown in bold.

5 CONCLUSÕES

A investigação apresentada destinou-se a investigar a influência genética na obesidade infantil utilizando marcadores genéticos e epigenéticos, através da avaliação de polimorfismos em genes de susceptibilidade identificados pelos estudos de varredura genômica e do perfil de expressão de miRNAs circulantes em crianças acompanhadas desde o nascimento. Dentro do capítulo 1, nos 5 subcapítulos, cada um com a sua especificidade, ora foi salientada a epidemiologia da obesidade infantil (subcapítulo 1.1), ora a avaliação nutricional na infância (subcapítulo 1.2), ora os aspectos genéticos e ambientais da obesidade (subcapítulo 1.3), para depois ser realizada uma breve revisão dos genes de susceptibilidade investigados (subcapítulo 1.4) e, finalmente, a contribuição dos microRNAs como reguladores transcricionais na obesidade e como novos potenciais biomarcadores minimamente invasivos encontrados na circulação sanguínea (subcapítulo 1.5).

Neste momento, a fim de concluir, torna-se útil ressaltar que as considerações desta Tese, no sentido de efetuar um contributo para a validação de *loci* de susceptibilidade à obesidade nas crianças Brasileiras, assim como fornecer o primeiro perfil de expressão de microRNAs, partem da crescente preocupação acerca da epidemia recente de obesidade infantil em nosso país, onde mais de 30% das crianças entre 5 e 9 anos apresentam excesso de peso. O Brasil enfrenta, atualmente, um fenômeno de transição nutricional, que se caracteriza pela presença da deficiência de micronutrientes, excesso de peso e outras doenças crônicas coexistindo nas mesmas comunidades e, muitas vezes, no mesmo domicílio.

Baseada nas consequências do excesso de peso na infância sobre a saúde clínica e pública na vida adulta, dentre elas o desenvolvimento de resistência à insulina, dislipidemia, hipertensão e doenças cardiovasculares, esta pesquisa utilizou-se de uma abordagem genética e epigenética, de modo a fornecer evidências tanto da contribuição genética na susceptibilidade à obesidade em crianças de idade precoce do sul do Brasil, em especial das cidades de Porto Alegre e São Leopoldo, quanto do potencial dos microRNAs como futuros alvos terapêuticos e marcadores precoces de processos metabólicos.

Excesso de peso e obesidade resultam de uma combinação de influência genética, comportamental e ambiental sobre metabolismo, dieta e atividade física. Quando existe predisposição genética para a obesidade, o ambiente e o estilo de vida do indivíduo potencializam o seu desenvolvimento. O estilo de vida da sociedade contemporânea é apontado como o principal responsável pelo rápido crescimento da prevalência de obesidade, onde a facilidade de acesso e o baixo custo de alimentos altamente palatáveis e de grande densidade energética, aliados ao menor requerimento de atividade física na vida diária favorecem o acúmulo de gordura. Neste sentido, as evidências atuais apontam que estratégias

de prevenção do ganho excessivo de peso, tanto no que diz respeito aos hábitos de alimentação saudáveis quanto em relação à prática frequente de atividade física, constituem a maneira mais eficaz de evitar o acúmulo excessivo de gordura corporal em indivíduos com predisposição genética, já que as preferências alimentares e os padrões dietéticos se estabelecem precocemente, principalmente em relação ao consumo de frutas, vegetais e alimentos de alta densidade energética e baixa densidade de nutrientes.

Neste sentido, é importante destacar a oportunidade única que existe através do acompanhamento das duas coortes de crianças investigadas nesta Tese e a interação entre nutricionistas e geneticistas. A criteriosa coleta de dados antropométricos e dietéticos (tanto da gestante quanto da criança em diferentes momentos durante o seu crescimento), assim como a avaliação nutricional realizada pela equipe de nutricionistas possibilitam que essas variáveis sejam confrontadas e discutidas juntamente com as informações moleculares geradas pela equipe da genética. A combinação de novas tecnologias de biologia molecular com estudos da nutrição clássica em uma abordagem integrada e multidisciplinar tem um objetivo comum: estudar a interação entre os alimentos e os genes. Atualmente, o impacto da variação genética na resposta aos alimentos, a nutrigenética, é uma área bastante promissora, pois tem por finalidade auxiliar a prevenção e o tratamento de doenças através da alimentação.

Este estudo validou as associações de cinco das dez variantes genéticas mais replicadas pelos estudos de varredura genômica realizados em adultos em uma coorte de crianças do Sul do Brasil acompanhadas desde o nascimento até os 3,5 anos de idade. As variantes investigadas nos genes *TMEM18, BDNF* e *NEGR1* foram associadas com índice de massa corporal e gordura subcutânea, e a variante no gene *SEC16B*, embora não foi associada a fenótipos antropométricos, mostrou associação com maior ingestão diária de energia proveniente de carboidratos e lipídios na idade de 3,5 anos. Os resultados também mostraram que o efeito das variantes no *BDNF* começa mais cedo, na idade de 1 ano, enquanto que os efeitos das variantes nos genes *NEGR1* e *TMEM18* parecem começar um pouco mais tarde, sendo detectados a partir dos 3,5 anos de idade.

Além disso, o presente trabalho identificou dois microRNAs circulantes diferentemente expressos em crianças magras e obesas, miR-16-5p e miR-19b, os quais mostraram concentrações diminuídas no grupo obeso e apresentaram correlações inversas significativas com medidas de gordura corporal. Estes resultados, embora iniciais, sugerem um papel potencial desses miRNAs na obesidade infantil como novos biomarcadores para a prevenção precoce e para melhorar o poder de diagnóstico das complicações metabólicas

associadas à obesidade. Torna-se evidente a necessidade de futuras investigações para determinar o valor prognóstico desses miRNAs circulantes, mas deve-se reconhecer que os resultados aqui gerados fornecem uma melhor compreensão sobre o papel dos miRNAs na fisiopatologia da obesidade, apontando para novas vias de sinalização. Ao nosso conhecimento, este estudo forneceu a primeira evidência de expressão de miRNAs circulantes associados à obesidade tanto em crianças quanto em adultos sul americanos.

Embora seja amplamente aceito que a obesidade é uma condição hereditária, os mais poderosos estudos de associação do genoma (GWAS) têm recentemente estimado que apenas 2,7% da variabilidade do IMC pode ser explicada pelos 97 *loci* associados à obesidade até o momento e sugerem que apenas 21% da variação no IMC seja explicada por variantes genéticas comuns (47). Apesar do pequeno tamanho de efeito das variantes genéticas associadas ao IMC, sabe-se que elas exercem efeitos cumulativos sobre o risco de obesidade.

A chamada *missing heritability* ou herdabilidade perdida das doenças complexas tem sido objeto de intenso debate entre os pesquisadores nos últimos anos. Zuk *et al*. reconhecem que, mesmo identificando todas as variantes genéticas relacionadas a qualquer característica, a compreensão total da sua herdabilidade não será atingida se não forem considerados os efeitos epistáticos (144). Para características com interações gene-gene e gene-ambiente (que se aplica à maioria das características complexas), mesmo quando todas as variantes que contribuem para o fenótipo forem identificadas, a *missing heritability* não será zero (145). Além disso, é provável que o cálculo de herdabilidade de muitas características complexas seja superestimado, criando-se a chamada *phantom heritability* (144). Os autores afirmam que as características com maior complexidade biológica devem apresentar a maiores frações de *missing heritability* e ressaltam que esse fenômeno não se deve apenas a interações genéticas. Outra hipótese proposta pelos autores grupo é que devem existir inúmeras variantes causais que seriam responsáveis por uma fração significante da *missing heritability*. Além disso, existe outra explicação plausível para a *missing heritability* da adiposidade, que pode estar relacionada com um nível de regulação gênica ainda pouco explorado, os microRNAs (miRNAs), considerado uma modificação epigenética, juntamente com a metilação do DNA e a modificação das histonas (146). A programação epigenética refere-se a mudanças na estrutura bioquímica do DNA, caracterizando um fenômeno hereditário, porém reversível que afeta a expressão gênica sem alterar a sequência do DNA (147). Evidências sugerem que mudanças epigenéticas associadas à má nutrição materna e ao estresse estão associadas com a programação fetal que promove obesidade, resistência à insulina e diabetes (146).

6 REFERÊNCIAS

1. Zhao J, Grant SF. Genetics of childhood obesity. Journal of obesity. 2011;2011:845148.
2. Ichihara S, Yamada Y. Genetic factors for human obesity. Cellular and molecular life sciences : CMLS. 2008;65(7-8):1086-98.
3. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. Jama. 2014;311(8):806-14.
4. Ministério da Saúde. VIGITEL: Vigilância de fatores de risco e proteção para doenças crônicas por inquérito telefônico. Brasília, Brasil2014.
5. Velloso LA, Schwartz MW. Altered hypothalamic function in diet-induced obesity. Int J Obes (Lond). 2011;35(12):1455-65.
6. van de Sande-Lee S, Velloso LA. [Hypothalamic dysfunction in obesity]. Arquivos brasileiros de endocrinologia e metabologia. 2012;56(6):341-50.
7. Keller P, Gburcik V, Petrovic N, Gallagher IJ, Nedergaard J, Cannon B, et al. Gene-chip studies of adipogenesis-regulated microRNAs in mouse primary adipocytes and human obesity. BMC Endocr Disord. 2011;11:7.
8. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. 2006;444(7121):847-53.
9. World Health Organization. Global Strategy on Diet, Physical Activity and Health. Childhood overweight and obesity 2015 [Available from: http://www.who.int/dietphysicalactivity/childhood/en.
10. Williams DE, Cadwell BL, Cheng YJ, Cowie CC, Gregg EW, Geiss LS, et al. Prevalence of impaired fasting glucose and its relationship with cardiovascular disease risk factors in US adolescents, 1999-2000. Pediatrics. 2005;116(5):1122-6.
11. Prats-Puig A, Ortega FJ, Mercader JM, Moreno-Navarrete JM, Moreno M, Bonet N, et al. Changes in circulating microRNAs are associated with childhood obesity. J Clin Endocrinol Metab. 2013;98(10):E1655-60.
12. Garver WS, Newman SB, Gonzales-Pacheco DM, Castillo JJ, Jelinek D, Heidenreich RA, et al. The genetics of childhood obesity and interaction with dietary macronutrients. Genes & nutrition. 2013;8(3):271-87.
13. Whitaker RC, Wright JA, Pepe MS, Seidel KD, Dietz WH. Predicting obesity in young adulthood from childhood and parental obesity. The New England journal of medicine. 1997;337(13):869-73.
14. Grant SF, Li M, Bradfield JP, Kim CE, Annaiah K, Santa E, et al. Association analysis of the FTO gene with obesity in children of Caucasian and African ancestry reveals a common tagging SNP. PloS one. 2008;3(3):e1746.
15. Mozaffari H, Nabaei B. Obesity and related risk factors. Indian J Pediatr. 2007;74(3):265-7.
16. Instituto Brasileiro de Geografia e Estatística (IBGE). Pesquisa de orçamentos familiares 2008-2009: antropometria e estado nutricional de crianças, adolescentes e adultos no Brasil. Rio de Janeiro, RJ2010.
17. Wang H, Gauthier BR, Hagenfeldt-Johansson KA, Iezzi M, Wollheim CB. Foxa2 (HNF3beta) controls multiple genes implicated in metabolism-secretion coupling of glucose-induced insulin release. J Biol Chem. 2002;277(20):17564-70.
18. Hakanen M, Lagstrom H, Kaitosaari T, Niinikoski H, Nanto-Salonen K, Jokinen E, et al. Development of overweight in an atherosclerosis prevention trial starting in early childhood. The STRIP study. Int J Obes (Lond). 2006;30(4):618-26.
19. Wang YC, Li Y, Wang XY, Zhang D, Zhang H, Wu Q, et al. Circulating miR-130b mediates metabolic crosstalk between fat and muscle in overweight/obesity. Diabetologia. 2013;56(10):2275-85.

