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Avaliação da Influência de Polimorfismos nos Genes *DRD4* e *SLC6A3* sobre Ingestão Alimentar e Parâmetros Antropométricos de Crianças

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RESUMO

A obesidade na infância é um crescente problema de saúde mundial, cujas causas podem ser atribuídas à predisposição genética, aliada à inatividade física e à alimentação inadequada. A dopamina está envolvida na regulação da ingestão alimentar sob comando do sistema nervoso central. O gene *DRD4* codifica o receptor de dopamina D4 e o gene *SLC6A3* codifica o transportador de dopamina (DAT), polimorfismos nesses genes podem influenciar em diferenças na recompensa alimentar. Os objetivos desse trabalho foram investigar a associação do polimorfismo exon 3 VNTR do gene *DRD4* e dos polimorfismos 3'UTR VNTR, rs2550948, rs2652511 e rs1048953 do gene *SLC6A3* com ingestão alimentar e parâmetros de adiposidade em crianças em três fases do desenvolvimento: no primeiro ano de vida, aos 3 a 4 anos e aos 7 a 8 anos. A análise genotípica dos polimorfismos VNTR foi realizada através de PCR seguida de eletroforese em gel de agarose. Os SNPs foram analisados em equipamento de automação laboratorial pela metodologia Taq Man©. As variáveis foram comparadas entre os grupos por General Linear Model, por ANOVA, pelo teste U de Mann-Whitney ou por Kruskal-Wallis. As frequências genotípicas encontradas estão de acordo com estudos prévios e em equilíbrio de Hardy-Weinberg. Na comparação entre os diferentes genótipos foram observadas associações entre os alelos de maior atividade dopaminérgica dos polimorfismos *DRD4* exon 3 VNTR e *SLC6A3* 3'UTR VNTR com maior ingestão de alimentos palatáveis e maiores medidas de circunferência da cintura das crianças aos 3 a 4 anos. O polimorfismo rs1048953 esteve associado à ingestão energética diária no mesmo período e à razão cintura-estatura das crianças aos 7 a 8 anos. Nossos resultados sugerem que portadores dos alelos de maior atividade dopaminérgica dos polimorfismos *DRD4* exon 3 VNTR e *SLC6A3* 3'UTR VNTR e portadores do genótipo T/T da variante rs1048953 podem apresentar risco aumentado para ingestão alimentar excessiva, visto que maior atividade dopaminérgica pode aumentar o valor motivador percebido pela recompensa alimentar e, possivelmente, levar a obesidade. Mais estudos são necessários para melhor suportar nossas observações.

PALAVRAS-CHAVE: dopamina. *DRD4*. *SLC6A3*. polimorfismos. ingestão alimentar. parâmetros de adiposidade.

ABSTRACT

Childhood obesity is a crescent world health problem, whose causes can be attributed to the genetic predisposition, allied to the physical inactivity and the inadequate feeding. Dopamine is involved in the food intake regulation under the central nervous system control. The *DRD4* gene encodes for D4 dopamine receptor and the *SLC6A3* gene encodes the dopamine transporter (DAT), polymorphisms in those genes can influence differences on food reward. The aim of this study was to investigate the association of the polymorphisms in the genes *DRD4* (exon 3 VNTR) and *SLC6A3* (3'UTR VNTR, rs2550948, rs2652511 and rs1048953) with food intake and nutritional status in children in three development phases: in the first year of life, at 3 to 4 years and at 7 to 8 years old. The genotype analysis of VNTR polymorphisms was accomplished through PCR following by eletroforese on agarose gel. SNPs were genotyped using automation laboratorial equipment by the Taq Man© methodology. Variables were compared among the groups by Lineal General Model, by ANOVA, by U test of Mann-Whitney or by Kruskal-Wallis. The genotypes frequencies observed were in agreement with previous studies and with those expected under Hardy–Weinberg equilibrium. When different genotypes were compared it was observed associations among the alleles of higher dopamine activity of the polymorphisms *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR with higher intake of palatable foods and higher waist circumference of children at 3 to 4 years. The rs1048953 polymorphism was associated with average energy intake daily in the same period and with ratio waist-to-height of children at 7 to 8 years. Our results suggest that the carriers of the high dopamine activity alleles of the polymorphisms *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR and carriers of T/T genotype of the variant rs1048953 can present increased risk for overeating, since the high dopamine activity can increase the perceived incentive value of food reward and possibly lead to obesity. More studies are necessary to best support this hypothesis.

KEY WORDS: dopamine. *DRD4*. *SLC6A3*. polymorphisms. food intake. nutritional status.

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1 INTRODUÇÃO

A obesidade está se tornando um sério problema de saúde mundial. A prevalência da obesidade continua a aumentar dramaticamente, visto que o número de crianças com sobrepeso mais do que triplicou desde a década de 1960 (Ogden, Flegal *et al.*, 2002). Em 2010, o mundo apresentava aproximadamente 42 milhões de crianças menores de cinco anos com sobrepeso, sendo que aproximadamente 35 milhões destas crianças residiam em países em desenvolvimento (Who, 2012). No mesmo ano, a proporção de adultos obesos nos Estados Unidos ultrapassou 30% da população (Flegal, Carroll *et al.*, 2010).

Obesidade pode ser definida por um acúmulo anormal ou excessivo de gordura que representa risco para a saúde. Essa deposição de adiposidade pode resultar da ingestão alimentar exagerada ou da diminuição do índice metabólico (O' Rahilly, 2009). Os indivíduos com excesso de peso frequentemente sofrem de desordens psicológicas, como mudanças de humor e depressão, e com estigmatização social (Luppino, Dewit *et al.*, 2010); além de significativas morbidade e mortalidade prematuras associadas a graves condições de saúde que a obesidade predispõe, incluindo diabetes tipo 2, hipertensão arterial, doenças da artéria coronária e muitas formas de câncer (Must, 1999; Calle, Rodriguez *et al.*, 2003; Bean, Stewart *et al.*, 2008).

O aumento da disponibilidade de alimentos palatáveis e de alta densidade calórica, aliado à reduzida necessidade de esforço físico durante o trabalho e em atividades domésticas contribuíram para um estado positivo no balanço de energia, o qual a longo prazo é suficiente para levar a uma mudança na média de peso da população (O' Rahilly e Farooqi, 2006).

A obesidade é causada por uma complexa interação de múltiplos fatores, primários e secundários. O acúmulo de adiposidade pode ser atribuído à pré-disposição genética, aliada à inatividade física e a uma alimentação inadequada (Horvath, 2005).

1.1 A ALIMENTAÇÃO INADEQUADA COMO UM DOS FATORES DA OBESIDADE

O ritmo agitado da vida moderna contribui para uma alimentação de má qualidade. É frequente a desatenção na alimentação no que diz respeito à escolha de alimentos que contemplem as necessidades energéticas e nutritivas do organismo de forma apropriada, que não alcancem o excesso e nem se reduzam à carência. Atualmente o mundo dispõe de uma ampla variedade e acessibilidade de alimentos e a publicidade maciça dos produtos industrializados aumenta a probabilidade de as pessoas ingerirem esses alimentos, mesmo sem necessidade, logo após uma refeição ou até mesmo quando a pessoa pretendia privar-se de alimentos ou comer moderadamente.

Influenciando as escolhas individuais (quando, o quê e o quanto consumir), essas sedutoras ofertas de alimentos contribuem, porção por porção, com o excesso de ingestão energética capaz de levar à obesidade (Berthoud e Morrison, 2008; Davis e Carter, 2009). A oferta de um alimento atraente e saboroso pode despertar um desejo súbito de ingeri-lo e a ingestão de pequenas porções pode desencadear o poder motivacional do alimento e com ele o desejo de comer mais (Berridge, Ho *et al.*, 2010).

As crianças são especialmente vulneráveis a essa atraente oferta de alimentos. Estudos já observaram que crianças obesas apresentam ingestão alimentar aumentada após exposição à oferta de alimentos (Jansen, Theunissen *et al.*, 2003), consumo elevado de lanches na ausência de fome (Birch e Fisher, 2000) e dificuldade em desenvolver um padrão normal de desaceleração da ingestão de alimentos durante as refeições (Barkeling, Ekman *et al.*, 1992; Lindgren, Barkeling *et al.*, 2000). Essa alimentação excessiva frequentemente ocorre na ausência de fome fisiológica verdadeira e as propriedades sensoriais dos alimentos palatáveis podem promover esse desejo de comer independentemente das necessidades de energia (Lowe e Butryn, 2007).

Alimentos palatáveis são definidos como alimentos saborosos e agradáveis ao paladar, sensações que agregam modalidades sensoriais como o sabor, o cheiro e a textura; esses alimentos geralmente apresentam alto teor de energia, visto que sua composição inclui quantidades significativas de açúcares e gorduras (De Araujo, Rolls *et al.*, 2003; Rolls, Verhagen *et al.*, 2003; Berthoud, Lenard *et al.*, 2011). Embora a natureza da palatabilidade ainda seja alvo de debate (Yeomans, 1998),

tem sido amplamente aceito que as propriedades sensoriais dos alimentos influenciam em sua percepção. (Hyde e Witherly, 1993). De fácil acesso, esses alimentos têm sido considerados o maior fator de risco ambiental para o desenvolvimento da obesidade (Volkow e Wise, 2005) e a elevada ingestão dos alimentos palatáveis é considerada o motivo que mais contribui para o atual crescimento dessa epidemia (Swinburn, Sacks *et al.*, 2009).

A maioria das teorias que tentam explicar a regulação da alimentação sugere que dois sistemas homeostáticos paralelos interagem para influenciar ingestão alimentar. O sistema metabólico compreende hormônios que regulam a fome, a saciedade e a deposição de adiposidade, tais como a leptina, a grelina e a insulina, os quais atuam em circuitos cerebrais para estimular ou inibir a ingestão alimentar com o objetivo de preservar o balanço energético apropriado (Kenny, 2011). Em adição, sistemas cerebrais de recompensa também desempenham um importante papel no comportamento alimentar, visto que a busca pelos prazerosos efeitos dos alimentos palatáveis é uma força motivadora poderosa que em certos indivíduos pode anular os sinais homeostáticos (Shomaker, Tanofsky-Kraff *et al.*, 2010).

Experimentos realizados há 60 anos já sugeriam que um desequilíbrio na homeostase de energia sob controle do sistema nervoso central estaria associado ao desenvolvimento da obesidade (Kennedy, 1953), já que diversos neurotransmissores cerebrais tais como a dopamina (DA), o GABA, a noradrenalina e a serotonina estão envolvidos na regulação da ingestão alimentar (Schwartz, Woods *et al.*, 2000).

1.2 O SISTEMA DOPAMINÉRGICO NO DESENVOLVIMENTO DA OBESIDADE

Décadas de pesquisa realizada por Hoebel e colaboradores forneceram informações essenciais sobre o papel do sistema dopaminérgico na regulação da ingestão alimentar, possibilitando o desenvolvimento do conceito de "*food reward*", a recompensa alimentar (Hernandez e Hoebel, 1988; Rada, Avena *et al.*, 2005; Avena, Rada *et al.*, 2006). Notavelmente, as pioneiras experiências de Hoebel e colaboradores estabeleceram o sistema dopaminérgico como um fator chave no processo que leva uma alimentação excessiva crônica ao desenvolvimento da obesidade (Hoebel, Hernandez *et al.*, 1981; Hernandez e Hoebel, 1982; Ahlskog, Randall *et al.*, 1984).

Diversas evidências mostram que o sistema dopaminérgico, envolvendo estruturas cerebrais como o núcleo *accumbens*, a amígdala e o córtex orbitofrontal, atua na regulação da recompensa e do prazer que experimentamos a partir de nossas ações (Wise, 2002; Kelley, Schiltz *et al.*, 2005). A atividade aumentada da DA está associada com melhor estado motivacional e resposta mais forte do reforço de recompensa natural (como ocorre na ingestão de alimentos palatáveis) ou farmacológico (como ocorre no consumo de drogas de abuso) (Kelley, 2004; Cota, Tschop *et al.*, 2006). No entanto, a sensibilidade ou a reatividade dessa rede neural é afetada por diversos fatores biológicos, tais como a disponibilidade de DA liberada na sinapse, seu transporte, sua ligação a receptores de DA na fenda sináptica, a quantidade de receptores de DA e a rapidez de sua re-captação pela célula pré-sináptica (Elsworth e Roth, 1997).

Reforçadores positivos, tais como alimentos palatáveis e drogas de abuso estimulam a liberação de DA no cérebro (Salamone, 1994; Volkow, Wang *et al.*, 2002). Estudos prévios já observaram que a ingestão alimentar aumenta as concentrações de DA no cérebro de animais (Hernandez e Hoebel, 1988; 1990) e de humanos (Small, Jones-Gotman *et al.*, 2003). Comer é uma atividade de alto reforço da recompensa (Wise, 2002) e existem diferenças individuais na eficácia do reforço de alimentos que podem estar relacionadas com diferenças na alimentação e na ingestão de energia (Epstein, Leddy *et al.*, 2007). Da mesma forma que a eficácia do reforço de uma droga de abuso está relacionada ao seu consumo (Bickel, Marsch *et al.*, 2000), os indivíduos que dispõem de alimentos de alto poder de reforço podem consumir mais energia do que realmente necessitam (Epstein, Wright *et al.*, 2004).

