Universidade de São Paulo

Faculdade de Saúde Pública

Papel da lipoproteína de baixa densidade eletronegativa, da proteína transportadora de éster de colesterol e da resistência à insulina no risco cardiometabólico de adolescentes obesos

Ana Paula de Queiroz Mello

Tese apresentada ao Programa de Pós-Graduação em Nutrição em Saúde Pública para obtenção do título de Doutor em Ciências

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Orientadora: Prof[a]. Dr[a]. Nágila Raquel Teixeira Damasceno

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Se procurar bem você acaba encontrando.
Não a explicação (duvidosa) da vida,
Mas a poesia (inexplicável) da vida.

Carlos Drummond de Andrade

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Primeiramente a Deus, que sempre me acompanha e me ilumina.

Ao meu pai, Flávio (in memorian), e à Dora, minha mãe, pela dedicação, amor e paciência. Ao Flavinho pela sua simplicidade e alegria de viver.

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Resumo

Mello, APQ. **Papel da lipoproteína de baixa densidade eletronegativa, da proteína transportadora de éster de colesterol e da resistência à insulina no risco cardiometabólico de adolescentes obesos.** [Tese de Doutorado]. São Paulo: Faculdade de Saúde Pública da Universidade de São Paulo; 2011.

Introdução: A obesidade é um importante problema de Saúde Pública e, segundo a Organização Mundial da Saúde, representa uma epidemia global. É considerada uma doença crônica, multifatorial, que depende não só de fatores genéticos e fisiopatológicos, mas também de variáveis culturais, sociais e psicológicas associadas à quantidade e a qualidade da alimentação. Nesse contexto, os adolescentes como foco de mudanças fisiológicas, anatômicas e sociais se tornam um grupo com elevada frequência de fatores de risco associados à obesidade. **Objetivo:** Avaliar o papel da lipoproteína de baixa densidade eletronegativa [LDL(-)], da proteína transportadora de éster de colesterol (CETP) e da resistência à insulina (RI) no risco cardiometabólico de adolescentes. **Métodos:** Foram recrutados adolescentes de ambos os sexos, com faixa etária de 10 a 19 anos e, regularmente matriculados em escolas da cidade de São Paulo. Medidas antropométricas, tais como, peso, altura, circunferência da cintura (CC) e composição corporal foram avaliadas, e classificadas considerando sexo e idade. Após jejum (12-15h), foi coletada uma amostra de sangue e a partir do plasma foram realizadas as seguintes análises: glicose, insulina e cálculo do HOMA, perfil lipídico, apolipoproteína A-I e B, atividade da paraoxonase 1 (PON1), ácidos graxos livres (AGL), atividade da CETP e LDL(-). Os resultados obtidos foram analisados por meio dos testes qui-quadrado, Kappa, Kolmogorov-Smirnov, t-student, ANOVA, Mann-Whitney, Kruskal-Wallis, Bonferroni e de tendência linear, com valor de significância de $p < 0,05$. **Resultados:** Os adolescentes eutróficos apresentaram menor atividade de CETP e conteúdo de LDL(-) em relação àqueles com excesso de peso. O IMC apresentou associação positiva e linear com o CT/HDL-C, TG/HDL-C, ApoB/ApoA-I, LDL(-) e LDL(-)/CT. Perfil inverso foi observado para HDL-C e ApoA-I. A CC mostrou associação positiva com TG, CT/HDL-C, LDL-C/HDL-C, TG/HDL-C, ApoB/ApoA-I, LDL(-), LDL(-)/CT e CETP. Associação negativa foi observada entre CC e HDL-C e ApoA-I. Após o ajuste pela atividade da CETP, associações entre o HDL-C, LDL-C/HDL-C, CT/HDL-

C e LDL(-)/LDL-C com o IMC ou CC foram modificadas. Ao ajustarmos pela concentração de LDL(-), nenhuma associação sofreu alteração, o que sugere um mecanismo independente para a regulação dessa partícula durante a obesidade.Ao analisarmos os adolescentes segundo CC, verificamos que os adolescentes com CC_{ALTA} apresentaram elevado IMC, % de gordura corporal, pressão arterial sistólica, conteúdo de insulina, HOMA, TG, TG/HDL-C, CT/HDL-C, LDL-C/HDL-C, ApoB/ApoA-I, AGL, LDL(-), LDL(-)/CT e atividade da CETP, quando comparados aqueles com CC_{BAIXA}. Perfil inverso foi observado para % de massa magra, HDL-C, ApoA-I, HDL/ApoA-I e PON-1. Quando classificados segundo HOMA (resistente à insulina - RI e sensível à insulina - SI), os adolescentes RI mostraram um impacto negativo sobre o IMC, CC, % de gordura corporal, pressão arterial, TG e TG/HDL-C, e resultado inverso para % de massa magra e PON1. O índice de risco cardiovascular proposto mostrou que o grupo CC_{ALTA}-RI = CC_{ALTA}-SI > CC_{BAIXA}-RI = CC_{BAIXA}SI. **Conclusões:** Portanto, a concentração de LDL(-) e a atividade da CETP associada à obesidade, principalmente abdominal, alteram o risco cardiometabólico de adolescentes.

Palavras-chave: nutrição, obesidade, adolescentes, fator de risco cardiovascular, LDL eletronegativa, CETP, resistência à insulina.

Abstract

Mello, APQ. **Role of the electronegative low-density lipoprotein, cholesteryl ester transfer protein and insulin resistance in the cardiometabolic risk of the adolescents.** [Doctoral thesis]. São Paulo: Faculdade de Saúde Pública da Universidade de São Paulo; 2011.

Introduction: Obesity is a major public health problem and, according to World Health Organization, represents a global epidemic. It is considered a chronic, multifactorial disease, which depends not only of genetic and pathophysiology factors, but also of cultural, social and psychological associated with diet profile variables. In this context, adolescents as the focus of physiological, anatomical and social changes become a group with high frequency of risk factor for obesity. **Objective:** To evaluate the role of the electronegative low-density lipoprotein [LDL(-)] concentration, cholesteryl ester transfer protein (CETP) activity and insulin resistance on cardiometabolic risk of adolescents. **Methods:** We recruited adolescents of both sexes, aged 10 to 19 years and enrolled in schools in the city of São Paulo. Anthropometric measurements such as weight, height, waist circumference (WC) and body composition were evaluated and classified according to sex and age. After fasting (12-15h) was analyzed from plasma: glucose, insulin and HOMA, lipid profile, apolipoprotein A-I and B, paraoxonase 1 activity (PON1), non-esterified fatty acids (NEFA), CETP activity and LDL(-). The results were analyzed by chi-square, Kappa, Kolmogorov-Smirnov, t-student, ANOVA, Mann-Whitney, Kruskal-Wallis, Bonferroni and linear tendency test, with $p< 0.05$. **Results:** The subjects with normal weight had lower CETP activity and content of LDL(-) than excess weight adolescents. BMI showed positive and linear association with TC/HDL-C, TG/HDL-C, ApoB/ApoA-I, LDL(-) and LDL(-)/TC. Profile opposite was observed for HDL-C and ApoA-I. The WC was positively associated with TG, TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, ApoB/ApoA-I, LDL(-), LDL(-)/CT and CETP. Negative association was observed between WC and HDL-C and ApoA-I. After adjustment for CETP, associations between HDL-C, LDL-C/HDL-C, TC/HDL-C and LDL(-)/LDL-C with BMI or WC were modified. Adjustment for LDL(-) content was not able to change these associations, suggesting an independent mechanism for regulation of the levels of this particle during obesity. Analysis second WC, it was found that adolescents

with WC_{HIGH} showed higher BMI, % body fat, systolic blood pressure, insulin, HOMA, TG, TG/HDL-C, TC/HDL-C, LDL-C/HDL-C, ApoB/ApoA-I, NEFA, LDL(-), LDL(-)/TC and CETP activity than WC_{LOW} group. Profile opposite was observed for % lean body mass, HDL-C, ApoA-I, HDL/ApoA-I and PON-1. When it classified according to HOMA (insulin resistant – IR and insulin sensitive – IS), IR group showed a negative impact on BMI, WC, % body fat, blood pressure, TG and TG/HDL-C, and contrary result for % mass lean and PON1. The cardiovascular risk index propose showed that WC_{HIGH} – IR = WC_{HIGH} – IS > WC_{LOW} – IR = WC_{LOW} – IS group. **Conclusions:** Therefore, LDL(-) content and CETP activity associated with obesity, mainly abdominal, alter the cardiometabolic risk of adolescents.

Keywords: nutrition, obesity, adolescents, cardiovascular risk factor, electronegative LDL, CETP, insulin resistance.

Índice

Siglas Utilizadas

AGL	Ácidos graxos livres
ANOVA	Análise de variância
ApoA-I	Apolipoproteína A-I
ApoA-II	Apolipoproteína A-II
ApoB	Apolipoproteína B
ApoC-III	Apolipoproteína C-III
ApoE	Apolipoproteína E
BHT	2,6-di-tert-butil-p-hidroxitolueno
BIA	Impedância bioelétrica
CC	Circunferência da cintura
CETP	Proteína transportadora de éster de colesterol
COEP	Comitê de Ética em Pesquisa
CT	Colesterol total
DAC	Doença arterial coronariana
DM2	Diabetes *mellitus* tipo 2
EDTA	Ácido etileno-diaminotetraacético
ELISA	*Enzyme-linked immunosorbent assay*
FCF	Faculdade de Ciências Farmacêuticas
FSP	Faculdade de Saúde Pública
HDL	Lipoproteína de alta densidade
HOMA	*Homeostasis Model Assessment*
IBGE	Instituto Brasileiro de Geografia e Estatística
IDF	*International Diabetes Federation*
IDL	Lipoproteína de densidade intermediária
IL-18	Interleucina - 18
IL-6	Interleucina - 6
IMC	Índice de massa corporal
LDL	Lipoproteína de baixa densidade
LDL(-)	Lipoproteína de baixa densidade eletronegativa
LDLox	Lipoproteína de baixa densidade oxidada
MDA	Malonaldeído
NHANES	*National Health and Nutrition Examination Survey*
OMS	Organização Mundial da Saúde
OPAS	Organização Pan-americana de Saúde
PAF-AH	Fator ativador de plaquetas acetil-hidrolase
PBS	Tampão fosfato salina
PCR	Proteína C reativa
PMSF	Fluoreto de fenilmetilsulfonila
POF	Pesquisa de Orçamentos Familiares

PON1	Paraoxonase 1
R	Resistência
RI	Resistência à insulina
SBC	Sociedade Brasileira de Cardiologia
SBD	Sociedade Brasileira de Diabetes
SBH	Sociedade Brasileira de Hipertensão
SM	Síndrome metabólica
TCLE	Termo de Consentimento Livre e Esclarecido
TG	Triacilgliceróis
TMB	Tetrametilbenzina
TNF-α	Fator de necrose tumoral alfa
USP	Universidade de São Paulo
VIGITEL	Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico
VLDL	Lipoproteína de muito baixa densidade
Xc	Reactância

Introdução

1. INTRODUÇÃO

1.1 Aspectos Epidemiológicos da Obesidade

A obesidade é um importante problema de Saúde Pública e, segundo a Organização Mundial da Saúde (OMS), representa uma epidemia global (OMS, 2005). É considerada uma doença crônica, multifatorial, caracterizada pelo acúmulo excessivo de tecido adiposo no organismo. Nesse contexto, tanto o aporte calórico, quanto o gasto energético dependem não só de fatores genéticos e fisiológicos, mas também de variáveis culturais, sociais e psicológicas associadas à quantidade e a qualidade da alimentação (WAITZBERG, 2000).

A prevalência da obesidade tem aumentado nos últimos anos, e resulta de uma complexa interação entre genes, consumo alimentar, atividade física e outros fatores ambientais (BIRO *et al.*, 2010).

Atualmente, há aproximadamente 1,6 bilhões de adultos com excesso de peso em todo o mundo, e pelo menos 400 milhões desses, são obesos. Se considerarmos as crianças menores de cinco anos, estima-se que 20 milhões delas apresentam excesso de peso (OMS, 2010). Ao analisarmos os estudos realizados nos Estados Unidos, nos períodos de 1963-5 e 1988-91, a proporção de crianças entre 6-11 anos que eram obesas aumentou de 3,9% para 11,4% nos meninos e de 4,3 para 9,9% nas meninas (EDMUNDS, 2001). De acordo com estudos mais recentes, a prevalência de excesso de peso em crianças teve uma mudança drástica entre 1980 e 2006, aumentando de 5,5% para 16,3% (NGUYEN *et al.*, 2010). Além disso, os dados da *National Health and Nutrition Examination Survey* (NHANES) mostraram que no período entre 2003 e 2006, 31,9% das crianças americanas de 2 a 19 anos apresentavam excesso de peso e 16,3% obesidade (OGDEN *et al.*, 2008). Segundo *Bogalusa Heart Study*, a obesidade infantil triplicou o risco de hipercolesterolemia associada à lipoproteína de baixa densidade (LDL), aumentou o risco de hipertrigliceridemia em 7,1 vezes e de hiperinsulinemia em 12,6 vezes. Nesse mesmo estudo, verificou-se que, aproximadamente, 58% dos adolescentes de 11 a 17 anos com sobrepeso ou obesidade tinham no mínimo um fator de risco cardiovascular (FREEDMAN *et al.*, 1999).

No Brasil, segundo estudo VIGITEL (Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico), foi encontrado um

aumento na prevalência de excesso de peso, diabetes e hipertensão arterial sistêmica em adultos (Ministério da Saúde, 2010). Dados da Pesquisa de Orçamentos Familiares (POF) de 2008-2009 também mostraram que o excesso de peso foi diagnosticado em aproximadamente um quinto dos adolescentes, ultrapassando em seis vezes a frequência do déficit de peso; e a obesidade correspondeu a cerca de um quarto do total de casos de excesso de peso (Instituto Brasileiro de Geografia e Estatística – IBGE, 2010). VEIGA *et al.* (2004) verificaram que a prevalência de excesso de peso mais do que quadriplicou nos meninos (2,6 para 11,8%) e ultrapassou o dobro nas meninas (5,8 para 15,3%) no período de 1975 a 1997. Posteriormente, em estudo realizado com adolescentes entre 11 e 13 anos, SUÑÉ *et al.* (2007) descreveram prevalência de 24,8% de sobrepeso e obesidade em um município do interior do Rio Grande do Sul (Brasil).

Vários fatores são importantes na gênese da obesidade, como os genéticos, os fisiológicos e os metabólicos. Prematuridade, acúmulo de adiposidade na infância e desenvolvimento puberal precoce têm sido implicados no desenvolvimento da obesidade na adolescência (ADAIR, 2008). No entanto, os fatores que poderiam explicar o aumento no número de adolescentes obesos parecem estar mais relacionados às mudanças no estilo de vida e nos hábitos alimentares (BOUCHARD, 2009). O aumento no consumo de alimentos ricos em açúcares simples, lipídeos e álcool, resultando num comportamento alimentar insalubre e o sedentarismo são os principais fatores relacionados ao meio ambiente que contribuem para o aumento da prevalência de sobrepeso e obesidade (RODRÍGUEZ-MARTÍN *et al.*, 2009). No estudo de YANNAKOULIA *et al.* (2010) verificou-se que a obesidade infantil foi inversamente associada com o consumo de verduras e com o hábito de jantar, confirmando a influência do meio ambiente sobre o desenvolvimento do excesso de peso.

Sabe-se que a obesidade na infância e adolescência tende a persistir na fase adulta se não for corretamente controlada, levando ao aumento da morbimortalidade e diminuição da expectativa de vida (MUST, 1996; VIRDIS *et al.*, 2009; OMS, 2010). Várias doenças estão associadas com a obesidade, tais como, as cardiovasculares, pulmonares, gastrointestinais, dermatológicas, neurológicas, musculoesqueléticas, metabólicas, câncer e psiquiátricas (BARLOW, 2007; CATENACCI *et al.*, 2009; BIRO *et al.*, 2010). De acordo com HASLAM *et al.* (2010), a prevalência de obesidade em crianças e adolescentes tem associação direta com risco de

desenvolver diabetes *mellitus* tipo 2 (DM2) na fase adulta. Esse fato tem sido foco de diversos estudos, pois a associação da obesidade com alterações metabólicas, tais como dislipidemias, hipertensão, intolerância à glicose e DM2, considerados fatores de risco para as doenças cardiovasculares, vem sendo detectada em segmentos cada vez mais jovens da população (CAMPOS *et al.*, 2004; VIRDIS *et al.*, 2009; FALASCHETTI *et al.*, 2010).

Segundo THOMPSON *et al.* (2007), a incidência de excesso de peso foi maior na faixa etária de 9 a 12 anos, do que em adolescentes com idade superior. Esse período coincide com a fase de maturação sexual, onde há uma rápida deposição de gordura, além de ser um período vulnerável para o aumento expressivo da chance de desenvolver a obesidade e suas co-morbidades. Esses mesmos autores verificaram que meninas que apresentavam excesso de peso tinham de 3 a 10 vezes mais risco de hipertensão arterial sistêmica, de redução na concentração de colesterol na lipoproteína de alta densidade (HDL-C) e de aumento nos triacilgliceróis (TG) plasmáticos; além de apresentarem o triplo do risco de terem concentração elevada de colesterol associado à LDL (LDL-C), quando comparadas às eutróficas. Além disso, MUST *et al.* (1992) sugeriram previamente que o tempo de duração da obesidade está diretamente associado à morbimortalidade por doenças cardiovasculares.

Considerando a elevada prevalência da obesidade e o desenvolvimento precoce de enfermidades relacionadas, tem sido observado que as consequências da obesidade têm relação direta e indireta nos custos hospitalares e influenciam negativamente as políticas de Saúde Pública. Esse fato torna-se ainda mais importante quando analisamos o aumento da obesidade nos grupos populacionais com nível socioeconômico mais baixo (Organização Pan-americana de Saúde – OPAS, 2000).

Portanto, considerando o impacto epidemiológico da obesidade na população em geral e, particularmente, nos adolescentes, torna-se importante avaliar os aspectos metabólicos associados a essa doença.

1.2 Aspectos Metabólicos da Obesidade

Segundo FALASCHETTI *et al.* (2010), há correlações positivas entre o índice de massa corporal (IMC), a circunferência da cintura (CC) e a porcentagem de gordura corporal com a concentração de colesterol total, triacilgliceróis, LDL-C e

apolipoproteína B. Ainda de acordo com esses autores, crianças obesas apresentam mais que o triplo de risco de hipertrigliceridemia e um risco sete vezes maior de apresentar baixa concentração plasmática de colesterol associado à HDL, quando comparadas às eutróficas.

Além do impacto direto da obesidade sobre o perfil lipídico, vários estudos encontraram associação do excesso de peso com a concentração de LDLox em adultos (LINNA *et al.*, 2007; BECK *et al.*, 2008; NJAJOU *et al.*, 2009) e crianças (KELISHADI *et al.*, 2008; KELLY *et al.*, 2010). Lipoproteínas modificadas por oxidação induzem o recrutamento de monócitos, diferenciação em macrófagos, proliferação de células musculares lisas, formação de células espumosas e acúmulo excessivo de lipídeos na parede arterial, que amplificam e mantêm os sinais pró-inflamatórios presentes na aterosclerose (FROSTEGARD *et al.*, 1990; BERLINER *et al.*, 1995; ROSS, 1999; GOTTLIEB *et al.*, 2005; YOSHIDA *et al.*, 2010).

Dentre as diversas formas de LDL modificada que tem sido monitorada, a participação da **lipoproteína de baixa densidade eletronegativa [LDL(-)]** tem sido foco de vários estudos. De acordo com SÁNCHEZ-QUESADA *et al.* (2004), a origem da LDL(-) na circulação pode não ser somente através de alterações oxidativas, mas também através da glicosilação não enzimática; pela ação do fator de ativação de plaquetas acetil-hidrolase (PAF-AH); alterações no catabolismo das lipoproteínas, promovendo aumento das ApoE e ApoC-III; enriquecimento de ácidos graxos livres (AGL) no plasma ou reação cruzada com hemoglobina e, possivelmente, outros mecanismos não identificados. Independente das vias de origem, diversos estudos na literatura propõem que a partícula de LDL(-), gerada tem propriedades pró-inflamatórias e pró-aterogênicas (SEVANIAN *et al.*, 1999; HULTHE e FAGERBERG, 2002; SÁNCHEZ-QUESADA *et al.*, 2004; SIQUEIRA *et al.*, 2006; DAMASCENO *et al.*, 2006). Em estudo recente, a LDL(-) apresentou associação positiva com o colesterol total e LDL-C e, negativa com HDL-C (MELLO *et al.*, 2010). Portanto, a LDL(-) tem potenciais características para ser um importante biomarcador para doença arterial coronária (DAC) (LOBO *et al.*, 2008; MELLO *et al.*, 2011). De acordo com HOPPS *et al.* (2010), estudos utilizando parâmetros de estresse oxidativo plasmático poderão contribuir na identificação de fatores de riscos associados à síndrome metabólica (SM) e DAC.

Esta partícula modificada apresenta em sua composição maior concentração de triacilgliceróis e AGL em comparação à LDL nativa, isto é, não modificada. O

enriquecimento da LDL(-) com AGL contribui para aumentar a aterogenicidade desta partícula (BLANCO *et al.*, 2010).

Os AGL participam da produção de citocinas, fatores de crescimento e ativação de células relacionadas à aterogênese, podem estar envolvidos na atividade pró-inflamatória da LDL(-) (CARLSSON *et al.*, 2000; BENÍTEZ *et al.*, 2002; BENÍTEZ *et al.*, 2004; BANCELLS *et al.*, 2010). Na LDL(-), o acúmulo de AGL pode ser devido à hidrólise enzimática de triacilgliceróis, colesterol esterificado ou fosfolipídios (BENÍTEZ *et al.*, 2004; BICKERTON *et al.*, 2007), diminuindo a resistência à oxidação e aumentando a carga negativa da LDL (BRASCHI *et al.*, 1997; BENÍTEZ *et al.*, 2002). KAMEI *et al.* (2005) observaram que estilo de vida ocidental proporcionou aumento da geração de AGL e secreção de insulina após sobrecarga de glicose em japoneses que viviam nos Estados Unidos.

Além das modificações descritas acima, na obesidade o desenvolvimento da **resistência à insulina (RI)** contribui para o aumento da lipólise e, consequentemente, aumento dos AGL (ZHANG *et al.*, 2008; FABBRINI *et al.*, 2009). Segundo KARPE *et al.* (2005), aproximadamente 20-50% dos AGL do plasma são provenientes do tecido adiposo visceral. A hipercolesterolemia e o elevado *turnover* dos AGL são as alterações primárias do metabolismo lipídico em obesos, e a associação do aumento da lipólise com a redução do *clearence* plasmático lipídico favorece a manutenção da dislipidemia (ZHANG *et al.*, 2008). A taxa de lipólise pode aumentar de 20-30% em obesos comparados com eutróficos (HOROWITZ *et al.*, 2000). De acordo com BUTTE *et al.* (2007), crianças com excesso de peso apresentaram maiores concentrações de glicose, triacilgliceróis, AGL e insulina do que crianças eutróficas. Portanto, a resistência à insulina está associada à alteração do metabolismo de lipídeos, principalmente, aumento da concentração de AGL e triacilgliceróis.

Considerando os aspectos citados acima, a obesidade se confirma como um elemento chave na etiologia da SM, pois influencia direta ou indiretamente todos os componentes associados (resistência à insulina, estados pró-inflamatórios e trombóticos e alterações no metabolismo lipídico, incluindo aumento de triacilgliceróis, diminuição de HDL-C, aumento de partículas de LDL pequenas e densas e AGL) (GERTOW *et al.*, 2006).

Nesse contexto, a **proteína transportadora de éster de colesterol (CETP)** pode representar um importante indicador da homeostase lipídica, pois sua atividade

e concentração apresentam forte associação com as modificações nos triacilgliceróis, LDL-C e HDL-C (QUINTÃO & CAZITA, 2010). A CETP é uma glicoproteína plasmática com característica hidrofóbica, sintetizada no fígado, tecido adiposo, baço e nos macrófagos. A CETP circula no sangue, predominantemente ligada á HDL, e é responsável pela transferência de éster de colesterol da HDL para as lipoproteínas ricas em apolipoproteína B (ApoB) em troca de triacilgliceróis, promovendo assim a transformação da HDL2 em HDL3. A inibição da atividade da CETP produz um aumento da concentração de HDL-C devido ao retardo do catabolismo das ApoA-I e ApoA-II, desta forma, exercendo importante ação moduladora no risco cardiovascular (IKEWAKI *et al.*, 1993; TALL, 1993; RADEAU, 1998; BARTER, 2003).