20. Dattilo AM, Birch L, Krebs NF, Lake A, Taveras EM, Saavedra JM. Need for early interventions in the prevention of pediatric overweight: a review and upcoming directions. Journal of obesity. 2012;2012:123023.
21. World Health Organization. Global Database on Body Mass Index 2015 [Available from: http://apps.who.int/bmi/index.jsp?introPage=intro_3.html.
22. World Health Organization W. Obesity: preventing and managing the global epidemic. Report of a WHO consultation on obesity, Geneva, 3 – 5 June 1997. Geneva: 1998.
23. Vitolo M. Nutrição: da gestação ao envelhecimento. . Avaliação Nutricional da Criança. Rio de Janeiro: Rubio; 2008. p. 177.
24. Despres JP, Lemieux I, Prud'homme D. Treatment of obesity: need to focus on high risk abdominally obese patients. Bmj. 2001;322(7288):716-20.
25. Botton J, Heude B, Kettaneh A, Borys JM, Lommez A, Bresson JL, et al. Cardiovascular risk factor levels and their relationships with overweight and fat distribution in children: the Fleurbaix Laventie Ville Sante II study. Metabolism: clinical and experimental. 2007;56(5):614-22.
26. Nathan BM, Moran A. Metabolic complications of obesity in childhood and adolescence: more than just diabetes. Curr Opin Endocrinol Diabetes Obes. 2008;15(1):21-9.
27. Pereira PF, Serrano HM, Carvalho GQ, Lamounier JA, Peluzio Mdo C, Franceschini Sdo C, et al. [Waist circumference as indicator of body fat and metabolic alterations in teenagers: comparison among four references]. Rev Assoc Med Bras. 2010;56(6):665-9.
28. Zambon MP, Zanolli Mde L, Marmo DB, Magna LA, Guimarey LM, Morcillo AM. [Body mass index and triceps skinfold correlation in children from Paulinia city, Sao Paulo, SP]. Rev Assoc Med Bras. 2003;49(2):137-40.
29. Sigulem DM, Devincenzi MU, Lessa AC. [Diagnosis of child and adolescent nutritional status]. Jornal de pediatria. 2000;76 Suppl 3:S275-84.
30. Junior GF, Osorio MM. Padrão alimentar de crianças menores de cinco anos Revista de Nutrição. 2005;18(6):793-802

31. Monteiro CA, Cannon G, Levy B, Claro RM, Moubarac JC. The Food System. Ultra-processing. The big issue for nutrition, disease, health, well-being. World Nutrition. 2012;3(12):527-69.
32. Núcleo de estudos e pesquisa em alimentação (NEPA). Universidade Estadual de Campinas. Tabela Brasileira de composição de alimentos (TACO). 4th ed. Campinas: NEPA/Unicamp; 2011. p. 161, (2011).
33. Guenther PM, Casavale KO, Reedy J, Kirkpatrick SI, Hiza HA, Kuczynski KJ, et al. Update of the Healthy Eating Index: HEI-2010. J Acad Nutr Diet. 2013;113(4):569-80.
34. Kopelman PG. Obesity as a medical problem. Nature. 2000;404(6778):635-43.
35. Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML, et al. The global obesity pandemic: shaped by global drivers and local environments. Lancet. 2011;378(9793):804-14.
36. Palou A, Serra F, Bonet ML, Pico C. Obesity: molecular bases of a multifactorial problem. Eur J Nutr. 2000;39(4):127-44.
37. Levin BE. Developmental gene x environment interactions affecting systems regulating energy homeostasis and obesity. Front Neuroendocrinol. 2010;31(3):270-83.
38. Maes HH, Neale MC, Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. Behavior genetics. 1997;27(4):325-51.
39. Wardle J, Carnell S, Haworth CM, Plomin R. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. The American journal of clinical nutrition. 2008;87(2):398-404.

40. Stunkard AJ, Foch TT, Hrubec Z. A twin study of human obesity. Jama. 1986;256(1):51-4.
41. Speliotes E. The Genetic Determinants of Common Human Obesity. Current Cardiovascular Risk Reports. 2009;3:411–7.
42. Cambuli VM, Musiu MC, Incani M, Paderi M, Serpe R, Marras V, et al. Assessment of adiponectin and leptin as biomarkers of positive metabolic outcomes after lifestyle intervention in overweight and obese children. J Clin Endocrinol Metab. 2008;93(8):3051-7.
43. Day FR, Loos RJ. Developments in obesity genetics in the era of genome-wide association studies. J Nutrigenet Nutrigenomics. 2011;4(4):222-38.
44. Skidmore PM, Cassidy A, Swaminathan R, Richards JB, Mangino M, Spector TD, et al. An obesogenic postnatal environment is more important than the fetal environment for the development of adult adiposity: a study of female twins. The American journal of clinical nutrition. 2009;90(2):401-6.
45. Chesi A, Grant SF. The Genetics of Pediatric Obesity. Trends Endocrinol Metab. 2015.
46. Burton PR CD, Cardon LR, Craddock N, Deloukas P, Duncanson A, Kwiatkowski DP et al. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447(7145):661-78.
47. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015;518(7538):197-206.
48. Bernhard F, Landgraf K, Kloting N, Berthold A, Buttner P, Friebe D, et al. Functional relevance of genes implicated by obesity genome-wide association study signals for human adipocyte biology. Diabetologia. 2013;56(2):311-22.
49. Skledar M, Nikolac M, Dodig-Curkovic K, Curkovic M, Borovecki F, Pivac N. Association between brain-derived neurotrophic factor Val66Met and obesity in children and adolescents. Progress in neuro-psychopharmacology & biological psychiatry. 2012;36(1):136-40.
50. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. 2007;316(5826):889-94.
51. Scuteri A, Sanna S, Chen WM, Uda M, Albai G, Strait J, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS genetics. 2007;3(7):e115.
52. Willer CJ, Speliotes EK, Loos RJ, Li S, Lindgren CM, Heid IM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. Nature genetics. 2009;41(1):25-34.
53. Bradfield JP, Taal HR, Timpson NJ, Scherag A, Lecoeur C, Warrington NM, et al. A genome-wide association meta-analysis identifies new childhood obesity loci. Nature genetics. 2012;44(5):526-31.
54. da Silva CF, Zandona MR, Vitolo MR, Campagnolo PD, Rotta LN, Almeida S, et al. Association between a frequent variant of the FTO gene and anthropometric phenotypes in Brazilian children. BMC medical genetics. 2013;14:34.
55. Ng MC, Tam CH, So WY, Ho JS, Chan AW, Lee HM, et al. Implication of genetic variants near NEGR1, SEC16B, TMEM18, ETV5/DGKG, GNPDA2, LIN7C/BDNF, MTCH2, BCDIN3D/FAIM2, SH2B1, FTO, MC4R, and KCTD15 with obesity and type 2 diabetes in 7705 Chinese. J Clin Endocrinol Metab. 2010;95(5):2418-25.
56. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nature genetics. 2010;42(11):937-48.

57. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. Nature genetics. 2009;41(1):18-24.
58. Herbert A, Gerry NP, McQueen MB, Heid IM, Pfeufer A, Illig T, et al. A common genetic variant is associated with adult and childhood obesity. Science. 2006;312(5771):279-83.
59. Meyre D, Delplanque J, Chevre JC, Lecoeur C, Lobbens S, Gallina S, et al. Genome-wide association study for early-onset and morbid adult obesity identifies three new risk loci in European populations. Nature genetics. 2009;41(2):157-9.
60. Gunstad J, Schofield P, Paul RH, Spitznagel MB, Cohen RA, Williams LM, et al. BDNF Val66Met polymorphism is associated with body mass index in healthy adults. Neuropsychobiology. 2006;53(3):153-6.
61. Zhou D, Liu H, Zhou M, Wang S, Zhang J, Liao L, et al. Common variant (rs9939609) in the FTO gene is associated with metabolic syndrome. Mol Biol Rep. 2012;39(6):6555-61.
62. Geller F, Reichwald K, Dempfle A, Illig T, Vollmert C, Herpertz S, et al. Melanocortin-4 receptor gene variant I103 is negatively associated with obesity. Am J Hum Genet. 2004;74(3):572-81.
63. Stutzmann F, Vatin V, Cauchi S, Morandi A, Jouret B, Landt O, et al. Non-synonymous polymorphisms in melanocortin-4 receptor protect against obesity: the two facets of a Janus obesity gene. Human molecular genetics. 2007;16(15):1837-44.
64. Saunders MA, Liang H, Li WH. Human polymorphism at microRNAs and microRNA target sites. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(9):3300-5.
65. Loos RJ, Lindgren CM, Li S, Wheeler E, Zhao JH, Prokopenko I, et al. Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nature genetics. 2008;40(6):768-75.
66. Grant SF, Bradfield JP, Zhang H, Wang K, Kim CE, Annaiah K, et al. Investigation of the locus near MC4R with childhood obesity in Americans of European and African ancestry. Obesity. 2009;17(7):1461-5.
67. Albuquerque D, Nobrega C, Rodriguez-Lopez R, Manco L. Association study of common polymorphisms in MSRA, TFAP2B, MC4R, NRXN3, PPARGC1A, TMEM18, SEC16B, HOXB5 and OLFM4 genes with obesity-related traits among Portuguese children. Journal of human genetics. 2014;59(6):307-13.
68. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell. 2005;123(3):493-505.
69. Adan RA, Tiesjema B, Hillebrand JJ, la Fleur SE, Kas MJ, de Krom M. The MC4 receptor and control of appetite. British journal of pharmacology. 2006;149(7):815-27.
70. Acosta A, Camilleri M, Shin A, Carlson P, Burton D, O'Neill J, et al. Association of melanocortin 4 receptor gene variation with satiation and gastric emptying in overweight and obese adults. Genes & nutrition. 2014;9(2):384.
71. Lazopoulou N, Gkioka E, Ntalla I, Pervanidou P, Magiakou AM, Roma-Giannikou E, et al. The combined effect of MC4R and FTO risk alleles on childhood obesity in Greece. Hormones (Athens). 2015;14(1):126-33.
72. Yilmaz Z, Davis C, Loxton NJ, Kaplan AS, Levitan RD, Carter JC, et al. Association between MC4R rs17782313 polymorphism and overeating behaviors. Int J Obes (Lond). 2015;39(1):114-20.
73. Wang K, Li WD, Zhang CK, Wang Z, Glessner JT, Grant SF, et al. A genome-wide association study on obesity and obesity-related traits. PloS one. 2011;6(4):e18939.