Se as diferenças individuais do reforço alimentar estão relacionadas a diferenças na ingestão de energia, a obesidade, que é caracterizada pela ingestão de excesso de energia, pode estar relacionada ao reforço alimentar; visto que ela pode ser considerada um tipo de desordem aditiva que envolve alterações na funcionalidade cerebral, caracterizada pela ingestão alimentar compulsiva e pela dificuldade em limitar a ingestão (Volkow e O' Brien, 2007). Dentro desse contexto, os indivíduos obesos podem estar encontrando na alimentação mais reforço de recompensa e, por isso, estariam mais motivados para comer do que indivíduos com peso normal (Saelens e Epstein, 1996).

Alguns trabalhos têm associado diferenças individuais na sensibilidade à recompensa ao comportamento compulsivo de comer em excesso (Davis e

Woodside, 2002; Davis, Patte *et al.*, 2007) e no desenvolvimento da obesidade (Wang, Volkow *et al.*, 2002; Epstein, Wright *et al.*, 2004; Kelley, Schiltz *et al.*, 2005; Pritchett e Hajnal, 2011).

Estudos baseados em imagens encontraram associação entre menor expressão de receptores dopaminérgicos e a diminuição do metabolismo do córtex pré-frontal em indivíduos obesos, nos quais também foi observada uma relação inversa entre o índice de massa corporal (IMC) e a disponibilidade desses receptores (Wang, Volkow *et al.*, 2001; Volkow, Wang *et al.*, 2008). Menor atividade metabólica pré-frontal também foi observada em adultos saudáveis com maior IMC (Volkow, Wang *et al.*, 2008; Willeumier, Taylor *et al.*, 2011). Dois estudos em fumantes relataram uma associação entre polimorfismos do gene transportador de DA (*SLC6A3* ou *DAT1*) com fenótipos de obesidade (Epstein, Jaroni *et al.*, 2002) e reforço alimentar (Epstein, Wright *et al.*, 2004).

Um estudo prévio realizado por nosso grupo na mesma amostra de crianças que compõe o presente trabalho avaliou a influência de polimorfismos em genes que codificam enzimas envolvidas no metabolismo da DA, a monoamina oxidade A (MAOA) e a catecol-o-metiltransferase (COMT) e observou associações significativas entre alelos de polimorfismos que proporcionam a metabolização mais rápida da DA com a ingestão mais elevada de alimentos de alto teor lipídico (Galvão, Krüger *et al.*, 2011).

Aitlhadj *et al.* (2011) resumiram bem a bioquímica do metabolismo dopaminérgico em uma recente revisão. A DA é uma monoamina derivada do aminoácido tirosina. Esse neurotransmissor é produzido pela conversão da tirosina em *1-dihydroxypheny-l-alanine* (l-DOPA), em uma reação mediada pela enzima tirosina hidroxilase, em seguida a l-DOPA é transformada em DA pela reação mediada pela aminoácido descarboxilase. A DA formada no citosol dos neurônios dopaminérgicos é rapidamente armazenada em vesículas sinápticas, onde fica estocada e de onde é liberada quando ocorre a despolarização neuronal. Após a exocitose do neurotransmissor para a fenda sináptica, a DA se liga a receptores específicos pré e pós-sinápticos (D1 a D5). A sinalização dopaminérgica é limitada pela recaptação pré-sináptica do neurotransmissor mediada pelo transportador de DA (DAT) e pela inativação metabólica mediada pela ação das enzimas monoamina oxidade (MAO) e COMT. Em uma via paralela, a ação da DA também pode ser

limitada através de sua conversão em noradrenalina, em uma reação mediada pela enzima DOPA β-hydroxylase (Aitlhadj, Ávila *et al.*, 2011).

É possível que variações em enzimas, transportadores, receptores ou em outros pontos dessa via metabólica sejam capazes de causar diferenças individuais no metabolismo dopaminérgico que resultem em diferentes comportamentos do sistema dopaminérgico.

Estudos convergem em evidências que sugerem que a atividade dopaminérgica está envolvida na regulação do comportamento alimentar em humanos (Volkow, Wang *et al.*, 2002; Small, Jones-Gotman *et al.*, 2003; Volkow, Wang *et al.*, 2003; Galvão, Krüger *et al.*, 2011; Pritchett e Hajnal, 2011) e está associada com a determinação do IMC (Wang, Volkow *et al.*, 2001; Volkow, Wang *et al.*, 2008; Willeumier, Taylor *et al.*, 2011). Assim, há evidências crescentes na direção de que o sistema dopaminérgico pode estar envolvido com fenótipos relacionados à obesidade.

Uma forma indireta de estudar diferenças individuais na capacidade constitutiva do sistema de recompensa em relação à disponibilidade de DA no cérebro é analisar polimorfismos em genes que codificam proteínas relacionadas à disponibilidade desse neurotransmissor e seus efeitos sobre o comportamento alimentar. Desta forma, genes envolvidos no metabolismo, transporte e em receptores celulares de DA são fortes candidatos a esses estudos.

1.3 GENE DO RECEPTOR DE DOPAMINA *DRD4*

O receptor de dopamina D4 pertence à classe 2 dos receptores dopaminérgicos acoplados à proteína G, caracterizados por inibir a enzima adenilato ciclase na produção de AMP cíclico e, por consequência, diminuir a sinalização intracelular (Rondou, Haegeman *et al.*, 2010). O receptor D4 é aplamente expresso no cortex pré-frontal e em outras regiões do cérebro envolvidas com circuitos de recompensa que medeiam o reforço alimentar, tais como o hipocampo, a amígdala e o hipotálamo (Meador-Woodruff, Grandy *et al.*, 1994; Meador-Woodruff, Damask *et al.*, 1996).

O gene do receptor de dopamina D4 (*DRD4*) tem sido foco de estudos devido ao seu potencial impacto nos mecanismos de recompensa, atenção e comportamento. Esse gene está localizado no cromossomo 11p15.5 (Van Tol,

Bunzow *et al.*, 1991), possui 3 introns (Gingrich e Caron, 1993) e apresenta um polimorfismo de número variável de repetições em *tandem* (VNTR - *Variable Number Tandem Repeat*) no exon 3, o qual vem sendo muito estudado em genética do comportamento.

O exon 3 codifica a terceira alça citoplasmática intracelular do receptor D4 (Van Tol, Bunzow *et al.*, 1991). O polimorfismo *DRD4* exon 3 VNTR, presente nesse exon, apresenta sequências repetitivas de 48 pb que variam de 2 (2R) a 11 (11R) repetições, como resultado, a proteína nessa posição pode apresentar de 32 a 176 aminoácidos (Ding, Chi *et al.*, 2002). Os alelos mais frequentes na maioria das populações apresentam sequências de 2R, 4R, e 7R (Van Tol, Wu *et al.*, 1992), com exceção das populações asiáticas em que o alelo de 7R apresenta uma frequência extremamente baixa e o alelo de 2R é o mais prevalente (Chang, Kidd *et al.*, 1996).

Alelos do polimorfismo *DRD4* exon 3 VNTR com número maior de repetições (alelos longos), em particular o alelo de 7R, foram associados com a diminuição da resposta à DA (Asghari, Sanyal *et al.*, 1995), traços de personalidade com comportamento de busca por novas sensações (*personality trait of novelty seeking)* (Benjamin, Li *et al.*, 1996; Ebstein, 2006), impulsividade (Congdon, Lesch *et al.*, 2008), raiva (Kang, Namkoong *et al.*, 2008), comportamento agressivo (Fresan, Camarena *et al.*, 2007) e distúrbio de déficit de atenção e hiperatividade (Brookes, Xu *et al.*, 2006; Li, Sham *et al.*, 2006).

A transmissão de sinal via receptores D4 é naturalmente inibitória (Hurd e Hall, 2005), em virtude da inibição da enzima adenilato ciclase (Rondou, Haegeman *et al.*, 2010), entretanto esse efeito de diminuição da sinalização intracelular é abrandado pela presença do alelo de 7R. O potencial da DA em inibir a formação de AMP cíclico em presença do alelo de 7R é reduzido quando comparado aos alelos de 2R e 4R (Asghari, Sanyal *et al.*, 1995; Ding, Chi *et al.*, 2002). A hipótese apresentada é que o número de repetições poderia afetar a transmissão de sinal no neurônio pós-sináptico, visto que indivíduos com pelo menos um alelo de 7R ou mais apresentariam diminuição da afinidade de ligação da DA ao receptor e da disponibilidade do mesmo, o que contribuiria para prejudicar a neurotransmissão (Schoots e Van Tol, 2003). Essa atenuada resposta à DA produzida pelo alelo de 7R possivelmente fornece suporte para a hipótese de associação do polimorfismo *DRD4* exon 3 VNTR com os fenótipos de adição acima mencionados (Mc Geary, 2009).

Ao nosso conhecimento, são poucos os estudos que investigaram possíveis associações entre o polimorfismo *DRD4* exon 3 VNTR e parâmetros antropométricos e nenhum investigou a associação do polimorfismo com comportamento alimentar. Pelo menos dois estudos mostraram que portadores do alelo 7R apresentaram em média IMC mais elevado do que os não portadores (Levitan, Masellis *et al.*, 2004; Guo, North *et al.*, 2006). Um destes estudos sugeriu que a associação foi influenciada pela etinia, já que foi observada entre afro-americanos e hispânicos, mas não em americanos caucasianos (Guo, North *et al.*, 2006).

1.4 GENE DO TRANSPORTADOR DE DOPAMINA *SLC6A3*

O DAT é uma proteína transmembrana que corresponde ao membro 3 da família 6 de carregadores de soluto (SLC6A3) (He, Vasiliou *et al.*, 2009). O DAT é expresso em neurônios dopaminérgicos do mesencéfalo (em projeções para o núcleo estriado e para o córtex pré-frontal), apresentando maior densidade nos ganglios basais (Hurd e Hall, 2005). Esse transportador regula a atividade de DA na fenda sináptica através da rápida recaptação do neurotransmissor nos terminais nervosos pré-sinápticos (Gainetdinov, Jones *et al.*, 1999; Bannon, 2005), limitando assim a magnitude e duração da ativação na transmissão dopaminérgica (Bannon, Poosch *et al.*, 1992; Bannon, Granneman *et al.*, 1995; Bannon e Whitty, 1995). Dessa forma, o DAT tem papel crítico na neurotransmissão mediada pela DA.

O gene do transportador de dopamina (*SLC6A3*) está localizado no cromossomo 5p15.3 e é constituido de 15 exons separados por 14 introns em uma extensão de mais de 60 kb. A porção codificadora inicia no exon 2 e termina próximo ao exon 15 e é bastante conservada, polimorfismos nessa região correspondem a alterações silenciosas ou raras substituições conservativas de aminoácidos (Grünhage, Schulze *et al.*, 2000), o que sugere que as diferenças individuais na expressão do gene *SLC6A3* podem originar-se de sequências regulatórias (Bannon, Michelhaugh *et al.*, 2001).

O *SLC6A3* apresenta um polimorfismo VNTR na região não traduzida 3' (3'UTR – *3' untranslated region*), o *SLC6A3* 3'UTR VNTR, que apresenta sequências repetitivas de 40 pb. Os alelos de 9R e 10R apresentam maior prevalência, mas a ocorrência de alelos de 2R a 13R também já foi descrita (Vandenbergh, Persico *et al.*, 1992; Mitchell, Howlett *et al.*, 2000; Cornish, Manly *et al.*, 2005; Lohoff, Bloch *et*

al., 2010). Apesar dessa variante não alterar a sequência de aminoácidos da proteína, por situar-se em uma região regulatória, ela tem mostrado efeitos sobre a expressão do gene (Mill, Asherson *et al.*, 2002). Em relação aos efeitos do polimorfismo *SLC6A3* 3'UTR VNTR sobre a função dopaminérgica, as observações dos estudos funcionais e de associação ainda são consideradas inconsistentes, devido a divergências entre os resultados.

Alguns estudos sugerem que o alelo de 10R está associado com reduzida expressão do *SLC6A3* quando comparado ao alelo de 9R, os indivíduos portadores desse alelo apresentariam um relativo acúmulo de DA no neurônio pré-sináptico, visto que a maior densidade de DAT representaria melhor desempenho na recaptação desse neurotransmissor (Jacobsen, Staley *et al.*, 2000; Michelhaugh, Fiskerstrand *et al.*, 2001; Van Dyck, Malison *et al.*, 2005). Com resultados que associam o alelo de 9R à maior disponibilidade de DAT no núcleo estriado um estudo analisou um modelo de dominância entre os alelos de 9R e 10R e encontrou associações significativas que sugerem que o alelo de 9R apresenta um efeito de dominância sobre o alelo de 10R (Giessen, Win *et al.*, 2009).

Outros estudos, por outro lado, têm relatado efeitos na direção oposta indicando que maiores concentrações de DAT estão disponíveis em indivíduos portadores do alelo de 10R (Heinz, Goldman *et al.*, 2000; Fuke, Suo *et al.*, 2001; Mill, Asherson *et al.*, 2002; Van Ness, Owens *et al.*, 2005). Heinz, Goldman *et al.* (2000) observaram que indivíduos portadores de pelo menos um alelo de 9R teriam uma redução de aproximadamente 20% da disponibilidade de DAT no núcleo estriado, em comparação com indivíduos homozigotos para o alelo de 10R. Os resultados de Mill, Asherson *et al.* (2002) indicam que o nível de expressão do *SLC6A3* no cerebelo, no lobo temporal e em linfócitos associa-se ao número de alelos de 10R, um modelo no qual indivíduos homozigotos 10R/10R apresentariam um nível de expressão do *SLC6A3* mais elevado que indivíduos homozigotos 9R/9R. Estudos adicionais não encontraram efeitos do polimorfismo sobre a densidade (Martinez, Gelernter *et al.*, 2001), a disponibilidade ou a função de DAT (Lynch, Mozley *et al.*, 2003).