A atividade da CETP apresenta importante papel na distribuição de éster de colesterol entre HDLs e LDLs, contribuindo de diversas maneiras para um fenótipo lipídico mais aterogênico, tais como, aumentando o conteúdo de LDLox e ésteres de colesterol na lipoproteína de muito alta densidade (VLDL), interagindo com a lipase lipoproteica na formação de LDL pequena e densa e formando partículas de HDL menores (RYE *et al.*, 1999; BARTER *et al.*, 2003; QUINTÃO & CAZITA, 2010). Avaliando a participação da CETP no risco cardiovascular, SANDHOFER *et al.* (2006) mostraram correlação inversa da concentração de CETP com o diâmetro da LDL e com a concentração de HDL-C. As partículas de LDL pequenas e densas (Fenótipo B) migram de maneira mais fácil para o espaço sub-endotelial, são menos resistentes à oxidação, têm menor afinidade pelo receptor B/E e menor concentração de antioxidantes em comparação às partículas de LDL maiores (Fenótipo A) (SEVANIAN *et al.*, 1996; RIZZO *et al.*, 2009). Recentemente, algumas dessas observações foram confirmadas por nosso grupo de pesquisa, ao evidenciar que o conteúdo de LDL(-) se encontra, prioritariamente, associado às partículas de LDL menores e mais densas (MELLO, 2007; MELLO *et al.*, 2010). Neste contexto, a CETP confirma seu efeito pró-aterogênico.

A CETP encontra-se aumentada em situações pró-aterogênicas, tais como, dislipidemia, obesidade e surgimento de aterosclerose prematura, além de correlacionar-se com marcadores pró-inflamatórios relacionados com DAC (QUINTÃO & CAZITA, 2010).

Diversos estudos mostram que polimorfismos no gene *TaqIB*, com a presença do alelo B2 (B1B2 ou B2B2), favorecem a diminuição da atividade da CETP que

promove o aumento da HDL-C e diminuição da concentração de insulina e HOMA (*homeostasis model assessment*) (THU *et al.*, 2005; SMART *et al.*, 2010; LÓPEZ-RÍOS *et al.*, 2011). De modo contrário, a atividade aumentada da CETP pode estar relacionada à obesidade, resistência à insulina e dislipidemia (HAYASHIBE *et al.*, 1997; BOEKHOLDT *et al.*, 2004). Sugere-se que a diminuição do tecido adiposo favoreça a redução da CETP (GREAVES *et al.*, 2003; LAIMER *et al.*, 2009; ASZTALOS *et al.*, 2010), estando a redução da concentração e da atividade da CETP associada a um perfil lipídico menos aterogênico, com partículas de LDL maiores aumentadas (EBENBICHLER, 2002, SANDHOFER *et al.*, 2008). Além disso, na hipertrigliceridemia, a elevada atividade da CETP proporciona a formação de LDL pequena e densa e HDL pequena e densa, ambas envolvidas na progressão da aterosclerose (KOLOVOU, 2009).

Em estudo realizado com crianças e adolescentes obesos de 5 a 14 anos, ASAYAMA *et al.* (2002) verificaram que a concentração da CETP foi inversamente relacionada com o colesterol associado à HDL. Nesse mesmo estudo, verificou-se que a redução do peso após intervenção nutricional promoveu diminuição na concentração da CETP, embora não tenha havido alterações no perfil lipídico dos indivíduos. Em estudo mais recente, também em adolescentes, foi observado que mutações genéticas associadas com a CETP promovem aumento da HDL-C e diminuição da relação colesterol total (CT) e HDL-C (SMART *et al.*, 2010).

De acordo com GREAVES *et al.* (2003), a atividade da CETP no plasma apresentou correlação negativa com HDL-C e positiva com colesterol total, LDL-C, CT/HDL-C, triacilgliceróis plasmáticos e susceptibilidade oxidativa da LDL. Observou-se ainda que a gordura abdominal apresenta correlação positiva com a concentração plasmática de CETP.

Segundo SANDHOFER *et al.* (2006), a concentração de CETP mostrou correlação negativa com o tamanho da LDL, independentemente do sexo, e com a concentração de HDL-C em indivíduos do sexo masculino. De acordo com esses autores, a diferença fisiológica na adiposidade entre homens e mulheres justificaria as distintas concentrações de CETP, pois essa é expressa, principalmente, no tecido adiposo subcutâneo. Portanto, sugere-se que apesar da concentração de CETP mostrar correlação negativa com a idade, apresentando menor conteúdo no período pós-menopausa, do que no pré-menopausa (ZHANG *et al.*, 2001), essa é um importante fator no processo aterogênico associado ou não à obesidade (INAZU *et*

al., 2000; ISHIKAWA *et al.*, 2001). Esse fato reforça a noção da complexidade do processo aterosclerótico e a importância da participação de outros componentes lipídicos.

As manifestações clínicas da aterosclerose, em geral, se iniciam a partir da meia-idade, entretanto, sabe-se que o processo aterosclerótico começa na infância (THOMPSON *et al.*, 2007). O ritmo de progressão está relacionado com a presença dos fatores de risco clássicos identificados no adulto, tais como obesidade, tabagismo, hipertensão arterial sistêmica, diabetes *mellitus*, história familiar, idade e nível de atividade física (MCGILL *et al.*, 2000). Portanto, avaliar o conteúdo de LDL(-), a atividade da CETP e a resistência à insulina em adolescentes obesos podem evidenciar, de modo precoce, o risco cardiometabólico dessa população. Destaca-se ainda, a possível influência dessas variáveis sobre a modulação de fatores de risco cardiovascular rotineiramente avaliados em adultos.

Hipótese

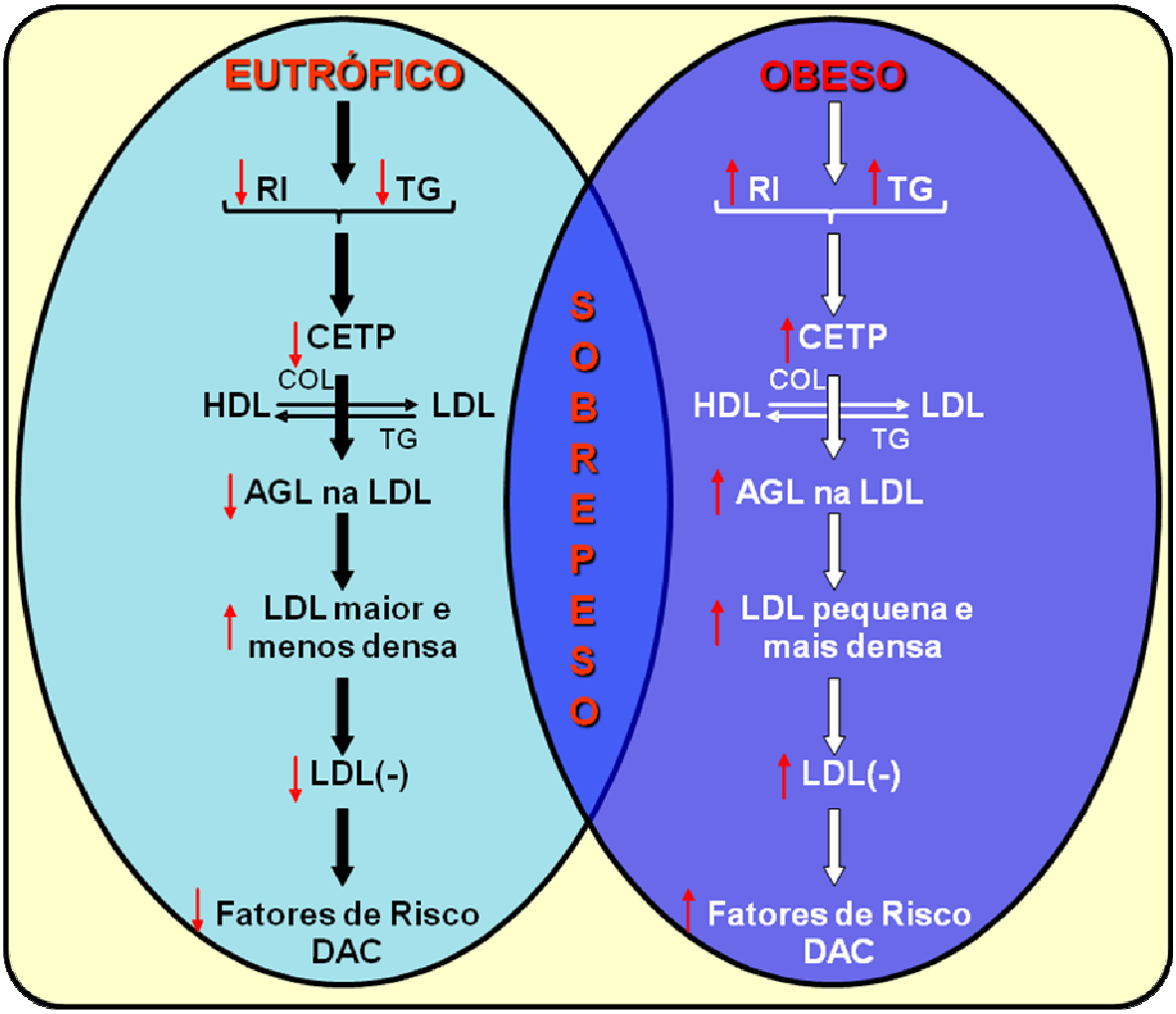
2. HIPÓTESE

Considerando os aspectos apresentados acima, este estudo propõe identificar componentes relacionados ao perfil nutricional associados direta e indiretamente à LDL(-), a CETP e a resistência à insulina em adolescentes. Tais componentes poderiam modificar de modo positivo ou não esses marcadores bioquímicos, modulando os fatores de risco cardiometabólicos nessa população. Além desses aspectos, reforça nosso estudo o fato de ser o primeiro a avaliar simultaneamente a relação da obesidade com a LDL(-), a CETP e a resistência à insulina em adolescentes.

De acordo com a **Figura 1**, o excesso de peso estaria associado à resistência à insulina, favorecendo o aumento na atividade da CETP. Esse fato contribuiria para um menor *clearance* de colesterol, com consequente aumento das partículas ricas em ApoB (VLDL, IDL e LDL). De modo simultâneo, esse perfil também favoreceria o aumento de AGL, diretamente relacionados à redução da carga positiva da LDL e a geração de LDL(-).

Nesse contexto, sugere-se que o perfil nutricional é o determinante central das modificações bioquímicas. Portanto, é provável que adolescentes com sobrepeso e obesidade tenham maior frequência de fatores de risco cardiovascular associado ao metabolismo lipídico e oxidativo comparado aos adolescentes eutróficos, e que modificações na CETP e na LDL(-) aumentam o risco cardiometabólico dos adolescentes com excesso de peso.

Figura 1: Influência da lipoproteína de baixa densidade eletronegativa, proteína transportadora de éster de colesterol e da resistência à insulina em parâmetros cardiometabólicos de adolescentes eutróficos e com excesso de peso.



Objetivos

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o papel da lipoproteína de baixa densidade eletronegativa, da proteína transportadora de éster de colesterol e da resistência à insulina no risco cardiometabólico de adolescentes.

3.2 Objetivos Específicos

Para a consecução do objetivo geral, os seguintes objetivos específicos foram propostos:

- Caracterizar a amostra quanto aos aspectos clínicos;
- Avaliar o estado nutricional dos adolescentes, através do índice de massa corporal, circunferência da cintura e composição corporal;
- Analisar a concentração de glicose e insulina plasmática, e resistência à insulina;
- Avaliar colesterol e triacilgliceróis no plasma e colesterol nas lipoproteínas (LDL e HDL);
- Determinar o conteúdo de apolipoproteína A-I e B no plasma;
- Determinar a atividade da paraoxonase 1 no plasma;
- Determinar a concentração de ácidos graxos livres no plasma;
- Determinar a atividade da proteína transportadora de ésteres de colesterol no plasma;
- Avaliar a concentração de lipoproteína de baixa densidade eletronegativa no plasma.

Metodologia

4. METODOLOGIA

4.1 Casuística

A amostra do estudo foi composta por adolescentes de ambos os sexos, com idade entre 10 – 19 anos (OMS, 1995), distribuída em três grupos: **Eutrófico** - composto por adolescentes eutróficos; **Sobrepeso** - formado por adolescentes com risco de obesidade e **Obeso** - formado por adolescentes com obesidade, segundo COLE *et al.* (2000).

Os participantes elegíveis foram selecionados em escolas públicas localizadas na zona centro-oeste da cidade de São Paulo - SP. A seleção das escolas e dos adolescentes foi realizada por meio de sorteio aleatório.

A partir das escolas públicas de ensino fundamental e médio localizadas na zona centro-oeste da cidade de São Paulo (n= 92), foram sorteadas cinco escolas. Após reunião com a Direção da unidade escolar, onde o projeto foi apresentado e discutido, cada escola ficou livre para decidir participar ou não da pesquisa. A região alvo (zona centro-oeste) foi selecionada devido aos fatores logísticos associados, tais como localização próxima à Faculdade de Saúde Pública (FSP/USP) e facilidade no transporte de amostras.

Após a definição das escolas, foram coletadas informações, tais como, sexo, data de nascimento e medidas de peso e altura de todos os adolescentes que aceitaram participar da triagem inicial. Com essas informações, foram sorteados adolescentes eutróficos, com sobrepeso e obesos, de acordo com o estado nutricional preconizado por COLE *et al.* (2000).

Todos os participantes e os responsáveis pelos adolescentes passaram pelo processo de esclarecimento, e após assinatura do Termo de Consentimento Livre e Esclarecido (TCLE), conforme **Anexo 1**, se realizou a coleta de dados. Todos os procedimentos de obtenção de amostras, análises e divulgação dos resultados seguiram as normas do Conselho Nacional de Saúde, no que se refere à ética em pesquisa com seres humanos (BRASIL, 1999).

4.2 Cálculo Amostral

O tamanho da amostra foi determinado para um estudo aleatorizado, utilizando 3 fatores: faixa etária (10 níveis: 10 – 19 anos), sexo (2 níveis: masculino e

feminino) e grupo (3 níveis: eutrófico, sobrepeso e obeso). Para detectar uma diferença mínima entre os valores médios dos extratos em torno de 3 unidades e para determinar o poder mínimo de 80% e nível de significância de $p < 0,05$, o tamanho da amostra encontrado foi de 6 indivíduos para cada extrato (combinação de níveis e fatores). Considerando todos os fatores, encontramos um total de 144, isto é, no mínimo, 48 adolescentes por grupo. A esse valor acrescentou-se 20% para possíveis perdas. Portanto, cada grupo deveria ser formado, no mínimo, por 58 adolescentes.

O cálculo do tamanho da amostra foi determinado por meio do programa Power Analysis Sample Size® (NCSS Statistical Software, 2005).

4.3 Delineamento Clínico do Estudo

Este estudo é do tipo caso-controle e inclui as seguintes etapas de coleta de dados:

ETAPA I – Identificação de adolescentes com características de interesse para o presente estudo. Entrega do TCLE, verificação da aceitação dos pais ou responsáveis e agendamento para a ETAPA II.

ETAPA II – Avaliação clínica, antropométrica e coleta de sangue.

ETAPA III – Entrega dos resultados referentes à avaliação nutricional e perfil lipídico (total e frações), assim, como orientações específicas (sugestão para procurar um profissional médico e/ou nutricionista) quando estes resultados indicaram risco à saúde destes indivíduos.

4.4 Critérios de Inclusão

Foram incluídos no estudo adolescentes de ambos os sexos, que apresentaram o Termo de Consentimento Livre e Esclarecido devidamente preenchido e assinado pelos pais ou responsáveis, e com as seguintes características:

- Idade entre 10 – 19 anos;
- Matriculados em escola pública;
- Clinicamente saudável;
- Não fumar;

- Sem uso frequente de álcool [utilizamos os valores estabelecidos para adultos: consumo ≤ 30,0 g de etanol/dia para homens e ≤ 15,0 g de etanol/dia para mulheres, segundo V Diretrizes Brasileira de Hipertensão da Sociedade Brasileira de Hipertensão (SBH, 2010)];
- Sem uso de medicamentos moduladores do metabolismo lipídico, antioxidantes e hormônios há pelo menos 2 meses.

4.5 Critérios de Não Inclusão

Adolescentes que preencheram algum dos critérios abaixo não participaram do estudo:

- Adolescentes desnutridos;
- Adolescentes grávidas ou lactantes;
- Presença de doenças crônicas, tais como, diabetes, neuropatias, endocrinopatias, nefropatias, doenças pulmonares, cardiopatias, doenças reumatológicas, imunodeficiência congênita ou adquirida, além de síndrome de *Cushing* e hipotiroidismo auto relatadas;
- Adolescentes que participam de outros protocolos de pesquisa.

4.6 Avaliação Sócio-Econômica, Cultural e Clínica

O perfil sócio-econômico dos adolescentes foi avaliado através de um questionário previamente estruturado, e que abordou os itens sexo, idade, raça, renda familiar e número de indivíduos que moram na casa, além do nível de escolaridade do adolescente e da família (pai e mãe). A avaliação clínica foi constituída pelas informações sobre história clínica atual, antecedentes familiares de doenças crônicas (pai e mãe), uso regular de medicamentos e/ou vitaminas, prática de atividade física relatada pelo adolescente ou responsável, além da aferição da pressão arterial sistêmica através de esfigmomanômetro (**Anexo 2**).

4.7 Avaliação Antropométrica

A avaliação dos parâmetros antropométricos foi feita através da coleta de peso (Kg), altura (m), CC (cm) e dados de composição corporal (porcentuais de massa gorda e massa magra).

O peso foi determinado com o adolescente utilizando o mínimo de roupa possível e descalço, através da balança digital Control II® (Plenna, São Paulo, Brasil) com limite de capacidade de 150,0 Kg e precisão de 100,0 g. A medida de altura foi determinada através do estadiômetro portátil Alturaexata® (TBW, São Paulo, Brasil) com limite de 2,1 m e precisão de 1,0 mm, onde o indivíduo permaneceu descalço, em posição ereta, com os pés paralelos, calcanhares, panturrilha, glúteos, ombros e cabeça encostados no estadiômetro, com a cabeça sob o plano horizontal de Frankfurt (LOHMAN *et al.*, 1988). Esses dados foram utilizados para cálculo do IMC, definido como peso em quilos divido pela estatura em metros quadrados (kg/m^2). O valor do IMC foi classificado de acordo com COLE *et al.* (2000), considerando sexo e idade, para a avaliação do estado nutricional.

Utilizando uma fita inelástica, flexível, com precisão 1,0 mm (TBW, São Paulo, Brasil), coletamos a medida da CC, adotando-se como referencial anatômico o ponto médio entre a última costela e a crista ilíaca. O indivíduo foi orientado a permanecer com o abdômen relaxado, ficar em pé, manter os braços paralelos ao corpo e pés unidos. Os valores de CC foram classificados, segundo FERNÁNDEZ *et al.* (2004) para indivíduos com até 18 anos, e para aqueles com idade superior consideramos os valores propostos pela IDF (*International Diabetes Federation*) (IDF, 2006).

Para a realização da impedância bioelétrica (BIA), utilizamos o aparelho tetrapolar Biodynamics®, modelo 450 (TBW, São Paulo, Brasil), através do qual foi aplicada uma corrente de 800 µA a uma frequência de 50 KHz. As medidas de impedância bioelétrica foram tomadas do lado direito do indivíduo, que foi orientado a manter-se deitado em posição supina, com os braços em ângulo de 30° em relação ao seu corpo e as pernas sem contato entre si. Objetos metálicos, calçados e meias foram retirados e, durante o teste, o indivíduo permaneceu imóvel. O equipamento apresentou os valores brutos de resistência (R, Ω), reactância (Xc, Ω) e ângulo de fase (°) de cada indivíduo.

O cálculo do porcentual de gordura corporal (%) e do porcentual de massa magra (%) foi determinado através do programa Biodynamics® (TBW, São Paulo, Brasil), onde consideramos sexo, idade, peso, altura, nível de atividade física, R e Xc. Os valores obtidos para porcentagem de gordura corporal foram classificados de acordo com os parâmetros estabelecidos por RODRÍGUEZ (2004).

4.8 Avaliação da Maturação Sexual

A maturação sexual foi analisada individualmente, por meio da auto-avaliação, seguindo os critérios de estadiamento puberal propostos por MARSHALL & TANNER (1970) e MARSHALL (1969). Foram fornecidas aos meninos figuras com diferentes graus de pilosidade pubiana e desenvolvimento genital (Anexo 3). Para as meninas foram apresentadas figuras que mostravam diferentes graus de desenvolvimento mamário, além de figuras com diferentes graus de pilosidade pubiana (Anexo 3). O adolescente realizou uma auto-classificação ao analisar as figuras, e anotou em um formulário o código correspondente ao seu estádio puberal, as meninas também anotaram a idade da menarca.

A classificação da maturação sexual como pré-púbere e púbere, para cada sexo, foi realizada segundo a OMS (1995), conforme quadro abaixo (**Quadro 1**).

Quadro 1: Classificação da Maturação Sexual.

Sexo	Estádio de Tanner	Classificação
Masculino	Genitália no estádio 1 ou 2	Pré-púbere
	Genitália a partir do estádio 3	Púbere
Feminino	Mamas no estádio 1	Pré-púbere
	Mamas a partir do estádio 2	Púbere

Fonte: Adaptada da OMS (1995).

4.9 Avaliação Bioquímica

4.9.1 Obtenção de sangue e lipoproteínas

Após um jejum de 12-15 horas foi coletada uma amostra de sangue (20,0 ml). O sangue foi coletado em tubos *vacutainer* contendo ácido etileno-diaminotetraacético-EDTA (1,0 mg/ml) (BD, Brasil), utilizado como anticoagulante e antioxidante. Os tubos foram refrigerados e protegidos da luz até a obtenção do plasma. Ao plasma (3000 rpm, 10 min, a 4°C) acrescentamos os seguintes inibidores de proteases: aprotinina (10,0 µg/ml), benzamidina (10,0 µM) e fluoreto de fenilmetilsulfonila-PMSF (5,0 µM), além do antioxidante 2,6-di-tert-butil-p-hidroxitolueno-BHT (100,0 µM).

Os ensaios bioquímicos realizados neste estudo foram testados quanto a sua precisão, devendo apresentar coeficiente de variação intra- e interensaio de no máximo 15% (SHAH *et al.*, 2000).

4.9.2 Detecção da concentração de Glicose e Insulina

A determinação da glicose plasmática foi realizada através do kit comercial, enzimático e colorimétrico Glicose PAP Liquiform® (Labtest, Minas Gerais, Brasil), sendo os resultados classificados, segundo as Diretrizes da Sociedade Brasileira de Diabetes - SBD (2009).

A detecção da insulina plasmática foi realizada por meio da técnica de radioimunoensaio utilizando o kit comercial Human Insulin-Specific RIA Kit® (Linco Research, St Charles, MO, USA). Essa etapa foi terceirizada junto ao Laboratório da empresa Gênese Produtos e Diagnósticos®.

A resistência à insulina foi calculada pelo índice HOMA (MATTHEWS *et al.*, 1985), onde:

$$\text{HOMA} = \frac{[\text{insulina de jejum } (\mu U/ml) \times \text{glicemia de jejum } (mmol/l)]}{22{,}5}$$

4.9.3 Definição do Perfil Lipídico e das Apolipoproteínas

Através da aplicação manual de reagentes enzimáticos, foram analisadas as concentrações de colesterol e triacilgliceróis no plasma e colesterol na HDL. Para a determinação das concentrações de colesterol total e associado à HDL foram utilizados os Kits Colesterol Liquiform® (Labtest, Minas Gerais, Brasil) e Colesterol HDL® (Labtest, Minas Gerais, Brasil). Para a determinação de triacilgliceróis plasmáticos utilizamos o Kit Triglicérides Liquiform® (Labtest, Minas Gerais, Brasil). O conteúdo de colesterol associado à LDL foi determinado por meio da fórmula de FRIEDEWALD (1972), onde: LDL-C= CT – HDL-C – (TG/5). Essa fórmula só foi aplicada para os indivíduos com TG< 400,0 mg/dl.

Para classificação dos adolescentes, segundo o perfil lipídico, foram utilizados os valores de referência propostos pela I Diretriz de Prevenção da Aterosclerose na Infância e na Adolescência (Sociedade Brasileira de Cardiologia - SBC, 2005) (**Quadro 2**).

Quadro 2: Valores de referência lipídica propostos para a faixa etária de 2 a 19 anos.

Lipídeos	Desejáveis (mg/dl)	Limítrofes (mg/dl)	Aumentados (mg/dl)
CT	<150	150-169	≥170
LDL-C	<100	100-129	≥130
HDL-C	≥45	-	-
TG	<100	100-129	≥130

Fonte: SBC (2005).

Além da análise da concentração de colesterol total no plasma e nas lipoproteínas isoladas, também verificamos as relações entre estas. O índice de Castelli I foi determinado pela razão entre o colesterol total e o HDL-C e o índice de Castelli II pela razão entre LDL-C e HDL-C (CASTELLI *et al.*, 1983).