74. Mejia-Benitez A, Klunder-Klunder M, Yengo L, Meyre D, Aradillas C, Cruz E, et al. Analysis of the contribution of FTO, NPC1, ENPP1, NEGR1, GNPDA2 and MC4R genes to obesity in Mexican children. BMC medical genetics. 2013;14:21.
75. Lv D, Zhang DD, Wang H, Zhang Y, Liang L, Fu JF, et al. Genetic variations in SEC16B, MC4R, MAP2K5 and KCTD15 were associated with childhood obesity and interacted with dietary behaviors in Chinese school-age population. Gene. 2015;560(2):149-55.
76. Stutzmann F, Cauchi S, Durand E, Calvacanti-Proenca C, Pigeyre M, Hartikainen AL, et al. Common genetic variation near MC4R is associated with eating behaviour patterns in European populations. Int J Obes (Lond). 2009;33(3):373-8.
77. Horstmann A, Kovacs P, Kabisch S, Boettcher Y, Schloegl H, Tonjes A, et al. Common genetic variation near MC4R has a sex-specific impact on human brain structure and eating behavior. PloS one. 2013;8(9):e74362.
78. Ho-Urriola J, Guzman-Guzman IP, Smalley SV, Gonzalez A, Weisstaub G, Dominguez-Vasquez P, et al. Melanocortin-4 receptor polymorphism rs17782313: association with obesity and eating in the absence of hunger in Chilean children. Nutrition. 2014;30(2):145-9.
79. Speakman JR. Functional analysis of seven genes linked to body mass index and adiposity by genome-wide association studies: a review. Human heredity. 2013;75(2-4):57-79.
80. Jurvansuu JM, Goldman A. Obesity risk gene TMEM18 encodes a sequence-specific DNA-binding protein. PloS one. 2011;6(9):e25317.
81. Almen MS, Jacobsson JA, Shaik JH, Olszewski PK, Cedernaes J, Alsio J, et al. The obesity gene, TMEM18, is of ancient origin, found in majority of neuronal cells in all major brain regions and associated with obesity in severely obese children. BMC medical genetics. 2010;11:58.
82. den Hoed M, Ekelund U, Brage S, Grontved A, Zhao JH, Sharp SJ, et al. Genetic susceptibility to obesity and related traits in childhood and adolescence: influence of loci identified by genome-wide association studies. Diabetes. 2010;59(11):2980-8.
83. Rask-Andersen M, Jacobsson JA, Moschonis G, Chavan RA, Sikder MA, Allzen E, et al. Association of TMEM18 variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats. European journal of human genetics : EJHG. 2012;20(2):192-7.
84. Noble EE, Billington CJ, Kotz CM, Wang C. The lighter side of BDNF. American journal of physiology Regulatory, integrative and comparative physiology. 2011;300(5):R1053-69.
85. Araki S, Yamamoto Y, Dobashi K, Asayama K, Kusuhara K. Decreased plasma levels of brain-derived neurotrophic factor and its relationship with obesity and birth weight in obese Japanese children. Obes Res Clin Pract. 2014;8(1):e63-9.
86. Hinney A, Vogel CI, Hebebrand J. From monogenic to polygenic obesity: recent advances. Eur Child Adolesc Psychiatry. 2010;19(3):297-310.
87. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003;112(2):257-69.
88. Zegers D, Hendrickx R, Verrijken A, Van Hoorenbeeck K, Van Camp JK, de Craemer V, et al. Screening for genetic variants in BDNF that contribute to childhood obesity. Pediatric obesity. 2014;9(1):36-42.
89. Gratacos M, Gonzalez JR, Mercader JM, de Cid R, Urretavizcaya M, Estivill X. Brain-derived neurotrophic factor Val66Met and psychiatric disorders: meta-analysis of case-

control studies confirm association to substance-related disorders, eating disorders, and schizophrenia. Biological psychiatry. 2007;61(7):911-22.
90. Mei H, Chen W, Jiang F, He J, Srinivasan S, Smith EN, et al. Longitudinal replication studies of GWAS risk SNPs influencing body mass index over the course of childhood and adulthood. PloS one. 2012;7(2):e31470.
91. Alharbi KK, Richardson TG, Khan IA, Syed R, Mohammed AK, Boustred CR, et al. Influence of adiposity-related genetic markers in a population of saudi arabians where other variables influencing obesity may be reduced. Dis Markers. 2014;2014:758232.
92. Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmao L, Amorim A, et al. Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. Hum Mutat. 2010;31(2):184-90.
93. Zarelli VE, Dawid IB. Inhibition of neural crest formation by Kctd15 involves regulation of transcription factor AP-2. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(8):2870-5.
94. Dupin E, Creuzet S, Le Douarin NM. The contribution of the neural crest to the vertebrate body. Adv Exp Med Biol. 2006;589:96-119.
95. Williams MJ, Goergen P, Rajendran J, Zheleznyakova G, Hagglund MG, Perland E, et al. Obesity-linked homologues TfAP-2 and Twz establish meal frequency in Drosophila melanogaster. PLoS genetics. 2014;10(9):e1004499.
96. Lee AW, Hengstler H, Schwald K, Berriel-Diaz M, Loreth D, Kirsch M, et al. Functional inactivation of the genome-wide association study obesity gene neuronal growth regulator 1 in mice causes a body mass phenotype. PloS one. 2012;7(7):e41537.
97. Walley AJ, Jacobson P, Falchi M, Bottolo L, Andersson JC, Petretto E, et al. Differential coexpression analysis of obesity-associated networks in human subcutaneous adipose tissue. Int J Obes (Lond). 2012;36(1):137-47.
98. Rukh G, Sonestedt E, Melander O, Hedblad B, Wirfalt E, Ericson U, et al. Genetic susceptibility to obesity and diet intakes: association and interaction analyses in the Malmo Diet and Cancer Study. Genes & nutrition. 2013;8(6):535-47.
99. Ntalla I, Panoutsopoulou K, Vlachou P, Southam L, William Rayner N, Zeggini E, et al. Replication of established common genetic variants for adult BMI and childhood obesity in Greek adolescents: the TEENAGE study. Annals of human genetics. 2013;77(3):268-74.
100. Magi R, Manning S, Yousseif A, Pucci A, Santini F, Karra E, et al. Contribution of 32 GWAS-identified common variants to severe obesity in European adults referred for bariatric surgery. PloS one. 2013;8(8):e70735.
101. Budnik A, Heesom KJ, Stephens DJ. Characterization of human Sec16B: indications of specialized, non-redundant functions. Scientific reports. 2011;1:77.
102. Warrington NM, Wu YY, Pennell CE, Marsh JA, Beilin LJ, Palmer LJ, et al. Modelling BMI trajectories in children for genetic association studies. PloS one. 2013;8(1):e53897.
103. Gonzalez JR, Estevez MN, Giralt PS, Caceres A, Perez LM, Gonzalez-Carpio M, et al. Genetic risk profiles for a childhood with severely overweight. Pediatric obesity. 2014;9(4):272-80.
104. Schmid PM, Heid I, Buechler C, Steege A, Resch M, Birner C, et al. Expression of fourteen novel obesity-related genes in Zucker diabetic fatty rats. Cardiovascular diabetology. 2012;11:48.
105. Hotta K, Kiura K, Fujiwara Y, Takigawa N, Oze I, Ochi N, et al. Association between incremental gains in the objective response rate and survival improvement in phase III trials of first-line chemotherapy for extensive disease small-cell lung cancer. Ann Oncol. 2009;20(5):829-34.

106. Zhao J, Bradfield JP, Li M, Wang K, Zhang H, Kim CE, et al. The role of obesity-associated loci identified in genome-wide association studies in the determination of pediatric BMI. Obesity. 2009;17(12):2254-7.
107. Mitchell JA, Hakonarson H, Rebbeck TR, Grant SF. Obesity-susceptibility loci and the tails of the pediatric BMI distribution. Obesity. 2013;21(6):1256-60.
108. Han DY, Murphy R, Morgan AR, Lam WJ, Thompson JM, Wall CR, et al. Reduced genetic influence on childhood obesity in small for gestational age children. BMC medical genetics. 2013;14:10.
109. Ren D, Zhou Y, Morris D, Li M, Li Z, Rui L. Neuronal SH2B1 is essential for controlling energy and glucose homeostasis. J Clin Invest. 2007;117(2):397-406.
110. Tang L, Ye H, Hong Q, Chen F, Wang Q, Xu L, et al. Meta-analyses between 18 candidate genetic markers and overweight/obesity. Diagn Pathol. 2014;9:56.
111. Pillay V, Crowther NJ, Ramsay M, Smith GD, Norris SA, Lombard Z. Exploring genetic markers of adult obesity risk in black adolescent South Africans-the Birth to Twenty Cohort. Nutr Diabetes. 2015;5:e157.
112. Volckmar AL, Bolze F, Jarick I, Knoll N, Scherag A, Reinehr T, et al. Mutation screen in the GWAS derived obesity gene SH2B1 including functional analyses of detected variants. BMC Med Genomics. 2012;5:65.
113. Liu W, Chen L, Zhu J, Rodgers GP. The glycoprotein hGC-1 binds to cadherin and lectins. Exp Cell Res. 2006;312(10):1785-97.
114. Li F, Zhao J, Yuan Z, Zhang X, Ji J, Xue F. A powerful latent variable method for detecting and characterizing gene-based gene-gene interaction on multiple quantitative traits. BMC Genet. 2013;14:89.
115. Liu RH, Yang MH, Xiang H, Bao LM, Yang HA, Yue LW, et al. Depletion of OLFM4 gene inhibits cell growth and increases sensitization to hydrogen peroxide and tumor necrosis factor-alpha induced-apoptosis in gastric cancer cells. Journal of biomedical science. 2012;19:38.
116. Warrington NM, Howe LD, Paternoster L, Kaakinen M, Herrala S, Huikari V, et al. A genome-wide association study of body mass index across early life and childhood. Int J Epidemiol. 2015;44(2):700-12.
117. Fessner A, Esser JS, Bluhm F, Grundmann S, Zhou Q, Patterson C, et al. The transcription factor HoxB5 stimulates vascular remodelling in a cytokine-dependent manner. Cardiovascular research. 2014;101(2):247-55.
118. Kam MK, Cheung MC, Zhu JJ, Cheng WW, Sat EW, Tam PK, et al. Perturbation of Hoxb5 signaling in vagal and trunk neural crest cells causes apoptosis and neurocristopathies in mice. Cell death and differentiation. 2014;21(2):278-89.
119. Fu M, Lui VC, Sham MH, Cheung AN, Tam PK. HOXB5 expression is spatially and temporarily regulated in human embryonic gut during neural crest cell colonization and differentiation of enteric neuroblasts. Dev Dyn. 2003;228(1):1-10.
120. Dankel SN, Fadnes DJ, Stavrum AK, Stansberg C, Holdhus R, Hoang T, et al. Switch from stress response to homeobox transcription factors in adipose tissue after profound fat loss. PloS one. 2010;5(6):e11033.
121. Mishra PJ, Bertino JR. MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. Pharmacogenomics. 2009;10(3):399-416.
122. Mattick JS, Taft RJ, Faulkner GJ. A global view of genomic information--moving beyond the gene and the master regulator. Trends Genet. 2010;26(1):21-8.
123. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000;408(6808):86-9.