O polimorfismo *SLC6A3* 3'UTR VNTR tem sido a variante do gene *SLC6A3* mais estudada. No entanto, o advento dos estudos de varredura do DNA tem permitido que novas variantes, principalmente as alterações de nucleotídeo único (SNPs - *Single Nucleotide Polimorphism*), possam ser eleitas como candidatas a

estudos que investigam a associação de variantes nesse gene com alterações no sistema dopaminérgico.

Os três SNPs incluídos nesse estudo pertencem a possíveis regiões regulatórias do gene *SLC6A3*, dois deles estão localizados na extremidade 5', rs2550948 e rs2652511, e um está localizado no intron 3, rs1048953 (Genro, Polanczyk *et al.*, 2008). Ao nosso conhecimento, ainda são poucos os estudos que avaliaram a associação desses SNPs com variações no sistema dopaminérgico. Um estudo que investigou variações gênicas na região promotora do *SLC6A3* em uma amostra de 119 caucasóides descendentes de germânicos sugeriu que variações nessa região estão relacionadas a sítios de ligação para fatores de transcrição e, portanto, poderiam ser funcionais (Rubie, Schmidt *et al.*, 2001).

Drgon, Lin *et al.* (2006), em uma extensa análise da extremidade 5' do gene *SLC6A3* em uma amostra de 15 americanos com ascendência europeia, observaram que a disponibilidade de DAT no núcleo estriado ventral era significativamente maior em portadores da combinação haplotípica C–G (rs2652511–rs2937639) quando comparada ao grupo de indivíduos portadores da combinação T–A (Drgon, Lin *et al.*, 2006). Resultados que não foram confirmados por Giessen, Lin *et al.* (2009) em um estudo com 81 caucasianos. Em outro estudo, o alelo C da variante rs2652511 foi associado a um maior risco para esquizofrenia em pacientes asiáticos diagnosticados com a doença (Huang, Chen *et al.*, 2010). Brookes *et al.* (2006), em uma amostra de europeus, e Genro *et al.* (2008), em uma amostra brasileira de descendentes de europeus, encontraram associação entre portadores do alelo C do polimorfismo rs2652511, com forte desequilíbrio de ligação (LD) com a variante rs2550948, e desordens de déficit de atenção e hiperatividade. Xu *et al.* (2009) não observaram essa associação em um estudo que incluiu uma amostra européia e outra asiática (Xu, Mill *et al.*, 2009).

Estudos com amostras caucasóides mostraram que o padrão de LD no gene *SLC6A3* é segmentado e que a região promotora não está no mesmo bloco haplotípico do intron 3 (Brookes, Xu *et al.*, 2006; Friedel, Saar *et al.*, 2007; Genro, Polanczyk *et al.*, 2008). Portanto, o polimorfismo rs1048953 pertenceria a um bloco haplotípico diferente, o que permite avaliar se o padrão de LD nesta amostra é similar aos já descritos.

2 JUSTIFICATIVA

O ambiente obesogênico moderno tem causado um significativo aumento médio de peso na população durante a última década, no entanto ainda se observa uma variabilidade de peso dentro das populações (James, Leach *et al.*, 2001; Hedleya, Ogden *et al.*, 2004). Essa discrepância sugere haver um componente genético atuando significantemente sobre esse parâmetro (Maes, Neale *et al.*, 1997; Wardle, Carnell *et al.*, 2008).

Embora a obesidade seja tratada como uma doença única, os mecanismos fisiológicos que causam ganho de peso nas pessoas podem ser diferentes. Além disso, visto que as respostas orgânicas ao ato de comer são diferentes entre os indivíduos, é possível supor que a abordagem na prevenção e no tratamento desses indivíduos deve ser diferente e apropriada a cada caso, embora atualmente o manejo não seja conduzido de forma distinta.

Este trabalho é inovador, pois apresenta um perfil amostral pouco explorado, nossa amostra é composta de crianças de faixa etária bastante estreita e que estão sendo acompanhadas desde o nascimento, adicionalmente, ao nosso conhecimento, poucos estudos investigaram a associação de variantes em genes do sistema dopaminérgico com parâmetros de adiposidade em crianças e nenhum com ingestão alimentar.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar a associação de variantes nos genes *DRD4* e *SLC6A3* com ingestão alimentar e parâmetros antropométricos em crianças.

3.2 OBJETIVOS ESPECÍFICOS

- Determinar as frequências dos polimorfismos 3'UTR VNTR, rs2550948, rs2652511 e rs1048953 do gene *SLC6A3* e do polimorfismo exon 3 VNTR do gene *DRD4* em uma amostra de crianças de São Leopoldo.
- Investigar a possível associação desses variantes nos genes *SLC6A3* e *DRD4* com a ingestão alimentar no primeiro ano de vida dessas crianças, aos 3 a 4 anos e aos 7 a 8 anos.
- Investigar a possível associação desses variantes nos genes *SLC6A3* e *DRD4* com parâmetros antropométricos no primeiro ano de vida dessas crianças, aos 3 a 4 anos e aos 7 a 8 anos.

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5 ARTIGO

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***DRD4* and *SLC6A3* gene polymorphisms are associate with food intake and nutritional status in children in early stage of development**[1-3]

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ABSTRACT

Background: Variants of dopamine system genes, as *DRD4* and *SLC6A3* genes, may be involved in the food intake regulation, because dopaminergic system has influence on food reward.

Objective: We investigate the association of the polymorphisms in the genes *DRD4* (exon 3 VNTR) and *SLC6A3* (3'UTR VNTR, rs2550948, rs2652511 and rs1048953) with food intake and nutritional status in children.

Design: This was a prospective cohort study in which 354 children were recruited to the birth. Dietary data and nutritional status were collected at the first year of life, at 3 to 4 years, and at 7 to 8 years old. Children were pooled in groups according with the genotypes: carriers (*DRD4*.7R+) or non-carriers (*DRD4*.7R-) of the 7-repeat allele of *DRD4* exon 3 VNTR, homozygous for the 10-repeat allele (*SLC6A3*.10R/10R) or homozygous for the 9-repeat or heterozygous 9-repeat/10-repeat (*SLC6A3*.9R*) of *SLC6A3* 3'UTR VNTR.

Results: At the first year of life *DRD4*.7R- children showed higher BMI Z-score (P=0.019). At 3 to 4 years *DRD4*.7R- and *SLC6A3*.10R/10R children (higher dopamine activity) showed higher intake of palatable foods (P=0.024) and higher waist circumference (P=0.017). The rs1048953 polymorphism was associated with higher average energy intake daily (P=0.003) in the same period and with ratio waist-to-height of children at 7 to 8 years (P=0.041).

Conclusions: Carriers of the high dopamine activity alleles of the VNTR studied polymorphisms and carriers of T/T genotype of the variant rs1048953 can present increased risk for overeating obesity, since the high dopamine activity can increase the perceived incentive value of food reward and possibly lead to obesity.

INTRODUCTION

Overeating often occurs in the absence of physiological hunger and the sensory properties of palatable food can promote the desire to eat independent of metabolism's energy needs (1). Eating is a highly reward action (2) and the reinforcing value of food is related to activity of the dopamine system once food consumption increases brain dopamine levels in animals (3-4) and humans (5). Individual differences in the reinforcing value of food may help to explain the excess energy intake responsible for obesity (6). Those individual differences on the degree of sensitivity to food reward include both the sensory pleasure associated with eating and the degree to which food elicits the motivation to eat (7). Studies have linked food reward sensitivity to stronger food cravings (8), preferences for sweet and fatty foods (9), and higher body weight in adults and children (6, 9-10).

Dopamine activity is related to both the density of dopamine receptors and the amount of the dopamine transporter, among other factors. Thus, one way to indirectly to study individual differences in brain dopamine activity is the investigation of polymorphisms in dopamine receptors and transporter genes. The *DRD4* gene is located at cromossome 11p15.5 region, there was described in this gene a functional polymorphism of variable number of tandem repeat (VNTR) in exon 3 with unit repetition sequence of 48 base pairs, the *DRD4* exon 3 VNTR (11). Long alleles of *DRD4* exon 3 VNTR, in matter the 7-repeat allele have been associated with decrease of the answer to the dopamine (12). The dopamine transporter (DAT) is responsible for regulating the dopamine release into the extracellular space by recapturing dopamine from presynaptic terminals (13). This protein is coded by *SLC6A3* gene, which is located at cromossome 5p15.3 region. In 3' untranslated region of *SLC6A3*, there is a VNTR polymorphism, the *SLC6A3* 3'UTR VNTR, which

display unit repetition sequence of 40 base pairs (14). Studies have been discordant regarding the polymorphism effect on *SLC6A3* expression. Some studies suggest that the 9-repeat allele is associated with a reduced concentration of DAT when compared to the 10-repeat allele (15-18), while other reports show effects in the opposite direction indicating that larger concentrations of DAT are available in individuals carriers of the 9-repeat allele (19-21). Among other variants described in *SLC6A3* gene, three single nucleotide polimorphisms (SNPs), two them were in 5' region of the gene (rs2550948 and rs2652511) and one in intron 3 (rs1048953), might be functional variants, because there are located in possible regulatory sequences of this gene (22).

To our knowledge, few studies analyzed the influence of those polimorphisms on food intake in humans. Epstein and colleagues (2002 and 2004) found association among *SLC6A3* 3'UTR VNTR genotypes and obesity phenotypes (23) and food reinforcement (24) in smokers. The eating behavior traits were associated with *DRD4* exon 3 VNTR genotypes when the spanish subjects were grouped in obese (body mass index [BMI] ≥ 30) and non obese (25). A recent study from our group evaluated the influence of polimorphisms in genes that encodes enzymes involved in dopamine metabolism, *MAOA* and *COMT*, and observed associations among alleles that increase the dopamine degradation rate with higher intake of lipid-dense foods (26).

The aim of this study was to evaluate the influence of polymorphisms in the genes *DRD4* (exon 3 VNTR) and *SLC6A3* (3'UTR VNTR, rs2550948, rs2652511 and rs1048953), related to the dopamine system on food intake and nutritional status in children at three different development phases: in the first year of life, at 3 to 4 years, and at 7 to 8 years old.

SUBJECTS AND METHODS

Subjects

This was a prospective cohort study undertaken with the Ten Steps in Action (BRATSA I) study (27). The subjects consisted of 354 children recruited to the birth from October 2001 until July 2002 at the Hospital Centenário, located in São Leopoldo city in southern Brazil. All eligible mothers were informed about both the overall aims of the study (advice on the feeding of preschoolers and its effects on the child's health) as well as all research procedures and then they were invited to participate of the study. The study protocol was approved by the Ethics Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre, and all parent/guardian of the participants provided written informed consent before start the study.

Data Collection

The data collection regarding children's anthropometric and dietary variables was accomplished during home visits in three different development phases: in the first year of life, at 3 to 4 years, and at 7 to 8 years old. The data from 5% of the participants were confirmed by telephone. At 3 to 4 years a clinical assessment was scheduled at a municipal health center, then blood samples were collected and brought to the Molecular Biology Laboratory of the Universidade Federal de Ciências da Saúde de Porto Alegre, where DNA analyses were performed. Race or ethnicity was self-defined by skin color (i.e., whites and non-whites) for children’s mothers, as officially used in demographic censuses in Brazil. More details of the traits studied are described in (28).

Nutritional Status

The child's nutritional status was assessed by means of anthropometric measurements in all visits. In the first year of life children's weight and length were evaluated. At 3 to 4 years and at 7 to 8 years were measured weight, height, waist circumference and tricipital and sub-scapular skin-folds thickness. Anthropometric measurements were taken as follows: wearing light clothing and unshod, each child was weighed on a digital balance (Filizola – Campo Grande, MS) with 100 g gradations, while height was measured using a stadiometer (Seca - México, D.F) fixed to a smooth wall, with the child in an erect posture, heels touching the wall. Waist circumference was measured at the minimum circumference between the iliac crest and the rib cage using an inflexible measuring tape. Triceps and sub-scapular skin-folds thickness were measured using a skin-fold caliper (Lange - Santa Cruz, CA) to the nearest 1.0 mm. The sum of the two individual skin-folds thickness and the ratio waist-to-height [waist(cm)/height(kg)] (29) of each children was computed. BMI was calculated: [weight(kg)/height^2(m^2)], and the values were transformed in Z-score. The BMI variation percentile of each children was computed by the formula $[(BMI_a - BMI_b)/BMI_b]x100$ (a = BMI final and b = BMI initial).