Com a finalidade de melhorarmos a quantificação das lipoproteínas aterogênicas circulantes, avaliamos o colesterol não associado à HDL. Esta variável foi baseada na subtração de HDL-C do colesterol total. Avaliamos ainda a relação dos TG com o HDL-C.

As apolipoproteínas A-I e B foram detectadas no soro, para tanto, alíquotas de sangue foram coletadas em tubo seco, sendo o soro obtido por completa retração do coágulo (após 30 min) e por centrifugação (3.000 rpm, 10 min, 4℃). A técnica utilizada para detecção das apolipoproteínas foi a turbidimetria. A amostra combinada com um anticorpo específico presente no reagente formou um complexo insolúvel que foi medido no sistema automatizado Cobas Mira Plus CC® (Roche Diagnostics, Mannheim, Alemanha), à 340nm. Para esta análise utilizamos os kits comerciais: Apolipoproteína A-I (LP2116), Apolipoproteína B (LP2117), Controle Lipide Nível 2 (LE2669) e Calibrador de Apolipoproteina (LP3023), todos da marca RANDOX® (Co, Antrim, Reino Unido). Os resultados foram expressos em mg/dl.

As concentrações de HDL-C e LDL-C foram expressas sob a forma bruta (mg/dl) e ajustadas pelo conteúdo de ApoA-I e ApoB, respectivamente.

4.9.4 Determinação da atividade da PON1

A atividade da PON1 foi determinada pela adição de 500 µl de tampão Tris-HCl (0,1M, pH 8,05), contendo 2 mmol/l de $CaCl_2$ e 1,1 mmol/l de paraoxon (Sigma Chemical Co.) em 25 µl de soro. Em seguida, foi distribuído 200 µl da solução em placas de fundo plano contendo 96 poços. A leitura foi feita em duplicata utilizando o leitor de microplacas (Bio-Rad®, Benchmark, Microplate Reader) com comprimento de onda de 405 nm, a 37°C. Para o cálculo da atividade, foram feitas 6 leituras em intervalos de um minuto cada. A atividade foi expressa em nmol/min/ml (MACKNESS *et al.*, 1991). O resultado final foi obtido mutiplicando-se a média da variação das absorbâncias pelo fator descrito abaixo:

Cálculo do Fator:

$$\text{Fator} = \frac{\text{VTR(ml)}}{\varepsilon_{405} \times \text{VA(ml)} \times \text{E(cm)}}$$

Onde:

VTR (volume total da reação)= 525 µl (500 µl solução + 25 µl soro)

ε_{405}= 18050 l . M^{-1} . cm^{-1} (SENTÍ *et al.* , 2003; AGACHAN *et al.*, 2004)

VA (volume da amostra)= 25 µl

E (espessura do poço da placa)= 1 cm

Substituindo os valores e ajustando para as unidades internacionais temos:

Fator = 1163,43 nmol/ml

Cálculo da Atividade:

Atividade da Paraoxonase = Fator x Δabs/min

= 1163,43 x Δabs nmol/ml/min

Onde:

Δabs/min= média da variação das absorbâncias medidas a cada 1 minuto.

4.9.5 Determinação da concentração dos AGL

A concentração dos AGL foi determinada através do kit comercial HR Series NEFA-HR(2)® (WAKO Diagnostics, Texas, EUA). Esse kit utiliza a metodologia baseada em ensaio enzimático colorimétrico. Os ácidos graxos são tratados com acil-CoA sintetase na presença de ATP e CoA. A acil-CoA produzida é oxidada pela

adição de acil-CoA oxidase. Em seguida, ocorre a geração de peróxido de hidrogênio, que permite a condensação oxidativa de 3 meti-N-etil-N (β-hidroxietil) - anilina com 4-aminoantipirina para formar um aduto de cor roxa. Os AGL são detectados por meio de reação de cor ($\lambda_{primário}$= 560nm e $\lambda_{secundário}$= 670nm). As amostras foram analisadas em duplicatas, sendo os resultados expressos em Eq/l.

4.9.6 Determinação da atividade da CETP

Para a determinação da atividade da CETP utilizamos o kit comercial CETP Activity Assay® (BioVision Research Products, CA, EUA). A metodologia empregada foi baseada na técnica de fluorescência ($_{Exc}$ 465nm e $_{Em}$ 535nm), onde uma molécula doadora contendo lipídeo fluorescente foi transferida para outra molécula na presença de CETP. Nesse sentido, o aumento da fluorescência manteve relação direta com a atividade da CETP presente no plasma dos adolescentes. As análises foram realizadas em duplicata, sendo os resultados expressos em pmol/µl/h.

4.9.7 Detecção da concentração de LDL(-)

A LDL(-) foi detectada através de ELISA (*Enzyme-Linked Immunosorbent Assay*) sanduíche, seguindo protocolo padronizado por nosso grupo de pesquisa. A sensibilização das placas (Costar®, modelo 3690, Corning, USA) foi feita com Anticorpo Monoclonal Anti-LDL(-) (MAb-1A3) (0,5 µg/poço, 50,0 µl/poço), diluído em tampão Carbonato-Bicarbonato, (0,1 M, pH 9,6), sendo as placas incubadas *overnight* a 4°C. Após esse período, os sítios livres foram bloqueados com leite desnatado (Molico®, Nestlé, São Paulo, Brasil), diluído a 5% em tampão Fosfato Salina 0,01mol/L (PBS - pH 7,4) e incubados a 37°C por 2 horas. Em seguida, as placas foram lavadas quatro vezes com PBS-Tween (0,05%). Foram adicionados 50,0 µl/poço de plasma diluído 1:1600 em tampão PBS, sendo as placas incubadas por 2 horas / 37°C. Após essa etapa, as placas foram lavadas, conforme descrito acima, e foram adicionados 50,0 µl/poço de anticorpo monoclonal anti-LDL(-) biotinilado (MAb-2C7) (0,12 µg/poço, 50,0 µl/poço). As placas foram incubadas a 37°C por 1 hora, em seguida, foram lavadas conforme descrito acima. Após essa etapa, foram adicionados 50,0 µl/poço de estreptoavidina-peroxidase (1:60000) diluída em PBS. As placas foram incubadas por 1 hora / 37°C e, novamente lavadas, conforme descrito acima. A reação de cor foi desenvolvida através da adição de

substrato composto por 3,3',5,5'-tetrametilbenzina (TMB), tampão citrato-fosfato (0,1 M, pH 4,2) e H_2O_2 (30%) (250/12/10, µl/ml/µl). As placas foram incubadas por 10 minutos / 37°C, protegidas da luz. A reação foi bloqueada com 50 µl/poço de H_2SO_4 (2,0 M) e a absorbância monitorada em 450 nm. Os resultados foram expressos a partir da média das absorbâncias das amostras menos o *background* e, posteriormente, aplicados à curva padrão e multiplicados pela respectiva diluição, sendo os resultados expressos em µg/ml. A calibração desta curva padrão foi feita com LDL(-) obtido de um *pool* de plasma, previamente descrito por DAMASCENO *et al.* (2006). Os anticorpos utilizados nessa análise foram gentilmente doados pela Prof[a]. Dr[a]. Dulcineia Saes Parra Abdalla, do Laboratório de Bioquímica Clínica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF/USP).

4.10 Análise Estatística

Todas as análises foram conduzidas em duplicata e são apresentadas sob a forma de tabelas e gráficos.

Para as variáveis qualitativas, utilizamos teste qui-quadrado e apresentamos as frequências no formato de valor absoluto seguido da sua respectiva porcentagem. A distribuição dos adolescentes, segundo IMC e CC foi comparada por meio do teste de concordância (Kappa) (LANDIS & KOCH, 1977).

Tanto para a forma de apresentação dos dados quantitativos, assim como para a determinação dos testes que foram utilizados, consideramos o tipo de distribuição dessas variáveis (teste Kolmogorov-Smirnov; $p > 0,05$). As variáveis com distribuição normal foram apresentadas sob a forma de média e intervalo de confiança e, realizado teste como t-student e ANOVA (análise de variância). Para as demais variáveis, a apresentação se deu por mediana (p50) e intervalos interquartis (p25 e p75) e, utilização de teste não paramétrico como Mann-Whitney e Kruskal-Wallis.

Sexo, idade e maturação sexual foram testados em modelos lineares univariados, sendo os grupos posteriormente comparados por meio do teste Bonferroni. Teste de tendência linear também foi utilizado para análise das variáveis em tercis, segundo IMC e CC.

Todos os testes estatísticos foram realizados com o auxílio do programa Statistical Package for the Social Sciences® (SPSS), versão 15.0 (SPSS Incorporation, 2006).

O valor de significância considerado foi de $p < 0,05$.

4.11 Aspectos Éticos

Este estudo foi submetido, analisado e aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Saúde Pública (COEP/FSP), sob o número 1722 (**Anexo 4**).

Esse estudo foi submetido e aprovado pelos diretores das seguintes escolas públicas localizadas na cidade de São Paulo: Escola Estadual Caetano de Campos, Escola Estadual Prof. Antonio Alves da Cruz, Escola Estadual Romeu de Moraes, Escola Estadual Prof. Alipio de Barros e Escola Municipal de Ensino Fundamental Prof^{a}. Ileusa Caetano da Silva.

Resultados e Discussão

5. RESULTADOS E DISCUSSÃO

No presente estudo, os resultados e discussão estão apresentados no formato de 3 artigos descritos nos tópicos seguintes, conforme proposta do Programa de Pós-graduação da FSP/USP.

5.1 Artigo 1

Electronegative low-density lipoprotein: Origin and impact on health and disease.

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Review

Electronegative low-density lipoprotein: Origin and impact on health and disease

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ABSTRACT

Oxidative modifications in lipoproteins (LP), especially in low-density lipoproteins (LDL), are associated with initiation and progression of atherosclerosis. The levels of a sub-fraction of LDL with oxidative characteristics, named electronegative LDL [LDL(−)], minimally oxidized LDL, and minus LDL, are known to be increased in subjects with familial hypercholesterolemia, hypertriglyceridemia, nonalcoholic steatohepatitis, diabetes mellitus, coronary artery disease, patients undergoing hemodialysis, and athletes after aerobic exercise. In addition to the oxidative profile, physical and biological characteristics of LDL(−) consist of nonenzymatic glycosylation, increased expression and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enriched NEFA content, hemoglobin and ApoB-100 cross-linking, and increase in ApoC-III and ApoE in LDL. Herein, we summarize the state of the art of the up-to-date body of knowledge on the possible origin and impact of LDL(−) in health and disease. Further, the potential perspectives of using LDL(−) as a biomarker in conditions under metabolic stress are also discussed.

Contents

1. Introduction

Oxidative modifications in lipoproteins (LP), especially in low-density lipoproteins (LDL), are associated with atherosclerosis and many studies have detected oxidatively modified LDL particles in humans [1–6], monkeys [7], and rabbits [8].

Oxidized LDL (oxLDL) results from exposure of LDL to a number of oxidizing agents (such as superoxide anion and hydrogen peroxide present in cells, especially macrophages present in the arterial wall), enzymes (such as lipoxygenases), and products of myeloperoxidase. This exposure may lead to depletion of antioxidant compounds and later oxidation of the lipid and protein components of LDL particles [9]. Previously, Esterbauer et al. found that oxLDL is an important atherogenic factor occurring in plasma, arteries, and plaques of humans and experimental animals [10]. According to Toshima et al., lack of association with hypertension, serum cholesterol, smoking, and sex suggested that oxLDL is an independent risk factor for cardiovascular heart disease (CHD) [11].

Avogaro et al. described a sub-fraction of LDL with oxidized characteristics that was named electronegative LDL [LDL(−)] [1]. Later, this particle was denominated as minimally oxidized LDL

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Table 1
Behavior of LDL(−) in disease and health conditions.

Study design	Endpoint (LDL(−) content)	References
Familial hypercholesterolemia		
FH × NL	↑ FH	[13,14,16]
FH × FH plus SIM (2 months)	↑ FH, basal	[29]
FH × FH plus SIM (3 months)	↑ FH, basal	[16]
FH × FH plus SIM (6 months)	↑ FH, basal	[16]
FH × FH plus SIM or SIM + α-tocopherol	↑ FH, basal	[29]
Other dyslipidemias		
HTG × NL	↑ HTG	[13]
HC × NL	↑ HC	[30]
Diabetes mellitus		
DM2 × control	↑ DM	[18,20,22]
DM2 with MA × control	↑ DM, with MA	[19]
DM2 with MA × DM2 without MA	↑ DM, with MA	[19]
DM2 × DM2 with acarbose	↑ DM, basal	[31]
DM1 with poor GC × control	↑ DM	[21]
DM1 with good GC × control	↑ DM	[21]
DM1 with poor GC × DM1 with good GC	↑ DM, with poor GC	[21]
Exercise		
Athletes before AE × athletes after AE	↑ After	[28]
Renal disease		
HD × control	↑ HD	[25]
HD × PD	↑ HD	[26]
HD × HD plus α-tocopherol	↑ HD, basal	[32]
Other situations		
ACS × control	↑ ACS	[24]
SA × control	↑ SA	[24]
CAD × control	↑ CAD	[23]
Nonalcoholic SH × alcoholic SH	↑ Nonalcoholic SH	[17]

ACS: acute coronary syndrome; AE: aerobic exercise; CAD: coronary artery disease; DM: diabetes mellitus; GC: glycemic control; HC: hypercholesterolemic; HD: hemodialysis; HTG: hypertriglyceridemic; MA: microalbuminuria; NL: normolipidemic; PD: peritoneal dialysis; SA: stable angina; SH: steatohepatitis; SIM: simvastatin.

Table 2
Physicochemical properties, composition, and oxidative parameters of electronegative LDL in comparison with native LDL.

Characteristics	Electronegative LDL	References
Total cholesterol	Higher	[14–16,35]
Triglycerides	Higher	[14–16,21,22,35,36]
Phospholipids	Lower	[14]
Apo B	Lower	[14–16,21,22,35,36]
Apo E	Higher	[14,16,21,22,35,36]
Apo CIII	Higher	[14,16,21,22,35,36]
NEFA	Higher	[14–16,21,22,35,36]
PAF-AH activity	Higher	[15,21,22,36]
IL-8	Higher	[16,35]
IL-6	Higher	[27]
MCP-I	Higher	[16,35]
GM-CSF	Higher	[34]
GROβ	Higher	[34]
GROγ	Higher	[34]
Lysophosphatidylcholine	Higher	[35]
TBARS	Higher	[37]
Conjugated dienes	Higher	[7,37,38]
Alpha-tocopherol	Lower	[37]
Sialic acid	Higher	[14,38]
Lycopene	Lower	[22]
Lag phase	Higher	[14,16,21,22]
Affinity to LDLr	Lower	[16]
Electrophoretic mobility	Higher	[6]
Loss of secondary structure of Apo B	Higher	[37]
Total PUFA	Lower	[7]
Total cholesterol	Higher	[14–16,35]

GM-CSF: granulocyte/monocyte colony-stimulating factor; GROβ: growth-related oncogene β; GROγ: growth-related oncogene γ; IL-6: interleukin-6; IL-8: interleukin-8; LDLr: low-density lipoprotein receptor; MCP-I: monocyte chemotactic protein-1; NEFA: non-esterified fatty acids; PAF-AH: platelet-activating factor acetylhydrolase; PUFA: polyunsaturated fatty acids; TBARS: thiobarbituric acid-reactive substances.

and LDL minus based on its properties of electric mobility. Afterwards, Fabjan et al. showed that LDL modifications induced by oxidizing agents lead to the formation of LDL(−), as well as to particles with higher degree of oxidation identified as LDL(2−), which is more electronegative than LDL(−) [12]. Since then, more than one hundred of articles in the literature have assessed the role of LDL(−) in different chronic diseases and physiological conditions. Similar to oxLDL, the LDL(−) content is increased in subjects with familial hypercholesterolemia [13–16], hypertriglyceridemia [13], nonalcoholic steatohepatitis [17], diabetes mellitus [18–22], and coronary artery disease [23,24], in addition to patients undergoing hemodialysis (HD) [25,26] and athletes after aerobic exercise [27,28] (Table 1). Despite the relevance of these results, the origin and biochemical role of LDL(−) in health and disease is not yet completely clear.

In this review, our aim was to evaluate the "*state of the art*" of LDL(−). Therefore, retrospective information on physicochemical, inflammatory, and immune characteristics, such as effects of life style (including diet, exercise) and drugs on modulation of LDL(−) in different physiological and pathological processes, will be discussed.

2. Physicochemical characteristics of LDL(−)

LDL(−) is a modified LDL sub-fraction present *in vivo*, which was first isolated by ion-exchange chromatography [1]. Usually, the plasma concentration of this particle is lower than 10% of total LDL in healthy subjects, and greater than 10% of total LDL in patients with high cardiovascular risk [13,14,16,22]. The mechanisms that explain the increase of LDL(−) generation in individuals with CHD are not entirely clear. According to Yologlu et al., it is likely that the LDL particles in dyslipidemic subjects are more prone to oxidation than in normolipidemic individuals. Therefore, the presence of a large number of lipoprotein particles with pro-inflammatory capacity would contribute to endothelial dysfunction thus increasing the cardiovascular risk [33]. In this context, the interaction of LDL(−) with endothelial cells promotes the release of granulocyte/monocyte colony-stimulating factor (GM-CSF) that contributes to increase foam cells formation, thus amplifying the oxidative stress in the intima and changing the quality and composition of the extracellular matrix in the atheroma [34].

Differences in physicochemical properties, composition, and oxidative parameters between native and electronegative LDL could explain the atherogenic and inflammatory actions of this particle (Table 2). LDL(−) has low levels of antioxidant vitamins and a high content of oxidized cholesterol, lipoperoxides, conjugated dienes, and thiobarbituric acid reactive substance (TBARS) [6,16,22,37]. LDL(−) from subjects with either familial hypercholesterolemia or normolipidemia is relatively rich in free cholesterol and triglycerides, and its ApoB content is lower than that in the non-electronegative LDL fraction [14]. LDL(−) also contains increased amounts of ApoE, ApoC-III, sialic acid, and non-esterified fatty acids (NEFA) [14]. LDL(−) and α-tocopherol levels showed an inverse and significant correlation [30]. Regarding lipid profile, the percentage of LDL(−) was positively correlated with LDL cholesterol [39].

Differently from *in vitro* oxidized LDL, LDL(−) particles show altered structure of the surface lipids and a denatured ApoB-100 backbone that appears to be buried into the lipophilic environment [11,37,40,41]. However, LDL(−) did not show ApoB fragmentation or other changes arising from excessive oxidation. This observation was confirmed by our group, who found structural modifications in ApoB of LDL(−) by circular dichroism spectroscopy [37]. According to our previous results, LDL(−) recognized by anti-LDL(−) monoclonal antibodies (clone 3D1036), shows a slight alterations of secondary structure. In addition, it is well established that differ-

ences in the lipid composition of LDL promote ApoB conformational changes that are relevant for LDL interaction with B/E receptor [42]. Binding of native LDL to its receptor is mediated by positively charged Lys residues in ApoB that have affinity for negatively charged Cys residues in the receptor binding domain. In contrast, LDL(−) has low binding affinity for the LDL receptor [43]. The increased electronegativity and low number of active surface-accessible Lys residues in this particle certainly contributed to its decreased affinity for the LDL receptor [35]. The unfolding of ApoB-100 and its sinking into the particle surface lipids can also contribute for this effect [40]. In addition, interaction with PGs is an essential step in the retention of LDL by subendothelial extracellular matrix, and it appears that this binding involves basic residues of amino acids (ApoB) and negatively charged proteoglycans (PGs). Previously, Borén et al. [44] developed a model based on mutagenesis of the large ApoB protein to assess its functional domains within native LDL. In short, recombinant LDL thus obtained showed chances in lysine to glutamic acid (K3363E), basic to neutral amino acids (RK3359-3369A) in site B, where arginine residues were changed to serine ones, and lysine to alanine or arginine was substituted with to glutamine (R3500Q). The same authors described that arginine at residue 3500 stabilizes the carboxyl terminus, permitting normal interaction between LDL and its receptor [45]. Nevertheless, only recombinant lysine (K3363E) LDL and lysine and arginine (RK3359-3369A) LDL were associated with decreased PGs binding [45]. Therefore, these amino acids residues appear to be important for the atherogenic potential of LDL. Previously, it was shown that native LDL has two lysine populations, i.e., "normal" Lys residues have a pK_a of 10.4 whereas "active" Lys residues, which have been suggested to be involved in receptor binding, have a pK_a of 8.8 [43]. Interestingly, LDL(−) sub-fraction showed a third type of Lys residues, named "intermediate" Lys, with a different microenvironment and higher basicity (pK_a 10.7) [43]. These differences between native LDL and LDL(−) indicate a distinct conformation of ApoB-100 with a possible loss of affinity of LDL(−) for the B/E receptor [43]. In fact, LDL(−) from subjects with normolipidemia and familial hypercholesterolemia shows impaired binding to the LDL receptor, a characteristic that could lead to decreased *in vivo* clearance of this particle [35]. Lower clearance of LDL(−) results in increased residence time in blood circulation, which in turn could favor further modifications of LDL(−) resulting in increased inflammatory and atherogenic potential.

As compared to native LDL, LDL(−) presents increased binding affinity to arterial PGs, the main component of the subendothelial extracellular matrix. This property would favor retention of LDL(−) on the PG-rich surface layer of the arterial intima thus contributing to progression of atherosclerosis [36]. The interaction occurs between positively charged residues of ApoB-100 and the negatively charged sulfate and carboxyl groups in the glycosaminoglycan (GAG) chains of PGs [46,47]. Regarding that the negative net charge of LDL(−) is a common characteristic in all LDL(−) subfractions, their interaction with PGs could be decreased. However, it has been previously reported that the LDL(−) subpopulation shows different size, density, and composition that contributes to distinct levels of binding to PGs [13,36].

In contrast to the oxidative origin of LDL(−), Sánchez-Quesada et al. verified that both native and electronegative LDL present low levels of lipid peroxidation products, an indication that LDL(−) is not only produced by oxidative modification [48]. Physical and biological characteristics of LDL(−) consist of nonenzymatic glycosylation, increased content and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enrichment of NEFA, hemoglobin and ApoB-100 cross-linking, and increase in ApoC-III and ApoE in LDL [48].

Glycation and oxidation of LDL reduces its affinity for the LDL receptor, leading to reduced hepatic catabolism, increased content of cholesteryl esters in macrophages, and altered endothelial function [49]. These events seem to be closely interrelated. In addition, as a result of hyperglycemia, tissue and plasma proteins are modified and their physiological function is disturbed [50]. Protein glycation that occurs in diabetic patients is regarded as one of the key factors in the pathogenesis of diabetic complications, including accelerated atherosclerosis [51]. Although nonenzymatic glycosylation and oxidation are increased in type 2 diabetes (DM2), these features would not be directly involved in the generation of LDL(−) [22]. LDL(−) properties suggest that high content of this particle in plasma could promote accelerated atherosclerosis in DM2 patients through both an increase in its residence time in plasma and induction of an inflammatory response in the arterial wall cells [22]. Optimization of glycemic control in DM2 subjects increased native LDL resistance to oxidation (longer lag-phase time) but no effect was observed in oxidizability of LDL(−) [22]. In a recent study conducted in patients with DM2, LDL(−) decreased significantly as compared to baseline levels after treatment with oral antidiabetic drugs (both pioglitazone and metformin for 12 weeks), suggesting that hypoglycemic drugs may have an antiatherosclerotic effect [52]. Previously, Sánchez-Quesada et al. [21] described that high level of glycation is necessary for LDL to achieve its electronegativity. These findings clearly show that further physicochemical changes in LDL contribute to the generation of LDL(−). Therefore, events such as aggregation [36] and high PAF-AH activity [21] contribute to the generation of LDL(−) by an oxidative-independent mechanism. Nevertheless, LDL(−) from diabetic patients shows inflammatory potential associated with chemokine release in endothelial cells. This proatherogenic effect could be related to the high PAF-AH activity observed in LDL(−) [21].

The lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as PAF-AH, is considered a member of the group of phospholipases A_2, which are specific for hydrolysis of phospholipids [53]. PAF-AH is produced by inflammatory cells and is mostly transported by LDL (85%), where it hydrolyzes oxidized phospholipids. Several studies propose a proinflammatory role for PAF-AH that acts by forming noxious bioactive lipid mediators (lysophosphatidylcholine and oxidized NEFA) in the lesion-prone vasculature [54]. Asatryan et al. observed that LDL incubated with low-molecular weight phospholipases A_2 (PLA_2) induced formation of LDL(−) without evidence of significant increase in lipid peroxidation [55]. The action of PAF-AH produces lysophospholipids and mainly lysophosphatidylcholine (LPC) that links this enzyme to early coronary atherosclerosis [56]. Müller et al. showed that presence of lysophosphatidylcholine implies that an additional way is available for the organism to modulate the intensity of production of reactive oxygen species at the inflammatory site [57]. Although increased PAF-AH activity appears to be strongly related to high cardiovascular risk [58], this particle displays an important role in the preventing additional oxidation of LDL(−) [21], thus reinforcing the non-oxidative mechanism for the generation of LDL(−). Recently, we observed that obese adolescents have increased PAF-AH activity, although it did not show correlation with LDL(−) levels (unpublished data). These results are consistent with the presence of other mechanisms contributing for the generation of LDL(−).