124. McGregor RA, Choi MS. microRNAs in the regulation of adipogenesis and obesity. Curr Mol Med. 2011;11(4):304-16.
125. Bengestrate L, Virtue S, Campbell M, Vidal-Puig A, Hadaschik D, Hahn P, et al. Genome-Wide Profiling of MicroRNAs in Adipose Mesenchymal Stem Cell Differentiation and Mouse Models of Obesity. PloS one. 2011;6(6):e21305.
126. Lee EK, Lee MJ, Abdelmohsen K, Kim W, Kim MM, Srikantan S, et al. miR-130 suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor gamma expression. Mol Cell Biol. 2011;31(4):626-38.
127. Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, et al. MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. PloS one. 2010;5(2):e9022.
128. Heneghan HM, Miller N, Kerin MJ. Role of microRNAs in obesity and the metabolic syndrome. Obes Rev. 2010;11(5):354-61.
129. Lee JH, Friso S, Choi SW. Epigenetic mechanisms underlying the link between non-alcoholic fatty liver diseases and nutrition. Nutrients. 2014;6(8):3303-25.
130. Saunders MA, Lim LP. (micro)Genomic medicine: microRNAs as therapeutics and biomarkers. RNA Biol. 2009;6(3):324-8.
131. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.
132. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215-33.
133. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PloS one. 2008;3(11):e3694.
134. Pescador N, Perez-Barba M, Ibarra JM, Corbaton A, Martinez-Larrad MT, Serrano-Rios M. Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. PloS one. 2013;8(10):e77251.
135. Ortega FJ, Mercader JM, Catalan V, Moreno-Navarrete JM, Pueyo N, Sabater M, et al. Targeting the circulating microRNA signature of obesity. Clin Chem. 2013;59(5):781-92.
136. Moran R. Evaluation and treatment of childhood obesity. Am Fam Physician. 1999;59(4):861-8, 71-3.
137. Johnson L, Mander AP, Jones LR, Emmett PM, Jebb SA. A prospective analysis of dietary energy density at age 5 and 7 years and fatness at 9 years among UK children. Int J Obes (Lond). 2008;32(4):586-93.
138. Wang Y, Monteiro C, Popkin BM. Trends of obesity and underweight in older children and adolescents in the United States, Brazil, China, and Russia. The American journal of clinical nutrition. 2002;75(6):971-7.
139. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(11):4402-7.
140. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18(10):997-1006.
141. Galvao AC, Kruger RC, Campagnolo PD, Mattevi VS, Vitolo MR, Almeida S. Association of MAOA and COMT gene polymorphisms with palatable food intake in children. J Nutr Biochem. 2012;23(3):272-7.
142. Zandona MR, Rodrigues RO, Albiero G, Campagnolo PD, Vitolo MR, Almeida S, et al. Polymorphisms in LEPR, PPARG and APM1 genes: associations with energy intake and metabolic traits in young children. Arquivos brasileiros de endocrinologia e metabologia. 2013;57(8):603-11.

143. Miranda RC, Vetter SB, Genro JP, Campagnolo PD, Mattevi VS, Vitolo MR, et al. SLC6A14 and 5-HTR2C polymorphisms are associated with food intake and nutritional status in children. Clin Biochem. 2015.
144. Zuk O, Hechter E, Sunyaev SR, Lander ES. The mystery of missing heritability: Genetic interactions create phantom heritability. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(4):1193-8.
145. Rovaris DL, Mota NR, Callegari-Jacques SM, Bau CH. Approaching "phantom heritability" in psychiatry by hypothesis-driven gene-gene interactions. Front Hum Neurosci. 2013;7:210.
146. Nistala R, Hayden MR, Demarco VG, Henriksen EJ, Lackland DT, Sowers JR. Prenatal Programming and Epigenetics in the Genesis of the Cardiorenal Syndrome. Cardiorenal Med. 2011;1(4):243-54.
147. Choi SW, Friso S. Epigenetics: A New Bridge between Nutrition and Health. Adv Nutr. 2010;1(1):8-16.

7 ANEXOS

7.1 Anexo A – Instruções aos Autores Periódico *Pediatric Obesity*

Author Guidelines

Pediatric Obesity is a peer-reviewed, bi-monthly journal devoted to research into obesity and its co-morbidities during neonatal development, infancy, childhood and adolescence. We are interested in papers that cover the broad spectrum of issues related to pediatric obesity including the following categories: Treatment & Prevention; Epidemiology and Global Prevalence; Measurement & Assessment; Disease Outcomes; Mechanisms; Behavior & Environment; Diet and Physical Activity. *Pediatric Obesity* is an official journal of the World Obesity Federation.
Submission is considered on the conditions that papers are previously unpublished, and are not offered simultaneously elsewhere; that all authors have read and approved the content, and all authors have also declared all competing interests; and that the work complies with the Ethical Policies of the journal, and has been conducted under internationally accepted ethical standards after relevant ethical review. It is highly recommended you read this policy and complete any necessary documentation prior to your submission.
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Acceptance of papers is based on the originality of the observation or investigation, the quality of the work described, the clarity of presentation, and the relevance to our readership. When submitting a manuscript it is with the understanding that the manuscript (or its essential substance) has not been published other than as an abstract in any language or format and has not been submitted elsewhere for print or electronic publication consideration.
The journal operates a stringent peer review process. All manuscripts will be reviewed by the Editor, members of the Editorial Board, or other expert reviewers. At the discretion of the Editor, the manuscript may be returned immediately without full review, if deemed not competitive or outside the realm of interests of the majority of the readership of the Journal. The decision (reject, invite revision, accept) letter will be conveyed through *Pediatric Obesity* ScholarOne Manuscripts, coming directly from the Editor who has assumed responsibility for the manuscript's review. Editorial decisions are based not just on technical merit of the work, but also on other factors such as the priority for publication and the relevance to the Journal's general readership. All papers are judged in relation to other submissions currently under consideration. Rebuttals to rejected manuscripts are strongly discouraged and requests for resubmission of rejected manuscripts are generally not granted.
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Pediatric Obesity is a member of the UK Committee on Publication Ethics and subscribes to its recommendations (Committee on Publication Ethics [COPE]: guidelines on good publication practice, www.publicationethics.org.uk). Our Best Practice Guidelines on Publication Ethics: A Publisher's Perspective. Second Edition are available athttp://exchanges.wiley.com/ethicsguidelines.
The Editors reserve the right to reject a paper on ethical grounds. All authors are responsible for adhering to guidelines on good publication practice.
No paper can be published in the Journal unless it meets all of these requirements.
The corresponding author must provide an e-mail address for communication with the Editors and the Publisher.
Article types
Original Articles which report on clinical, population health and laboratory investigations and observations from both human and animal studies in all areas relevant to the broad area of child and adolescent obesity including its critical periods of development from the neonatal period to young adulthood. Manuscripts should be between 1,500 and 3,000 words in length (i.e. up to 6 typewritten double-spaced pages), not including tables, figure legends, and references necessary to support the data and their interpretation. Manuscripts should generally follow the IMRAD (Introduction, Methods,

Results, Discussion) format. They should include hypothesis testing, appropriate statistical methods, a clear reporting of results, and conclusions that are supported by the results.

Short Communications Studies that fall short of the criteria for full research papers (e.g. preliminary experiments limited by sample size or duration, novel hypotheses, commentaries) may be submitted as Short Communications, **which will be published online-only**. They should generally contain no more than 1,000 words of text, a maximum of two display items (tables and/or figures) and a maximum of 20 references. Apart from the Abstract (one paragraph of maximum 150 words) and Keywords, the text does not need to be divided into sections. In all other respects, the directions for full papers should be followed.

Review Articles Please contact the Editor-in-Chief before submission of a review article in order to ensure that the proposed topic falls within the journal guidelines and that a review on that topic is not currently under preparation by another author. Reviews should be a maximum of 5000 words, excluding references.

Letters to the Editor are considered for publication (subject to editing and abridgment) provided they do not contain material that has been submitted or published elsewhere, **and will be published online-only**. The text, not including references, must not exceed 250 words if it is in reference to a recent Journal article, or 400 words in all other cases. A letter must have no more than five references and one figure or table. Letters referring to a recent Journal article must be received within one month of its publication.

Specific Types of Studies

Epidemiological reports

Authors should include the following information in their reports:

Details of study

• Population sampled. National, regional, or specific selected group. Indicate if the sample population is representative of a national or regional population. If neither, state from what population the sample was drawn (e.g. children from an ethnic minority group, children from lower socio-economic status families, children from an urban obesity clinic), giving details and stating why this group may be of significance.

• Time of data collection. Indicate the time period when data were collected (e.g. at school entry autumn 2003, or recruited between January 2002 and July 2002).

• Anthropometric data recorded. Indicate what measures were taken and how (e.g. self reported in interview, reported by parents, measured by school nurse). If measured, indicate whether weight included clothing, shoes etc, height was in shoes or not, waist circumference included clothing, and also indicate definitions of waist, hip, thigh etc). Skinfold measures should also be described carefully.

Defining overweight and obesity

• The prevalence of overweight and obesity should be defined according to cut-off criteria.

• If using national or local definitions, a reference to the source tables giving the cut-off criteria should be provided (also cite this in the Reference list).

• For studies reporting the prevalence of childhood overweight and/or obesity in their population characteristics, the journal requests that these are shown using both the IOTF and WHO definitions. Although these definitions produce somewhat different prevalence rates, both definitions are being used for international comparisons at this stage and sufficient numbers of published studies which report both prevalence values will be needed to generate the algorithms to estimate one from the other.

The IOTF reference for children aged 2-18 years is: Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000; 320: 1240-5. Available at http://bmj.bmjjournals.com/cgi/reprint/320/7244/1240

The WHO reference for children aged 0-5 years is: WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development. Geneva: World Health Organization, 2006. Available at:http://www.who.int/childgrowth/standards/en/

The WHO reference for children aged 5-19 years is: de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. Bulletin of the World Health Organization 2007; 85: 660-7. Available at:http://www.who.int/growthref/growthref_who_bull/en/index.html

• In all cases, please state clearly whether or not the figures for ‘overweight’ include those for ‘obese’.