Dietary Data

In the first year of life one 24-hour dietary recall was collected for each child. At 3 to 4 years and at 7 to 8 years, two 24-hour dietary recalls were collected on two nonconsecutive days (at an interval of 15 to 30 days). Mothers were asked about all food and drink consumed by the child on the previous day. The interviewers asked detailed questions about the types of foods, quantities, brands and preparation methods. Portion sizes were confirmed with the aid of an album, specially designed

for this study, containing photographs of utensils and foods and based on domestic measures, such as cups, tablespoons, and teaspoons. Calculations to estimate nutritional intake were performed using NutWin, version 1.5 (Nutritional Support Program from the Federal University of São Paulo) supplemented with information from tables of the chemical composition of foods (30-31) and/or provided by foods manufacturers. The food consumption mean (among the two days) was used to analyze average energy intake daily. For assessment of the intake of sugar-dense foods (SDF) and lipid-dense foods (LDF), the items listed in response to the dietary recall were classified as SDF if there was 50% or more sugar per 100 g in their composition (soda, Jell-O, candies and artificial juice) and as LDF if they contained more than 30% fat per 100 g (fried pastries, cookies with fillings, cold cuts and sausages, fried foods and chocolate). The means of the results of SDF and LDF both recalls was taken and used for analyses. The sum of the mean consumption of SDF and LDF at two days was used to evaluate daily intake of energy-dense foods (EDF).

DNA Analysis

Genomic DNA was extracted from peripheral blood leukocytes by a standard salting-out procedure (32). The *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR polymorphisms were genotyped using the polymerase chain reaction (PCR). The primers sequences for *DRD4* exon 3 VNTR were: forward 5' –AGG ACC CTC ATG GCC TTG – 3' and reverse 5' – GCG ACT ACG TGG TCT ACT CG – 3' (33) and for *SLC6A3* 3'UTR VNTR were: forward 5' – TGT GGT GTA GGG AAC GGC CTG AG – 3' and reverse 5' – CTT CCT GGA GGT CAC GCT CAA GG – 3' (34) (with adaptations). The PCR products containing the tandem repeat polymorphism were resolved by electrophoresis on 2.0% agarose gel with ethidium bromide, visualized

under UV using the 100 bp DNA ladder by comparison. A quality control was accomplished in *DRD4* exon 3 pb VNTR polymorphism genotyping through the confirmation of the genotypes cross electrophoresis on 6.0% polyacrylamide gel in sixty six samples (18.6%); for that, the samples with less frequent genotypes were chosen and samples with the most frequent genotypes were used as control. The analyses of *SLC6A3* SNPs (rs2550948, rs2652511 and rs1048953) were accomplished through real-time PCR in equipment of automation laboratorial Applied Biosystems© StepOnePlus Real-Time PCR System using the allelic discrimination methodology (Taq Man©).

Statistical Analysis

Allele frequencies were estimated by gene counting. A chi-square goodness-of-fit test was used to determine whether the distribution of observed genotype frequencies agreed with those expected under Hardy-Weinberg equilibrium. Variables were tested for the normal distribution through the Kolmogorov-Smirnov test and through the histogram graphics evaluation. For analyses of VNTR polymorphisms children were pooled in two groups. With regard to *DRD4* exon 3 VNTR children were classified as carriers of the allele of 7-repeat (*DRD4.*7R+ i.e., homozygous or heterozygous for the 7-repeat allele) or non-carriers of the allele of 7-repeat (or *DRD4.*7R- i.e., neither allele is 7-repeat). With regard to *SLC6A3* 3'UTR VNTR polymorphism children either were included into the group of homozygous for the 10-repeat (*SLC6A3*.10R/10R), either into the group of homozygous for the 9-repeat or heterozygous 9-repeat/10-repeat (*SLC6A3*.9R*); children carriers of other genotypes were excluded of the *SLC6A3* 3'UTR VNTR association analyses. For *SLC6A3* SNPs variants analyses it was maintained the three groups of genotypes.

The means of the parametric variables were compared among the groups by the General Linear Model test, considering that the children were part of the BRATSA I study (27), or by ANOVA following by Scheffé test of multiple comparisons. The median of the non parametric variables were compared among the groups by the U test of Mann-Whitney or by Kruskal-Wallis test. All tests were performed using the Statistical Package for Social Sciences, version 16.0 (SPSS, Chicago, IL, USA).

RESULTS

The mean age of children at each study phase its show in **Table 1**, its show also that 74.8% of children were white, and that percentage of boys among the subjects was 57.2%.

Eight alleles of the *DRD4* exon 3 VNTR (2R, 3R, 4R, 5R, 6R, 7R, 8R and 10R) were detected, 4R was the most frequent (59.4%) following the 7R (24.2%) and the 2R (7.8%). We have genotyping failures for twenty nine samples (9.3%). For *SLC6A3* 3'UTR VNTR 10R allele was the most common (75.6%) following the 9R (21.3%) among the six length variants detected (3R, 6R, 8R, 9R,10R, and 11R). Twenty subjects had rare *SLC6A3* 3'UTR VNTR genotype (two 3R/9R, two 6R/9R, two 8R/9R, four 3R/10R, five 8R/10R, one 9R/11R, and four 10R/11R) and were excluded of association analysis. For this polymorphisms we have genotyping failures in twenty eight samples and the losses totaled 13.5%. Allele frequencies for *SLC6A3* SNPs were, rs2550948 presented 50% of allele A and 50% of allele G, for rs2652511 the minor allele frequency (MAF) was T (49.4%) and for rs1048953 the MAF was T (26.2%). For SNPs analysis the same twenty eight samples failed to the being genotyping. Genotype frequencies after genotypes were pooled and excluded are presented in **Table 2.**

Genotype frequencies distributions observed were in agreement with those expected under Hardy–Weinberg equilibrium for *DRD4* exon 3 VNTR ($\chi^2_{df=17}$=10.29, P=0.890) for *SLC6A3* 3'UTR VNTR ($\chi^2_{df=9}$=4.21, P=0.920), and for *SLC6A3* SNPs rs2550948 ($\chi^2_{df=2}$=2.65, P=0.266), rs2652511 ($\chi^2_{df=2}$=1.75, P=0.417) and rs1048953 ($\chi^2_{df=2}$=5.90, P=0.052). No differences in genotype frequency distributions were detected between white and non-white subjects (data not shown). Haplotype analysis showed linkage disequilibrium only between rs2550948 and rs2652511 polymorphisms of *SLC6A3* gene (D': 0.994; r^2=0.029).

In the first year of life *DRD4*.7R- children showed BMI Z-score higher (mean=0.67, SD=1.06) when compared to *DRD4*.7R+ children (mean=0.50, SD=1.08, P=0.019), no differences were observed about others anthropometric measurements or food intake when compared among genotypes in this period.

The association analysis for VNTR polymorphisms in both genes and the interaction analysis between these two variants when the children were at 3 to 4 years are demonstrated in **Table 3** and **Table 4**, respectively. *DRD4*.7R- children at 3 to 4 years presented higher average energy intake daily (mean=1,522.74 kcal, SD=379.95 kcal) and higher waist circumference (median=51.00 cm, interquartile range=49.00 to 53.00 cm) when compared to *DRD4*.7R+ children (mean=1,513.28 kcal, SD=425.19 kcal, P=0.043 and median=50.00 cm, interquartile range=48.50 to 52.00 cm, P=0.026, respectively) (Table 3). In this same period of the children's life *SLC6A3*.10R/10R showed higher intake of LDF (median=138.00 kcal, interquartile range=28.32 to 267.64 kcal) and higher intake of EDF (median=253.25 kcal, interquartile range=151.49 to 406.27 kcal) when compared to *SLC6A3*.9R* (median=82.97 kcal, interquartile range=3.16 to 229.88 kcal, P=0.036 and median=193.62 kcal, interquartile range=108.59 to 344.98 kcal, P=0.014,

respectively). Still about this period children carriers of T/T genotype of rs1048953 variant demonstrated higher average energy intake daily (mean=1,813.02 kcal, SD=510.80 kcal) when compared to children carriers of genotypes T/C (mean=1,487.18 kcal, SD=368.66 kcal, Post Hoc multiple comparison test P=0.003) and C/C (mean=1,503.19 kcal, SD=397.00 kcal, Post Hoc multiple comparison test P=0.004). Others SNPs do not showed to be associated with anthropometric measurements or food intake in this period.

At 7 to 8 years, rs1048953 variant of *SLC6A3* gene was associated with ratio waist-to-height (P=0.041), T/T: median=0.45 cm [interquartile range=0.44 to 0.47 cm], T/C: median=0.44 cm [interquartile range=0.42 to 0.48 cm] and C/C: median=0.43 cm [interquartile range=0.41 to 0.46 cm]. Others polymorphisms in both analyzed genes do not showed to be associated with anthropometric measurements or food intake in this life period of children (data not shown).

DISCUSSION

While southern Brazilian population is European descendent, the higher frequency of 4-repeat allele (59.4%) of the polymorphism *DRD4* exon 3 VNTR found in our study was in agreement with European populations which the ancestral 4-repeat allele is most common (35). Our results in regard allelic frequencies of this variant also are similar to worldwide population frequencies determined by PCR analysis (36). The results from analysis of *SLC6A3* 3'UTR VNTR showed that 10-repeat (75.6%) following the 9R (21.3%) alleles are the most frequent, as well has been observed in studies involving other worldwide populations (14, 37-39). The allelic frequencies of the SNPs analyzed in this cohort were similar to a study developed in another southern Brazilian population (22). Our results about the

haplotype analysis of *SLC6A3* polymorphisms are consistent with previous studies that indicate a segmented pattern of linkage disequilibrium in this gene (22, 40-41), as only polymorphisms in 5' region of the gene (rs2550948 and rs2652511) are in the same haplotype block in our cohort.

Studies converge in evidences that suggest the dopamine activity is involved in eating behavior regulation in humans (5, 26, 42-44). Previous reports show the food intake increases dopamine levels in both animal (3-4) and human brain, whose amount of feeding-induced released dopamine was correlated with the degree of experienced pleasure (5), as feeding is an activity of higher reward reinforcement (45). However, there are individual differences in the reinforcing efficacy of food that may relate to differences in eating and energy intake (46). This prospective cohort study is a modesty attempt to produce more robust date on genetic association studies.

Our findings suggest that *DRD4*.7R- children, especially, present increased risk for obesity and higher food intake, since on first year of life these children showed higher BMI Z-score (P=0.019), at 3 to 4 years they presented higher average energy intake daily (P=0.043) and higher waist circumference (P=0.026) when compared to *DRD4*.7R+ children (Table 3). This results become on opposite direction of previous studies that observed association between 7-repeat carriers and BMI mean higher in adults and adolescents when compared to non-carriers of this allele (47-48). However, these studies are not totally comparable with our study, because the previous works evaluated adolescents and adults and we evaluated children. This apparent discrepancy would it is possible by lack of direct linkage between food intake and BMI in childhood and obesity in adulthood. Although, it was shown that

the risk of being obese in adulthood is increased by 3-fold among overweight/obese children and by 4-fold among overweight/obese adolescents (49).

Stice and colleagues (2010) showed that the activation in a region of the frontal operculum, a cerebral region involved in food craving and anticipated reward from food (50-51), was positively related to predicted values of future weight gain in *DRD4*.7R- subjects. The authors yet suggest that subjects that show elevated responsivity of these food reward region may be at increased risk for weight gain if they are not at genetic risk for compromised dopamine signaling (52). The same authors also suggest two possible pathways to unhealthy weight gain and appear to provide support for both the hypo-responsivity model and the hyper-responsivity model. Experiencing too little or too much reward from food may both paradoxically increase risk for obesity and while experiencing moderate reward from food would be a protective factor. This interpretation contemplate a previous theory that hint a quadratic relation between dopamine activity and reward sensitivity, where individuals with too little or too much dopamine activity show disturbances in reward sensitivity (53).

When we analyzed children at 7 to 8 years, these associations were not replicate, is possible that *DRD4* exon 3 VNTR variant display different actions at different development stages. Previous reports led the suggestion that *DRD4* is a ‘plasticity gene’ that makes individuals more susceptible to environmental influences. These reports have associated the 7-repeat allele with differential susceptibility thereby making individuals more responsive to both positive and negative environmental influences (54-56). Additional work is needed to identify the potential complex gene by gene and gene by environment interactions that may further characterize these main effects.

DRD4 exon 3 VNTR variant display repetitive sequences of 48 base pairs at exon 3, region that code of third cytoplasmatic loop of the dopamine receptor D4 (11). The number of repeats has been hypothesized to affect the transmitted signal in the postsynaptic neuron. Individuals with at least one allele containing seven or more repeats show both reduced binding affinities and receptor densities for dopamine neurotransmission (57). So what, *DRD4*.7R+ children could show decrease of the reward, since 7-repeat allele was associated with reduced postsynaptic inhibition, as compared to the 2-repeat and 4-repeat alleles (12, 35). Thus it is possible that *DRD4*.7R- preschool children are more sensible for reward reinforcement of food and, consequently, have higher BMI and waist circumference.