In addition, Benítez et al. [35] observed that enrichment of LDL with NEFA promotes a concentration-dependent loss of affinity for its receptor, although PLA_2 treatment had been more effective in generating LDL(−) than NEFA-induced modification. *In vitro* modification of LDL by PLA_2 or NEFA enhanced its electronegativity and resulted in an increase in the lysophosphatidylcholine content [34,35]. NEFA enrichment of LDL and apolipoprotein released by lipolysis could also increase LDL electronegativity [48]. In humans, increased NEFA content in LDL(−) from subjects with familial

hypercholesterolemia and diabetes is likely to play a major role in the loss of affinity of LDL(−) for the LDL receptor [22].

In addition, Ziouzenkova et al. observed that high degree of ApoB-100 modification resulted from the formation of covalent bond between hemoglobin (Hb) and LDL, which promoted formation of dityrosine but not malondialdehyde epitope [25]. Hb-mediated reactions can be implicated in the oxidative stress that arises during hemodialysis (HD). These modifications were probably induced by inflammatory processes occurring after contact between the blood and the HD membranes [25]. Hb-mediated oxidation induces the formation of cross-linking between ApoB and Hb backbones and increase in LDL electrophoretic mobility. These reactions also yielded a marked dose-dependent increase in the levels of LDL(−) and LDL(2−) with a preferential conversion to LDL(−), whereas the proportion of LDL(2−) was approximately 10 times lower than that of LDL(−) [25]. Interaction between LDL(−) and free Hb (total and metHb) promotes modifications in ApoB and an increase in the negative net charge of LDL, although with not strong lipid oxidation of LDL (TBARS and hydroperoxides). Therefore, modification of LDL by Hb occurs through a lipid peroxidation-independent mechanism [59]. These observations were recently reinforced after analysis of LDL(−) from patients undergoing hemodialysis. These subjects showed high levels of LDL(−) when compared with both patients undergoing peritoneal dialysis and normal healthy individuals. On the other hand, most pronounced lipid abnormalities were shown by patients undergoing peritoneal dialysis [26,32].

Furthermore, recent evidence suggests that enrichment of LDL with ApoC-III contributes to the generation of electronegative, proinflammatory and atherogenic particles, which are compatible with the properties of LDL(−) [60]. This possibility was reinforced by Mauger et al. [61] who verified a strong correlation of ApoC-III_1 and ApoC-III_2 with the small dense LDL phenotype. Recently, Mello et al. [62] observed that LDL(−) is mainly associated with smaller and denser LDL particles. Furthermore, Flood et al. [63], Hiukka et al. [60] and Camejo et al. [46] proposed that interaction of modified LDL and small dense LDL with PGs is influenced by ApoC-III. Regarding the presence of sialic acid in LDL(−) it is likely that binding of this particle to the arterial PGs depends on the degree of sialylation of ApoC-III. However, recently Bancells et al. [64] showed that presence of ApoC-III and ApoE do not influence LDL(−) binding affinity for PGs.

In addition to the biochemical mechanisms described above, Védie et al. previously proposed that variations observed in the genes coding for apolipoproteins (ApoB and ApoC-III) could change the electrophoretic behavior of LDL [65]. As ApoB is the main structural apolipoprotein of LDL, variations in the ApoB gene could result in differences in the electric charge of this particle in individuals with similar LDL lipid profile.

According to the response-to-retention hypothesis proposed by Williams and Tabas [47,66], lipoprotein retention is a key event in provoking initial damage to the normal artery and thus promoting atherosclerotic lesions. Regarding this hypothesis, retention of LDL(−) could contribute to atherosclerotic process by different mechanisms related to both the oxidative properties of the particle and modification-induced conformational changes (size and density) affecting lipids and proteins [67]. Recently, Bancells et al. [36] proposed that aggregation appears to cause increased LDL(−) binding affinity to PGs favoring the retention of LDL(−). In addition, previous results support the idea that retained LDL(−) contributes to the development of atherosclerosis by different ways, such as, apoptosis, inflammation, cytokines release and cytotoxicity [39,48].

Therefore, independently of the multiple and complex origin of the LDL(−), its interaction with PGs is essential for its retention and its participation in the atherosclerotic process.

3. Inflammatory and immune response

Both LDL oxidized *in vitro* by different agents and LDL(−) show pro-inflammatory characteristics associated with immune response activation. Presence of these modified lipoproteins in the bloodstream stimulates components of the immune system that are related to the acute and chronic phases of many diseases, especially atherosclerosis. These biomarkers include macrophages, T cells, monocyte chemotactic protein-1 (MCP-1), autoantibodies and autoantigens related to modified lipoproteins, interleukins (IL-1, IL-2, IL-6, IL-8, IL-12, and IL-10), tumor-necrosis factor (TNF), gamma-interferon (γ-IFN), and platelet-derived growth factor [41,68–70]. This profile is consistent with previous studies conducted by Sanchéz-Quesada et al. [14] and De Castellarnau et al. [68], who observed that LDL(−) isolated from plasma of both normolipidemic subjects and patients with FH has pro-inflammatory actions.

Regarding the immune nature of atherosclerosis, adhesion of monocytes to the arterial wall endothelium appears be one of the key events in the early development of atherosclerotic plaques. Frostegård et al. found a strong increase in the amount of adhesion molecules adhered to endothelial cells after their exposure to oxidized LDL (when compared to native LDL), suggesting that oxLDL induces adhesion as well as monocyte differentiation [71]. In this context, Fukumoto et al. [72] and Shoji et al. [73] observed that the immune system generates antibodies to oxLDL in presence of oxLDL. According to Inoue et al. [74], and Monaco et al. [75], anti-oxLDL antibodies show a deleterious effect. Afterwards, Faviou et al. [76] found that the concentration of anti-oxLDL antibodies in patients with unstable or stable angina was higher than in healthy subjects, reinforcing the idea of potential negative effect of these antibodies. In contrary, Karvonen et al. [77] showed that autoantibodies (IgM isotype) have an inverse association with carotid atherosclerosis, suggesting that activation of the humoral immune response to oxidized LDL may be beneficial. Recently, Chou et al. [78] described that oxidation-specific epitopes are the major target of natural antibodies.

Similarly to the profile of oxLDL described above, LDL(−) shows proinflammatory and immunogenic properties. According to Siqueira et al. [79], there is growing experimental evidence for the participation of acquired immunity in atherosclerosis. However, few studies link the immune response to oxLDL and the cardiovascular risk conferred by the metabolic syndrome.

Using an animal model, we previously observed that diet supplementation with soy isoflavones decreased the amount of IgG autoantibodies reactive to LDL(−) as compared to the group without supplementation. This event could be related to a lower generation of LDL(−) and, consequently, lower stimulation of the humoral immune response [80].

Further, after treating LDLr−/− mice with anti-LDL(−) monoclonal antibody (clone 31036), Grosso et al. [81] observed that their levels of circulating free LDL(−) were lower than those in either non-immunized mice or those immunized with irrelevant monoclonal antibody. This indicates that passive immunization with anti-LDL(−) monoclonal antibody had a protective effect on atherosclerotic plaque development. It is possible that the decreased levels of free LDL(−) in blood plasma were due to the formation of immune complexes between LDL(−) and the monoclonal antibody injected into mice. If formation of immune complexes had actually occurred, it could be concluded that the monoclonal antibody neutralized the circulating LDL(−) particles and their atherogenic and inflammatory effects were avoided. In addition, the authors demonstrated that mice treated with the anti-LDL(−) monoclonal antibody had less foam cells in the subintimal layer of atherosclerotic lesions than the control mice.

In subjects with CHD, Oliveira et al. [24] and Siqueira et al. [82] found that the titers of antibodies anti-LDL(−) were higher than in the control group. However, Barros et al. [83] found an opposite profile in children and adolescents. The concentration of anti-LDL(−) autoantibodies in normocholesterolemic individuals was higher than that in hypercholesterolemic subjects with or without family history of acute cardiovascular event.

In summary, LDL(−), as oxLDL, is able to activate inflammatory and immune responses, but the real impact of anti-LDL(−) autoantibodies in the atherosclerotic process and other chronic diseases is not yet clear. Fig. 1 shows a possible mechanism that links LDL(−) and inflammatory and immune responses to atherosclerosis. According to this hypothesis, native LDL (nLDL) may either be modified in blood plasma under inflammatory conditions or migrate into the sub-endothelial space where it undergoes oxidative and possibly other structural modifications that result in LDL(−). LDL(−) is internalized by macrophages through scavenger receptors generating foam cells. Further, epitopes from LDL(−) are presented to B cells by macrophages and anti-LDL(−) antibodies are produced. After this step, immune complexes to LDL(−) [IC-LDL(−)] could precipitate and stimulate the maintenance of the inflammatory and immune responses. In this condition, free LDL(−), antibodies to LDL(−), and IC-LDL(−) remain in the intima and can be effused to the lumen space. This view is compatible with the presence and detection of these biomarkers in the lumen and atherosclerotic lesions.

4. Pathophysiological properties of LDL(−)

LDL(−) is considered an important factor in the initiation and progression of atherosclerotic plaques. *In vitro* studies showed that LDL(−) in cultured endothelial cells has cytotoxic effect and stimulates apoptosis and production of leukocyte recruitment mediators, such as interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) [6,14,84].

The interaction of LDL(−) with endothelial cells and the consequent release of granulocyte/macrophage colony-stimulating factor (GM-CSF) could contribute to increase the formation of foam cells, both changing oxidative stress in the intima and the characteristics and composition of extracellular matrix in atheromatous plaques [34]. The *in vitro* susceptibility of LDL to oxidative modification has been positively associated with the amount of LDL(−), which shows lipid peroxides, necessary to initiate copper-catalyzed LDL oxidation [85]. However, mechanisms independent of oxidative modifications are able to generate LDL(−) with atherogenic potential.

Besides the *in vitro* studies, considerable *in vivo* evidence has shown that LDL(−) is present in plasma and atherosclerotic lesions of humans [7], rabbits [8] and mouse [81].

De Castellarnau et al. [68] and Sánchez-Quesada et al. [14] showed that LDL(−) isolated from either normocholesterolemic or hypercholesterolemic subjects induced release of IL-8 and MCP-1 in endothelial cells, supporting the hypothesis that this particle is proinflammatory and atherogenic in humans.

In type 1 and 2 diabetes mellitus (DM1 and DM2), LDL could be differently modified. Whereas subjects with DM1 show a favorable lipid profile and presence of microangiopathy, those with DM2 exhibit a profile related to dyslipidemia and macroangiopathy. This profile is reinforced by LDL analysis, in which LDL from subjects with DM1 shows the highest electrophoretic mobility, compatible with LDL(−) content; whereas differently, subjects with DM2 show LDL(−) is in a state of higher susceptibility to oxidation and with a higher content of diene conjugates [86]. Moro et al. [18] studied patients with DM2 and found that LDL was more glycated, more susceptible to *in vitro* oxidation, and contained a higher percentage of LDL(−) when compared with native LDL. Glycation of ApoB is proposed to be associated with a significant increase in the production of *in vivo* and *in vitro* oxidized LDL.

Sánchez-Quesada et al. [13] found that LDL(−) from normocholesterolemic individuals was predominant in the dense sub-fraction (Phenotype B), whereas most of LDL(−) from patients with familial hypercholesterolemia (FH) was present in the light LDL subclasses (Phenotype A). It is likely that the differences between contents of LDL sub-fractions found in this study reflect a change in triglyceride content in these sub-fractions. A similar profile was previously described by Sevanian et al. [85]. In contrast, Chappey et al. [87] found a bimodal distribution, in which LDL(−) was present in both denser and lighter LDL particles. The increase in the production of LDL(−) is closely related to the increase in the levels of oxLDL and small and dense LDL [31]. This observation is reinforced by the association observed between negative charge in LDL and inflammatory markers of atherosclerosis [14,88].

Regarding renal disease, the levels of LDL(−) in renal patients undergoing dialysis are higher than in normal subjects. LDL(−) may be a useful marker of oxidative stress, and Lobo et al. suggested that patients undergoing hemodialysis are more susceptible to cardiovascular disease due to this condition [26].

Therefore, LDL(−) is a potential marker present in pathophysiological processes related to cardiovascular disease, diabetes mellitus, renal disease, and possibly other diseases.

5. Methods for detection of LDL(−)

Firstly, Avogaro et al. [1] isolated LDL(−) using anion-exchange chromatography in a HPLC system, and this technique was later optimized in a FPLC system [3,5,7,14]. Although these methods generate semi-quantitative results, they remain the principal tool used in studies on LDL(−).

In the late years, capillary isotachophoresis (cITP) has also been used as a technique to characterize plasma lipoprotein sub-fractions according to their net electric charges [89]. The cITP technique allowed separation of two major LDL sub-fractions, fast- (fLDL) and slow-migrating LDL (sLDL), according to their electrophoretic mobilities. The fLDL fractions correspond to LDL(−), β-VLDL, and small dense LDL. After the light LDL fraction was precipitated from whole serum with heparin-Mg^{2+}, electronegative LDL could be measured using cITP in the small dense LDL fraction [90,91]. Therefore, the analytical cITP technique may be useful in the routine analysis of lipoprotein profiles. It was previously shown that the absolute levels of lipoprotein sub-fractions can be determined as the peak area relative to that of an internal marker, and the levels of fLDL and sLDL were proportional to the LDL protein content [91]. These authors reported that the fLDL and sLDL levels are associated with the carotid-artery intima-media thickness and that fLDL is significantly related to the level of serum triglycerides (TG). This observation was previously demonstrated by Sánchez-Quesada et al. [13], who reported that patients with hypertriglyceridemia have an increased proportion of LDL(−). Therefore, high levels of TG could contribute to increased LDL electronegativity. Difficulties due to lack of standardized assays to measure circulating LDL(−) have been overcome by the development of monoclonal antibodies (MAb 3D1036) [37]. Our laboratory has developed an assay to measure LDL(−) in plasma, total LDL and LDL sub-fractions and tissues using a monoclonal antibody MAb (3D1036) that recognizes epitopes in LDL(−) but not in native LDL (cross-linking <1.0%).

Recently, Faulin et al. [92] developed and validated a sandwich enzyme-linked immunosorbent assay (ELISA) to measure LDL(−) in human plasma using two different monoclonal antibodies (free and biotinylated, MAb-1A3 and MAb-2C7, respectively).

Regarding use of antibody (in comparison with other techniques), its main advantages are (I) specificity and sensitivity, (II)

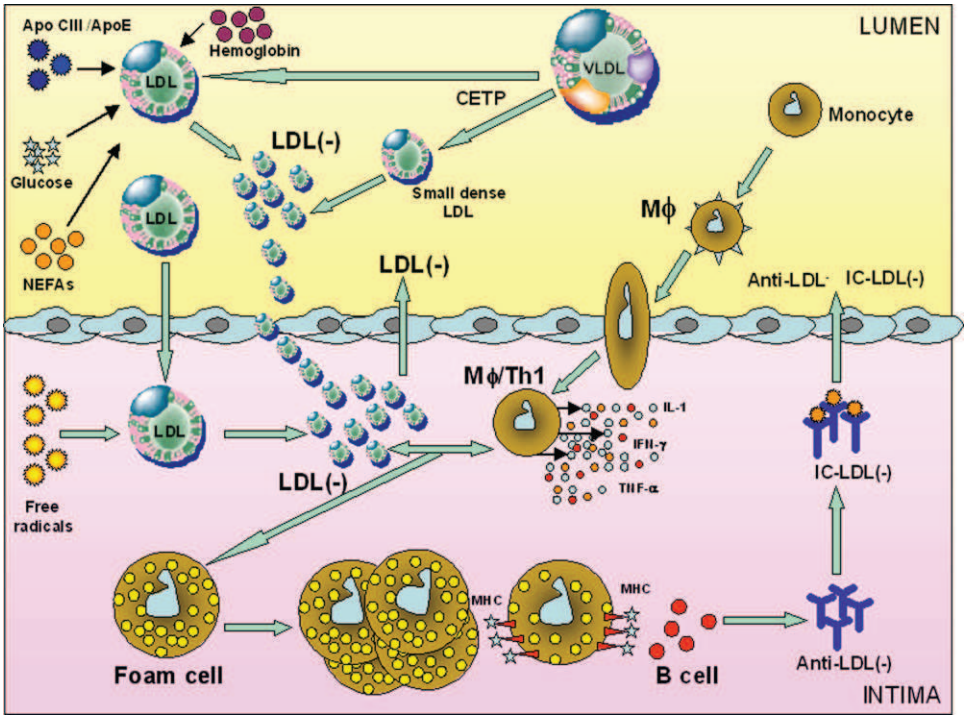

Fig. 1. Immune and cellular mechanisms involving electronegative low-density lipoprotein [LDL(−)]. Anti-LDL(−): antibodies to LDL(−), IC-LDL(−): immune complexes to LDL(−), IL-1: interleukin-1, LDL(−): electronegative LDL, MHC: major histocompatibility complex, MΦ: macrophages, MΦ/Th1: activated macrophages, nLDL: native LDL, TNF: tumor-necrosis factor, and Φ-IFN: gamma-interferon. For more information, please see text.

simultaneous analysis of large number of samples, (III) reduced time of analysis per sample, and (IV) direct detection in different biological fluids.

Therefore, the ELISA technique is a very practical tool to measure LDL(−) in human blood for both widespread research and clinical diagnosis.

The current methods used to monitor LDL(−) are specific for electronegative LDL independently of its origin. These methods are not able to discriminate LDL(−) generated by non-enzymatic glycosylation, increased expression and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enriched NEFA, hemoglobin and ApoB-100 cross-linking, increase in ApoC-III and ApoE or oxidation reactions in LDL. In addition, while chromatographic and electrophoretic methods evaluate total LDL(−) particle regarding its electronegativity, ELISA (based on monoclonal antibodies – MAb) utilizes FPLC-purified human LDL(−) which is used as antigen to generate the MAbs [37]. These MAbs recognize epitopes presents in LDL(−) that was also isolated as a function of its net electric charge.

Although most studies show increased levels of LDL(−) in subjects with high cardiovascular risk, previously Barros et al. [83] and Córdoba-Porras et al. [93] did not show significant differences relative to controls. These differences are probably associated with the distinct design of the studies, in which clinical and demographic characteristics of subjects are a crucial point. Current studies in the literature are limited to a reduced number of subjects ($n < 100$), and distinct methods of detection. In addition, there are few validation and reproducible studies.

6. Effect of drugs and life style

From the classical studies conducted by Anitschkow (1913) on diet and cardiovascular disease [94], it is accepted that components of the diet are important in the development, prevention, and treatment of cardiovascular diseases. Whereas cholesterol [95], the saturated and trans fatty acids stimulate the atherogenic process [96], consumption of fiber and monounsaturated (w-9) and polyunsaturated fatty acids (w-3 and w-6) [97–100] modulate lipid metabolism and reduce the cardiovascular risk. Besides the well-established role of the nutrients describe above, involvement of isolated soy protein [101,102], isoflavones [8], phenolic components [103], phytosterols [104], and antioxidants [105–107] has shown that other diet components display important effects on the development of atherosclerosis and other diseases in which modification of LDL is present.

Soy isoflavones can both prevent lipid peroxidation by scavenging lipid-derived peroxyl radicals and inhibit copper-dependent LDL oxidation [108,109]. Damasceno et al. [80] observed that isoflavones decreased the amount of LDL(−) in plasma and aorta of dyslipidemic rabbits. This effect occurred for both high and low intake of isoflavones (7.3 and 0.73 mg/kg of body per day, respectively), but with different intensity. Previously, Wiseman et al. [110] reported that isoflavone-containing soy protein is more effective in inhibiting LDL oxidation than isoflavone-depleted soy protein. Similarly, Damasceno et al. [102] worked with rabbits and verified that consumption of a diet rich in cholesterol and casein caused an increase in the atherosclerotic lesion size in the aorta when compared to animals that received a hypercholesterolemic diet containing soy protein instead of casein. This increase observed in the casein group may be associated with an increase in the generation of LDL(−).

In addition, Natella et al. [111] reported that supplementation with selenium for a 10-day period was able to prevent both the postprandial increase in LDL(−) and susceptibility to oxidative modification in LDL. In another study, experimental data showed a postprandial increase in LDL(−) concentration after ingestion of a meal containing oxidizable lipids [112].

In vitro studies have shown that phenolic components present in coffee are able to modify lipoprotein oxidative susceptibility. In 2007, it was observed that resistance of LDL to oxidative modification significantly increased and LDL(−) concentration did not

change after coffee drinking. It is likely that these results indicate incorporation of coffee's phenolic acids into LDL particles [113].

Regarding the antioxidant potential of nutrients and the bioactive components present in diet, α-tocopherol is the most investigated of them. It is considered a chief antioxidant for the prevention of experimental atherosclerosis. It acts as a scavenger of lipid peroxyl radicals in lipoproteins protecting them against oxidation and avoiding generation of oxLDL [114]. The effect of α-tocopherol supplementation on LDL(−) content in hemodialysis (HD) patients was previously investigated by Mafra et al. [32] who observed decreased LDL(−) levels after supplementation. On the other hand, Pereira et al. [29] observed that simvastatin decreased LDL(−) levels independent of its association with α-tocopherol, suggesting that α-tocopherol does not affect the antioxidant action of simvastatin in terms of protein nitration or generation of LDL(−) in hypercholesterolemic subjects.

In fact, simvastatin therapy induced a progressive decrease in the proportion of LDL(−). Simvastatin not only decreases plasma cholesterol but also modifies the qualitative characteristics of LDL, e.g., improvement of LDL(−) affinity for LDL receptor and increase in light LDL in comparison with dense LDL [115]. A similar profile was described by Zhang et al. [90], who evaluated low-dose rosuvastatin showing that this drug reduced LDL(−) content and the small and dense LDL sub-fractions in hypercholesterolemic patients with CHD. Although reduction in the content of modified LDL may represent a novel pleiotropic effect of rosuvastatin, the mechanism of these effects is not yet clear. These authors propose that upregulation in the number of LDL receptors is due to inhibition of cellular cholesterol synthesis in patients under statin therapy. Similarly, simvastatin therapy has been shown to increase the affinity of LDL(−) for LDL receptors in patients with familial hypercholesterolemia [16].

Recently, Tang et al. [116] evaluated the effect of LDL(−) from smokers on differentiation of endothelial progenitor cells (EPC) and observed that the most electronegative fraction (L5) was associated with upregulation of lectin-like oxLDL receptor 1 (LOX-1 receptor) and inhibition of EPC.

Besides the effect of diet and drugs, the protective role of regular exercise against atherosclerosis is well established. However, information on the effect of exercise on LDL(−) is insufficient. According to Sánchez-Quesada et al. [117], high levels of HDL in trained subjects could explain the increased resistance of LDL to oxidation and decreased generation of LDL(−) observed in these subjects. This possibility was reinforced by a study conducted by Benítez et al. [28], in which high levels of HDL were related to reduced LDL oxidation (approximately 20%). In athletes, however, LDL(−) content after aerobic exercise was higher than before [27,28]. According to Gutteridge, intense exercise promotes an increase in O_2 consumption in skeletal muscle and this event favors oxidative modification of LDL [118].

Therefore, drugs and life style components (diet, smoke, and exercise) are able to modify LDL(−) generation, possibly reducing the cardiovascular risk.

7. Conclusion

Classical risk factors for CHD include levels of total- (TC) and LDL cholesterol (LDL(−)C), low levels of HDL cholesterol (HDL-C), as well as elevated blood pressure, smoking habit, age, and recently, obesity, familial history of premature CHD, and physical inactivity.

Currently, news risk factors were added to these parameters. Qualitative characteristics of lipoproteins, such as physicochemical properties (size, electrophoretic mobility) and oxidative profile, have been the goal of many studies. In this context, LDL(−) is a potential marker. In this review, the major points focusing this particle showed that:

(I) Origin of LDL(−) is multiple and complex, and includes the oxidative process;
(II) LDL(−) is able to activate the inflammatory and immune responses;
(III) Currently, no accessible commercial "gold standard" method is available to evaluate LDL(−), and there is not any study showing correlation between methods;
(IV) Although LDL(−) is present in health and disease, its content during pathological processes is higher than 10% of total LDL;
(V) Drugs, diet, cigarette smoking, and exercise modify the content of LDL(−) in humans;

In conclusion, LDL(−) is a potential metabolic stress biomarker, which is present in health and disease. Regarding the open problems relative to this particle, we propose that evaluation of LDL(−) be included in prospective, randomized, and crossover trials, since only with large-scale information its clinical relevance will be safely analyzed.