Study results

The presentation of results should include, where appropriate, age- and sex-specific results and an indication of sample size in sub-groups.

Clinical Trials

Trial registration

•All clinical trials published in the Journal must have been prospectively registered in a public trials registry. The details of this policy are contained in the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (http://www.ICMJE.org/)

•The trial registration number should be given at the end of the Abstract.

Reporting of trials

Trials should be reported in accordance with the CONSORT (Consolidated Standards of Reporting Trials) statement (http://www.consort-statement.org/). Please also submit a checklist for editors and reviewers (not for publication) showing that you have covered each of the main CONSORT reporting points within the text of the manuscript (http://www.consort-statement.org/Downloads/Checklist.doc)

Manuscript format

Authors must provide their entire manuscript (in English) in electronic format.

General advice about the presentation of manuscripts:

- Provide a clear, concise and interesting title, and abstract, this helps readers quickly see the value of your work.
- The full contact details of the corresponding author must be included on the title page and the covering letter.
- All pages should be numbered.
- Avoid, as much as possible, the use of abbreviations.
- All scientific units should be expressed in SI units.
- Read these Author Guidelines carefully and follow them as closely as you can.

Title Page

The title page should contain: (1) the title of the article, (2) the name of each author (first name and surname preferred), (3) the name of the department(s) and institution(s) to which the authors belong, (4) three to six keywords, (5) a running title, (6) full address including e-mail of the corresponding author.

Main text

Original research papers should be divided into (1) 'What is already known about this subject' and 'What this study adds' (up to three short bullet points for each), (2) structured abstract (200 words) comprising Background; Objectives; Methods; Results; Conclusions, (3) introduction, (4) methods, (5) results, (6) discussion, (7) conflicts of interest statement, (8) acknowledgements (including author contributions), (9) references.

For guidance on the content and style of the introduction, materials and methods, results and discussion, please follow the International Committee of Medical Journal Editors (ICJME) Uniform Requirements for Manuscripts Submitted to Biomedical Journals:http://www.icmje.org/manuscript_1prepare.html

Reviews should be divided into: (1) structured abstract (200 words), (2) introduction, (3) text subdivided into paragraphs, (4) conclusion or discussion, (5) conflicts of interest statement, (6) author contributions, (7) acknowledgements, (8) references. Review authors are particularly encouraged to use tables, diagrams and figures. Personal conclusions and practical applications are welcome.

Abbreviations

Abbreviations should be explained at the beginning of the manuscript and listed in the order in which they appear. Avoid abbreviations in the title and in the abstract.

Drug Names

Generic names should, in general, be used. If an author so desires, brand names may be inserted in parentheses.

Acknowledgements

This section should outline the contribution of each author to the manuscript e.g.: study design, data collection, data analysis, data interpretation, literature search, generation of figures, writing of the manuscript. An example that authors might like to follow is:

XY and NM conceived and carried out experiments, AB and GH conceived experiments and analysed data. OP carried out experiments. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Any contributors who did not meet the authorship criteria should also be listed, such as colleagues who provided only technical support, writing assistance or general support. Financial and material support must always be acknowledged, with a clear statement defining all funding sources. This should include grants, equipment, drugs and other reagents, or gifts of materials.

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- Maher VMG, Thompson GR. Analysis of evidence from cholesterol-lowering and regression trials. *J Drug Dev* Suppl 1990; **3/1:** 199-203.

Examples of book references:

- Lissner L, Bengtsson C, Lapidus L, Larson B, Bengtsson B, Brownell KD. Body weight variability and mortality in the Gothenburg Prospective Studies on men and women. In: Bjorntorp P, Rossner S (eds). *Obesity in Europe 88: Proceedings of the First European Congress on Obesity*. Libbey: London, 1989, pp 55-60.
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Please note that Blackwell Publishing will dispose of all hardcopy or electronic material submitted by authors 2 months after publication.

7.2 Anexo B – Instruções aos Autores Periódico *International Journal of Obesity*

Aims and Scope: The International Journal of Obesity is a multidisciplinary forum for basic, clinical and applied studies of the biochemical, physiological, genetic, molecular, metabolic, nutritional, psychological and epidemiological aspects of obesity and related disorders.
Topics Covered Molecular, cellular, animal, human experimental and clinical studies, which address issues related to the development and treatment of obesity, and the functional impairments associated with the obese state. The problems of obesity are multifactorial, and the International Journal of Obesity will expect to publish articles with biological, psychological, clinical, sociological and environmental approaches to these problems.
Due to the high volume of submissions that the Journal receives, the following manuscripts will be deemed low priority: Simple prevalence studies involving a single country at a single time-point. Studies that merely confirm established facts from previous publications and that contain little new information. For example, it is hard to justify publication space for studies that report obesity is associated with known health risks. Therefore, studies that replicate the findings of previously published papers will tend to have a lower priority. If similar data are already published, it will be critical for authors to explain the novelty of their manuscript in the covering letter to the editor. Those that involve co-morbidities of obesity (e.g. diabetes, cardiovascular disease), without having obesity-specific components to them. Recent examples include manuscripts that look at associations between inflammatory markers and diabetes or cardiovascular disease. This information is clearly of medical relevance, but it is not necessarily a high priority for a journal devoted to obesity research. Those that report the absence of links between obesity and a specific genotype or polymorphism; it is possible that such work could be considered in the form of a Short Communication, but a full manuscript is not justified. Those that describe anthropometric indices of obesity that might correlate with plasma markers of co-morbidities, but do not include any data relating to outcome of the co-morbidities. Retrospective studies, secondary analyses of data that arise from studies that were not primarily concerned with obesity or body weight, or clinical “audits” (for example of surgical interventions) that were not designed as appropriately
controlled clinical research interventions, unless there is particularly novel information presented that is of importance to the medical literature. Those that claim to be pediatric articles but which do not deal specifically with children and adolescents up to the age of 18 years. Case reports that do not describe a critical finding or major addition to the literature.
If authors wish to submit articles to the International Journal of Obesity in the above areas, they would need to state clearly in the covering letter and introduction to the manuscript what is novel and informative about the study and why is it a valuable addition to the scientific literature.
Original Articles: Please see ‘Preparation of Original Articles’ below for further details. Structured abstract: Max 300 words. 4,000 words max excluding abstract, references, figures and tables. Max of 6 tables/figues. Max of 60 references.
Short Communications: These are studies that fall short of the criteria for full Original Articles (e.g. preliminary experiments limited by sample size or duration, or novel hypotheses). Apart from including an abstract, there is no obligation to divide the text into sections. Unstructured abstract: Max 200 words. 1,500 words max excluding abstract, references, figures and tables. Max of 2 tables. Max of 20 references.
Reviews (by Editor invitation only): Reviews are comprehensive analyses of specific topics that are solicited by the Editor. Proposals for reviews may be submitted via the online submission system as a presubmission enquiry. PLEASE NOTE: All reviews should include search criteria and selection criteria in a Methods Section, along with the total number of articles identified and the total number selected for inclusion in the review. All invited reviews will undergo peer review prior to acceptance. Unstructured abstract However, if your Review is systematic, please provide a structured abstract. Max 300 words. 6,000 words max excluding abstract, references, figures and tables.
Max of 8 Max of 120
Letters to the Editor: Letters to the Editor will be considered for publication, subject to editing. Letters must contain information critical to a certain area or must be referencing data recently

published in IJO. A Letter must reference the original source but can use an arbitrary title. No abstract required. 500 words max excluding references, figures and tables. Max of 2 Max of 10.

Editorials (by Editor invitation only): Proposals for Editorials may be submitted; authors should only send an outline of the proposed paper for initial consideration.

No abstract required. 1,000 words excluding references, figures and tables. Max of 2 Max of 10 references.

Commentaries (by Editor invitation only): Commentaries discuss a paper published in a specific issue and should set the problems addressed by the paper in the wider context of the field. No abstract required. 1,500 words excluding references, figures and tables. Max of 1tabel. Max of 10 references.

Debates: Debates address an area of research which is of major present interest, and for which there are substantially different views. Subject and authors are chosen by the editors but proposals are welcome. Unstructured abstract Max 100 words. 2,000 words excluding references, figures and tables. Max of 1 Max of 20

Technical Reports Technical: Reports are original articles that address areas of more methodological interest. The content of these Reports must have direct relevance to the field of Obesity and have the same level of scientific rigour expected of the normal original articles. Structured abstract Max 300 words. 2,500 words excluding references, figures and tables. Tables: Max of 4. References: Max of 25.

Expert Reports Expert: Reports are articles submitted by a consensus of individuals expert in a given field that opine on a topic in the field of obesity. The article specifications listed are a guide and prospective authors are encouraged to contact Richard Atkinson via the International Journal of Obesity Editorial Office (ijo@nature.com) to discuss their report before submission. Unstructured abstract Max 100 words. 2,000 words excluding references, figures and tables. Max of 2 tables. Max of 20 references.

Clinical Trials: The International Journal of Obesity is interested in attracting the submission of manuscripts describing new therapeutic approaches to obesity treatment. These human intervention trials of new therapies can be pharmacological, surgical, dietary, physical activity, nutraceutical (including herbal preparations), behavioural or some other relevant intervention, but must be novel, include an appropriate control group and be of a sufficiently long duration to generate results of clinical relevance. Trials which also consider maintenance of weight loss would be of particular interest. With regard to the duration of such trials, the following will apply:

1. Diet / lifestyle /nutraceutical interventions. The total duration (weight loss plus weight maintenance) must be at least 1 year. Anything less than this is of little practical value and is highly unlikely to reveal any novel mechanistic findings. The only exception would be if a shorter period of intervention was accompanied by a truly novel mechanistic approach. Even then the study should be at least 3 months in duration and such papers normally would be submitted as Short Communications. 2. Surgery Short term, post-surgery studies are of minimal value as many are likely to be in the rapid phase of weight loss and unlikely to achieve a state of weight maintenance. Thus, surgical studies should be 1 year or more in duration and linked with novel mechanistic/physiological measurements. The only exceptions would be if a shorter period of intervention were accompanied by a truly novel mechanistic approach and such papers normally would be submitted as Short Communications. 3. Drug studies 1 year or longer studies with truly novel agents are unrealistic. However, a 3 month study with a truly novel agent would not normally deserve to be published as a full paper and should be submitted as a Short Communication if it is of less than 1 year duration. Any established drug being applied to obesity (e.g. the recent application of anti-depressants to an obesity target) or any obesity drug which has already produced publications demonstrating efficacy in humans MUST be studied for at least 1 year.

In addition to these trials of new therapeutic approaches, the International Journal of Obesity is also interested in publishing systematic reviews of weight loss and weight maintenance interventions in human subjects. However, these reviews and any associated meta-analyses should only be concerned with studies that are of a duration of at least 1 year.

See the Editorial Policy section for further information on requirements when submitting a clinical trial.