Also at 3 to 4 years *SLC6A3*.10R/10R children showed higher intake of LDF (P=0.036) and EDF (P=0.014) when compared to *SLC6A3*.9R* children. With results in the same direction, Collins and Fuemmeler (2011), in a nationally representative study of adolescents that evaluated a cohort of 20,745 students in United States, related that *SLC6A3*.10R/10R females with high depressive symptoms reported greater intake of high-calorie sweet foods when compared to *SLC6A3*.9R* females with high depressive symptoms ($p<0.05$) (58). However in the same direction of our results, this study and results are not totally comparable with our study and results, because the age and psychiatric complications of the analyzed subjects and for this polymorphism the association detected was with palatable food intake (lipids and sugar dense foods). The *SLC6A3* 3'UTR VNTR variant do not alter the protein amino acids sequence, but it is localized in an regulatory region, it has been showing effects on the expression of the gene. The 9-repeats allele was related to increased of dopamine transporter density, leading to greater dopamine reuptake and lower synaptic concentration of dopamine, the 10-repeat allele on the other hand, would be

associated with smaller availability of dopamine transporter, taking a increase of dopamine levels in the synapse (19-21, 59). However other studies showed results in the opposite direction (15-18). In support of these findings, Peciña and colleagues (2003) examined elevated synaptic dopamine on spontaneous food and water intake and incentive motivation to obtain a palatable sweet reward in mutant DAT knockdown mice and found that the hyperdopaminergic mice had higher energy intakes and preference for a sweet reward than did the controls (60). As the authors, DAT knockdown mutation elevates dopamine neurotransmission and can increases the perceived incentive value of reward stimuli, what appears to be supported best by the incentive salience hypothesis of dopamine function.

The interaction analysis between the variants *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR show that *DRD4*.7R- and *SLC6A3*.10R/10R children presented higher intake of EDF (P=0.024) and higher waist circumference (P=0.017). This combination of genotypes for both genes has been associated with higher dopamine activity when compared to their counterparts (21, 57). The incentive salience hypothesis posits that dopamine systems modulate the perceived incentive value of reward stimuli, so that more dopamine makes rewards 'wanted' more without necessarily being more 'liked' (61). 'Wanting' most relevantly influences food intake (7). So what, in agreement with the results of van Dyck and colleagues (2005) *SLC6A3*.10R/10R subjects, that showed decrease availability of DAT and consequently smaller dopamine intake (21) and *DRD4*.7R-, that show increase dopamine binding affinities and higher receptor densities, taking a possible increases the perceived incentive value of reward stimuli (57). Palatable foods are positive reinforcement that stimulate dopamine liberation in the brain (43, 62). Repeated stimulation of the reward pathways through highly palatable food may lead to

dopamine maladjustment and overeating (63). Further research is needed to confirm associations and to elucidate potential mechanisms.

With regard to the associations observed between rs1048953 polymorphism and average energy intake daily when children were at 3 to 4 years and ratio waist-to-height when the children were at 7 to 8 years, we cannot meet a reasonable explanation, since we could not find studies that evaluate the functionality of this variant located in intron 3, a possible regulatory sequence of *SLC6A3*. However we can suggest that carriers of T/T genotype display increased risk for obesity since at 3 to 4 years these children demonstrated higher average energy intake daily that can been shown at 7 to 8 years when the same children showed higher ratio waist-to-height.

In our study, the majority of associations among genetic variants with food intake and nutritional status were found at two initial phases of childhood development, at first year of life and at 3 to 4 years, period that coincides with the pre-school-aged children. Children at school (7 to 8 years) have many opportunities to eat without parental supervision and can easily access energy dense foods in their social environment (64). Therefore it is possible that the environment may play a stronger effect, independent of genotypes, on eating palatable foods among older children.

There are, however, some potential limitations to this study. The moderate sample size may not have enough power to detect an association of polymorphisms with small effects on food intake and anthropometric measurements, such as rs2550948 and rs2652511. However, we believe that the size of our sample was sufficient to detect genetic effects, reinforcing the importance of our findings relating the *DRD4* exon 3 VNTR polymorphism to higher average energy intake daily and to

higher waist circumference, as well the *SLC6A3* 3'UTR VNTR polymorphism to higher intake of LDF and EDF. Another apparent limitation of our study is that when the subjects were on the pre-school-aged (at first year of life and at 3 to 4 years) they might not have free access to food. However, as discussed before the free access to food, may to mask the genetic variants influence. Another limitation of our study could be indicated by the fact that our analyses were not controlled for the child's sensorial sensibility. In the eating behavior literature, sensory sensitivity has been associated with selective eating in children (e.g., (65)), besides the sensory properties of food influence how rewarding a food is (66), consequently, for Naish and Harris (2012) it is possible that people who are high in sensory sensitivity are more perceptive of the rewarding effects of palatable food (67).

In summary, our results suggest that the *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR polymorphisms might have an impact on children's eating behavior since the presence of alleles related with higher dopamine activity (21, 57) was associated with higher palatable food intake. Our study and findings are innovative because we detected candidate genes for childhood eating patterns and found that polymorphisms already influence palatable food intake in early life. It is possible that genetic variations in other parts of these (or other) genes in the dopamine system are associated with obesity in this population-based sample. However, it will require future studies to further examine this hypothesis. Thus, replication is needed before definitive conclusions can be made about the role of these genes on risk of obesity. Although in this study we have focused on dopamine, it is important to point out that the food intake regulation is complex and involves other physiological mechanisms and other neurotransmitters, for example the brain serotonergic and noradrenergic systems (68).

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CF conducted the laboratorial analyses, analyzed the data and wrote the paper manuscript. MRV and PDBC coordinated the collected data, and were responsible for data management. VNM revised the manuscript. JPG helped with the genetic variants select and revised the manuscript. SA designed the study, revised the data analysis and the manuscript, and received the grants that supported the present work. Both authors participated in data interpretation. Neither of the authors had any personal or financial conflicts of interest.

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TABLE 1

Characteristics of children cohort

Characteristics		
Age (months)	first year of life	12.9 ± 1.1[1]
	at 3 to 4 years old	47.7 ± 2.6
	at 7 to 8 years old	92.5 ± 8.6
Gender	boys	186 (57.2)[2]
	girls	139 (42.8)
Skin color	white	234 (74.8)[2]
	non-white	74 (23.6)

[1] Mean ± SD

[2] Number of subjects (%) (partial data due to data loss)

TABLE 2

Genotype frequencies for the polymorphisms studied

Polymorphisms	Genotypes	N	(%)
DRD4 exon 3 VNTR	7R-	196	(60.3)
	7R+	129	(39.7)
SLC6A3 3'UTR VNTR	10R/10R	183	(59.8)
	9R/10R	114	(37.2)
	9R/9R	9	(3.0)
rs2550948	A/A	79	(24.2)
	A/G	168	(50.6)
	G/G	79	(24.2)
rs2652511	C/C	80	(24.5)
	C/T	170	(52.2)
	T/T	76	(23.3)
rs1048953	C/C	175	(53.7)
	C/T	131	(40.2)
	T/T	20	(6.1)

TABLE 3

Food intake and anthropometric measurements according to *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR polymorphisms genotype in children at 3 to 4 years old

	DRD4 exon 3 VNTR			*SLC6A3* 3'UTR VNTR[1]		
	Children 7R- (n=196)	Children 7R+ (n=129)	*P*	Homozygotes 10/10 (n=183)	Carriers of the genotypes 9/9 or 9/10 (n=123)	*P*
SDF (kcal)	97.62 (45.00 to 164.00)[2]	88.16 (40.25 to 153.75)[2]	0.412[3]	102.89 (45.00 to 161.70)[2]	89.70 (36.24 to 152.76)[2]	0.255[3]
LDF (kcal)	123.50 (26.17 to 256.51)[2]	114.92 (19.71 to 258.74)[2]	0.701[3]	138.00 (28.32 to 267.64)[2]	82.97 (3.16 to 229.88)[2]	0.036[3]
EDF (kcal)[4]	239.73 (134.96 to 403.70)[2]	227.17 (131.87 to 370.54)[2]	0.059[5]	253.25 (151.49 to 406.27)[2]	193.62 (108.59 to 344.98)[2]	0.014[5]
Energy intake (kcal)	1522.74 ± 379.95[6]	1513.28 ± 425.19[6]	0.043[5]	1528.25 ± 396.81[6]	1499.31 ± 420.76[6]	0.859[5]
BMI Z-score	0.27 (-0.39 to 0.86)[2]	0.12 (-0.52 to 0.64)[2]	0.247[3]	0.28 (-0.39 to 0.94)[2]	0.06 (-0.52 to 0.70)[2]	0.099[3]
Sum of skin-folds (mm)	12.50 (11.00 to 15.00)[2]	12.50 (11.00 to 15.00)[2]	0.807[3]	12.5 (10.62 to 16.00)[2]	12.50 (11.00 to 15.00)[2]	0.877[3]
Waist circumference (cm)	51.00 (49.00 to 53.00)[2]	50.00 (48.50 to 52.00)[2]	0.026[3]	50.50 (49.00 to 53.00)[2]	50.00 (48.50 to 53.00)[2]	0.544[3]
Ratio waist-to-height	0.49 ± 0.04[6]	0.49 ± 0.03[6]	0.393[5]	0.50 ± 0.03[6]	0.49 ± 0.03[6]	0.151[5]
BMI variation[7]	-10.21 (-14.58 to -5.85)[2]	-9.27 (-15.60 to -3.85)[2]	0.741[3]	-9.30 (-14.94 to -4.36)[2]	-10.66 (-15.55 to -7.31)[2]	0.078[3]

[1] Subjects with different genotypes were excluded of the *SLC6A3* 3'UTR VNTR analyses.

[2] Median (Interquartile Range).

[3] U Test of Mann-Whitney.

[4] Analyzed from natural logarithm of the sum SDF and LDF.

[5] General Linear Model.

[6] Mean ± Standard Deviation.

[7] Calculate by the difference among BMI at 3 to 4 years and BMI in the first year of life (%).

EDF, energy-dense food; LDF, lipid-dense foods; SDF, sugar-dense foods.

TABLE 4

Food intake and anthropometric measurements according to interaction between *DRD4* exon 3 VNTR and *SLC6A3* 3'UTR VNTR polymorphisms genotype in children at 3 to 4 years old

	Children 7R- and homozygotes 10/10 (n=103)	Children 7R+ and 9/9 or 9/10 (n=43)	*P*
SDF (kcal)	103.13 (45.01 to 164.00)[1]	84.60 (37.20 to 131.82)[1]	0.196[2]
LDF (kcal)	152.75 (29.14 to 276.02)[1]	81.20 (6.37 to 255.66)[1]	0.160[2]
EDF (kcal)[3]	270.15 (158.36 to 415.07)[1]	219.58 (97.79 to 329.29)[1]	0.024[4]
Energy intake (kcal)	1540.42 ± 410.82[5]	1526.21 ± 513.23[5]	0.747[4]
BMI Z-score	0.28 (-0.39 to 0.99)[1]	0.01 (-0.50 to 0.50)[1]	0.115[2]
Sum of skin-folds (mm)	12.50 (10.50 to 16.00)[1]	13.00 (11.50 to 15.00)[1]	0.729[2]
Waist circumference (cm)	50.50 (49.00 to 53.00)[1]	49.75 (48.00 to 51.00)[1]	0.017[2]
Ratio waist-to-height	0.50 ± 0.04[5]	0.48 ± 0.03[5]	0.212[4]
BMI variation[6]	-9.59 (-14.42 to -5.47)[1]	-11.21 (-16.52 to -5.44)[1]	0.417[2]

[1] Median (Interquartile Range).

[2] U Test of Mann-Whitney.

[3] Analyzed from natural logarithm of the sum SDF and LDF.

[4] General Linear Model.

[5] Mean ± SD.

[6] Calculate by the difference among BMI at 3 to 4 years and BMI in the first year of life (%).

EDF, energy-dense food; LDF, lipid-dense foods; SDF, sugar-dense foods.

6 CONCLUSÃO

A obesidade é uma síndrome complexa determinada por inúmeros fatores, dentre eles o comportamento alimentar. O qual, por sua vez é influenciado por diversos sistemas fisiológicos homeostáticos, nos quais atuam hormônios e neurotransmissores que agem sobre circuitos cerebrais com o objetivo de preservar o balanço energético apropriado, regulando estímulos como a fome e a saciedade. Diferenças individuais nesses sistemas podem influenciar e prejudicar a manutenção do balanço energético.

As frequências alélicas e genotípicas observadas em nossa amostra de crianças estiveram de acordo com as frequências encontradas em trabalhos prévios que analisaram amostras de populações globalmente distribuídas e um estudo que analisou uma amostra proveniente do sul do Brasil. Esses resultados semelhantes indicam o adequado desenvolvimento das técnicas de determinação de genótipos empregadas em nosso estudo. A análise haplotípica também foi consistente com resultados de estudos prévios que indicaram um padrão segmentado de desequilíbrio de ligação do gene *SLC6A3*.

Como amplamente discutido, a alimentação é uma atividade de alto poder de recompensa, cuja regulação está intimamente relacionada à função dopaminérgica. Estímulos externos como a oferta de alimentos saborosos representam uma força motivadora que pode suplantar os sinais homeostáticos e permitir que as pessoas ingiram mais energia do que o metabolismo necessita.

Estudos prévios indicam que variantes nos genes *DRD4* e *SLC6A3* influenciam a sensibilidade ou a reatividade do sistema dopaminérgico. Nossos resultados sugerem que os polimorfismos *DRD4* exon 3 VNTR e *SLC6A3* 3'UTR VNTR podem influenciar o comportamento alimentar de crianças, visto que os alelos associados à maior atividade dopaminérgica, em nosso estudo estiveram associados à maior ingestão de alimentos palatáveis e a maiores medidas antropométricas.