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5.2 Artigo 2

Can CETP activity and LDL(-) modulate the impact of obesity on lipid risk factors in adolescents?

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Can CETP activity and LDL(-) modulate the impact of obesity on lipid risk factors in adolescents?

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Running Title: CETP and LDL(-) modulate cardiometabolic risk

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Abbreviations used: ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BFM%: body fat mass percentage; BMI: body mass index; CETP: cholesterol ester transfer protein; CHD: cardiovascular heart disease; HDL-C: hight density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LDL(-): electronegative LDL; MAb: monoclonal antibody; NW: Normal weight; OB: Obese; OW: Overweight; SPSS: Statistical Package for the Social Sciences; TC: total cholesterol; WC: waist circumference.

ABSTRACT

Background: Obesity has been associated with increased cholesterol ester transfer protein (CETP) and possibly with electronegative LDL [LDL(-)]. However, the impact of these particles on the other lipid risk factors in adolescents is not clear. **Objective:** To investigate whether CETP and LDL(-) are able to change the existing association between obesity and lipid risk factors in adolescents. **Study design:** Both genders, anthropometria, and sexual maturation were monitored in 242 adolescents. The glucose and lipid profile, apolipoproteins, and LDL(-) and CETP activity were analyzed. **Results:** CETP in normal weight-NW ($P<0.001$) and overweight-OW ($P<0.001$) was lower than in obese-OB adolescents. LDL(-) in NW was lower than in OW ($P=0.017$) and OB ($P=0.049$). BMI exhibited positive and linear association with TC/HDL-C ($P=0.026$), TG/HDL-C ($P=0.007$), ApoB/ApoA-I ($P=0.035$), LDL(-) ($P=0.019$), and LDL(-)/TC ($P=0.027$). An inverse profile was observed between HDL-C ($P=0.040$) and ApoA-I ($P<0.001$) with BMI. Waist circumference showed positive association with TG ($P=0.031$), TC/HDL-C ($P=0.011$), LDL-C/HDL-C ($P=0.031$), TG/HDL-C ($P=0.002$), ApoB/ApoA-I ($P=0.001$), LDL(-) ($P=0.033$), LDL(-)/TC ($P=0.044$) and CETP ($P=0.007$). Negative association was observed between WC and HDL-C ($P=0.013$) and ApoA-I ($P=0.004$). After adjustment for CETP, associations between the HDL-C, LDL-C/HDL-C, TC/HDL-C and LDL(-)/LDL-C with BMI or WC were modified. Adjustment for LDL(-) content was not able to change these associations, suggesting an independent mechanism for regulation of the levels of this particle during obesity. **Conclusions:** CETP activity, but not LDL(-) was able to modulate associations of BMI and WC on lipid biomarkers in adolescents.

Keywords: Adolescent, obesity, CETP, electronegative low density lipoprotein, cardiovascular heart disease.

INTRODUCTION

In childhood, obesity is associated with presence of hypertension, dyslipidemia, impaired glucose tolerance, and vascular abnormalities. These factors either isolated or associated represent a plausible mechanism linking childhood obesity with increased risk of cardiovascular heart disease (CHD) in adults (1,2). In addition, central obesity shows strongest association with risk for CHD and co-morbidities (3).

It is known that the atherosclerotic process begins early in the life and its progression depends on inherited and environmental factors (4,5). Therefore, identification of young subjects with high levels for cardiovascular risk factors is important. Dyslipidemias are the major cause for atherosclerosis, especially high cholesterol content in low-density lipoprotein (LDL-C) (6). However, qualitative changes in LDL are also associated with coronary events (7).

Electronegative LDL [LDL(-)] is a minimally-modified form of LDL that can be identified in dyslipidemia and atherosclerosis primary process (8). Early, electronegative LDL was described as an oxidized LDL sub-fraction (9) but the origin of this particle has been also associated with non-oxidative mechanisms (10). Recently, the atherogenic potential of LDL(-) was related mainly with smaller and denser LDL particles (11). These particles can also be generated by enzymatic activity of cholesteryl ester transfer protein (CETP) that transfers cholesteryl esters from high-density lipoprotein (HDL) to ApoB-containing particles (very low-density lipoprotein - VLDL and LDL), thus increasing the amount of small and dense low-density and small high-density lipoproteins (12,13). Furthermore, it has been shown that adults with smaller and denser LDL (phenotype B) have increased cardiovascular risk and accelerated progression of coronary and carotid atherosclerosis (14,15).

In adults, the negative effect of obesity on CETP is widely described (16,17). However, no study was found linking the role of LDL(-) with overweight and obesity in childhood and adolescence. We hypothesized that obesity modifies many cardiometabolic biomarkers in adolescents and both CETP and LDL(-) can have an important role in modulation of these factors.

With this possibility in the mind, we evaluated the impact of obesity on classical cardiovascular risk factors in adolescents (primary endpoint) and

investigated whether CETP activity and LDL(-) levels are able to modulate the association between these parameters (secondary endpoint).

MATERIALS AND METHODS

Briefly, a random sample of adolescents belonging to five public schools at Sao Paulo (SP, Brazil) was invited to join the present study in the years 2008 and 2009. Eligible participants were nonsmokers, non-alcoholics, non-lactants and non-pregnants and at least two months of washout for medicaments. Acute or chronic previous diseases were considered. Age and sex were also investigated. Underweight adolescents were excluded. Clinical evaluation was performed by direct interview, and included familial clinical history and regular use of drugs and nutritional supplements. The protocol for the present study was approved by the local Ethics Committee (# 1722). All adolescents who were enrolled in the study had assented, and their parents (or guardians) provided written informed consent. The degree of sexual maturation of adolescents was assessed by self-administration of appropriate line drawings representing different Tanner stages (18,19). Body mass of subjects was measured with a digital balance (Control II®, Plenna®, Sao Paulo, SP, Brazil; to the nearest 0.1 kg). Their height was measured by using a portable stadiometer (Alturaexata®, TBW®, Sao Paulo, SP, Brazil; to the nearest 0.1 cm). Body mass index (BMI) was classified by using cut-off points as proposed by Cole *et al.* (20) for age and sex and adolescents were classified in normal weight (NW), overweight (OW) and obese (OB). Waist circumference (WC) was measured in the supine position, and in the midway between the lowest rib and the upper prominence of the iliac crest at the end of gentle expiration by using an unstretchable measuring tape to the nearest 0.1 cm. Body fat mass percentage (BFM%) was assessed using the bioelectrical impedance technique (Biodynamics®, model 450, TBW, Sao Paulo, Brazil). After an overnight fast, blood samples were collected and plasma glucose was measured by the glucose-oxidase method (Labtest®, MG, Brazil). Cholesterol (TC) and triglycerides (TG) were determined by standard enzymatic methods (Labtest®, MG, Brazil). High-density lipoprotein cholesterol (HDL-C) was measured by a precipitation technique (Labtest®, MG, Brazil). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald equation (21) except in samples with TG ≥ 400 mg/dl. Apolipoprotein A-I (ApoA-I) and B (ApoB) were

measured by a turbidimetric method (Randox®, Antrim, UK). The plasma levels of LDL(-) were determined by sandwich ELISA using anti-LDL(-) human monoclonal antibody (MAb) (1A3 and 2C7 clones). Microplates (Costar®, model 3690, Cambridge, MA, USA) were coated with MAb1A3 (0.5 µg/well, 50 µl, 0.1 M carbonate-bicarbonate buffer, pH 9.6) and incubated (4 °C, overnight). The microplates were then washed with PBS buffer (with 0.05% Tween 20, pH 7.4, four times) and blocked with nonfat dry milk (5%, 37 °C, 2 h). Afterward, the microplates were washed and incubated with plasma (diluted 1:1600 in PBS buffer, 50 µl, 37 °C, 2 h). The plates were washed and incubated (37 °C, 1 h) with biotinylated anti-LDL(-) MAb (MAb2C7, in PBS buffer, 0.12 µg/well). After washing, the microplates were incubated (37 °C, 1 h) with horseradish peroxidase conjugated streptavidin (Thermo Fisher Scientific®, Rockford, USA; 1:60,000) in PBS buffer. After the plates were washed, a solution containing the substrate 3,3',5,5'-tetramethylbenzidine (250.0 µl), phosphate-citrate buffer (0.1 M, 12.0 ml, pH 4.2), and hydrogen peroxide (10.0 µl) was added (50.0 µl/well). After color development (37 °C, 10 min), the reaction was stopped (H_2SO_4, 2.0 M, 50 µl/well). Color intensity was measured by light absorption (Bio-Tek® Instruments, Winooski, VT, USA; λ = 450 nm) and the LDL(-) concentrations were determined in a standard curve [optical density (OD) *vs* titer after background correction (OD_{sample} - $OD_{background}$)]. The standard curve was constructed for LDL(-) from human plasma pool as described by Damasceno *et al.* (8).

CETP activity was analyzed with the CETP activity assay kit® (BioVision Research Products®, CA, USA) and measured by a fluorescence method (λ_{exc} = 465 nm and λ_{em} = 535 nm). All samples and standards were run in triplicate.

Statistical Analysis

All analyses were performed with the use of the Statistical Package for the Social Sciences® (SPSS), version 15.0. Mean and median values were used to characterize the study samples. After analysis by the Kolmogorov-Smirnov test, the parametric variables were showed as means (95% confidence interval, $CI_{95\%}$), and non-parametric variables as median (interquartile range). Regarding that WC is an anthropometric measure with strong correlation with cardiovascular risk and that the values for this parameter changed as a function of BMI in the present

study, we evaluated the association of the BMI and WC with risk factors for CHD using linear tendency analysis in three models: crude (1), CETP-adjusted (2), and LDL(-)-adjusted (3). The level of concordance between BMI classification and WC tertiles was evaluated by Kappa test. A level of significance of $P < 0.05$ was adopted.

RESULTS

Initially, 2746 adolescents were enrolled in the anthropometric evaluation by BMI. From this population, 1372 adolescents accepted to continue the next phase of the study, but only 261 of them completed all information included in the study protocol. Later, 19 adolescents were excluded because their data and biochemical profile showed misinformation. Therefore, the sample size included the NW (n = 77), OW (n = 82) and OB (n = 83) groups, classified according to the BMI.

Regarding gender and sexual maturation, the number of adolescents showed to be similar when distributed according to BMI. Adolescents in the OW group were younger than those in the NW ($P = 0.001$) and OB groups ($P = 0.048$). As expected, differences in BMI among groups were confirmed by WC ($P < 0.001$), BFM% ($P < 0.001$) and BLM% values ($P < 0.001$), and differences between groups were maintained after sex stratification. The levels of glucose, TG, TC, LDL-C, and non-HDL-C were similar between groups. However, the TC/HDL-C ($P = 0.015$), LDL-C/HDL-C ($P = 0.026$), TG/HDL-C ($P < 0.001$) and ApoB/ApoA-I ($P = 0.002$) ratios were higher in the OB group in comparison with the NW group, and these ratios in the OW group were similar to those in the NW group. An exception was the NW group, in which the TG/HDL-C ratio was lower than in the OW group ($P = 0.039$). The levels of HDL-C in the OW ($P = 0.027$) and OB groups ($P < 0.001$) were lower than in the NW group. In addition, ApoA-I in the OB group was lower than in the NW group ($P = 0.001$). When prevalence of dyslipidemia was investigated, 79% of NW, 79% of OW, and 87% of OB group presented at least one altered lipid parameter (SBC, 2005). However, the groups showed similar profiles ($P = 0.356$) (**Table 1**).

CETP activity was lower in the NW ($P < 0.001$) and OW ($P < 0.001$) groups than in the OB group (**Fig. 1**). The levels of LDL(-) in the OW ($P = 0.017$) and OB ($P = 0.049$) groups were higher than in the NW group (**Fig. 2**). Validity of the values

for LDL(-) was confirmed by reduced intra-assay (2.5%) and inter-assay (6.8%) coefficients of variation.

Association of BMI with lipid risk factors showed that the TC/HDL-C (P = 0.026), TG/HDL-C (P = 0.007), ApoB/ApoA-I (P = 0.035), LDL(-) (P = 0.019), and LDL(-)/TC (P = 0.027) ratios increased proportionally to the BMI tertiles. In HDL-C (P = 0.040) and ApoA-I (P < 0.001), an inverse profile was observed (model 1). After adjustment for CETP activity (model 2), association of HDL-C (P = 0.054) and the TC/HDL-C ratio (P = 0.052) with BMI was lost. Adjustment for LDL(-) (model 3) showed similar results when compared with the crude analysis (model 1) (**Table 2**).

The results shown above were reinforced by the WC analysis. The values for WC showed robust associations with those for TG (P = 0.031), TC/HDL-C (P = 0.011), LDL-C/HDL-C (P = 0.031), TG/HDL-C (P = 0.002), ApoB/ApoA-I (P = 0.001), LDL(-) (P = 0.033), and LDL(-)/TC (P = 0.044) ratios, and CETP activity (P = 0.007), in which the adolescents in 3rd tertile showed higher values than in the 1st tertile. A negative association of the HDL-C (P = 0.013) and ApoA-I (P = 0.004) values was observed with WC measurements (model 1). After adjustment for CETP activity (model 2), the previous association with the LDL-C/HDL-C ratio (P = 0.106) was lost, while the LDL(-)/LDL-C ratio showed significant association (P = 0.043). Adjustment for LDL(-) levels (model 3) did not cause any change in the association between WC and the lipid risk factors (**Table 3**).

Regarding that the negative effect of obesity on lipid risk factors changed as a function of BMI and WC, concordance analysis between these anthropometric measurements were tested. This analysis showed that 88% of the adolescents in the NW group were in the 1st tertile, 27% of the adolescents in the OW group were in 2nd tertile, and 95% of the adolescents in the OB group were in the 3rd tertile of WC (**Fig. 3**). The kappa-index showed agreement of 0.548 (moderate agreement, P < 0.001) between BMI and WC. The nutritional profile differed depending on the screening tool used.

DISCUSSION

In the present study, we verified that obesity in adolescents can induce changes in their cardiovascular risk factors that are compatible with a pro-atherogenic profile. However, the impact of weight/adiposity excess on these risk factors was changed by CETP activity, but not by LDL(-) levels. The profile suggests that LDL(-) has an independent and negative action in cardiometabolic risk of obese adolescents.

A previous study with 262 obese children observed that 83.4% of them present at least one cardiovascular risk factor, and most of these factors were significantly associated with increased WC that reflects central adiposity (22). In addition, Sun *et al.*, (23) described that obese children with high values for both BMI and WC have an increased risk for the adult metabolic syndrome. This association reinforces the notion that BMI itself does not reflect all changes that occur in obese adolescence thus making BMI-based evaluation insufficient to both diagnose the nutritional status and detail the components of body composition (24). Moreover, BMI may be an imprecise measure of adiposity in children because it does not distinguish between lean and fat mass (25). Similar to previous studies, our results confirm that more than 75% adolescents showed at least one lipid parameter altered, independent of BMI or WC. Overweight/obese individuals present significantly lower levels of HDL-C (26,27,28,29). Besides their low HDL-C levels, these individuals frequently show simultaneous low levels of ApoA-I as compared to subjects with normal weight (30). In the present study, low levels of HDL-C and ApoA-I in overweight/obese adolescents were opposed to high TG/HDL-C ratio. Independent of other factors, HDL-C is an important anti-atherogenic component (31). Decreased levels of HDL-C accelerate the progression of atherosclerosis because its main function (reverse cholesterol transport) is impaired (32). Moreover, the anti-atherogenic role of HDL is linked to pleiotropic actions [e.g., antioxidant properties (33) and anti-inflammatory role (34)].

In the present study, the high TG/HDL-C ratio in overweight/obese adolescents was reinforced by WC tertiles. An increased TG/HDL-C ratio might have additional importance because it is a marker of insulin resistance, increased number of smaller and denser LDL particles, and a predictor of a first coronary event (35). Similar results were found in another study that investigated

association of lipid abnormalities with severe adiposity in adolescents (36). However, our results show that LDL-C remained unchanged as a function of BMI and WC. LDL-C elevation in children may not be related only to obesity, but it may be a consequence of familial hypercholesterolemia (36). Therefore, evaluation in other potential biomarker can be important in adolescents.

CETP activity is essential to a positive balance between HDL-C and LDL-C (13). The most important established action ascribed to CETP is its role in the efflux of cholesteryl ester from HDL to the accumulating triglyceride-rich apoB-containing lipoproteins (37). Our results confirmed this main function and showed that the CETP activity changes according to BMI and WC values in adolescents. Obese subjects had 20-30% higher plasma CETP activity than lean subjects, and total both body fat and abdominal fat were positively associated with CETP activity (17). Overweight and obese adolescents included in the present study showed CETP activities that were higher (9% and 60%, respectively) than in NW adolescents. Similar results were recently described, in which CETP activity in obese adolescents was higher than in normal and overweight adolescents (29).

In addition, many studies have shown that density and size of LDL particles are considerably important for cardiovascular risk (11,38,39). However, the impact of LDL(-) on other cardiovascular risk factors was not yet described. CETP activity favors the generation of dense and small LDL (phenotype B) (13). According to Mello *et al.* (11), more than 50% of LDL(-) particles are related to phenotype B. In the present study, the OW and OB adolescents showed increased LDL(-) content in comparison with the NW adolescents. Subjects with both CETP deficiency and high levels of HDL-C have a lower incidence of coronary heart disease than those with normal CETP activity (40). Inhibition of CETP was shown to increase both the levels of HDL-associated antioxidant enzyme (paraoxonase) and formation of large buoyant LDL, and to decrease LDL oxidation (12,41). LDL particles incubated in plasma containing a CETP-inhibiting antibody were more resistant to oxidation, indicating that CETP inhibition might reduce oxidative modification of lipoproteins (42). These observations are in agreement with the present study that showed a synergistic contribution of CETP and HDL-C to increase of the LDL(-) content in adolescents with high values for BMI and WC.

Although our results show that the main lipid risk factors changed as a function of BMI and WC values, WC is considered the most sensitive and specific parameter

for accumulation of body fat in the upper body. It is a measure that can be used isolated to determine the risk of developing metabolic disorders in young people, including children and adolescents (43). The use of WC as a predictor of cardiovascular disease is related to the role of the central location of body fat. Adipocytes located in the central region are more resistant to the antilipolytic effect of insulin. In addition, the waist region is closer to the portal circulation, which releases high levels of free fatty acids. These can contribute to increased synthesis of VLDL, thus promoting the development of atherosclerosis (44).

Central adiposity was the only predictor of all risk factors for CHD, indicating a 1.3-to 7.2-fold risk of dyslipidemia, hypertension, and fasting hyperglycemia (3).

In the present study, high WC values were found to be associated with an increase in TG and LDL(-) levels, the TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, ApoB/ApoA-I, LDL(-)/TC, and LDL(-)/LDL-C ratios, and CETP activity. Contrarily, high WC values were associated with decreased levels of HDL-C and ApoA-I. This profile suggests an increased cardiometabolic risk in obese adolescents.

Overweight children are more likely than thinner children and adolescents to have both adverse levels of high cardiovascular disease risk factors and become obese adults (45).

One particular feature of our study is that both association of WC with the LDL-C/HDL-C ratio disappeared and an association of WC with the LDL(-)/LDL-C ratio appeared after CETP activity adjustment. This profile reinforces the notion that CETP activity has an important role in lipid metabolism and contributes to the generation of LDL(-) in adolescents.

Consistently with this lipid metabolic cascade, we observed that the level of LDL(-) in NW adolescents is lower than in the OW and OB adolescents.

However, the impact of LDL(-) on the association of BMI and WC with lipid factors was unchanged by adjustment. This analysis suggests that LDL(-) is an independent risk factor for CHD. In another study, the lack of association of oxidized LDL (oxLDL) with hypertension, serum cholesterol, smoking, and sex suggests that this is an independent risk factor for CHD (46). Similar to oxLDL, LDL(-) content is increased in subjects with dyslipidemia (47,48), diabetes mellitus (49), and coronary artery disease (7). Thus, LDL(-) is a potential marker that is present in pathophysiological processes related to cardiovascular disease, diabetes mellitus, renal disease, and obesity.

Electronegative LDL has distinct origins and any of them are compatible with non-lipid cardiovascular risk factors, such nonenzymatic glycosylation, increased content and activity of platelet-activating factor acetylhydrolase, enrichment of non-esterified fatty acids, oxidative reaction, hemoglobin and ApoB-100 cross-linking, and increase in ApoC-III and ApoE in LDL (50). In obese subjects, it is likely that generation of LDL(-) occurs by these ways.

The present study has two limitations. First, the cross-sectional design of study does not establish a causal relationship between the monitored events. Second, the number of adolescent in the groups was paired and, therefore, this fact could lead to overestimation of differences between adolescents.

In conclusion, our data indicate that CETP activity was able to modulate association of BMI and WC with lipid risk factors. Although obesity has induced an increase in LDL(-) of adolescents, this particle did not modify association between their anthropometric and lipid parameters.

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APQM and NRTD designed the research, APQM and NRTD conducted the research, APQM and NRTD analyzed the data, APQM and NRTD wrote the paper, and NRTD had primary responsibility for the final content. All authors read and approved the final manuscript. The present study were financially supported by grants from FAPESP (# 07/57602-1 and 07/57601-5). The authors acknowledge Paulo Boschcov, PhD, former professor at UNIFESP, for his revision of the final version of the manuscript.

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Table 1: Characteristics of subjects.

	Normal weight - NW	Overweight - OW	Obese - OB
n (%)	77 (100)	82 (100)	83 (100)
Male, n (%)	26 (33.8)	37 (45.1)	31 (37.3)
Age, years	15.0 (13.0 - 16.0)	13.0 (12.0 - 14.0)*	14.0 (12.0 - 16.0)**
Postpubertal stage, n (%)	73 (94.8)	75 (91.5)	77 (92.8)
Male, n (%)	23 (88.5)	31 (83.8)	25 (80.6)
Female, n (%)	50 (98.0)	44 (97.8)	52 (100)
BMI, kg/m^2	20.7 (19.1 - 21.8)	25.0 (23.6 - 26.4)*	31.1 (29.0 - 35.8)*,**
WC, cm	69.1 (67.7 - 70.4)	80.7 (79.5 - 82.0)*	98.1 (94.5 - 101.7)*,**
Male, cm	70.8 (68.5 - 73.2)	82.9 (80.8 - 85.0)*	102.2 (97.2 - 107.2)*,**
Female, cm	68.2 (66.6 - 69.8)	78.9 (77.6 - 80.3)*	93.8 (90.6 - 96.9)*,**
BFM, %	19.4 (17.6 - 21.2)	24.5 (23.1 - 26.0)*	32.3 (30.8 - 33.8)*,**
Male, %	13.5 (10.3 - 16.8)	21.5 (19.3 - 23.7)*	29.6 (27.3 - 31.9)*,**
Female, %	22.4 (20.8 - 24.1)	27.1 (25.4 - 28.7)*	33.8 (31.9 - 35.8)*,**
BLM, %	77.4 (72.4 - 84.7)	72.3 (67.8 - 76.7)*	66.7 (62.5 - 70.1)*,**
Male, %	88.1 (78.6 - 92.6)	75.1 (80.4 - 69.8)*	70.0 (75.9 - 65.3)*,**
Female, %	74.4 (71.5 - 79.0)	70.0 (67.5 - 73.9)*	65.4 (61.0 - 68.4)*,**
Glucose, mg/dl	79.3 (76.4 - 82.2)	78.0 (75.3 - 80.8)	82.0 (79.1 - 84.9)
TG, mg/dl	71.0 (50.0 - 91.7)	76.1 (54.7 - 101.7)	81.7 (57.4 - 113.0)
TC, mg/dl	143.0 (135.7 - 150.3)	140.0 (132.5 - 147.6)	142.0 (133.3 - 150.7)
HDL-C, mg/dl	38.0 (33.5 - 47.1)	34.0 (27.8 - 43.2)*	32.6 (26.4 - 40.8)*
LDL-C, mg/dl	86.0 (78.4 - 93.6)	85.3 (77.4 - 93.1)	88.9 (80.1 - 97.6)
non-HDL-C, mg/dl	100.9 (93.2 - 108.6)	102.6 (94.2 - 111.0)	107.1 (97.7 - 116.6)
TC/HDL-C	3.4 (2.9 - 4.1)	4.0 (2.7 - 5.3)	4.2 (3.2 - 56)*
LDL-C/HDL-C	2.1 (1.5 - 2.7)	2.6 (1.4 - 3.6)	2.4 (1.7 - 3.7)*
TG/HDL-C	1.9 (1.1 - 2.3)	2.3 (1.3 - 3.1)*	2.2 (1.5 - 3.9)*
ApoA-I, mg/dl	121.0 (116.7 - 125.2)	113.7 (109.4 - 118.0)	109.1 (104.4 - 113.8)*
HDL-C/ApoA-I	0.3 (0.3 - 0.4)	0.3 (0.3 - 0.4)	0.3 (0.3 - 0.4)
ApoB, mg/dl	62.8 (60.3 - 65.3)	65.2 (61.7 - 68.7)	65.2 (61.3 - 69.1)

LDL-C/ApoB	1.4 (1.3 - 1.5)	1.3 (1.2 - 1.4)	1.4 (1.3 - 1.5)
ApoB/ApoA-I	0.5 (0.5 - 0.6)	0.6 (0.6 - 0.6)	0.6 (0.6 - 0.7)*
Dyslipidemia diagnosis, n (%)	61 (79.2)	65 (79.3)	72 (86.8)

* vs NW group, ** vs OW group. ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BFM: body fat mass; BLM: body lean mass; BMI: body mass index; HDL-C: high density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglycerides; WC: waist circumference.