Special Issues: Special issues are comprised of a group of high quality, peerreviewed manuscripts about a single specific theme / topic. Although the individual manuscripts are stand alone, they

collectively make an important point by offering a comprehensive view, or by providing a diverse perspective. The number of manuscripts in a special issue is determined on case by case basis. Special Issues are commissioned only by invitation or upon consultation with IJO editorial staff. Please contact the Editor, Special Issues, Nikhil Dhurandhar (nikhil.dhurandhar@pbrc.edu) for preliminary inquiries about special issues. Usually, a person willing to be the Guest Editor of special issue should initiate this process. This Guest Editor will act as the point of contact between IJO and the individual authors submitting manuscripts.

PREPARATION OF ARTICLES

Please note that original articles must contain the following components. Please see below for further details.

 Cover letter Title page (excluding acknowledgements) Abstract Introduction Materials (or Subjects) and Methods Results Discussion Acknowledgements Conflict of Interest References Figure legends Tables Figures

Cover Letter: The uploaded covering letter must state the material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration. If the manuscript has been previously considered for publication in another journal, please include the previous reviewer comments, to help expedite the decision by the Editorial team. Add note about including conflict of interest statement.

Title Page: The title page should bear the title of the paper, the full names of all the authors and their affiliations, together with the name, full postal address, telephone and fax numbers and e-mail address of the author to whom correspondence and offprint requests are to be sent (this information is also asked for on the

electronic submission form). The title page must also contain a Conflict of Interest statement (see Editorial Policy section).

 The title should be brief, informative, of 150 characters or less and should not make a statement or conclusion. The running title should consist of no more than 50 letters and spaces. It should be as brief as possible, convey the essential message of the paper and contain no abbreviations. Authors should disclose the sources of any support for the work, received in the form of grants and/or equipment and drugs. If authors regard it as essential to indicate that two or more co-authors are equal in status, they may be identified by an asterisk symbol with the caption 'These authors contributed equally to this work' immediately under the address list.

Abstract: Original Articles must be prepared with a structured abstract designed to summarise the essential features of the paper in a logical and concise sequence under the following mandatory headings:

 Background/Objectives: What was the main question or hypothesis tested? Subjects/Methods: How many subjects were recruited, how many dropped out? Was the study randomised, casecontrolled etc? Interventions/methods used and duration of administration. Results: Indicate 95% confidence intervals and exact P value for effects. Conclusions: Answer (significant or not) to main question.

Introduction: The Introduction should assume that the reader is knowledgeable in the field and should therefore be as brief as possible but can include a short historical review where desirable.

Materials/Subjects and Methods: This section should contain sufficient detail, so that all experimental procedures can be reproduced, and include references. Methods, however, that have been published in detail elsewhere should not be described in detail. Authors should provide the name of the manufacturer and their location for any specifically named medical equipment and instruments, and all drugs should be identified by their pharmaceutical names, and by their trade name if relevant.

Results and Discussion: The Results section should briefly present the experimental data in text, tables or figures. Tables and figures should not be described extensively in the text, either. The discussion should focus on the interpretation and the significance of the findings with concise objective comments that describe their relation to other work in the area. It should not repeat information in the results. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgements: These should be brief, and should include sources of support including sponsorship (e.g. university, charity, commercial organisation) and sources of material (e.g. novel drugs) not available commercially.

Conflict of Interest: Authors must declare whether or not there are any competing financial interests in relation to the work described. This information must be included at this stage and will be published as part of the paper. Conflict of interest should be noted in the cover letter and also on the title page. Please see the Conflict of Interest documentation in the Editorial Policy section for detailed information.

References: Only papers directly related to the article should be cited. Exhaustive lists should be avoided. References should follow the Vancouver format. In the text they should appear as numbers starting at one and at the end of the paper they should be listed (double-spaced) in numerical order corresponding to the order of citation in the text. Where a reference is to appear next to a number in the text, for example following an equation, chemical formula or biological acronym, citations should be written as (ref. X) and not as superscript. Example. “detectable levels of endogenous Bcl-2 (ref. 3), as confirmed by western blot”

All authors should be listed for papers with up to six authors; for papers with more than six authors, the first six only should be listed, followed by et al. Abbreviations for titles of medical periodicals should conform to those used in the latest edition of Index Medicus. The first and last page numbers for each reference should be provided. Abstracts and letters must be identified as such. Papers in press may be included in the list of references.

Personal communications must be allocated a number and included in the list of references in the usual way or simply referred to in the text; the authors may choose which method to use. In either case authors must obtain permission from the individual concerned to quote his/her unpublished work.

Examples: Journal article, up to six authors: Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol 2005; 6: 353–360.

Journal article, e-pub ahead of print: Bonin M, Pursche S, Bergeman T, Leopold T, Illmer T, Ehninger G et al. F-ara-A pharmacokinetics during reduced-intensity conditioning therapy with fludarabine and busulfan. Bone Marrow Transplant 2007; e-pub ahead of print 8 January 2007; doi:10.1038/sj.bmt.1705565

Journal article, in press: Gallardo RL, Juneja HS, Gardner FH. Normal human marrow stromal cells induce clonal growth of human malignant Tlymphoblasts. Int J Cell Cloning (in press).

Complete book: Atkinson K, Champlin R, Ritz J, Fibbe W, Ljungman P, Brenner MK (eds). Clinical Bone Marrow and Blood Stem Cell Transplantation, 3rd edn. Cambridge University Press: Cambridge, UK, 2004.

Chapter in book: Coccia PF. Hematopoietic cell transplantation for osteopetrosis. In: Blume KG, Forman SJ, Appelbaum FR (eds). Thomas' Hematopoietic Cell Transplantation, 3rd edn. Blackwell Publishing Ltd: Malden, MA, USA, 2004, pp 1443–1454.

Abstract: Syrjala KL, Abrams JR, Storer B, Heiman JR. Prospective risk factors for five-year sexuality late effects in men and women after haematopoietic cell transplantation. Bone Marrow Transplant 2006; 37(Suppl 1): S4 (abstract 107).

Correspondence: Caocci G, Pisu S. Overcoming scientific barriers and human prudence [letter]. Bone Marrow Transplant 2006; 38: 829–830.

Figure Legends: These should be brief, specific and appear on a separate manuscript page after the References section.

Tables: Tables should only be used to present essential data; they should not duplicate what is written in the text. It is imperative that any tables used are editable, ideally presented in Excel. Each must be uploaded as a separate workbook with a title or caption and be clearly labelled, sequentially. Please make sure each table is cited within the text and in the correct order, e.g. (Table 3). Please save the files with extensions .xls / .xlsx / .ods / or .doc or .docx. Please ensure that you provide a 'flat' file, with single values in each cell with no macros or links to other workbooks or worksheets and no calculations or functions.

Figures: Figures and images should be labelled sequentially and cited in the text. Figures should not be embedded within the text but rather uploaded as separate files. Detailed guidelines for submitting artwork can be found by downloading our Artwork Guidelines. The use of three-dimensional histograms is strongly discouraged when the addition of the third dimension gives no extra information.

Reuse of Display Items: See the Editorial Policy section for information on using previously published tables or figures.

Revised 20/8/2014 5

Supplementary Information: Supplementary information (SI) is peer-reviewed material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. The article must be complete and selfexplanatory without the SI, which is posted on the journal's website and linked to the article. SI may consist of data files, graphics, movies or extensive tables. Please see our Artwork Guidelines for information on accepted file types.

Authors should submit supplementary information files in the FINAL format as they are not edited, typeset or changed, and will appear online exactly as submitted. When submitting SI, authors are required to:

 Include a text summary (no more than 50 words) to describe the contents of each file. Identify the types of files (file formats) submitted. Include the text "Supplementary information is available at (journal name)'s website" at the end of the article and before the references.

Availability of Data and Materials: Please see our Editorial Policies for information regarding data, protocols, sequences, or structures.

Subject Ontology: Choosing the most relevant and specific subject terms from our subject ontology will ensure that your article will be more discoverable and will appear on appropriate subject specific pages on nature.com, in addition to the journal's own pages. Your article should be indexed with at least one, and up to four unique subject terms that describe the key subjects and concepts in your manuscript. Click here for help with this.

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 Colour should be distinct when being used as an identifying tool. Spaces, not commas should be used to separate thousands. At first mention of a manufacturer, the town (and state if USA) and country should be provided. Statistical methods: For normally distributed data, mean (SD) is the preferred summary statistic. Relative risks should be expressed as odds ratios with 95% confidence interval. To compare two methods for measuring a variable the method of Bland & Altman (1986, Lancet 1, 307–310) should be used; for this, calculation of P only is not appropriate. Units: Use metric units (SI units) as fully as possible. Preferably give measurements of energy in kiloJoules or MegaJoules with kilocalories in parentheses (1 kcal = 4.186kJ). Use % throughout. Abbreviations: On first using an abbreviation place it in parentheses after the full item. Very common abbreviations such as FFA, RNA, need not be defined. Note these abbreviations: gram g; litre l; milligram mg; kilogram kg; kilojoule kJ; megajoule MJ; weight wt; seconds s; minutes min; hours h. Do not add s for plural units.

LANGUAGE EDITING

Authors who are not native speakers of English sometimes receive negative comments from referees or editors about the language and grammar usage in their manuscripts, which can contribute to a paper being rejected. To reduce the possibility of such problems, we strongly encourage such authors to take at least one of the following steps:

 Have your manuscript reviewed for clarity by a colleague whose native language is English. Review the tips for technical writing here: http://www.nature.com/authors/author_resources/how_write .html. Use an English language editing service such as Nature Publishing Group Language Editing. An editor will improve the English to ensure that your meaning is clear and to identify problems that require your review.

Please note that the use of a language editing service is at the author's own expense and does not guarantee that the article will be selected for peer review or accepted.

Conflict of Interest

In the interests of transparency and to help readers form their own judgments of potential bias, authors must declare whether or not there are any competing financial interests in relation to the work described. This information must be included in their cover letter and on the title page of their manuscript. In cases where the authors declare a competing financial interest, a statement to that effect is published as part of the article. If no such conflict exists, the statement will simply read that the

authors have nothing to disclose. For the purposes of this statement, competing interests are defined as those of a financial nature that, through their potential influence on behaviour or content, or from perception of such potential influences, could undermine the objectivity, integrity or perceived value of a publication. They can include any of the following:

 Funding: Research support (including salaries, equipment, supplies, reimbursement for attending symposia, and other expenses) by organizations that may gain or lose financially through this publication. The role of the funding body in the design of the study, collection and analysis of data and decision to publish should be stated. Employment: Recent (while engaged in the research project), present or anticipated employment by any organization that may gain or lose financially through this publication. Personal financial interests: Stocks or shares in companies that may gain or lose financially through publication; consultation fees or other forms of remuneration from organizations that may gain or lose financially; patents or patent applications whose value may be affected by publication.