Em relação ao polimorfismo *DRD4* exon 3 VNTR, as crianças que não eram portadoras de alelos de 7 repetições apresentaram maior IMC no primeiro ano de vida e maiores ingestão energética diária e circunferência da cintura aos 3 a 4 anos. Como essas crianças apresentariam elevada sinalização dopaminérgica via receptores D4 (visto que estudos indicam que a presença do alelo de 7 repetições

estaria associada a reduzidas disponibilidade e afinidade de ligação do receptor de DA) elas podem estar em risco para obesidade devido à elevada responsividade do sistema dopaminérgico à recompensa alimentar, embora alguns trabalhos também observaram uma associação entre a menor sinalização dopaminérgica e o risco da obesidade. A teoria que sugere uma relação quadrática entre a atividade dopaminérgica e a sensibilidade à recompensa alimentar parece explicar esses resultados aparentemente controversos. Segundo essa hipótese os indivíduos que apresentam distúrbios na sensibilidade à recompensa, devido à sinalização dopaminérgica muito deficiente ou muito intensa, apresentariam risco aumentado para obesidade, enquanto experimentar sensibilidade moderada à recompensa representaria um fator de proteção.

A sinalização dopaminérgica elevada também esteve associada à maior ingestão de alimentos de alto teor lipídico e de alto teor energético em nosso estudo, à medida que crianças portadoras do alelo de 10 repetições do polimorfismo *SLC6A3* 3'UTR VNTR apresentaram maior ingestão desses alimentos aos 3 a 4 anos. Segundo estudos prévios o alelo de 10 repetições estaria associado à menor disponibilidade de transportadores de DA, resultando em maior concentração de DA na fenda sináptica e, possivelmente, promovendo maior sinalização dopaminérgica. Essa elevada sinalização dopaminérgica pode promover um aumento do valor motivador percebido do estímulo da recompensa. Essa hipótese postula que o sistema dopaminérgico modula o valor motivador percebido do estímulo da recompensa, assim a maior sinalização dopaminérgica seria capaz de aumentar o desejo por um alimento por torná-lo mais atrativo e o estímulo repetido das vias de recompensa pelos alimentos palatáveis pode levar a distúrbios no sistema dopaminérgico e à alimentação excessiva.

Em relação aos três SNPs do gene *SLC6A3* incluídos nesse estudo, apenas um apresentou associação com ingestão de alimentos palatáveis e com medidas antropométricas na amostra estudada. O polimorfismo rs1048953 foi associado à ingestão energética diária das crianças aos 3 a 4 anos e à razão cintura-estatura aos 7 e 8 anos. Embora não tenha sido possível encontrar uma explicação razoável para essas associações, visto que não dispomos de estudos que avaliaram a funcionalidade dessa variante localizada em uma possível região regulatória do gene *SLC6A3*, nós sugerimos que as criancas portadoras de genótipo T/T apresentam risco aumentado para a obesidade já que aos 3 a 4 anos elas apresentaram maior

ingestão energética diária que pode ter se refletido nas maiores medidas de razão cintura-estatura observadas aos 7 a 8 anos nessas mesmas crianças.

A maior parte das associações entre variantes gênicas e ingestão alimentar e medidas antropométricas encontradas em nosso estudo foram observadas nas duas primeiras fases do desenvolvimento das crianças, no primeiro ano de vida e aos 3 a 4 anos. Quando as crianças estavam em idade escolar, aos 7 a 8 anos, não foram observadas diferenças entre os grupos genotípicos em nosso estudo. Nessa fase as crianças têm mais oportunidades de comer sem a supervisão dos pais, podendo acessar alimentos com maior facilidade, além disso, ficam mais expostos à oferta de alimentos com alto teor energético. Assim, é possível que o ambiente tenha um efeito significativo sobre a ingestão de alimentos palatáveis entre crianças com mais idade, independentemente de seus genótipos.

Tendo em vista a complexidade do mecanismo de recompensa, é possível que outras variações nesses genes, ou em outros genes do sistema dopaminérgico estejam associados com obesidade nessa amostra específica. No entanto, novos estudos são necessários para melhor avaliar essas hipóteses e suportar nossos resultados. Nossos estudo e resultados são inovadores porque encontramos genes candidatos para avaliar o comportamento alimentar na infância sugerindo que esses polimorfismos influenciam a ingestão de alimentos palatáveis logo no início da vida. Apesar disso, a replicação desse modelo de estudo é necessária antes de conclusões definitivas sobre o papel que essas variantes gênicas exercem no risco para obesidade. Embora nesse estudo o enfoque principal no sistema dopaminérgico seja visível, é importante ressaltar que a regulação da ingestão alimentar, é complexa e envolve outros mecanismos fisiológicos e outros neurotransmissores.

O envolvimento de recentes mudanças ambientais, principalmente a grande oferta de alimentos palatáveis e reduzidas oportunidades para a realização de tarefas que exigem o consumo de energia, parecem inegáveis. Esse quadro, aliado à ocorrência de diferenças individuais e à complexidade dos mecanismos de recompensa que sugerem a ausência de uma única via ou molécula responsáveis por sua regulação torna a investigação nessa área bastante promissora. Estudos que enfoquem outras variantes nesses genes ou em outros genes relacionados podem ajudar a elucidar os mecanismos que influenciam na decisão da ingestão de

alimentos, seja por resultado de raciocínio consciente ou processo emocional subconsciente.

ANEXO A – PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA

Título do Projeto: Incidência de obesidade e anemia em uma coorte de nascimento acompanhada até 4 anos de idade: avaliação do componente genético

Pesquisador Responsável : Márcia Regina Vitolo **Parecer 736/08**

Data da Versão	Cadastro 411/08	Data do Parecer 13/11/2008

Grupo e Área Temática **Classificação utilizada pela CONEP**

Objetivos do Projeto
Geral: Investigar a associação de variantes de genes envolvidos nas diferentes vias de controle e regulação do peso corporal e parâmetros antropométricos e de ingesta alimentar em uma coorte de crianças entre 3 e 4 anos de idade.

Sumário do Projeto

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Outro (citar no comentário)
Outras instituições envolvidas	Sim
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Instituto de Cardiologia do RS; Laboratório de Biologia Molecular da UFCSPA.

Introdução	Adequada

Comentários sobre a Introdução

Objetivos	Adequados

Comentários sobre os Objetivos

Não apresenta o objetivo no protocolo de pesquisa.

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 397 Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Menores de 18 anos
Seleção eqüitativa dos indivíduos participantes	Adequada
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Adequado
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Adequado
Data de início prevista	2008
Data de término prevista	2010
Orçamento	Adequado
Fonte de financiamento externa	Agência de fomento

Comentários sobre o Cronograma e o Orçamento

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Após análise do projeto acima descrito recomenda-se aprovar. A pesquisa atende as exigências, em seus aspectos éticos e metodológicos, as Diretrizes e Normas, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

ANEXO B – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

O presente estudo (**Investigação dos Fatores de Risco para Obesidade Precoce e Anemia em uma Coorte de Crianças Submetidas a um Programa de Intervenção Nutricional no Primeiro Ano de Vida**) pretende dar continuidade ao trabalho realizado no 1.º ano de vida de seu filho, visando acompanhar as condições de crescimento e desenvolvimento por meio das medidas de peso, altura, quantidade de gordura corporal, as quais não conferem riscos nem dor para seu filho.

Utilizaremos um questionário para fazer-lhe perguntas sobre sua família, o qual conterá: condições de vida (sociais e econômicas), moradia, práticas alimentares de seu filho, atividades diárias e presença de doenças. Em data marcada com o pesquisador, será verificada a pressão arterial e será realizada coleta de sangue por profissional treinado com agulhas descartáveis, sem risco de contaminação, para análise dos níveis de colesterol, LDL, triglicerídeos, proteína-C reativa e glicemia, além disso, será avaliado alterações genéticas que podem estar associadas à obesidade e anemia. A criança sentirá um pequeno desconforto o momento da picada, porém não haverá riscos a sua saúde. Entretanto, não há outra forma de verificação que possa fornecer resultados mais precisos. Essas informações serão transformadas em números e a identidade da sua família não será divulgada em nenhum momento.

Este estudo é importante para se conhecer os fatores que são responsáveis pela obesidade e anemia na infância e dessa forma intervir de forma mais ampla na população. A senhora receberá todos os resultados das avaliações e orientações ou encaminhamentos se necessário para o melhor bem estar seu e de seu filho. A

senhora também terá toda a liberdade de interromper a entrevista em qualquer momento ou de pedir maiores esclarecimentos caso tenha alguma dúvida.

Assinará duas cópias desse consentimento, ficando uma em seu poder e a outra com a responsável do programa.

São Leopoldo, ____ de _____ de 200___.

Nome_____

Assinatura_____

Eu, Profa. Márcia Regina Vitolo, nutricionista, estou realizando a pesquisa: "INCIDÊNCIA DE OBESIDADE E ANEMIA EM UMA COORTE DE NASCIMENTO ACOMPANHADA ATÉ 4 ANOS DE IDADE: AVALIAÇÃO DO COMPONENTE GENÉTICO". Esta pesquisa visa esclarecer como variações genéticas normais podem influenciar no desenvolvimento da obesidade infantil, assim como, no desenvolvimento de anemia. Para que tal pesquisa possa ser realizada peço sua colaboração, autorizando que seja realizado o estudo das variantes genéticas nas amostras de sangue que já foram coletadas de seu filho.

Quais os riscos em participar? Como não se fará nenhuma picada a mais do que aquelas necessárias para os exames que já foram realizados não há risco para a paciente em participar deste projeto.

O que o paciente ganha com este estudo? Com a análise, poderemos saber quais crianças podem ter maior predisposição ao desenvolvimento de obesidade e anemia. No entanto, os benefícios deste estudo poderão ser obtidos apenas em longo prazo.

Quais são os seus direitos? Os seus registros médicos serão sempre tratados confidencialmente. Os resultados deste estudo poderão ser usados para fins científicos, mas você não será identificado por nome. Sua participação

no estudo é voluntária, de forma que, caso você decida não participar, isto não afetará no tratamento normal que você tem direito.

Assinará duas cópias desse consentimento, ficando uma em seu poder e a outra com a responsável do programa.

Nome: .....

Assinatura: .....

Núm de Identificação: .....

Assinantura do Responsável: ..... Data: ___/___/_____

Em caso de qualquer dúvida quanto à pesquisa ou sobre os seus direitos, você poderá contatar com Prof. Márcia Regina Vitolo – tel 8162 9929 – 3224 8822 (ramal 153).

ANEXO C – QUESTIONÁRIO SOCIOECONÔMICO E DIETÉTICO

FICHA DA CRIANÇA

Entrevistador_____

_____

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

Identificação (criança):

2.Telefones para contato_____

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

Dados Maternos e Socioeconômicos:

8.Qual a sua idade? _____anos	IdMae:_____
09.Data de nascimento da mãe _____/_____/_____	DNm_____
10.Qual o seu estado civil? Casada/ou mora junto (1) Viúva (2) Solteira (3) Separada (4)	EstCivil _____
11.Você teve outros filhos? (1) Sim (2) Não (pule para a questão 14)	Filhos: _____ Quant:_____ DNf:___/___/___ DNf:___/___/___ DNf:___/___/___
12. Se sim: Quantos:_____ DN_____/_____/_____ DN_____/_____/_____- DN_____/_____/_____	
13.Quantas pessoas moram na sua casa? _____	Famí:_____ Adul:_____
14.Qual o grau de parentesco? (1) Família nuclear (2) Família não nuclear	Parentes: ___

15.Qual a sua ocupação? (1) Desempregada (2) Empregada c/ carteira assinada (3) Empregada s/ carteira assinada (4) Do lar (5) Estudante	OcupaMae:____
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	OcupaPai:_____
17.Qual a renda total da família? R$ _____	RendaT:_____
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)	Vcfum:_____

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

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

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)	Fgest:_____

Se sim:

25.Quantos cigarros você fumava por dia?_____	Ncfum:_____

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: ___ ColAlto: _____

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	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:___
	PA: _____ PApai: ___

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	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)	Exam:_____

<u>Se sim anotar:</u>

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: 29.Qual o nome do suplemento?_____ 30.Qual a quantidade? _____gotas ou _____drágeas 31.O seu filho realmente recebe o suplemento? (1) Sim (2) nao 32.Que idade a crianca tinha quando iniciou com o uso desse suplemento?_____meses 33. Tempo de uso:_____semanas	Suple:_____ Qual:_____ Qgotas:___ Qdrag:_____ Receb:_____ Idade:_____ Tempu:_____

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)	Intern:_____
35.Seu (sua) filho (a) teve episódios de diarréia no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Diarré:_____
36. Seu (sua) filho (a) apresentou febre importante no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Febre:_ _____
37. Seu (sua) filho (a) teve infecção no últimos 6 meses? Sim (1) Não (2) Não sabe (3)	Infecç:_____

38. Seu (sua) filho (a) teve infecção urinária nos últimos 6 meses? Sim (1) Não (2) Não sabe (3) 39.O seu (sua) filho (a) apresentou algum problema respiratório? Sim (1) Não (2) (pule para a 67)	InfUri:_____ Resp:_____

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	Tosse:____ Coriza:____ Obstru:_____ Respd:_____

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)	Vol:_____ 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)	Creche:_____ Períod:_____ Temp:_____ Idcre:_____ Ondfica:_____

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

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)	Quant:_____ Tagua:_____

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	Objet:_____

a 53)	

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?)	Quais:_____

Estado Nutricional: 53. Peso_____gramas 54. Comprimento_____cm	Peso:_____ Compri:_____

Atividades diárias(ontem) : 55. Que horas foi dormir ontem___ Que horas acordou hoje_____/ horas de sono:_____ 56. O que fez ontem pela manha: ( ) Creche / Tempo_____ ( ) Assistiu TV / Tempo:_____ ( ) Brincou fora de casa / Tempo:_____ ( ) Brincou dentro de casa / Tempo:_____ ( ) Dormiu / Tempo:_____ 57. O que fez ontem de tarde:	Hsonoit:_____ MCrecheT:___ MTvT:_____ MBrifT:_____ MBridT:_____ MDormT:_____

( ) 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	ConFi:_____
3. ativo	
4. muito ativo	
5. agitado	

ANEXO D – NORMAS DE EDITORAÇÃO DA REVISTA

THE AMERICAN JOURNAL OF CLINICAL NUTRITION

The American Journal of CLINICAL NUTRITION

Information for Authors

INTRODUCTION

The purpose of *The American Journal of Clinical Nutrition* (*AJCN*) is to publish original research studies relevant to human and clinical nutrition. Well-controlled clinical studies that describe scientific mechanisms, efficacy, and safety of dietary interventions in the context of disease prevention or a health benefit will be considered. Public health and epidemiologic studies relevant to human nutrition, and innovative investigations of nutritional questions that employ epigenetic, genomic, proteomic, and metabolomic approaches are encouraged. Solicited editorials,book reviews, solicited or unsolicited review articles, invited controversy position papers, and letters to the Editor that relate to prior *AJCN* articles are essential components of the *AJCN*. All submitted material with scientific content will undergo peer review by the Editors or their designees before acceptance for publication.