Table 2: Biochemical parameters in adolescents according body mass index tertiles.

	1st Tertile	2nd Tertile	3rdTertile	Model 1	Model 2	Model 3
BMI, kg/m^2	≤ 21.8	21.9 - 22.1	≥ 22.2			
n (%)	59 (100)	7 (100)	168 (100)			
Age, years	14.0 (12.0 - 16.0)	13.0 (12.0 - 16.0)	14.0 (12.0 - 16.0)	0.844	0.688	0.838
Glucose, mg/dl	78.9 (75.5 - 82.3)	76.5 (64.8 - 88.2)	80.3 (78.3 - 82.4)	0.394	0.521	0.524
TG, mg/dl	71.4 (53.3 - 99.3)	76.4 (65.7 - 91.9)	76.9 (54.8 - 105.1)	0.130	0.146	0.083
TC, mg/dl	143.6 (134.8 - 152.3)	139.5 (115.5 - 163.5)	142.5 (136.7 - 148.3)	0.949	0.858	0.981
HDL-C, mg/dl	38.2 (31.9 - 45.7)	50.3 (30.4 - 76.1)	34.2 (27.4 - 41.7)	0.040	0.054	0.046
LDL-C, mg/dl	87.2 (78.3 - 96.0)	70.5 (43.5 - 97.4)	88.3 (82.4 - 94.2)	0.656	0.866	0.737
non-HDL-C, mg/dl	102.8 (93.8 - 111.8)	86.5 (62.7 - 110.2)	105.8 (99.4 - 112.1)	0.449	0.628	0.489
TC/HDL-C	3.5 (2.9 - 4.2)	3.4 (1.8 - 3.9)	3.9 (3.0 - 5.5)	0.026	0.052	0.041
LDL-C/HDL-C	2.1 (1.5 - 2.7)	2.0 (0.6 - 2.5)	2.4 (1.7 - 3.6)	0.058	0.112	0.088
TG/HDL-C	1.9 (1.2 - 2.6)	2.0 (1.0 - 2.0)	2.2 (1.3 - 3.4)	0.007	0.011	0.008
ApoA-I, mg/dl	121.3 (116.4 - 126.2)	123.5 (105.4 - 141.6)	112.2 (109.0 - 115.3)	< 0.001	0.003	0.002
HDL-C/ApoA-I	0.3 (0.3 - 0.4)	0.4 (0.3 - 0.5)	0.3 (0.3 - 0.4)	0.110	0.797	0.624
ApoB, mg/dl	62.2 (59.1 - 65.4)	61.3 (56.5 - 66.1)	65.4 (62.8 - 67.9)	0.256	0.247	0.241
LDL-C/ApoB	1.4 (1.3 - 1.5)	1.1 (0.7 - 1.5)	1.4 (1.3 -1.4)	0.957	0.537	0.636
ApoB/ApoA-I	0.5 (0.5 - 0.6)	0.5 (0.4 - 0.6)	0.6 (0.6 - 0.6)	0.035	0.026	0.033
LDL(-), µg/ml	804.4 (448.6 - 1307.0)	1032.9 (236.1 - 1382.0)	1243.9 (677.8 - 1507.0)	0.019	0.022	-
LDL(-)/TC	0.65 (0.54 - 0.76)	0.64 (0.34 - 0.94)	0.79 (0.73 - 0.86)	0.027	0.027	-
LDL(-)/LDL-C	0.95 (0.55 - 1.57)	1.12 (0.82 - 2.22)	1.25 (0.74 - 1.84)	0.061	0.051	-
CETP, pmol/µl/h	31.5 (17.2 - 46.7)	16.5 (10.0 - 25.9)	41.0 (20.8 - 53.0)	0.196	-	0.231

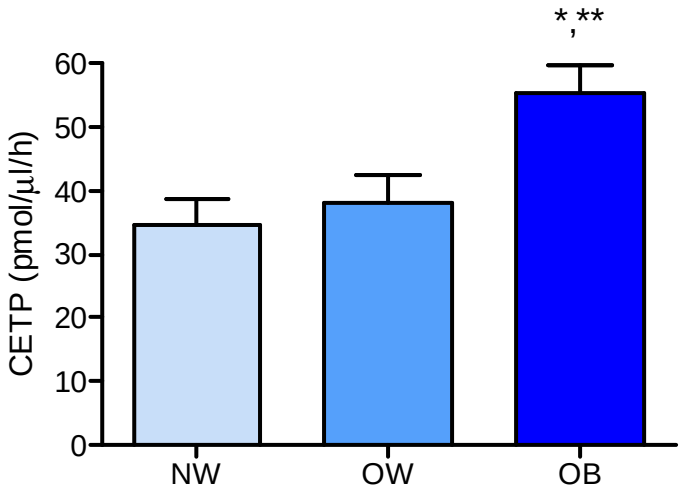
Model 1: crude; Model 2: CETP-adjusted; Model 3: LDL(-)-adjusted. ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BFM: body fat mass; BLM: body lean mass; BMI: body mass index; CETP: cholesterol ester transfer protein; HDL-C: high density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LDL(-): electronegative LDL; TC: total cholesterol; TG: triglycerides; WC: waist circumference.

Table 3: Biochemical parameters in adolescents according waist circumference tertiles.

	1st Tertile	2nd Tertile	3rdTertile	Model 1	Model 2	Model 3
Waist circumference, cm	≤ 76.3	76.4 - 80.1	≥ 80.2			
n (%)	92 (100)	32 (100)	118 (100)			
Age, years	14.0 (12.0 - 16.0)	13.0 (12.0 - 14.0)	14.0 (12.0 - 16.0)	0,329	0,457	0,365
Glucose, mg/dl	78.5 (76.0 - 81.0)	78.0 (72.3 - 83.8)	81.3 (79.0 - 83.6)	0,114	0,136	0,115
TG, mg/dl	71.2 (49.6 - 98.8)	75.9 (58.9 - 101.7)	79.7 (56.5 - 110,5)	0,031	0,040	0,005
TC, mg/dl	142.3 (135.0 - 149.6)	136.9 (125.9 - 147.9)	142.5 (135.7 - 149.2)	0,974	0,795	0,924
HDL-C, mg/dl	37.3 (32.4 - 47.4)	33.6 (25.6 - 38.9)	33.3 (27.8 - 41.7)	0,013	0,038	0,009
LDL-C, mg/dl	85.9 (78.4 - 93.3)	84.3 (72.9 - 95.7)	88.1 (81.1 - 95.0)	0,668	0,993	0,696
non-HDL-C, mg/dl	101.3 (93.7 - 109.0)	101.4 (89.6 - 113.1)	106.1 (98.5 - 113.7)	0,378	0,640	0,329
TC/HDL-C	3.5 (2.7 - 4.3)	3.8 (3.2 - 5.1)	4.3 (3.0 - 5.6)	0,011	0,044	0,009
LDL-C/HDL-C	2.2 (1.4 - 2.8)	2.4 (1.7 - 2.8)	2.5 (1.7 - 3.7)	0,031	0,106	0,033
TG/HDL-C	1.9 (1.2 - 2.5)	2.3 (1.8 - 4.0)	2.2 (1.4 - 3.5)	0,002	0,005	>0,001
ApoA-I, mg/dl	119.0 (115.0 - 123.0)	114.4 (106.0 - 122.7)	110.8 (107.1 - 114.5)	0,004	0,008	0,004
HDL-C/ApoA-I	0.3 (0.3 - 0.4)	0.3 (0.3 - 0.4)	0.3 (0.2 - 0.4)	0,246	0,478	0,199
ApoB, mg/dl	62.8 (59.8 - 65.9)	63.1 (57.5 - 68.5)	66.1 (63.1 - 69.0)	0,137	0,188	0,159
LDL-C/ApoB	1.4 (1.3 - 1.5)	1.4 (1.2 - 1.6)	1.3 (1.2 - 1.4)	0,573	0,427	0,624
ApoB/ApoA-I	0.5 (0.5 - 0.6)	0.6 (0.5 - 0.6)	0.6 (0.6 - 0.6)	0,001	0,003	0,001
LDL(-), µg/ml	791.1 (495.1 - 1374.8)	1273.0 (828.9 - 1573.7)	1246.2 (654.2 - 1495.4)	0,033	0,039	-
LDL(-)/TC	0.67 (0.59 - 0.76)	0.85 (0.73 - 0.98)	0.80 (0.71 - 0.88)	0,044	0,043	-
LDL(-)/LDL-C	1.23 (1.04 - 1.42)	1.47 (1.21 - 1.74)	1.52 (1.28 - 1.76)	0,066	0,043	-
CETP, pmol/µl/h	29.7 (10.3 - 41.7)	31.4 (14.3 - 46.7)	46.5 (23.0 - 54.8)	0,007	-	0,010

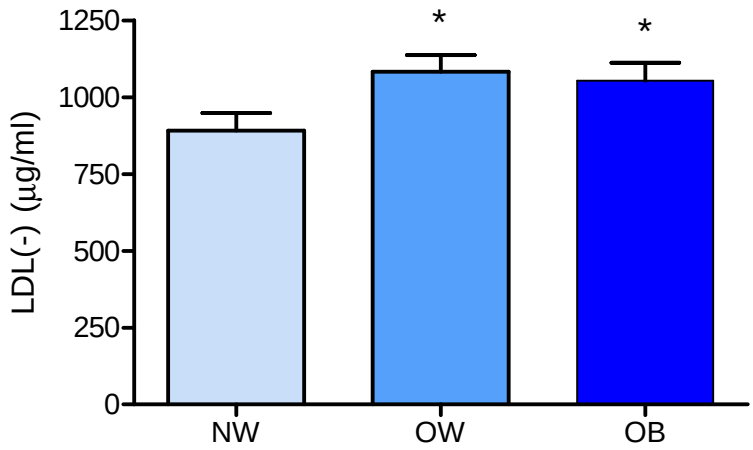
Model 1: crude; Model 2: CETP-adjusted; Model 3: LDL(-)-adjusted. ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BFM: body fat mass; BLM: body lean mass; BMI: body mass index; CETP: cholesterol ester transfer protein; HDL-C: high density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LDL(-): electronegative LDL; TC: total cholesterol; TG: triglycerides; WC: waist circumference.

Figure 1: CETP activity in adolescents according body mass index groups.



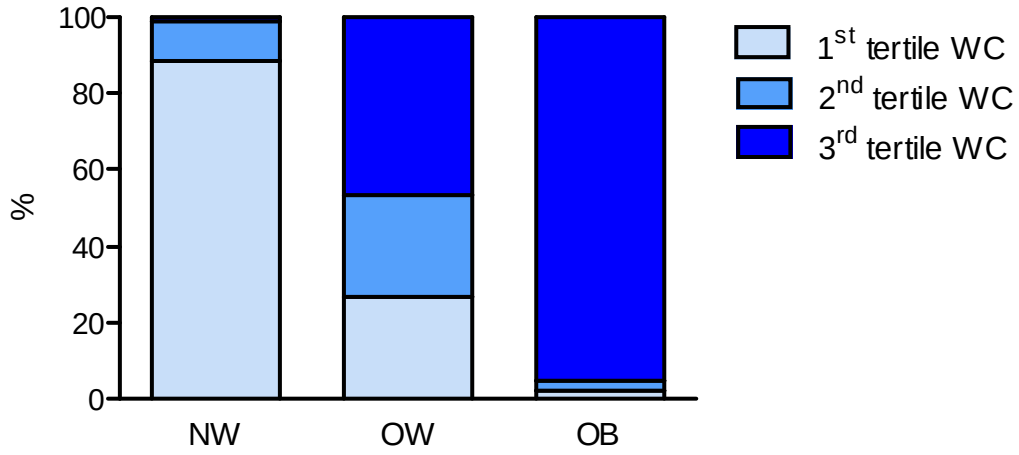
* vs NW group, ** vs OW group. CETP: cholesterol ester transfer protein; NW: Normal weight group; OB: Obese group; OW: Overweight group.

Figure 2: LDL(-) levels in adolescents according body mass index.



* vs NW group. LDL(-): electronegative LDL; NW: Normal weight group; OB: Obese group; OW: Overweight group.

Figure 3: Proportional distribution of waist circumference tertile in relation to body mass index classification.



NW: Normal weight group; OB: Obese group; OW: Overweight group; WC: waist circumference.

5.3 Artigo 3

Waist circumference is more important than insulin resistance in the evaluation of the cardiometabolic risk index in adolescents.

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Waist circumference is more important than insulin resistance in the evaluation of the cardiometabolic risk index in adolescents

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Running Title: Evaluation of cardiometabolic risk in adolescents

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Abbreviations used: ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BFM%: body fat mass percentage; BLM%: lean mass percentage; BMI: body mass index; BP: blood pressure; CETP: cholesteryl ester transfer protein; CRI: cardiometabolic risk index; CVD: cardiovascular disease DBP: diastolic blood pressure; HDL-C: HDL-cholesterol; HOMA: homeostasis model assessment; IR: insulin resistance (or resistant); IS: insulin sensitivity (or sensitive); LDL(-): electronegative LDL; LDL-C: LDL-cholesterol; MetS: metabolic syndrome; NEFA: non-esterified fatty acids; OD: optical density; PON1: paraoxonase 1; SBP: sysolic blood pressure; TC: total cholesterol; TG: triglycerides; WC: waist circumference.

ABSTRACT

Background: Overweight is a serious public health concern. Its development during adolescence generates an impact on health in the adulthood, which is mainly related to metabolic complications. **Objective:** To investigate the impact of waist circumference (WC) and insulin resistance (HOMA) on the cardiometabolic risk index (CRI) of adolescents. **Study design:** Body mass index (BMI) was calculated and WC was measured in adolescents (n=242; 10-18 y) of both sexes. Glucose, insulin, triglycerides (TG), cholesterol (C), total cholesterol (TC), apolipoprotein A-I (ApoA-I) and B (ApoB), non-esterified fatty acids (NEFA), and electronegative LDL [LDL(−)] concentrations and paraoxonase 1 (PON1) and cholesteryl-ester transfer protein (CETP) activities were determined in plasma. Adolescents were distributed by low or high WC (WC_{LOW}, WC_{HIGH}) and HOMA values (insulin sensitivity - IS and insulin resistance - IR). **Results:** Group WC_{HIGH} showed high values for BMI (P<0.001), body fat mass percentage (BFM%) (P<0.001), blood pressure (BP) (P<0.001), insulin (P<0.001), HOMA (P<0.001), TG (P<0.029), NEFA (P<0.039), CETP (P<0.001), LDL(-) (P<0.020), and TG/HDL-C (P<0.001), TC/HDL-C (P<0.002), LDL-C/HDL-C (P<0.012), ApoB/ApoA-I (P<0.017), and LDL(-)/TC (P<0.022) ratios in comparison to WC_{LOW}. Inverse profile to HDL-C (P<0.001), ApoA-I (P=0.028), HDL-C/APOA-I (P=0.005), and PON1 activity (P<0.001). Adolescents classified as IR showed negative impact on BMI (P<0.001), WC (P<0.001), BFM% (P<0.001), BP (P=0.008), TG (P<0.001), TG/HDL-C ratio (P=0.020), and low results for PON1 (P<0.001) than IS group. CRI showed that WC_{HIGH}-IR = WC_{HIGH}-IS > WC_{LOW}-IR = WC_{LOW}-IS (P<0.001). **Conclusions:** WC and IR can identify many cardiometabolic parameters associated with risk of adolescents to develop early cardiovascular disease. However, adolescents with WC_{HIGH} have a more negative cardiometabolic profile as compared with IR.

Keywords: Obesity, waist circumference, insulin resistance, lipid metabolism, adolescents.

INTRODUCTION

The dramatic increase in the prevalence of overweight and obesity in childhood is related to comorbidities that favor precocious development of the MetS (1,2). The MetS is a cluster of risk factors for cardiovascular disease, including visceral obesity, dyslipidemia, type-2 diabetes, and hypertension (3). In 2004, a report of the International Obesity Task Force showed that, worldwide, 1 in 10 children was overweight and at least 155 million school-aged children were overweight or obese (4).

In Brazil, prevalence of excess weight in adolescents had a sixfold increase in the male (3.7-21.7%) and almost threefold increase in the female (7.6-19.4%) populations from 1974 to 2009 (5). Regarding all deaths occurred in Brazil in 2007, 72% of them were attributed to noncommunicable diseases (cardiovascular diseases, chronic respiratory diseases, diabetes, cancer, and others, including renal diseases) and obesity appears to show an important association with these diseases (6). Furthermore, it was shown that changes in visceral fat were mainly related to an increase in fasting blood glucose and triglyceride concentrations, reduction in HDL-C concentrations and increase in blood pressure. Likewise, it was observed that the frequency of MetS was higher in the overweight and obese individuals (7). Besides the fact that MetS is a multifactorial event, obesity is a critical aspect in its development. Therefore, its prevention in childhood needs to be addressed (8).

Anthropometric markers are used in monitoring MetS. Waist circumference, which seems to be a simple and efficient tool, is used in clinical practice for the evaluation of cardiovascular risk factors (9). According to Hirschler (10), WC is more sensitive than BMI in predicting metabolic disorders. Recent evidence suggests that excessive deposition of adipose tissue contributes to an unfavorable metabolic profile, especially when this tissue is accumulated in the abdominal region (11,12). Visceral obesity increases the probability of impaired fasting glucose, glucose intolerance, and doubles prevalence of dyslipidemia (13). In addition, changes in lipid metabolism such as hypertriglyceridemia, increased levels of total and LDL-C, decreased levels of HDL-C, and changes in blood pressure have been linked to obesity (11). In these alterations, insulin resistance is a common and essential element for the

initiation and maintenance of MetS. However, prevalence of these alterations in children and adolescents is barely described.

Individuals with abdominal obesity, but without diagnosis of MetS, have increased cardiovascular risk. In addition, insulin resistance also occurs in lean individuals. However, the mechanisms contributing to impaired insulin signaling in the absence of obesity are much less studied (14). It has been reported that, even in subjects with normal body weight, presence of insulin resistance is associated with greater whole-body fat stores as well as higher lipid levels (15). Thus, it is not clear whether a relatively small expansion of adipose tissue can transmit an adiposity signal to insulin target tissues (16). On the other hand, it is accepted that obesity *per se* cannot fully explain the development of insulin resistance.

Therefore, the purpose of this study was to identify the impact of isolated or simultaneous diferents values of WC and insulin resistance on classical and emergence risk factors for cardiovascular disease in adolescents.

SUBJECTS AND METHODS

Briefly, adolescents from public schools of Sao Paulo (SP, Brazil) were invited to participate in this study. This study included adolescents who met the following criteria: nonsmoker, nonalcoholic, nonlactant, and nonpregnant individuals, and those who were at least two months after washout of drugs. Previous acute and chronic diseases were collected, and underweight adolescents were excluded. The study protocol was approved by the Ethics Committee of the School of Public Health, and University of Sao Paulo (Sao Paulo, SP, Brazil). All adolescents who agreed to participate in the study were enrolled, and written informed consent was provided by their parents (or guardians).

Information on sex and age were obtained by direct interview. Sexual maturation was assessed by self-administration of appropriate line drawings of different Tanner stages and the maturation stage of the adolescents was classified by sex according to the World Health Organization (17).

Body weight was measured to the nearest 0.1 kg with a digital balance (Control II®, Plenna®, Sao Paulo, Brazil) while subjects wore light clothing. Height was measured to the nearest 0.1 cm with a portable stadiometer (Alturaexata®, TBW®, Sao Paulo, Brazil). From these measurements, BMI (weight/height^2) was calculated. Waist circumference was measured to the nearest 0.1 cm in the supine position midway between the lowest rib and the iliac crest at the end of gentle expiration. Body fat mass percentage and BLM% were calculated by using the bioelectrical impedance technique (Biodynamics®, model 450, TBW, Sao Paulo, Brazil). Both SBP and DBP were measured with an indirect arm-cuff sphygmomanometer with subjects in the seated position at rest for 5 min.

After an overnight fast, blood samples were collected. Plasma glucose was measured by the glucose-oxidase method (Labtest®, MG, Brazil). Plasma insulin concentrations were measured by radioimmunoassay with a commercial kit (Linco Research®, St Charles, MO, USA). Insulin resistance was estimated by using a widely used prediction equation, the HOMA {insulin resistance = [fasting insulin (µU/ml) × fasting glucose (mmol/l)] / 22.5}. Triglycerides, TC, and HDL-C were determined by standard enzymatic methods (Labtest®, MG, Brazil). HDL-C was measured by a precipitation technique (Labtest®, MG, Brazil). LDL-C was calculated with the Friedewald equation, except in samples with TG ≥ 400 mg/dl. Apolipoprotein A-I and ApoB were measured by turbidimetric method (Randox®, Antrim, UK).

Paraoxonase 1 activity was measured by adding serum to Tris-HCl buffer (1 ml, 100 mmol/l, pH 8.0) containing $CaCl_2$ (2 mmol/l) and Paraoxon (diethyl-*p*-nitrophenyl phosphate, 1.1 mmol/l; Sigma Chemical Company®, London, Eng). Generation of *p*-nitrophenol was measured (λ = 405 nm, 37 ℃) in a microplate reader (Bio-Rad®, Benchmark, JPN).

Concentrations of NEFA were measured by colorimetric method with a commercial kit (WAKO Diagnostics®, TX, EUA). Cholesteryl ester transfer protein activity was measured by fluorescence method (λ_{exc} = 465 nm; λ_{em} = 535 nm) with a commercial kit (BioVision Research Products®, CA, USA).

Concentration of plasma LDL(−) was determined by sandwich ELISA using anti-LDL(−) human monoclonal antibody (MAb; 1A3 and 2C7 clones).

Briefly, microplates (Costar®, model 3690, Cambridge, MA, USA) were coated with MAb1A3 (0.5 µg/well) in carbonate-bicarbonate buffer (pH 9.6, 0.1 mol/l) and incubated (overnight, 4 °C). Then, the microplates were washed (four times) with PBS (pH 7.4) containing Tween 20 (0.05%), and blocked with nonfat dry milk (5%, 2 h, 37 °C). Afterward, the microplates were washed again and incubated with plasma (50 µl/well) diluted (1:1600) in PBS (2 h, 37 °C). Subsequently, the plates were washed and incubated with the anti-LDL(−) biotinylated MAb2C7 (0.12 µg/well) in PBS (1 h, 37 °C). After washing, the microplates were incubated with horseradish peroxidase-conjugated streptavidin (Thermo Fisher Scientific®, Rockford, IL, USA) diluted (1:60,000) in PBS (1 h, 37 °C). After the plates were washed, a solution containing the substrate 3,3',5,5'-tetramethylbenzidine (250 µl), phosphate-citrate buffer (12.0 ml, pH 4.2), and hydrogen peroxide (10.0 µl) was added (50.0 µl/well). After a delay (10 min, 37 °C) for color development, H_2SO_4 (2.0 M) was added to stop the reaction (50 µl/well). Color intensity was measured by light absorption (λ = 450 nm; Bio-Tek® Instruments, Winooski, VT, USA) and concentrations of LDL(−) were measured as a function of OD readings $(OD_{sample} - OD_{background})_{450}$ in a standard curve. The calibration curve was prepared with LDL(−) obtained from human plasma pool as previously described by Damasceno *et al.* (18). All samples and standards were run in triplicate.

The adolescents were distributed according to WC classification system (19), in which adolescents without or with risk of comorbidities related to obesity were named WC_{LOW} and WC_{HIGH}, respectively. Regarding HOMA, the adolescents were also classified as IS and IR according to Keskin *et al.* (20), and a cut-off value of 3.16 was adopted.

Furthermore, cluster analysis was performed using the cardiometabolic parameters described as following. Firstly, criteria previously established for BMI (3 levels) (21), BFM% (3 levels) (22), TG (cut-off: 130 mg/dl) (23), TC (cut-off: 170 mg/dl) (23), HDL-C (cut-off: 45 mg/dl) (23), LDL-C (cut-off: 130 mg/dl) (23) were adopted. For SBP/DBP, ApoA-I, ApoB, PON1, NEFA, CETP, and LDL(-) the 75 percentile was assumed, and a cut-off value was chosen to separate adolescents into high and low risk. Regarding these parameters, a CRI was obtained for the adolescents by summing the values for each variable;

then, differences between mean values for the groups (WC_{LOW}-IS, WC_{LOW}-IR, WC_{HIGH}-IS, and WC_{HIGH}-IR) were compared.