It is difficult to specify a threshold at which a financial interest becomes significant, but note that many US universities require faculty members to disclose interests exceeding $10,000 or 5% equity in a company. Any such figure is arbitrary, so we offer as one possible practical alternative guideline: "Declare all interests that could embarrass you were they to become publicly known after your work was published." We do not consider diversified mutual funds or investment trusts to constitute a competing financial interest.

The statement must contain an explicit and unambiguous statement describing any potential conflict of interest, or lack thereof, for any of the authors as it relates to the subject of the report. Examples include “Dr. Smith receives compensation as a consultant for XYZ Company,” “Dr. Jones and Dr. Smith have financial holdings in ABC Company,” or “Dr. Jones owns a patent on the diagnostic device described in this report.” These statements acknowledging or denying conflicts of interest must be included in the manuscript under the heading Conflict of Interest. The Conflict of Interest disclosure appears in the cover letter, in the manuscript submission process and before the References section in the manuscript.

Following the Conflict of Interest heading, there must be a listing for each author, detailing the professional services relevant to the submission. Neither the precise amount received from each entity nor the aggregate income from these sources needs to be provided. Professional services include any activities for which the individual is, has been, or will be compensated with cash, royalties, fees, stock or stock options in exchange for work performed, advice or counsel provided, or for other services related to the author’s professional knowledge and skills. This would include, but not necessarily be limited to, the identification of organizations from which the author received contracts or in which he or she holds an equity stake if professional services were provided in conjunction with the transaction.

Examples of declarations are:

 Conflict of interest. The authors declare no conflict of interest. Conflict of interest. Dr Caron's work has been funded by the NIH. He has received compensation as a member of the scientific advisory board of Acadia Pharmaceutical and owns stock in the company. He also has consulted for Lundbeck and received compensation. Dr Rothman and Dr Jensen declare no potential conflict of interest.

Authorship

Requirements for all categories of articles should conform to the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals,” developed by the ICMJE (www.icmje.org).

Each author must have contributed sufficiently to the intellectual content of the submission. The corresponding author should list all authors and their contributions to the work. Any changes to the author list after submission, such as a change in the order of the authors, or the deletion or addition of authors, must be approved by a signed letter from every author. The corresponding author must confirm that he or she has had full access to the data in the study and final responsibility for the decision to submit for publication. To qualify as a contributing author, one must meet all of the following criteria:

1) Conceived and/or designed the work that led to the submission, acquired data, and/or played an important role in interpreting the results. 2) Drafted or revised the manuscript. 3) Approved the final version. Contributions by individuals who made direct contributions to the work but do not meet all of the above criteria should be noted in the Acknowledgments section of the manuscript. Medical writers

and industry employees can be contributors. Their roles, affiliations, and potential conflicts of interest should be included in the author list or noted in the Acknowledgments and/or Contributors section concurrent with their contribution to the work submitted. Signed statements from any medical writers or editors declaring that they have given permission to be named as an author, as a contributor, or in the Acknowledgments section is also required. Failure to acknowledge these contributors can be considered inappropriate, which conflicts with the journal's editorial policy.

Statement of Ethics

As of March 2015, International Journal of Obesity requires authors of papers that are sent for external review to include in their manuscripts relevant details about several elements of experimental and analytical design. This initiative aims to improve the transparency of reporting and the reproducibility of published results, focusing on elements of methodological information that are frequently poorly reported. Authors being asked to resubmit a manuscript will be asked to confirm that these elements are included by filling out a checklist that will be made available to the editor and reviewers.

7.3 Anexo C – Aprovação do Comitê de Ética em Pesquisa

UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMITÊ DE ÉTICA EM PESQUISA CEP
UFCSPA

O Comitê de Ética em Pesquisa da UFCSPA, registrado na Comissão Nacional de Ética em Pesquisa (CONEP) sob o nº 075/05 em 23/07/04, analisou o Projeto:

Projeto: 11-817 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
MÁRCIA REGINA VITOLO
SILVANA DE ALMEIDA
MARÍLIA REMUZZI ZANDONÁ
CRISCIELE FONTANA
CARMELA FARIAS DA SILVA
RAQUEL CHRISTINE KRÖGER
SÍLVIA VALIM
VANESSA FIESTAUER
MARIANA EICK
VANESSA SUÑE MATTEVI

Título: INVESTIGAÇÃO DA ASSOCIAÇÃO DE VARIANTES GENÉTICAS COM A INGESTÃO ALIMENTAR E OBESIDADE

Esse projeto foi aprovado em seus aspectos éticos e metodológicos conforme as Resoluções 196/09 e demais Resoluções complementares. Toda e qualquer alteração do projeto, assim como eventos adversos graves, deverão ser comunicados a este CEP. Os TCLE, quando necessários, somente poderão ser utilizados após prévia e explícita aprovação (carimbo) de sua redação por este CEP"

Porto Alegre, 23 de abril de 2014.

Júlia S. Pereira Lima
Vice-Coordenadora CEP/UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da associação de variantes genéticas com a ingestão alimentar e o status nutricional, bioquímico e hematológico em crianças

Pesquisador: Silvana de Almeida

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 36424014.5.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 836.210

Data da Relatoria: 16/10/2014

Apresentação do Projeto:

Os presente estudo consiste em analisar os efeitos de variantes genéticas e epigenéticas dos genes LEPR, APM1, PPARG, FTO, MC4R, SEC16B, TMEM18, NEGR1, KCTD15, SH2B1, BDNF, OLFM4, HOXB5, DRD2, DRD4, MAOA, COMT, SLC3A4, SL6A14, SL6A4, 5-HTR2A, 5-HTR2C, TAS1R2, TAS1R3, HFE, FPN1, DMT1, HAMP, TFR, TF, TMPRSS6 e outros possíveis genes candidatos em relação a parâmetros antropométricos, ingestão alimentar e parâmetros bioquímicos e hematológicos em 600 crianças de 6-7 anos do município de Porto Alegre, RS.

As crianças estudas participarão do estudo "Impacto nas condições nutricionais e de saúde de crianças na idade de 6-7 anos que participaram de um ensaio de campo randomizado por conglomerados no primeiro ano de vida", avaliado e aprovado pelo Comitê de Ética em Pesquisa da UFCSPA conforme parecer consubstanciado nº 689.596 em 12/06/2014.

A análise molecular será realizada no Laboratório de Biologia Molecular da UFCSPA, o qual já possui a infraestrutura necessária para a realização das mesmas e anuência de realização do projeto pelo gerente de Laboratórios da Pós-Graduação. A extração de DNA será realizada segundo um protocolo padrão para extração de amostras de sangue total. O DNA de cada um dos tubos será dissolvido em 500l de TE e armazenado em freezer a -20°C

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Continuação do Parecer: 836.210

identificados apenas números. Genotipagem e análise de microRNAs: Os fragmentos de interesse serão analisados por reação em cadeia da polimerase (PCR) em tempo real, utilizando metodologia TaqMan, a qual utiliza o sistema de sondas de hidrólise. O padrão de metilação será avaliado através da metodologia de enzyme-linked immunosorbent assay (methDNA-ELISA).

Objetivo da Pesquisa:

Os objetivo primários são: investigar a associação de marcadores genéticos e epigenéticos envolvidos nas diferentes vias de controle e regulação do peso corporal e no metabolismo de micronutrientes, como o ferro, com fenótipos relacionados à obesidade, ingestão
alimentar, parâmetros bioquímicos e hematológicos em uma amostra de crianças de 6-7 anos do município de Porto Alegre, RS.

Como objetivos secundários estão os seguintes: 1 Determinar os genótipos de polimorfismos nos genes LEPR, APM1, PPARG, FTO, MC4R, SEC16B, TMEM18, NEGR1, KCTD15, SH2B1,
BDNF, OLFM4, HOXB5, DRD2, DRD4, MAOA, COMT, SLC3A4, SL6A14, SL6A4, 5-HTT, 5-HTR2A, 5-HTR2C, TAS1R2, TAS1R3 e HFE, FPN1, DMT1, HAMP, TFR, TF, TMPRSS6 e outros possíveis genes candidatos em uma amostra de crianças. 2 Analisar a associação dessas variantes
nos genes acima descritos com medidas antropométricas e dietéticas das crianças investigadas. 3 Analisar a associação dessas variantes nos genes acima descritos com parâmetros bioquímicos e hematológicos das crianças investigadas. 4. Analisar os padrões globais de metilação do genoma e em regiões regulatórias dos genes candidatos e sua associação com a amamentação e a parâmetros antropométricos nas crianças investigadas. 5. Avaliar a expressão gênica de microRNAs controladores dos genes candidatos e a sua associação com parâmetros antropométricos nas crianças investigadas.

Avaliação dos Riscos e Benefícios:

Os riscos em participar da pesquisa são mínimos e estão relacionados à coleta de sangue já autorizada anteriormente, no projeto já aprovado pelo CEP, o qual prevê a coleta de amostra biológica e de dados. A coleta será feita cuidadosamente por profissional treinado e poderá causar um pequeno desconforto durante a coleta e um pequeno hematoma que desaparecerá naturalmente. Os benefícios referidos são os da melhor compreensão dos mecanismos relacionados com obesidade e anemia infantil.

Comentários e Considerações sobre a Pesquisa:

Estudo bastante claro com metodologia adequada e de alta relevância.

Endereço: Rua Sarmento Leite ,245
Bairro: **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
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UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Considerações sobre os Termos de apresentação obrigatória:

Termo de consentimento livre e esclarecido adequado. Apresenta cartas de anuência do local de realização das análises.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Projeto em condições de ser aprovado pelo CEP.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Término do projeto 12/2017.