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All material to be considered for publication in a regular or supplement issue should be submitted electronically at the following website: http://submit.ajcn.org/. See "Tips for authors submitting manuscripts to the *AJCN*" for helpful advice regarding electronic submission.

Original manuscripts will be considered with the understanding that no part has been published, simultaneously submitted, or already accepted for publication elsewhere, other than in abstract form. Papers will be screened for similarity to previously published papers using iThenticate.

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Secretariat Office
Annals of Internal Medicine
American College of Physicians
Independence Mall West
Sixth Street at Race
Philadelphia, PA 19106-1572.

SUMMARY OF REQUIREMENTS

List of forms required by authors submitting to AJCN

Each manuscript component should begin on a new page in the following sequence:

Title page
Abstract
Text
Acknowledgments
References
Tables: each table on a separate page, complete with title and footnotes

Legends for figures
Figures

Identify on the title page the author who will be responsible for correspondence regarding the manuscript. The signed Authors' Agreement form and copies of any documents granting permission needed to reproduce material in print and electronic form or to use illustrations of identifiable subjects should be scanned and e-mailed to ajcnsubmit@nutrition.org. If scanning is not possible, then the Authors' Agreement form and any necessary documents may be faxed to (301) 634-7892. Authors should keep copies of all submitted material.

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Review Articles
Special Invited Articles, including Controversies and Perspectives
Original Research Communications (including formal systematic review/meta-analysis)
Letters to the Editor
Book Reviews
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ASN Announcements
Calendar of Events

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AJCN requires registration in an appropriate public trials registry of all clinical trials and observational studies that began after July 1, 2008. Such registries include ICMJE-approved public trials registries (http://www.clinicaltrials.gov, http://www.actr.org.au, http://www.isrctn.org, http://www.umin.ac.jp, http://www.trialregister.nl). It is highly desirable that studies begun before July 1, 2008 also be registered. Please report the study ID number and the website where the clinical trial is registered on the title page of the paper.

Depending on the design of the study, one of the health research reporting checklists referenced at the Equator Network (http://www.equator-network.org/resource-centre/library-of-health-research-reporting/) must accompany each manuscript as "supplemental files" in the online manuscript submission system. Page or line numbers must be included to indicate where the checklist items are located in your paper.

If none of the checklists apply, please explain in your cover letter why none are needed.

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Articles are copyedited according to *AJCN* style policy, the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals," and the style manual of the Council of Science Editors (Scientific style and format: the CSE manual for authors, editors, and publishers. 7th ed. Reston, VA: The Council, 2006).

Authorship

Scientific conduct
Each author must have participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and conduct of the experiment, and for the data interpretation. An article with corporate (collective) authorship must specify the key persons responsible for the article; others contributing to the work should be recognized separately. The Editors may require authors to justify the assignment of authorship. All authors must sign a statement agreeing to all the requirements for authorship with the transfer of copyright (http://www.ajcn.org/site/misc/Authors'_Agreement_Form.pdf). A Change in Authorship Form must be submitted if an author's name is added to the manuscript, there is a change in the author order, or an author wishes to remove his/her name. In the last case, a letter requesting the removal of his/her name and signed by the author must accompany the form.

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Authors must disclose in the Acknowledgment section any possible conflicts of interest. For detailed guidelines, please see the ASN Journals' Conflict of Interest Guidelines.

Instructions for manuscript preparation

The manuscript should be formatted as follows: 216 x 279 mm (8½ x 11 in) or ISO A4 (212 x 297 mm), with margins of at least 2.5 cm; use double-spacing and 12-point type throughout. Do not justify the right margin. **The abstract and text pages should have consecutive line numbers in the left margin beginning in the abstract and ending before the reference section.** Number pages consecutively in the upper right-hand corner of each page, beginning with the title page. Foreign authors are advised to have their manuscripts reviewed by a scientific colleague who is fluent in English so that the manuscripts will conform to US English usage and grammar.

Title page

The title page should contain:

1. the title of the article, beginning with a key word if possible, with only the first letter of the first word capitalized;
2. the names of all authors (first name, middle initial, last name) and their departmental and institutional affiliations at the time the research was done. Indicate which authors are associated with which institutions by listing the appropriate author initials in parentheses after each affiliation listed.
3. The last name of each author for the purpose of PubMed indexing
4. If an author has changed affiliations and wants this information in the article, then this information should be included in a separate line on the title page.

5. disclaimers, if any;
6. the name, mailing address, telephone and fax numbers, and e-mail address of the author responsible for correspondence about the manuscript;
7. the name and mailing and e-mail addresses of the author to whom requests for reprints should be addressed or a statement that reprints will not be available from the author;
8. sources of support, including grants, fellowships, and gifts of materials (eg, chemicals, experimental diets);
9. a short running head of **not more than 50 characters** (count letters and spaces);
10. a list of abbreviations and their definitions for all abbreviations used in the text if there are 3 or more; and
11. information pertinent to any clinical trial registry in which the trial is registered.

Abstract

A properly constructed and informative abstract is helpful for the initial editorial review of the submitted manuscript. Original research articles must include a structured abstract that contains no more than 250 words, is written **in complete sentences**, and includes the following headings:

Background: Provide 1 or 2 sentences that explain the context of the study.
Objective: State the precise objective, the specific hypothesis to be tested, or both.
Design: Describe the study design, including the use of cells, animal models, or human subjects. Identify the control group. Identify specific methods and procedures. Describe interventions, if used.
Results: Report the most important findings, including results of statistical analyses.
Conclusions:Summarize in 1 or 2 sentences the primary outcomes of the study, including their potential clinical importance, if relevant (avoid generalizations).

Review articles, special articles, and reports should include an unstructured abstract (no more than 250 words) that states the purpose of the article and emphasizes the major concepts and conclusions. Any abbreviations used in the abstract should be defined in the abstract at first mention.

Text

Use active voice whenever possible. Use past tense when describing and discussing the experimental work on which the article is based. Reserve present tense for reference to existing knowledge or prevailing concepts and for stating conclusions from the experimental work. Clearly differentiate previous knowledge and new contributions. Do not use *level* when referring to a concentration. Use metric units of measure; SI units are no longer required.

The text of observational and experimental articles should be divided into sections with the following headings: Introduction, Subjects (or Materials, for cell or animal studies) and Methods, Results, and Discussion. Long articles may require subheadings within some sections. Authors should consult recent issues of the *AJCN* for guidance on the formatting of other types of articles, book reviews, and editorials.

Introduction

Clearly state the purpose of the article. Summarize the rationale and background for the study or observation, giving only strictly pertinent references. Do not include methods, data, results, or conclusions from the work being reported. The Introduction should be limited to 1.5 manuscript pages.

Subjects (or Materials) and Methods
Describe clearly your selection of the experimental and control subjects and provide eligibility and exclusion criteria and details of randomization. Describe the methods for, and success of, any masking (blinding) of observations. Report any complications of experimental treatments. Identify the methods, apparatus (manufacturer's name in parentheses), and procedures in sufficient detail to allow other researchers to reproduce the results. Define all group designations parenthetically at first mention [for example, "control (CON) and high-fat (HF) groups"] and include definitions for these abbreviations in the abbreviation footnote on the title page. Do not use trademark names, such as Teflon, as generic terms. Give references for established methods, including statistical methods; provide references and brief descriptions of methods that have been published but are not well known; and describe new or substantially modified methods, giving reasons for using them and evaluating their limitations. Identify precisely all drugs and chemicals used, including generic names, dosages, and routes of administration. If trade names for drugs and chemicals are included, give the manufacturer's name and location.

Ethics. When reporting experiments on human subjects, indicate that the procedures followed were in accordance with the ethical standards of the responsible institutional or regional committee on human experimentation or in accordance with the Helsinki Declaration of 1975 as revised in 1983. Do not use patients' names, initials, or hospital identification numbers. When reporting experiments on animals, indicate approval by the institution's animal welfare committee and state whether the National Research Council's guide for the care and use of laboratory animals was followed.

Statistics. Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (eg, CIs, SDs, or SEs), even for differences that were not significant. Report the numbers of observations. Specify any general-use computer programs used, including the version number and the manufacturer's name and location. Include general descriptions of statistical methods in the Subjects (or Materials) and Methods section and specific descriptions in each table and figure legend. Indicate whether variables were transformed for analysis. Provide details about what hypotheses were tested, what statistical tests were used, and what the outcome and explanatory variables were (where appropriate). Indicate the level of significance used in tests if different from the conventional 2-sided 5% alpha error and whether or what type of adjustment is made for multiple comparisons.

When data are summarized in the Results section, specify the statistical methods used to analyze them. Avoid nontechnical uses of technical statistical terms, such as *random* (which implies a randomizing device), *normal*, *significant*, *correlation*, *sample*, and *parameter*. Define statistical terms, abbreviations, and symbols not listed under "Abbreviations for statistical terms." If there are 3 or more abbreviations used in the text, prepare an abbreviation footnote. The footnote should be associated with the first abbreviated term in the text and should be an alphabetized listing of all author-defined abbreviations and their definitions. Detailed statistical analyses, mathematical derivations, and the like may sometimes be suitably presented as one or more appendixes.

Results
Present your results in a logical sequence in the text, tables, and figures. Do not present specifics

of data more than once and do not duplicate data from tables or figures in the text; emphasize or summarize only important observations. Do not present data from individual subjects except for very compelling reasons. Report losses to observation (such as dropouts from a clinical trial). Use boldface for the first mention of each table or figure.

Discussion
The Discussion should not exceed 4 typewritten pages except in unusual circumstances as approved by the Editor. Emphasize concisely the novel and important aspects of the study and the conclusions that follow from them. Do not repeat in detail data or other material given in the Introduction or Results. Include the implications of the findings and their limitations and relate the observations to other relevant studies. Link conclusions with the goals of the study and avoid unqualified statements and conclusions that are not completely supported by the data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses and recommendations when warranted by the results and label them clearly as such.

Acknowledgments
Acknowledge only persons who have made substantive contributions to the study. Authors are responsible for obtaining written permission from everyone acknowledged by name and for providing to the Editor a copy of the permission, if requested. Authors must disclose any financial or personal relationships with the company or organization sponsoring the research at the time the research was done. Such relationships may include employment, sharing in a patent, serving on an advisory board or speakers' panel, or owning shares in the company. If an author or authors have no potential conflicts of interest, please state this. The source of support for the research reported in the paper should be listed on the title page, not as an acknowledgement. Each author is required to list his or her contribution to the work.

Authors' contributions to manuscript
A description of the contribution of each author must be provided in the Acknowledgment Section. Please use the following descriptors:

1. designed research (project conception, development of overall research plan, and study oversight);
2. conducted research (hands-on conduct of the experiments and data collection);
3. provided essential reagents or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc, necessary for research);
4. analyzed data or performed statistical analysis;
5. wrote paper (only authors who made a major contribution);
6. had primary responsibility for final content;
7. other (use only if categories above are not applicable; describe briefly);
8. for single-authored papers, please state: The sole author had responsibility for all parts of the manuscript.

Please do not include "obtained funding" (the initials of authors who received grants may be included in the footnote regarding support on the manuscript's title page). Although not all manuscripts will necessarily include all descriptors, all manuscripts, including reviews, must indicate who is responsible for design, writing, and final content. An example of a properly formatted author contribution statement is as follows: "AX, RFG, and PGY designed research; RFG

and QC conducted research; PT analyzed data; AX, PGY, and QC wrote the paper; PGY had primary responsibility for final content. All authors read and approved the final manuscript."

References

Number references consecutively in the order in which they are first mentioned in the text. Identify references by Arabic numerals in parentheses. References cited in tables or in legends to figures should be numbered according to the first citation of the table or figure in the text. Appendixes should have a separate reference section.