All tests were performed by using the Statistical Package for the Social Sciences (SPSS), version 15.0. Normal distribution of data was tested with the Kolmogorov-Smirnov test (P >0.05). Parametric variables were expressed as mean (confidence interval) and non-parametric variables as median (interquartile range) values. Univariate comparison for continuous variables were made by using the Student's *t*-test for parametric and the Mann-Whitney's U-test for non-parametric data. All quantitative variables were adjustment for pubertal development by Bonferroni test. Categorical variables were analyzed by χ^2 test and the results were expressed as frequency. The level of significance was set at $P < 0.05$.

RESULTS

Sex distribution was similar between the WC_{HIGH} and WC_{LOW} groups. A difference was observed regarding age, and presence of younger adolescents ($P < 0.001$) in the WC_{HIGH} group was higher in comparison to WC_{LOW} group. Regarding sexual maturation, the number of adolescents in the pubertal stage in the WC_{HIGH} group was proportionally lower than in the WC_{LOW} group ($P = 0.018$). As expected, the values for BMI ($P < 0.001$) and BFM% ($P < 0.001$) in the WC_{HIGH} group were higher than in the WC_{LOW} group. Regarding BLM%, lower values were observed ($P < 0.001$). Differently, the values for SBP ($P < 0.001$), insulin ($P < 0.001$) and HOMA ($P < 0.001$) in adolescents with WC_{HIGH} were higher than in those with WC_{LOW}. Regarding lipid profile, adolescents in the WC_{HIGH} group showed higher values for TG ($P = 0.029$) than those in the WC_{LOW} group, whereas adolescents in WC_{LOW} group showed higher values for HDL-C ($P < 0.001$) and ApoA-I ($P = 0.028$), HDL-C/ApoA-I ratio ($P = 0.005$), and PON1 activity ($P < 0.001$) than those in the WC_{HIGH} group. CETP activity ($P < 0.001$), NEFA ($P = 0.039$), LDL(-) ($P = 0.020$), and LDL(-)/TC ratio ($P = 0.022$) were also elevated in adolescent in the WC_{HIGH} group (**Table 1**).

The impact of WC_{HIGH} on lipid profile was reinforced by the results shown in **Figure 1,** where the TG/HDL-C ($P = 0.001$), TC/HDL-C ($P = 0.002$), LDL-

C/HDL-C (P = 0.012), and ApoB/ApoA-I (P = 0.017) ratios showed high values in the WC_{HIGH} group when compared with the WC_{LOW} group.

Table 2 shows differences between IS and IR adolescents. As expected, more pubertal adolescents (P = 0.005) were in the IR group. Regarding anthropometric parameters, BMI (P < 0.001), WC (P < 0.001), and BFM% (P < 0.001) showed high values in the IR group when compared with those in the IS group; on the other hand, BLM% showed a contrary profile (P = 0.001). Adolescents with IR showed higher values for SBP (P = 0.001) and DBP (P = 0.008), and TG (P = 0.010) concentrations. An inverse profile was found for PON1 activity (P < 0.001). However, TC, HDL-C, LDL-C, ApoA-I, ApoB, NEFA, and LDL(-) concentrations, and CETP activity were similar between groups. In addition, TG/HDL-C ratio (P = 0.020) in the IR group was higher than in the IS group (**Figure 2**).

Data on WC_{LOW}-IS, WC_{LOW}-IR, WC_{HIGH}-IS, and WC_{HIGH}-IR are shown in **Table 3**. Adolescents in the WC_{LOW} and IR groups show higher values for WC (P = 0.034) and BFM% (P = 0.027) than the group with both WC_{LOW} and IS. Contrarily, lower values are shown for CETP activity (P = 0.004) in the IR and WC_{LOW} groups. Comparison between IR and IS in the WC_{HIGH} group showed higher number of adolescents in the pubertal stage (P = 0.001), higher BMI (P = 0.006), WC (P = 0.002), SBP (P = 0.034), and DBP (P = 0.001), TG (P = 0.040) and TC (P = 0.012) concentrations in WC_{HIGH}-IR group.

When the influence of WC on the IS group was evaluated, it was shown that BMI (P < 0.001), BFM% (P < 0.001), SBP (P = 0.022) and the LDL(-)/TC ratio (P = 0.043) in the WC_{HIGH}-IS group were higher than in the WC_{LOW}-IS group. Inverse results were found for age (P = 0.001), pubertal stage (P = 0.008), BLM% (P < 0.001), HDL-C concentrations (P = 0.029) and PON1 activity (P < 0.001). In addition, when the impact of high and low WC on insulin resistance was evaluated, higher values were found for BMI (P < 0.001), BFM% (P < 0.001), SBP (P = 0.003), insulin concentration (P = 0.002), HOMA (P = 0.005) and CETP activity (P < 0.001), and lower values were found for age (P = 0.015), BLM% (P < 0.001), HDL-C concentration (P = 0.002), the HDL-C/ApoA-I ratio (P = 0.036) and PON1 activity (P < 0.001) in the WC_{HIGH}-IR group in comparison with the WC_{LOW}-IR group (**Table 3)**.

Lipid ratios in the 4 groups are shown in **Figure 3**. The values for the TG/HDL-C (P = 0.007), TC/HDL-C (P = 0.010), LDL-C/HDL-C (P = 0.040), and ApoB/ApoA-I (P = 0.023) ratios in the WC_{HIGH}-IR group were higher than those in the WC_{LOW}-IR group.

Regarding CRI, adolescents in the WC_{HIGH} group have higher risk of cardiovascular disease (WC_{HIGH}-IR = WC_{HIGH}-IS > WC_{LOW}-IR = WC_{LOW}-IS) than adolescents with WC_{LOW} ($P < 0.001$) independently of the presence of insulin resistance (**Figure 4**).

DISCUSSION

In this study, we found that adolescents with elevated WC and IR show a negative cardiometabolic profile. However, elevated WC exerts a higher impact on changes in classical and emergence cardiovascular risk factors than IR.

Previously, the Fels Longitudinal Study showed that children with altered values for WC and BMI are more susceptible to develop MetS (24). Waist circumference is frequently used as a surrogate for visceral adiposity and is reported to be more closely associated with negative health outcomes in comparison with subcutaneous abdominal adipose tissue or BMI (25). Recently, Schwandt (26) showed that central obesity was the only anthropometric variable that significantly predicted increasing risk of CVD.

According to Budak, overweight and obesity have an important impact on the development of components present in MetS among adolescents (27). In his sense, childhood obesity is associated with several metabolic and endocrine disorders (including hyperinsulinemia, glucose intolerance, hypertension, and dyslipidemia) that predispose individuals to early development of CVD and type 2 diabetes (11,28). Such events appear to have IR as a common point linking obesity to these diseases (13).

Hyperinsulinemia is considered an independent risk factor for CVD, as it has an important role in the development of other components of the MetS, such as dyslipidemia and hypertension (29). Regarding antropometric parameters, WC could be used to identify the overweight/obese adolescent in risk of developing IR (30). This possibility was confirmed by our results, where

insulin and HOMA were significantly higher in the WC_{HIGH} group, contributing to the notion that insulin release and impaired glucose are simultaneously affected by visceral obesity.

In addition, Obarzanek (31) found association between BP and mortality due to atherosclerotic disease in young adults after a 30-year follow up. The mechanisms that could explain association between arterial hypertension and MetS are mainly related to the presence of visceral fat, which results in IR. Subsequent hyperinsulinemia promotes an increase in sodium retention by the kidneys, which favors arterial hypertension (32). Our results showed that BP increased proportionally when WC and IR were analyzed either isolated or simultaneously.

Besides the negative influence of obesity on BP, Denney-Wilson (33) found significant changes in HDL-C concentration related to increased BMI and WC. In this study, we found that dyslipidemia is characterized by hypertriglyceridemia and low HDL-C concentrations in adolescents with WC_{HIGH} and IR, either isolatedly or in association. According to Allister (34), the overproduction of chylomicrons and reduced receptor mediated clearance are primarily responsible for the hyperchylomicronaemia and post-prandial dyslipidaemia commonly found in subjects with insulin resistance and dyslipidemia. Although the classical analysis of the lipid profile reveals an important relationship with cardiovascular risk, ratios between them appear to amplify their predictive value. More recently, the TG/HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios as well as TG and HDL-C concentrations were consistently associated with IR and MetS (35). Our results confirm this hypothesis, indicating that IR and high WC exert a negative effect on these ratios, and TG and HDL-C concentrations. Prediction of increased TG/HDL-C ratio might have additional importance for primary prevention of CVD since this ratio is a marker of IR, presence of smaller and denser LDL particles, and a predictor of a first coronary event in men (36).

Insulin resistance is associated with dysfunction in HDL biogenesis in men with MetS (37). Our results reinforce this observation showing that increased WC, associated or not with IR, promoted a significant reduction in HDL-C and ApoA-I concentrations, HDL-C/ApoA-I ratio, and PON1 activity.

Decreased PON1 activity and lower capacity of cholesterol transference to the liver favor oxidative reactions in lipid particles, which will contribute to accelerated cellular oxidative damage and arteriosclerosis in obesity (38). According to this author, HDL from obese patients repaired erythrocyte membranes against oxidative damage less efficiently than HDL from healthy subjects. PON1, found only in HDL particles, protects LDL from oxidation enhancing cholesterol efflux from macrophages and, as such, may play a dual role in the protection against atherosclerosis (39). Our results indicated that the WC_{HIGH} group had increased oxidative stress [high LDL(-) concentration] and decreased PON1 activity, which might contribute to the onset of CVD and accelerated atherosclerosis. However, Peti (40) did not find any change in PON1 activity related to HOMA and WC.

In obese individuals, TC and LDL-C concentrations are normally increased; however, our results showed a similar profile when adolescents were grouped according to WC or IR. Previously, Pereira (41) found similar results in the lipid profile, when subjects were distributed according to abdominal obesity. It is important to highlight that minor changes in the structure (density and size) of LDL particles can occur early in adolescents, suggesting that analysis of LDL subclasses (phenotype A and B) could be important to monitorate disfunction in lipid metabolism in this phase of life.

Dyslipidemia, characterized by high cholesterol and TG and low HDL-C concentrations, is often associated with oxidative stress (42). Currently, increase in oxidized LDL (oxLDL) together with inflammation represent an important risk factor for atherosclerosis (43). In children and adolescents, extreme obesity compared to milder forms of adiposity and normal weight, according to BMI, is associated with higher concentrations of oxLDL, C-reactive protein and interleukin-6. This association suggests that markers of early CVD and type 2 diabetes mellitus are already present in this young population (44). Thus, LDL(-), a sub-fraction of LDL with oxidative characteristics, was monitored in this study. Electronegative LDL increased the proportionality with WC, but not with IR. The negative impact of LDL(-) content observed in this study was previously shown in subjects with familial hypercholesterolemia (45), hypertriglyceridemia (45), nonalcoholic steatohepatitis (46), diabetes (47), and

coronary artery disease (48). It was observed that LDL(-) is mainly associated with smaller and denser LDL, reinforcing the atherogenic action of these particles (49). In a recent review, our group showed that LDL(-) is a potential marker present in pathophysiological processes related to CVD, diabetes, renal disease, and possibly other chronic diseases (50). Therefore, elevated LDL(-) observed in adolescents with high WC confirms the metabolic risk of this population.

Besides the oxidative origin of LDL(-), high CETP activity intensify the transfer of cholesterol from HDL to ApoB-rich particles, thus contributing to the generation of LDL(-). Magkos (51) found nearly 20% higher CETP activity when obese subjects were compared with lean ones. This profile was confirmed by present study, in which adolescents with high WC showed increased CETP activity and LDL(-) content. However, a similar profile was not observed when adolescents were grouped according to IR. In addition, adolescent in the WC_{HIGH} group showed CETP activity nearly 50% higher than in the WC_{LOW} group. When the influence of IR was added to WC_{HIGH}, the percent rate increased to about 80%. According to Quintão (52), there is a trend for CETP activity to vary inversely with HDL-C concentration and directly with the degree of atherosclerosis. The impact of IR on CETP activity is sparsely described in the literature. According to Bajnok (53), CETP activity showed a positive correlation with insulin concentration and HOMA. These results support the concept that adiposity and IR in adolescents contribute to a proatherogenic profile associated with changes in lipid metabolism. In addition to the negative impact of high WC on cholesterol metabolism, our results show that visceral adiposity promoted an unbalance in NEFA homeostasis. Increased concentrations of NEFA in subjects with visceral obesity were reported to contribute to the development of various disorders related to MetS, such as hepatic and peripheral IR, dyslipidemia, β-cell apoptosis, and endothelial dysfunction (54). Adipose tissue mass delivers more NEFA to the systemic circulation where they compete as substrate for use in skeletal muscles, which in turn reduce glucose utilization. This increase in blood glucose concentration provides a stimulus for increased insulin secretion and hyperinsulinaemia (55).

Although the negative action of IR and visceral obesity is widely observed in obesity, our results show that high values for insulin concentration are not able to explain all events associated with elevated WC in adolescents. This hypothesis was confirmed by analysis of the CRI proposed in the present study. According to this index, the metabolic risk in adolescents was better discriminated by high values for WC than by high values for IR. The cross-sectional design of this study is a potential limitation that precludes causal relations to be determined.

In conclusion, our data suggest that WC and IR are able to identify many cardiometabolic parameters associated with risk of adolescents to develop an early CVD. However, more variables were found to be altered in adolescents with high values for WC than in those with high values for IR. This observation suggests that WC, a quick, easy, and simple anthropometric measure, could indicate early cardiovascular risk in adolescents. Therefore, we propose that WC be included in the screening for cardiometabolic risk of adolescents.

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APQ Mello designed the experiment, collected the data, analyzed the data, wrote the manuscript, provided significant advice/consultation. JR Natale collected the data, analyzed the data, provided significant advice/consultation. NRT Damasceno designed the experiment, collected the data, analyzed the data, wrote the manuscript, provided significant advice/consultation.

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Table 1: Clinical and biochemical data according to waist circumference.

	WC_{LOW} (*n* = 90)	WC_{HIGH} (*n* = 152)
Boys, *n* (%)	30 (33.3)	64 (42.1)
Age, years	15.0 (13.0 - 16.0)	13.0 (12.0 - 15.0)[1]
Puberty, *n* (%)	88 (97.8)	137 (90.1)[1]
BMI, kg/m^2	20.8 (19.3 - 22.9)	27.3 (24.7 - 31.4)[1]
WC, cm	70.2 (68.8 - 71.5)	89.9 (87.8 - 91.9)[1]
BFM, %	18.5 (16.7 - 20.2)	29.5 (28.5 - 30.6)[1]
BLM, %	78.2 (72.4 - 86.4)	69.4 (64.8 - 73.4)[1]
SBP, mm Hg	105 (100 - 110)	115 (110 - 120)[1]
DBP, mm Hg	75 (70 - 83)	75 (70 - 80)
Glucose, mg/dl	78.9 (76.3 - 81.6)	80.4 (78.2 - 82.7)
Insulin, µU/ml	15.1 (11.2 - 19.0)	20.7 (15.2 -27.8)[1]
HOMA	2.87 (2.10 - 3.90)	4.00 (2.97 - 5.88)[1]
TG, mg/dl	71.4 (50.0 - 92.4)	79.2 (56.5 - 112.6)[1]
TC, mg/dl	143.7 (135.8 - 151.5)	142.3 (136.3 - 148.3)
HDL-C, mg/dl	37.9 (32.7 - 49.6)	33.3 (27.0 - 41.6)[1]
LDL-C, mg/dl	86.0 (77.8 - 94.1)	88.9 (82.8 - 94.9)
ApoA-I, mg/dl	118.8 (114.8 - 122.8)	112.6 (109.1 - 116.1)[1]
HDL-C/ApoA-I	0.33 (0.28 - 0.42)	0.30 (0.25 - 0.37)[1]
ApoB, mg/dl	62.7 (59.9 - 65.5)	65.7 (63.0 - 68.4)
LDL-C/ApoB	1.36 (1.25 - 1.47)	1.37 (1.29 - 1.45)
PON1, nmol/min/ml	120.2 (110.2 - 130.2)	65.7 (59.4 - 71.9)[1]
NEFA, Eq/l	507.2 (460.5 - 553.9)	608.7 (554.3 - 663.0)[1]
CETP, pmol/µl/h	28.2 (12.2 - 44.7)	42.0 (23.6 - 53.7)[1]
LDL(-), µg/ml	894.8 (495.1 - 1374.8)	1250.0 (701.6 - 1506.8)[1]
LDL(-)/TC	0.67 (0.59 - 0.76)	0.81 (0.73 - 0.88)[1]
LDL(-)/LDL-C	1.03 (0.64 - 1,57)	1.23 (0.69 - 1.90)

[1]Significantly different from WC_{LOW} ($P < 0.05$).

Table 2: Clinical and biochemical data according to HOMA.

	IS (*n* = 99)	IR (*n* = 140)
Boys, *n* (%)	44 (44.4)	50 (35.7)
Age, years	14.0 (12.0 - 16.0)	13.0 (12.0 - 15.8)
Puberty, *n* (%)	87 (87.9)	136 (97.1)[1]
BMI, kg/m^2	23.2 (20.7 - 26.5)	26.4 (22.4 - 30.8)[1]
WC, cm	77.4 (75.0 - 79.8)	86.3 (83.7 - 88.8)[1]
BFM, %	22.4 (20.6 - 24.3)	27.6 (26.2 - 29.1)[1]
BLM, %	74.1 (67.7 - 80.7)	70.0 (65.4 - 75.6)[1]
SBP, mm Hg	110 (100 - 115)	118 (110 - 120)[1]
DBP, mm Hg	70 (70 - 80)	80 (70 - 80)[1]
Glucose, mg/dl	73.8 (71.3 - 76.3)	84.1 (82.1 - 86.2)[1]
Insulin, µU/ml	13.0 (9.5 - 14.9)	23.5 (18.7 - 30.0)[1]
HOMA	2.31 (1.80 - 2.78)	4.68 (3.76 - 6.25)[1]
TG, mg/dl	67.5 (52.5 - 90.9)	80.7 (57.6 - 108.4)[1]
TC, mg/dl	137.4 (130.6 - 144.3)	146.9 (140.3 - 153.5)
HDL-C, mg/dl	36.6 (29.7 - 43.4)	34.7 (28.3 - 43.1)
LDL-C, mg/dl	84.0 (77.1 - 90.9)	90.9 (84.1 - 97.7)
ApoA-I, mg/dl	117.1 (113.4 - 120.8)	113.4 (109.6 - 117.3)
HDL-C/ApoA-I	0.31 (0.27 - 0.38)	0.32 (0.26 - 0.38)
ApoB, mg/dl	63.8 (61.1 - 66.6)	65.3 (62.4 - 68.2)
LDL-C/ApoB	1.32 (1.23 - 1.40)	1.40 (1.31 - 1.50)
PON1, nmol/min/ml	99.0 (88.8 - 109.1)	76.4 (68.4 - 84.4)[1]
NEFA, Eq/l	572.3 (522.3 - 622.2)	572.4 (514.7 - 630.0)
CETP, pmol/µl/h	37.0 (22.5 - 49.5)	33.4 (16.6 - 49.0)
LDL(-), µg/ml	949.3 (549.3 - 1372.8)	1215.0 (600.8 - 1504. 8)
LDL(-)/TC	0.74 (0.65 - 0.83)	0.77 (0.69 - 0.84)
LDL(-)/LDL-C	1.11 (0.65 - 1.77)	1.18 (0.66 - 1.84)

[1]Significantly different from IS ($P < 0.05$).

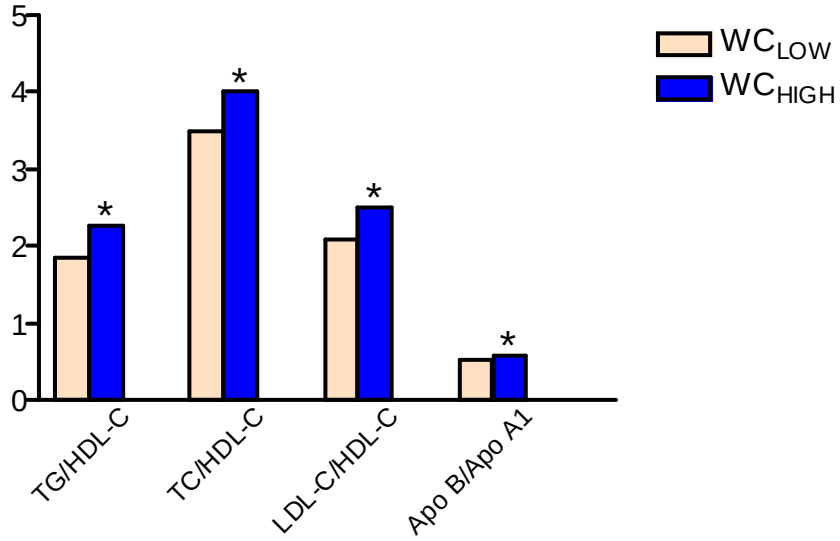
Table 3: Clinical and biochemical data according to waist circumference / HOMA.

	WC_{LOW}		WC_{HIGH}	
	IS (*n* = 52)	IR (*n* = 37)	IS (*n* = 47)	IR (*n* = 103)
Boys, *n* (%)	19 (36.5)	11 (29.7)	25 (53.2)	39 (37.9)
Age, years	15.0 (14.0 - 16.8)	14.0 (13.0 - 16.0)	13.0 (12.0 - 14.0)[3]	13.0 (12.0 - 15.0)[4]
Puberty, *n* (%)	50 (96.2)	37 (100)	37 (78.7)[3]	99 (96.1)[2]
BMI, kg/m^2	20.7 (19.1 - 23.2)	21.4 (19.8 - 22.8)	26.3 (23.4 - 30.3)[3]	28.7 (25.4 - 32.1)[2,4]
WC, cm	69.4 (67.6 - 71.2)	71.8 (69.6 - 73.9)[1]	85.7 (82.9 - 88.6)[3]	91.5 (88.8 - 94.2)[2,4]
BFM, %	17.0 (14.7 - 19.3)	20.6 (17.8 - 23.3)[1]	28.1 (26.3 - 29.9)[3]	30.2 (28.8 - 31.5)[4]
BLM, %	78.9 (74.0 - 87.9)	78.0 (71.6 - 80.8)	70.2 (66.4 - 74.0)[3]	68.5 (63.4 - 73.4)[4]
SBP, mm Hg	105 (100 - 104)	108 (101 - 110)	110 (109 - 120)[3]	120 (110 - 120)[2,4]
DBP, mm Hg	75 (70 - 84)	73 (70 - 88)	70 (70 - 76)	80 (70 - 80)[2]
Glucose, mg/dl	75.4 (71.7 - 79.1)	83.9 (80.6 - 87.2)[1]	72.2 (68.7 - 75.6)	84.2 (81.7 - 86.8)[2]
Insulin, µU/ml	12.0 (8.6 - 14.6)	20.0 (17.9 - 24.5)[1]	13.3 (10.6 - 15.1)	25.0 (20.3 - 32.2)[2,4]
HOMA	2.18 (1.71 - 2.65)	4.13 (3.43 - 5.05)[1]	2.35 (1.81 - 2.83)	5.00 (3.81 - 6.55)[2,4]
TG, mg/dl	67.3 (48.1 - 87.7)	75.4 (55.9 - 98.7)	67.9 (53.7 - 97.3)	81.7 (57.4 - 113.4)[2]
TC, mg/dl	142.5 (131.4 - 153.6)	145.3 (134.0 - 156.7)	132.2 (124.1 - 140.2)	147.5 (139.4 - 155.5)[2]
HDL-C, mg/dl	37.4 (32.4 - 48.9)	40.0 (34.4 - 56.1)	35.2 (27.1 - 41.6)[3]	32.9 (27.3 - 41.7)[4]
LDL-C, mg/dl	86.4 (75.1 - 97.8)	85.3 (73.3 - 97.4)	81.4 (73.3 - 89.4)	92.9 (84.6 - 101.2)
ApoA-I, mg/dl	118.4 (113.0 - 123.7)	119.4 (113.1 - 125.8)	115.8 (110.6 - 121.0)	111.2 (106.6 - 115.9)

HDL-C/Apo -I	0.32 (0.28 - 0.41)	0.34 (0.28 - 0.48)	0.30 (0.25 - 0.37)	0.31 (0.25 - 0.37)[4]
ApoB, mg/dl	62.6 (58.5 - 66.7)	62.7 (59.0 - 66.4)	65.1 (61.3 - 68.9)	66.2 (62.6 - 69.9)
LDL-C/ApoB	1.36 (1.22 - 1.50)	1.36 (1.19 - 1.53)	1.27 (1.16 - 1.38)	1.42 (1.31 - 1.53)
PON1, nmol/min/ml	124.7 (112.0 - 137.4)	113.8 (97.1 - 130.4)	72.1 (60.1 - 84.1)[3]	62.9 (55.4 - 70.3)[4]
NEFA, Eq/l	514.2 (451.9 - 576.5)	497.2 (423.0 - 571.5)	632.8 (555.8 - 709.9)	599.5 (525.8 - 673.2)
CETP, pmol/µl/h	32.1 (22.2 - 49.7)	22.6 (10.3 - 30.2)[1]	45.8 (24.1 - 49.5)	41.2 (23.5 - 54.2)[4]
LDL(-), µg/ml	774.7 (520.6 - 1327.3)	988.3 (471.3 - 1496.8)	1027.1 (787.8 - 1486.0)	1255.7 (654.2 - 1512.4)
LDL(-)/TC	0.65 (0.54 - 0.76)	0.71 (0.57 - 0.84)	0.83 (0.69 - 0.98)[3]	0.79 (0.70 – 0.87)
LDL(-)/LDL-C	0.96 (0.64 - 1.49)	1.14 (0.54 - 1.82)	1.31 (0.81 - 1.95)	1.23 (0.68 - 1.87)

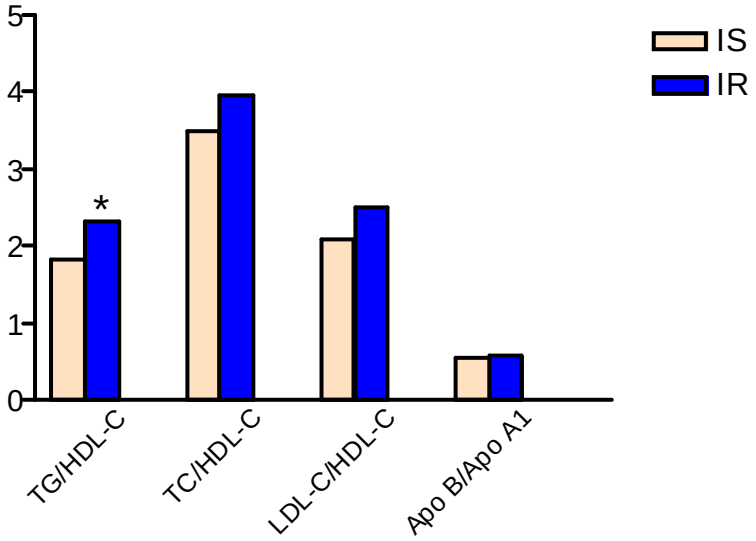
[1]Significantly different from WC_{LOW}-IS ($P < 0.05$); [2]Significantly different from WC_{HIGH}-IS ($P < 0.05$); [3]Significantly different from WC_{LOW}-IS ($P < 0.05$); [4]Significantly different from WC_{LOW}-IR ($P < 0.05$).