PORTO ALEGRE, 17 de Outubro de 2014

Assinado por:
Julia Fernanda Semmelmann Pereira Lima
(Coordenador)

7.4 Anexo D – Questionários Aplicados

FICHA DA CRIANÇA

Entrevistador_____

1. Data _____/_____/_____	Data3: ___/___/__

Identificação (criança):

2.Telefones para contato_____

3.Numero de identificação _____ 4.Nome da criança_____ 5.Nome da mãe_____ 6.Endereço: _____ _____ _____ _____ 7.Data de Nascimento: _____/_____/_____	Ident3: ___

Dados Maternos e Socioeconômicos:

8.Qual a sua idade? _____anos	IdMae3:_____
09.Data de nascimento da mãe ____/____/_____	DNm3_____
10.Qual o seu estado civil? Casada/ou mora junto (1) Viúva (2) Solteira (3) Separada (4)	EstCivil3 _____
11.Você teve outros filhos? (1) Sim (2) Não (pule para a questão 14)	Filhos3 _____ Quant3:_____ DNf1:___/___/___
12. Se sim: Quantos:_____ DN____/____/_____ DN____/____/____- DN____/____/____	DNf2___/___/___ DNf3___/___/___
13.Quantas pessoas moram na sua casa? _____	Famí3:_____
14.Qual o grau de parentesco?	Adul3:__ __

(1) Família nuclear (2) Família não nuclear	Parente3 ___

15.Qual a sua ocupação? (1) Desempregada (2) Empregada c/ carteira assinada (3) Empregada s/ carteira assinada (4) Do lar (5) Estudante	OcupaMae3:____
16.Qual a ocupação do pai do seu (sua) filho (a)? (1) Desempregado (2) Empregado c/ carteira assinada (3) Empregado s/ carteira assinada (4) Aposentado (5) Estudante	OcupaPai3:_____
17.Qual a renda total da família? R$ _____	RendaT3:_____
18.Qual o gasto familiar mensal com alimentação? R$_____	GFA:_____
19.Qual o gasto familiar mensal com transporte? R$_____	GFT: _____

20.Você é fumante? (1) Sim (2) Não (pule para a 22) (3) Parou de fumar (pule para a 22)	Vcfum3:_____

21.Quantos cigarros você fuma por dia? _____	Ncd3:_____

22.Alguém que mora na sua casa é fumante? Sim (1) Não (2) (Pule para a pergunta 24)	Ncd3:_____

Se sim:

23.Quem é fumante na sua casa? Pai (1) Outros moradores da casa (2) Anotar quantos (3)Pai e outros	QuemFuma: _____

24.Você fumou durante a gestação do seu filho que participou do projeto? (1) Sim (2) Não (pule para a 26)	Fgest3:_____

<u>Se sim:</u>

25.Quantos cigarros você fumava por dia?_____	Ncfum3_____

26. Alguém na família tem ou teve? (**referente a criança**) **Para a pergunta quem: coloque 1 quando sim e 2 quando não** 26.a Obesidade: (1) Sim (2) Não ou (3) Não Sabe (9) IGN ***Se sim:***Quem? ( ) Pai ( ) Mãe ( ) Avós ( ) Tios ( )Irmãos (88) NSA (99) IGN	Obesi: ____ Obpai: ___ Obmãe: ___ Obavós: ___ Obatio: ___ Obairm: ___
26.b Colesterol Alto: (1) Sim (2) Não ou (3) Não Sabe (9) IGN ***Se sim:***Quem?( ) Pai ( ) Mãe ( ) Avós ( ) Tios ( )Irmãos (88)NSA (99) IGN	ColAlto: ____ Colpai: ___ Colmãe: ___ Colavós: ___ Colatio: ___ Colairm: ___
26.c Doença cardiovascular: (1) Sim (2) Não (3) Não Sabe (9) IGN ***Se sim***:Quem?( ) Pai ( ) Mãe ( ) Avós ( ) Tios ( )Irmãos (88)NSA (99) IGN	DCV: ____ DCVpai: ___ DCVmãe: ___ DCVavós: ___ DCVtio: ___ DCVirm: ___
26.d Diabetes Melitus: (1) Sim (2) Não ou (3) Não Sabe) (9) IGN ***Se sim:***Quem?( ) Pai ( ) Mãe ( ) Avós ( ) Tios ( )Irmãos (88)NSA (99) IGN	DM: ____ DMpai:___ DMmãe:___ DMavós:___ DMtio:___ DMirm:___
26.e Hipertensão (Pressão Alta):(1) Sim (2) Não (3) Não Sabe (9) IGN ***Se sim:***Quem?( ) Pai ( ) Mãe ( ) Avós ( ) Tios ( )Irmãos (88)NSA (99) IGN	PA: _____ PApai: ___ PAmãe:____ PAavós:____ PAtio:____ PAirm:____

27. A criança realizou algum exame de sangue após o realizado quando o seu filho estava com 1 ano de idade, através do nosso projeto? Sim (1) Não (2)	Exam3_____

Se sim anotar:

Data:___/___/___ Data:___/___/___

Hb:_____g/dl Hb:_____g/dl

Ht:_____g/dl Ht:_____g/dl

VCM:_____fl VCM:_____fl

HCM:_____pg HCM:_____pg

28. Atualmente o seu filho esta recebendo algum suplemento de ferro? (1)Sim (2) Não Se sim:	Suple3_____
29.Qual o nome do suplemento?_____	Qual3_____
30.Qual a quantidade? _____gotas ou _____drágeas	Qgotas___ Qdrag____
31.O seu filho realmente recebe o suplemento? (1) Sim (2) nao	Receb3____
32.Que idade a criança tinha quando iniciou com o uso desse suplemento?_____meses	Idade:____ Tempu3____
33. Tempo de uso:_____semanas	

CONDIÇÕES DE SAÚDE NOS ÚLTIMOS 6 MESES

34. Seu (sua) filho (a) foi internado no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Intern3_____
35.Seu (sua) filho (a) teve episódios de diarréia no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Diarré3_____
36. Seu (sua) filho (a) apresentou febre importante nos últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Febre3_____
37. Seu (sua) filho (a) teve infecção no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Infecç3_____
38. Seu (sua) filho (a) teve infecção urinária nos últimos 6 meses? Sim (1) Não (2) Não sabe (3)	InfUri3_____
39.O seu (sua) filho (a) apresentou algum problema respiratório? Sim (1) Não (2) (pule para a 67)	Resp3_____

Leia as alternativas para o entrevistado

40. Qual ou quais problema (s) que seu (sua) filho (a) apresenta? Tosse ( ) Coriza ( ) Obstrução Nasal ( ) Respiração rápida ou difícil ( ) Para o quadro ao lado preencher 1 para sim e 2 para não	Tosse3____ Coriza3____ Obstru3_____ Respd3_____

Preencher se a criança recebe leite de vaca: (se não recebe pule para a 72)

41. Qual o volume da preparação?_____ml 42. Qual a freqüência que seu filho toma leite no dia? _____ vezes 43. Volume total de leite ingerido no dia: _____ml (descontar se sobra)	Vol3_____ freqleit_____ Volleite_____

44. A criança vai a creche? Sim (1) Não (2) 45. Período: meio turno (1) dia inteiro (2) 46 Desde que idade (em meses):_____ 47.Se **não**, no lugar onde ela fica, tem outras crianças junto? Sim (1) Não (2)	Creche3_____ Períod3_____ temp3_____ idcre3_____ ondfica3_____

48. O (a) seu (sua) filho (a) bebe água? Sim (1) Não (2) (Pule para a pergunta 51)	Água3 _____

Se sim:

49. Quanto bebe? _____ 50. Qual o "tipo" da água? Filtrada / Fervida / Torneira tratada e fervida / Mineral (próprias para o consumo) (1) Torneira não tratada (impróprias para o consumo) (2)	Quant3_____ Tagua3 _____

51. Seu (sua) filho (a) comeu/come terra ou objetos não alimentares? Sim (1) Não (2) (pule para a 80) Não sabe (3) (pule para a 53)	Objet3 _____

Se sim:

52. Quais os objetos não alimentares que seu (sua) filho (a) comeu? (1) Terra (2) Sabão/Sabonete (3) Terra + sabão (5) casca da mandioca (4) Outras substancias _____(qual?)	Quais3_____

Estado Nutricional:	
53. Peso _____ gramas	Peso3_____
54. Comprimento _____ cm	Compri3_____
Dobra cutânea subescapular: _____ mm	Subscap3 _____
Dobra cutânea triciptal: _____ mm	Tricip3

Hemoglobina	
Valor 1:	Hb13_____
Valor 2:	Hb23_____
Avaliação da saúde bucal	
Número de cáries:	Carie3_____
Número de superfícies atingidas:	SAi3_____

Atividades diárias(ontem) :	
55. Que horas foi dormir ontem ____Que horas acordou hoje _____/ horas de sono:_____	Hsonoit____
56. O que fez ontem pela manha:	
( ) Creche / Tempo_____	MCrecheT___
( ) Assistiu TV / Tempo:_____	MTvT_____
( ) Brincou fora de casa / Tempo:_____	MBrifT_____
( ) Brincou dentro de casa / Tempo:_____	MBridT____
( ) Dormiu / Tempo:____	MDormT_____
57. O que fez ontem de tarde:	
( ) Creche / Tempo_____	TCrecheT___
( ) Assistiu TV / Tempo:_____	TTvT_____
( ) Brincou fora de casa / Tempo:_____	TBrifT_____
( ) Brincou dentro de casa / Tempo:_____	TBridT____
( ) Dormiu / Tempo:____	TDormT_____
58. O que fez ontem de noite:	
( ) Assistiu TV / Tempo:_____	NTvT_____
( ) Brincou dentro de casa / Tempo:_____	NbrinT_____
( ) Dormiu / Tempo:____	NdormT_____
OBS:Colocar sempre o tempo de HORAS	
59. Tem alguma atividade física regular na semana:	
( ) Sim ( )Não	Ativ_____
Se sim qual:_____	Qual_____
Freqüência na semana:_____	FreqS____

60. Você considera seu filho: 1. muito calmo 2. calmo 3. ativo 4. muito ativo 5. agitado	ConFi_____

INQUÉRITO DE FREQÜÊNCIA DE ALIMENTOS

Com que freqüência o seu filho consome os seguintes alimentos?

Alimentos	Diária (vezes)	Quantidade (total)	Semanal (vezes)	Quantidade (total)	Esporádica	Sabor	Marca
Pão							
Bolo							
Biscoito recheado							
Biscoito sem recheio							
Mucilon/Neston/Farinha Láctea							
Petit Suisse							
Chocolate							
Sorvete							
Açúcar							
Chiclete							
Balas							
Doces							
Gelatina ( ) Diet ( ) Normal							
Salgadinho							
Carne de vaca							
Carne de frango							
Carne de peixe							
Fígado							
Refrigerante ( ) Diet ( ) Normal							
Suco artificial							

( ) Diet ( )Normal							
Suco natural							
Folhas verdes escuras							
Alimentos amarelos							
Frutas							

Inquérito recordatório de 24 horas

Horário	**Alimentos/e ou Preparação**	**Medidas caseiras**

LEMBRE-SE: ANOTE NO VERSO DESTA FOLHA A COMPOSIÇÃO DOS PRODUTOS INDUSTRIALIZADOS "MENOS CONHECIDOS"

Critério ABIPEME (Nova Proposta para o Critério ABA/ABIPEME)

			Nº de ítens possuídos						
Ítem			0	1	2	3	4	5	6 e +
Televisor			0	4	7	11	14	18	22
Rádio			0	2	3	5	6	8	9
Banheiro			0	2	5	7	10	12	15
Automóvel			0	4	9	13	18	22	26
Empregada Mensalista			0	5	11	16	21	26	32
Posse de:								Pontos	
Aspirador de Pó								6	
Máquina de Lavar Roupa								28	
VCR - vídeo cassete								10	
Geladeira								7	
Grau de instrução do chefe da família								Pontos	

Analfabeto ou primário incompleto								0	
Primário completo								5	
Ginásio Completo								10	
Colégial Completo								15	
Superior Completo								21	
Classe A (rica)	89 ou +								
Classe B (média alta)	59 a 88								
Classe C (média média)	35 a 58								
Classe D (média pobre)	20 a 34								
Classe E (pobre)	0 a 19								