It is rarely necessary to cite more than 50 references in an original research article. Try to avoid citing published abstracts as references [if a published abstract is cited, include "(abstr)" at the end of the reference]. Abstracts from scientific meetings not published in peer-reviewed journals may not be used as references. Unpublished observations and personal communications (written, not oral) may not be used as references but may be inserted in parentheses with the names of the responsible researchers and the year of the observation or communication. Authors are responsible for obtaining written permission from everyone so cited and for providing to the Editor a copy of the permission, if requested. Doctoral dissertations may be used as references. Include manuscripts accepted but not yet published; designate journal name followed by "(in press)." Report foreign titles in the original language, identify the language, and provide the English translation in parentheses. The references must be verified by the author against the original documents.

Journals

1. Journal article with DOI: If an article has a DOI number ("digital object identifier" number unique to the publication), it may be included at the end of the reference.
   Hamer M, Steptoe A. Prospective study of physical fitness, adiposity, and inflammatory markers in healthy middle-aged men and women. Am J Clin Nutr 2009;89:85–89. doi:10.3945/ajcn.2008.26779
2. Standard journal article: list all authors when 10 or fewer; when >10, list only the first 10 and add "et al." Abbreviate journal titles according to *Index Medicus* style, which is used in MEDLINE citations.
   Jeffery RW, Wing RR, Sherwood NE, Tate DF. Physical activity and weight loss: does prescribing higher physical activity goals improve outcome? Am J Clin Nutr 2003;78:684–9.
3. Corporate author
   National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 2002;106:3143–421.

   Books and other monographs

4. Personal authors
   Shils M, Shike M, Olson J, Ross AC. Modern nutrition in health and disease. 9th ed. Baltimore: Lippincott Williams & Wilkins, 1998.
5. Committee report or corporate author
   National Research Council. Recommended dietary allowances. 10th ed. Washington, DC:

National Academy Press, 1989.
Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for vitamin C, vitamin E, selenium and carotenoids. Washington, DC: National Academy Press, 2000.

6. Chapter in book
Young VR, Tharakan JF. Nutritional essentiality of amino acids and amino acid requirements in healthy adults. 2nd. ed. In: Cynober LA, ed. Metabolic and therapeutic aspects of amino acids in clinical nutrition. Boca Raton, FL: CRC Press, 2004:439–70.
7. Agency publication
US Department of Agriculture, US Department of Health and Human Services. Nutrition and your health: dietary guidelines for Americans. Washington, DC: US Government Printing Office, 2000. [USDA Home and Garden Bulletin no. 232.]

Internet references

8. Website
National Center for Health Statistics. National Health and Nutrition Examination Survey. Version current 1 October 2003. Internet: http://www.cdc.gov/nchs/nhanes.htm (accessed 13 October 2003).
9. Online journal article
Sinha A, Madden J, Ross-Degnan D, Soumerai S, Platt R. Reduced risk of neonatal respiratory infections among breastfed girls but not boys. Pediatrics [serial online] 2003;112:e303. Internet: http://pediatrics.aappublications.org/cgi/content/full/112/4/e303 (accessed 14 October 2003).

Tables

Tables must be included in the text file, and each should appear one per page. Double-spacing of tables is preferred but not required. Number tables consecutively with Arabic numerals (do not use 1A, 1B, etc) and supply a brief descriptive title for each. Give each column a short or abbreviated heading. Place explanatory matter in footnotes, not in the heading or table title. Each table should contain enough detail (including statistics) that the table is intelligible without reference to the text. All nonstandard abbreviations, including group designations, used in a table or table title should be defined in a footnote to the table title, and the abbreviations should be listed in alphabetic order. If the footnote to the table title contains multiple items, the definitions of the abbreviations should be the last item. If a table contains only one abbreviated term in the body of the table, then a separate footnote placed after that abbreviation should be used to define that term. Commonly used approved abbreviations (*see* Units and Abbreviations) may be used without explanation. Additionally, explanations are not needed for ANOVA, BMI, F (females), and M (males). For footnotes, use superscript Arabic numerals. For reporting results of statistical analyses, superscript letters can be used if explaining the results in the usual manner would be too complicated (*see* a recent issue of the *AJCN* for examples). The first appearance in a horizontal row determines the order of the footnotes. Identify statistical measures of variation, such as SD and SE. **Omit internal horizontal and vertical rules.** Cite each table in the text in consecutive order. Use boldface for the first mention of each table. If you use data from another published source, acknowledge the source fully. Number references in tables according to the location of the first citation of each table in the text.

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Cite each figure in consecutive order in the text. Use boldface for the first mention of each figure. Spell out the word "Figure"; do not use "Fig." If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce or adapt the material in print and electronic format. Except for documents in the public domain, permission is required from the copyright holder, regardless of authorship or publisher.

Legends for all figures should be included within the manuscript text file on a separate page and be typed with double-spacing (legends should not be included on the figures themselves). Each legend should contain enough detail, including statistics, to make the figure intelligible without reference to the text. All nonstandard abbreviations, including group designations, used in a figure or figure legend (*see* Units and Abbreviations for list of standard abbreviations) should be defined at the end of the figure legend and listed in alphabetic order. When symbols, arrows, numbers, or letters are used to identify parts of the figures, identify and explain each one clearly in the legend. Explain internal scale and identify the method of staining in photomicrographs.

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Supplemental material

Supplemental material may be included with manuscript submissions. Supplemental files for upload may include required research checklists, articles published/in press elsewhere, reports or technical briefs related to manuscript submission, figure source files, questionnaires, permissions, videos, etc. All supplemental data should be clearly labeled either as "Supplemental Data for Reviewers Only" or as "Online Supplemental Material" if it is submitted for online publication only in *The AJCN*. Therefore, please upload supplemental files for review-only separately from supplementals files for online publication. Online Supplemental Material (OSM) is not edited before being posted online.

UNITS AND ABBREVIATIONS

Use only standard abbreviations. Consult the following sources for standard abbreviations: *Scientific Style and Format* and *Standard for Use of the International System of Units (SI): the Modern Metric System* (American Society for Testing and Materials. IEEE/ASTM SI 10-1997. West Conshohocken, PA: ASTM, 1997) or www.ieee.org/web/publications/PSPB/index.html. Avoid abbreviations in the title, and avoid the use of abbreviations for single words. Each abbreviation should be defined in the text at first mention. If there are 3 or more abbreviations used in the text, prepare an abbreviation footnote. The footnote should be associated with the first abbreviated term in the text and should be an alphabetized listing of all author-defined abbreviations and their definitions. Note that group designations (for example, "CON" for control) should also be included in the abbreviation footnote. Standard units of measurement, chemical

compounds preceded by a digit, and the following standard abbreviations do not require definition: ADP, AIDS, AMP, ASN, ATP, AUC, BMI, BOLD, CDC, CFU, CoA, CTP, DHA, DMEM, DMSO, DNA, EDTA, eg, EGTA, ELISA, EPA, FAD, FAO, FMN, fMRI, GAPDH, GDP, GTP, HCl, HDL, HEPES, HIV, HOMA-IR, HPLC, ie, Ig, IL, LDL, In, LPS, MEM, MOPS, MRI, MUFA, *m/z*, NAD, NADH, NADP, NADPH, NHANES, NIH, PUFA, RNA, SDS-PAGE, SFA, TNF, tris, UDP, UNICEF, USDA, UTP, UV, VLDL, vol:vol, WHO, and wt:vol.

Abbreviations for statistical terms

analysis of covariance, ANCOVA
analysis of variance, ANOVA
coefficient of correlation, sample, *r*
coefficient of multiple correlation, *R*
coefficient of variation, CV
confidence interval, CI
degrees of freedom, df
hazard ratio, HR
interquartile range, IQR
not significant, NS
number of observations, *n*
odds ratio, OR
probability, *P*
risk ratio, RR
standard deviation, SD
standard error of the estimate, SEE
standard error of the mean, SEM
variance ratio, *F*

Metric units are required and the use of the International System of Units (SI units) is optional. For a comprehensive listing of SI conversion factors, consult *SI Units for Clinical Measurement* (Young DS, Huth EJ. Philadelphia: American College of Physicians, 1998) or Am J Clin Nutr 1998;67:166-81 or J Nutr 1990;120:20-35. Dosage forms and dietary ingredients may be expressed in gram or mole quantities. Energy may be expressed in kilocalories or joules; the conversion factor for converting kilocalories to kilojoules is 4.184. Do not report energy in Calories with a capital C; use kcal, MJ, or kJ instead. Temperatures should be reported in degrees Celsius. Blood pressures should be reported in millimeters of mercury. Use of katals to report enzyme activity is optional.

Commonly used approved abbreviations

Standard units of measurement

ampere, A	liter, L
becquerel, Bq	meter, m
coulomb, C	minute, min
curie, Ci	mole, mol
day, d	month, mo

Combining prefixes

tera- (10^{12}), T	micro- (10^{-6}), μ
giga- (10^{9}), G	nano- (10^{-9}), n
mega- (10^{6}), M	pico- (10^{-12}), p
kilo- (10^{3}), k	femto- (10^{-15}), f
milli- (10^{-3}), m	atto- (10^{-18}), a

degree Celsius, °C	ohm, Ω
farad, F	pascal, Pa
gram, g	second, s
hertz, Hz	sievert, Sv
hour, h	volt, V
joule, J	watt, W
katal, kat	week, wk
kelvin, K	year, y
kilocalorie, kcal	

Acceptable standard units	Unacceptable units
length: m, mm, μm	*length*: not acceptable: in, ft, yd, Å, mμ
area: m^2, mm^2, μm^2	*length*: not acceptable: sq in, in^2, μ^2
volume: L, mL, μL, pL	*volume*: not acceptable: pint, gallon, cc, ccm, λ, $\mu\mu$L
mass: kg, g, mg, μg, ng, pg	*mass*: not acceptable: oz, lb, gr, gm, gms, mgm, mgms, mgs
mass concentration: kg/L, g/L, mg/L, μg/L	*mass concentration*: not acceptable: mg %
substance concentration: mol/L, mmol/L, μmol/L, nmol/L	*substance concentration*: not acceptable: M, N

NOMENCLATURE

In general, the *AJCN* follows the nomenclature policies of the IUPAC–IUB Joint Commission on Biochemical Nomenclature. The vitamin nomenclature is summarized at J Nutr 1990;120:12–19, and the amino acid nomenclature is summarized at J Nutr 1987;117:15. Both articles can be accessed at http://jn.nutrition.org. Aurthors are responsible for ensuring that their terminology conforms with these policies. For guidelines on gene and protein nomenclature, authors should consult the following websites: http://www.informatics.jax.org/ (mouse), http://rgd.mcw.edu/ (rat), http://www.genenames.org (human and other species), and http://au.expasy.org/ (proteins).

As recommended by the American Society for Microbiology, the spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al. ed., ASM Press, Washington, DC 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *International Journal of Systematic Bacteriology*). Further information on currently approved bacterial names can be found at: Bacterial Nomenclature Up-to-Date and at List of Prokaryotic Names with Standing in Nomenclature. If authors must use a name that does not have standing nomenclature, the name should be enclosed in quotation marks in the title, when appropriate, and at its first use in the abstract and the text. Correspondingly, an appropriate statement concerning the nomenclaturla status of the name should be made in the text.

MICROBIOLOGICAL CULTURE DEPOSITION

The American Journal of Clinical Nutrition (AJCN)) expects authors to deposit microbial strains used in any study to be published in publicly accessible culture collections, for example, the American Type Culture Collection (ATCC) and to refer to the collectsion and strain numbers in the text (e.g. ATCC 53103). Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory strain designations and donor sources as well as original culture collection identification numbers. More information on the ATCC is accessible at http://www.lgcpromochem-atcc.com/.

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Initial manuscript submissions

Prepare your manuscript, including figure legends and tables, in Word format. Tables must be included in the text file; do not submit tables in separate files. Submit each figure in a separate file. Preferred formats for image (figure) files are PDF and EPS. Files must conform to the minimum-resolution specifications listed under Image resolution. If you wish to include OSM (*see* Supplemental material) with your submission, it should be clearly labeled and marked with an "Online Supplemental Material" header on each page. Online-only figures and tables should be labeled "Supplemental Figure 1," "Supplemental Table 1," etc.

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Submit manuscript text, including figure legends and tables, in a Word file; tables must be included in the text file; do not submit tables in separate files. Submit each figure in a separate file. Preferred formats for image (figure) files are PDF and EPS. Files must conform to the minimum-resolution specifications listed under Image resolution. Figures that are part of the regular manuscript submission and not part of OSM must be uploaded as separate files. OSM pages must be marked with an "Online Supplemental Material" header on each page. Online-only figures and tables should be labeled "Supplemental Figure 1," "Supplemental Table 1," etc. Upload the OSM in PDF format as supplemental file(s) in the upload area. OSM files will not be edited; therefore, please be sure that *The American Journal of Clinical Nutrition* format is used and that the files are accurate.

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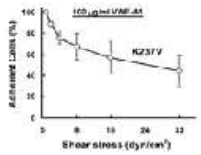
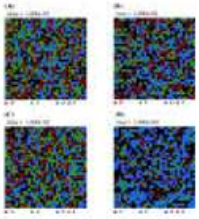
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2. If a figure is very small in the system-generated PDF file, the resolution of the figure file was not high enough. A higher resolution figure should be uploaded before the PDF is approved.
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