Figure 1: Lipids ratio according to waist circumference.



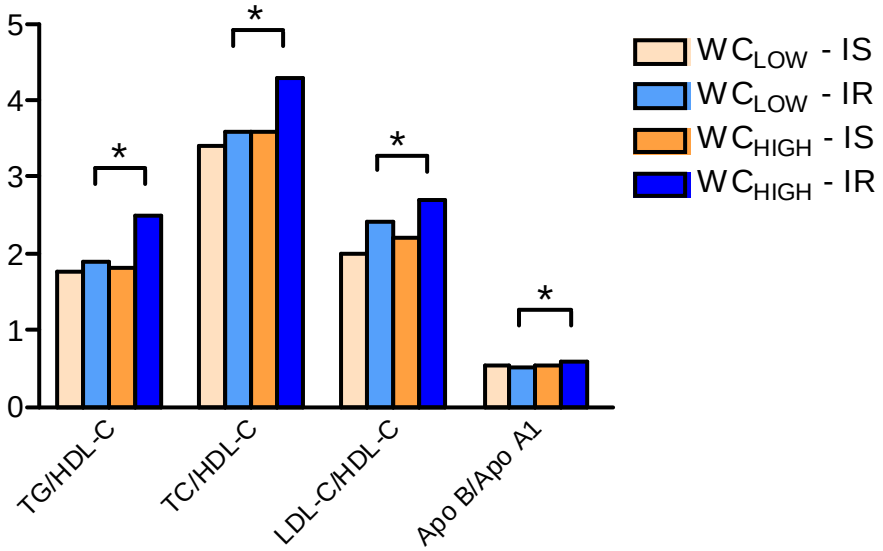
* Significantly different from WC_{LOW} ($P < 0.05$).

Figure 2: Lipids ratio according to HOMA.



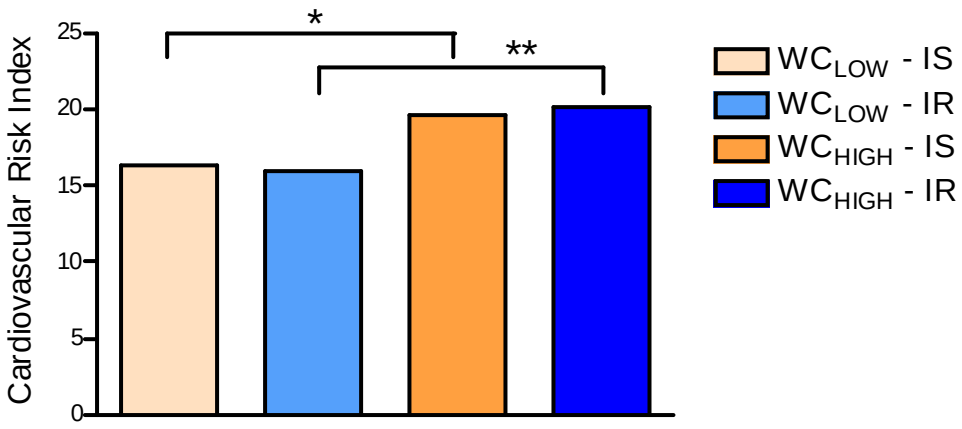
* Significantly different from IS ($P < 0.05$).

Figure 3: Lipids ratio according to waist circumference and HOMA.



* WC$_{HIGH}$-IR significantly different from WC$_{LOW}$-IR ($P < 0.05$).

Figure 4: Cardiovascular risk index according to waist circumference and HOMA.



* WC_{HIGH}-IS significantly different from WC_{LOW}-IS, $P < 0.05$. ** WC_{HIGH}-IR significantly different from WC_{LOW}-IR ($P < 0.05$).

Conclusões

6. CONCLUSÕES

Considerando os objetivos do presente estudo, os resultados obtidos nos permitem apresentar as seguintes conclusões:

- A partícula LDL(-) tem origem complexa e variada. Está presente em indivíduos saudáveis e doentes, entretanto, seu conteúdo em situações patológicas encontra-se acima de 10% do total de LDL-C.
- A LDL(-) aumenta de acordo com o IMC e a CC. Entretanto, esta partícula não modifica a associação entre os parâmetros antropométricos e lipídicos. Esse fato sugere um papel independente e negativo da LDL(-) sobre o risco cardiometabólico de adolescentes.
- A atividade da CETP aumenta de acordo com o IMC e a CC. Além disso, a CETP altera discretamente as associações do IMC e da CC com biomarcadores lipídicos em adolescentes.
- A CC é melhor preditor de parâmetros cardiometabólicos alterados que a resistência à insulina em adolescentes.

Portanto, a concentração de LDL(-) e a atividade da CETP associada à obesidade, principalmente abdominal, alteram o risco cardiometabólico de adolescentes.

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Anexos

Anexo 1: Termo de Consentimento Livre e Esclarecido

O estudo: Papel da lipoproteína de baixa densidade eletronegativa, da proteína transportadora de éster de colesterol e da resistência à insulina no risco cardiometabólico de adolescentes obesos tem como objetivo avaliar a influência do estado nutricional no transporte de colesterol e seu efeito sobre o estresse oxidativo. Assim, pretende-se identificar possíveis hábitos relacionados à saúde que possam minimizar o estresse oxidativo. Para a realização do mesmo, será coletada uma amostra de sangue (20,0 ml), o adolescente responderá a um questionário sobre aspectos clínicos e passará por aferições antropométricas. Todos os resultados obtidos estarão a sua disposição, sendo que sua identidade será mantida em total sigilo. O adolescente terá o direito de se retirar do projeto a qualquer momento. Sua participação ou não neste estudo não o comprometerá de qualquer forma.

Este projeto será desenvolvido pelo Departamento de Nutrição da Faculdade de Saúde Pública da Universidade São Paulo. A pesquisa tem caráter de diagnóstico e não de intervenção. Portanto, o risco é considerado mínimo. Contudo devido ao jejum para coleta de sangue ocasionalmente podem ocorrer tonturas ou enjôos. Os materiais de coleta de sangue são totalmente descartáveis, porém a coleta de sangue pode raramente gerar um pequeno hematoma (manchas roxas) no local de punção. Os benefícios deste estudo são a identificação da influência do estado nutricional sobre o perfil lipídico e o estresse oxidativo. Neste contexto, os benefícios para a população inclusa no estudo ainda são experimentais, mas visualizam a identificação de condições mais saudáveis para a população adolescente. Todos os resultados serão entregues aos participantes do estudo, sendo que orientações específicas (sugestão para procurar um profissional médico e/ou nutricionista) serão dadas quando estes resultados indicarem risco à saúde destes indivíduos.

Declaro que, após ter sido convenientemente esclarecido pelo pesquisador, e ter entendido o que me foi explicado, autorizo _____ _____ participar do presente Protocolo de Pesquisa.

São Paulo, ____de _____ de 20___.

Responsável:
Natureza (grau de parentesco, tutor, curador, etc.):
Sexo: Data de Nascimento:
RG: CPF:
Endereço:
Telefone:

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Telefone para contato: (11) 3061-7865 / Fax: (11) 3061-7130

Anexo 2: Protocolo de avaliação sócio-econômica, cultural, clínica e antropométrica

AVALIAÇÃO SÓCIO-ECONÔMICA CULTURAL, CLÍNICA E ANTROPOMÉTRICA

1. AVALIAÇÃO SÓCIO-ECONÔMICA E CULTURAL

1.1 Nome:
Responsável:

1.2 Sexo: 1 ( )F
2 ( )M

1.3 Data de Nascimento: **1.4 Idade (anos):**

1.5 Raça: 1( ) Branco 2( ) Negro 3( ) Pardo 4( ) Amarelo 5( ) Indígena

1.6 Endereço:
Bairro: CEP:

1.7 Telefone: Res. - Cel. - Tel. recado -
e-mail -

1.8 Escolaridade do Adolescente: 1( )Ensino Fundamental Incompleto – 8ª série 2( )Ensino Médio Incompleto – Colegial

1.9 Membros na Família: 1( ) 2 2( ) 3-4 3( )> 4

1.10 Escolaridade dos Pais:

	Pai	**Mãe**	
	1 ( )	( )	Analfabeto
	2 ( )	( )	Ensino Fundamental Incompleto
	3 ( )	( )	Ensino Fundamental Completo
	4 ( )	( )	Ensino Médio Incompleto
	5 ( )	( )	Ensino Médio Completo
	6 ( )	( )	Ensino Superior Incompleto
	7 ( )	( )	Ensino Superior Completo
	8 ( )	( )	Não conhece / Não sabe

1.11 Renda Média Mensal: 1( ) <1 SM 2( ) 1 - 5 SM 3( ) 6 - 10 SM 4( ) >10 SM 5( ) Não sabe

2. AVALIAÇÂO CLÌNICA

2.1 Faz uso de medicamento e/ou vitaminas regularmente? 1( ) Sim 2( )Não
Qual?_____
Frequência?_____
Indicação?_____

2.2 É portador de doença? 1( ) Sim 2( )Não
Qual? _____

2.4 Pratica algum esporte regularmente? 1( ) Sim 2( )Não
Qual? _____
Qual a frequência e duração?_____
Pratica esporte desde quando?_____

3. AVALIAÇÃO ANTROPOMÉTRICA

	1ª medida	**2ª medida**	**Medida Final**
3.1 Altura (m)			
3.2 Peso (kg)			
3.3 % de gordura – balança			
3.4 IMC (kg/m^2)			
3.5 Circunferência da Cintura (cm)			
3.6 Resistência (Ω) - bioimpedância			
3.7 Reactância (Ω) - bioimpedância			
3.8 % Gordura - bioimpedância			
3.9 % Massa Magra - bioimpedância			
4.0 Ângulo de fase (º) - bioimpedância			

Anexo 3: Modelos de Estádio Puberal – Masculino

Desenvolvimento Puberal Masculino
Tabelas de Tanner

Genitália

Pré-Púbere.

Aumento do escroto e dos testículos, sem aumento do pênis. Pele da bolsa escrotal fina e rosada.

Aumento do pênis em comprimento. Continua o aumento de testículos e escroto.

Aumento do diâmetro e desenvolvimento da glande. Continua o aumento de testículos e escroto, cuja pele escurece e engrossa.

Genital adulto em tamanho e forma.

Pêlos Pubianos

Pré-Púbere.

Pêlos longos, finos e lisos na base do pênis.

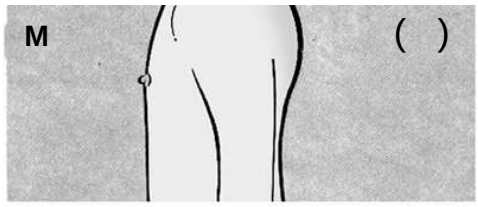
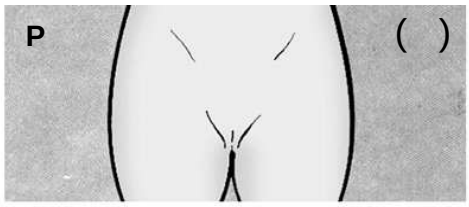
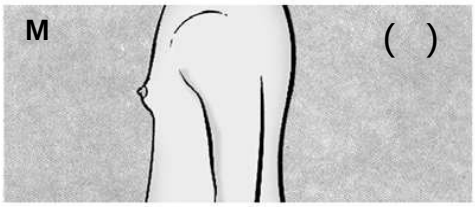
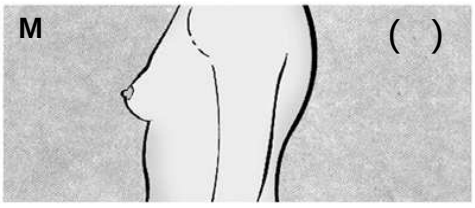
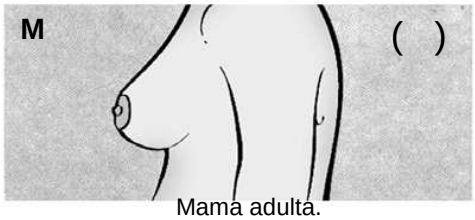
Pêlos mais escuros, mais espessos e encaracolados sobre o púbis.

Pêlos escuros, espessos e encaracolados cobrindo totalmente o púbis, sem atingir as raízes das coxas.

Pêlos estendendo-se até as raízes das coxas.

Anexo 3: Modelos de Estádio Puberal – Feminino

Desenvolvimento Puberal
Feminino

Mamas	Pêlos Pubianos
	
Pré-Púbere (somente elevação da papila).	Pré-Púbere (ausência de pêlos).
	
Broto mamário sub-areolar.	Pêlos longos, finos e lisos ao longo dos grandes lábios.
	
Maior aumento da mama e da aréola, sem separação dos seus contornos.	Pêlos mais escuros, mais espessos e encaracolados parcialmente sobre o púbis.
	
Projeção da aréola e da papila, com aréola saliente em relação ao contorno da mama.	Pêlos escuros, espessos e encaracolados cobrindo totalmente o púbis, sem atingir as raízes das coxas.
	
Mama adulta.	Pêlos estendendo-se até as raízes das coxas.

Anexo 4: Aprovação do Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA – COEP/FSP
Universidade de São Paulo
Faculdade de Saúde Pública

Of.COEP/ 122 / 08

Protocolo	1722
Projeto de Pesquisa	ASSOCIAÇÃO ENTRE O CONSUMO ALIMENTAR E A ATIVIDADE DA PROTEÍNA TRANSPORTADORA DE ÉSTER DE COLESTEROL E DA LIPOPROTEÍNA DE BAIXA DENSIDADE ELETRONEGATIVA EM ADOLESCENTES
Pesquisador(a)	Nágila Raquel Teixeira Damasceno

13 de JUNHO de 2008.

Prezado(a) Orientador(a),

O Comitê de Ética em Pesquisa da Faculdade de Saúde Pública da Universidade de São Paulo - COEP **analisou,** em sua 2.ª/08 Sessão EXTRAORDINÁRIA, realizada em 30/05/2008, de acordo com os requisitos da Resolução CNS/196/96 e suas complementares, o **protocolo** de pesquisa acima intitulada e o considerou **APROVADO.**

Cabe lembrar que conforme Resolução CNS/196/96 são deveres do (a) pesquisador (a):

1. Comunicar, de imediato, qualquer alteração no projeto e aguardar manifestação deste CEP (Comitê de Ética em Pesquisa), para dar continuidade à pesquisa;
2. Manter sob sua guarda e em local seguro, pelo prazo de 5 (cinco) anos, os dados da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo CEP, no caso eventual auditoria;
3. Comunicar, formalmente a este Comitê, quando do encerramento deste projeto;
4. Elaborar e apresentar relatórios parciais e finais;
5. Justificar, perante o CEP, interrupção do projeto ou a não publicação dos resultados.

Atenciosamente,

Cláudio Leone
Professor Associado
Coordenador do Comitê de Ética em Pesquisa – FSP/COEP

Ilm.ª Sr.ª
Prof.ª Dr.ª NÁGILA RAQUEL TEIXEIRA DAMASCENO
DEPARTAMENTO DE NUTRIÇÃO

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Currículo Lattes

Ana Paula de Queiroz Mello

Possui graduação em Nutrição pela Universidade Federal de Ouro Preto - UFOP (2002). Realizou Aprimoramento em Saúde, Nutrição e Alimentação Infantil pela Universidade Federal de São Paulo - UNIFESP (2003). Concluiu Mestrado em Saúde Pública, com ênfase na área de Nutrição, pela Faculdade de Saúde Pública - FSP/USP, 2007. Iniciou Doutorado em Nutrição em Saúde Pública, pela FSP/USP, em 2007. Tem experiência na área de Nutrição, com destaque para NUTRIÇÃO CLÍNICA, atuando principalmente nos seguintes temas: nutrição e obesidade, dislipidemia, consumo alimentar, avaliação nutricional e nutrição infantil.
(Texto informado pelo autor)

Rede de Colaboração

SciELO - artigos em texto completo

Última atualização do currículo em 04/10/2011

Endereço para acessar este CV:
http://lattes.cnpq.br/2856206643237239

Certificado pelo autor em 04/10/11

Dados pessoais

Nome Ana Paula de Queiroz Mello

Nome em citações bibliográficas MELLO, APQ

Sexo Feminino

Formação acadêmica/Titulação

2007
Doutorado em andamento em Doutorado em Nutrição em Saúde Pública .
Faculdade de Saúde Pública.
Título: Influência da Alimentação no Estress Oxidativo e na geração de Ácidos graxos não esterificados,
Orientador: Nágila raquel Teixeira Damasceno.

2005 - 2007
Mestrado em Mestrado em Saúde Pública .
Faculdade de Saúde Pública - Universidade de São Paulo.
Título: Lipoproteína de Baixa Densidade Eletronegativa (LDL-) em indivíduos com diferentes níveis de risco cardiovascular: Parâmetros Nutricionais e Bioquímicos, *Ano de Obtenção:* 2007.
Orientador: Nágila Raquel Teixeira Damasceno.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior .
Palavras-chave: Dislipidemia; Doença Coronariana; Estress Oxidativo; Nutrição.

2003 - 2004
Aperfeiçoamento em Saúde, Nutrição e Alimentação Infantil .
Universidade Federal de São Paulo, UNIFESP, Brasil. Ano de finalização: 2004.
Bolsista do(a): Fundação do Desenvolvimento Administrativo .

1997 - 2002
Graduação em Nutrição .
Universidade Federal de Ouro Preto, UFOP, Brasil.

Atuação profissional

Casa de Saúde Santa Marcelina, CSSM, Brasil.

Vínculo institucional

2011 - Atual Vínculo: Celetista formal, Enquadramento Funcional: Nutricionista, Carga horária: 30

Centro Universitário Adventista de São Paulo, UNASP, Brasil.

Vínculo institucional

2011 - Atual Vínculo: Professor vistante, Enquadramento Funcional: Professora do curso de pós-graduação, Carga horária: 4

Universidade de São Paulo - Faculdade de Saúde Pública, USP - FSP, Brasil.

Vínculo institucional

2007 - Atual Vínculo: Aluna de Doutorado em Nutrição, Enquadramento Funcional: Aluna de Doutorado em Nutrição

Vínculo institucional

2005 - 2007 Vínculo: Bolsista - Mestrado, Enquadramento Funcional: Aluna de Mestrado em Saúde Pública, Carga horária: 8, Regime: Dedicação exclusiva.

Vínculo institucional

Nágila Raquel Teixeira Damasceno

Graduada em Nutrição pela Universidade Estadual do Ceará (1995). Fez Mestrado (1997) e Doutorado (2001) em Ciências dos Alimentos na Universidade de São Paulo. Realizou estágios de Pós-doutoramento em Imunologia (2005) na Universidade de São Paulo e em Nutrição e Endocrinologia na Universidade de Barcelona (2010). Recebeu prêmios nacionais e internacionaos, além de ser co-autora de uma patente. Atualmente é professora doutora de Nutrição junto ao Depto. de Nutrição (FSP-USP). Nos últimos anos tem dedicado suas pesquisas aos efeitos dos componentes nutricionais sobre as doenças crônicas não transmissíveis (aterosclerose, obesidade, câncer, etc) com ênfase nas variáveis lipídicas, oxidativas, inflamatórias e imunológicas.
(Texto informado pelo autor)

Rede de Colaboração

Diretório de grupos de pesquisa

SciELO - artigos em texto completo

Última atualização do currículo em 17/08/2011

Endereço para acessar este CV:
http://lattes.cnpq.br/8729581028091781

Certificado pelo autor em 17/08/11

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Nome Nágila Raquel Teixeira Damasceno

Nome em citações bibliográficas DAMASCENO, N. R. T.

Sexo Feminino

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Formação acadêmica/Titulação

2009 - 2010 Pós-Doutorado .
Universidade de Barcelona.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico ,CNPq ,Brasil .
Grande área: Ciências da Saúde / *Área:* Nutrição.
Grande área: Ciências da Saúde / *Área:* Nutrição / *Subárea:* Bioquímica da Nutrição.

2003 - 2004 Pós-Doutorado .
Universidade de São Paulo, USP, Brasil.
Bolsista do(a): Instituto Danone .
Grande área: Ciências da Saúde / *Área:* Nutrição.
Grande área: Ciências da Saúde / *Área:* Nutrição / *Subárea:* Bioquímica da Nutrição.

1997 - 2001 Doutorado em Ciências dos Alimentos (Conceito CAPES 7) .
Universidade de São Paulo, USP, Brasil.
Título: Influência das Isoflavonas Extraídas da Soja na Hipercolesterolemia e na Aterosclerose Experimental Induzida pela Caseína, *Ano de Obtenção:* 2001.
Orientador: Dulcineia Saes Parra Abdalla.
Bolsista do(a): Fundação de Amparo à Pesquisa do Estado de São Paulo ,FAPESP ,Brasil .
Palavras-chave: Isoflavonas; Caseína; Oxidação; Lipoproteínas; Anticorpos; Aterosclerose.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Cardiologia.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Alergologia e Imunologia Clínica.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Endocrinologia.
Setores de atividade: Fabricação de Produtos Químicos; Fabricação de Produtos Farmacêuticos; Desenvolvimento de Produtos Tecnológicos Voltados Para A Saúde Humana.

1995 - 1997 Mestrado em Ciências dos Alimentos (Conceito CAPES 7) .
Universidade de São Paulo, USP, Brasil.
Título: Influência da Proteína Isolada da Soja e da Caseína no Desenvolvimento da Aterosclerose Experimental em Coelhos, Induzida pela Dieta Rica em Colesterol, *Ano de Obtenção:* 1997.
Orientador: Dulcineia Saes Parra Abdalla.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior ,CAPES ,Brasil .
Palavras-chave: Soja; Caseína; Aterosclerose; Colesterol; Anticorpos; hipercolesterolemia.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Cardiologia.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Alergologia e Imunologia Clínica.
Grande área: Ciências da Saúde / *Área:* Medicina / *Subárea:* Clínica Médica / *Especialidade:* Endocrinologia.