FÁBIO LIMA SODRÉ

BIOMARCADORES PÓS-PRANDIAIS DE OXIDABILIDADE PLASMÁTICA EM ADULTOS SAUDÁVEIS

Associação entre dieta, estresse oxidativo mitocondrial e aterosclerose

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Orientadora: Profa. Dra. Eliana Cotta de Faria

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"Queira!
Basta ser sincero
E desejar profundo
Você será capaz
De sacudir o mundo."

Raul Seixas / Paulo Coelho

"Se eu vi mais longe, foi por estar de pé sobre
ombros de gigantes."

Isaac Newton

Resumo

A aterosclerose é uma doença crônica degenerativa das artérias, caracterizada pelo acúmulo de lipídios na parede arterial. Na década de setenta, Zilversmit postulou que o fenômeno pós-prandial era promotor da aterosclerose. O mecanismo que conecta o estado pósprandial com a doença cardiovascular (DCV) inclui a disfunção endotelial, estresse oxidativo e inflamação. No presente estudo, foi estabelecida a composição química das subfrações das lipoproteínas de alta densidade (HDLs) em uma população adulta normolipidêmica brasileira e, comparada esta com outras populações. Além disto, as modificações na composição química desta partícula em indivíduos saudáveis normolipidêmicos foram descritas após a ingestão de uma dieta rica em lipídios. Estes dados demonstraram uma diminuição do conteúdo de ésteres de colesterol e aumento de triglicérides nas partículas de HDL, os quais refletem o aumento da atividade da enzima transferidora de ésteres de colesterol (CETP). Estas mudanças levam a uma atividade anti-oxidante reduzida da HDL. Por fim, este estudo evidenciou que a geração intracelular de espécies reativas ao oxigênio (ROS) está reduzida durante o período pósprandial. A diminuição do consumo de oxigênio celular e suas correlações com a geração de ROS indicam que a mitocôndria participa ativamente deste fenômeno. Disfunção endotelial foi outro achado durante este período. No mundo ocidental, o período pósprandial representa uma parte significativa do dia, ressaltando a importância deste estado no desenvolvimento da aterosclerose. A completa elucidação dos mecanismos envolvidos pode melhorar as intervenções dietéticas e levar a efeitos benéficos no combate ao estresse oxidativo e disfunção endotelial no período pós-prandial.

Abstract

Atherosclerosis is a chronic degenerative disease of the arteries, characterized by an accumulation of lipids in arterial walls. In 1970s, Zilversmit postulated that postprandial phenomenon was a promoter of atherosclerosis. The mechanisms that link the postprandial state with CVD include endothelial dysfunction, oxidative stress and inflammation. In present study, the chemical composition of high density lipoproteins (HDLs) subfractions in a Brazilian adult normolipidemic population was determinated and compared it with others populations. In addition, the modifications in chemical composition of this particle in healthy normolipidemic subjects after an intake of a fat-rich meal, was described. This data presents a diminished core content of cholesteryl ester (CE) and elevated triglyceride (TG) of postprandial HDL particles, which reflected enhanced activity of cholesteryl ester transfer protein (CETP). These changes lead to an impaired antioxidative activity of dense HDL. Finally, this study provides evidence that intracellular reactive oxygen species (ROS) generation is reduced during the postprandial period. The reduction of oxygen consumption and it correlation with ROS generation suggests that mitochondria plays a pivotal role in this phenomenon. Endothelial dysfunction was also found during this period. In western societies, a significant part of the day is spent in the postprandial state, further emphasising the importance of this period in the development of atherosclerosis. The complete elucidation of the involved mechanisms may improve the appropriateness of such dietary intervention supported by beneficial effects on postprandial oxidative stress and endothelial dysfunction.

Lista de abreviaturas

ABC	-	*ATP-binding cassete transpoters*
AHA	-	American Heart Association
apoA-I	-	apolipoproteína A-I
AVC	-	acidente vascular cerebral
CDC	-	Center for Disease Control
CE	-	ésteres de colesterol
CETP	-	enzima transferidora de ésteres de colesterol
CSPM	-	células sanguíneas periféricas mononucleares
CVD	-	*cardiovascular disease*
DAC	-	doença arterial coronariana
DCF	-	diclorofluresceína
DCV	-	doença cardiovascular
DHE	-	dihidroetídio
DM	-	diabetes melito
HDL	-	lipoproteína de alta densidade
IAM	-	infarto agudo do miocárdio
LCAT	-	lecitina-colesterol-acil-transferase
LDL	-	lipoproteína de baixa densidade
LDL-ox	-	lipoproteína de baixa densidade oxidada
NRS	-	espécies reativas de nitrogênio
OMS	-	Organização Mundial da Saúde

PAI-1	-	inibidor do ativador do plasminogênio-1
PCR-as	-	proteína C reativa de alta sensibilidade
PAF-AH	-	fator ativador de plaqueta – acetil hidrolase
PON 1	-	paroxonase 1
RLP	-	partículas de colesterol similares a remanecentes
ROS	-	espécies reativas ao oxigênio
SR-BI	-	*scavenger receptor B type I*
TG	-	triglicérides
TRL	-	lipoproteínas ricas em triglicérides

Sumário

Introdução Geral

ATEROSCLEROSE

A aterosclerose é uma doença crônica inflamatória que se desenvolve ao longo de vários anos. Esta doença se inicia no espaço subendotelial com a deposição de lipídios e a atração de células inflamatórias, musculares lisas e fibroblastos que levam à lesão endotelial e à exposição da matriz de colágeno, com a subseqüente a oclusão arterial que pode ocasionar vários desfechos clínicos como o acidente vascular cerebral (AVC) e o infarto agudo do miocárdio (IAM) (1)

De acordo com a Organização Mundial de Saúde (OMS), as DCVs são as principais causas de mortalidade no mundo (2,3), sendo responsáveis por 16,6 milhões de morte/ano, e contribuindo aproximadamente com um terço de todos eventos fatais. Neste contexto, as doenças do coração correspondem a 7,2 milhões dos óbitos/ano e o AVC a 5,5 milhões.

O custo dos cuidados diretos da DCV é alto, e, quando este é associado à prevalência desta patologia, tem-se a dimensão do grande problema de saúde pública que se afigura. Dados da Faculdade de Saúde Pública de Harvard (4) estimam um gasto direto de 12 mil dólares/ano para cada paciente tratado com IAM. A doença, além de apresentar um elevado custo direto, afeta também indiretamente a economia mundial, tendo em vista o acometimento de pessoas na meia idade, um período de alta produtividade, reduzindo sua força e capacidade de trabalho. Neste aspecto, o custo indireto da doença arterial coronariana, apenas nos Estados Unidos da América, consome aproximadamente 60 bilhões de dólares/ano (5), valor superior aos investimentos públicos na área de saúde de muitos países.

O esforço da comunidade científica médica internacional para o estudo do mecanismo de desenvolvimento da aterosclerose, principal causa de DCV, visando à prevenção e ao tratamento das doenças cardiovasculares, é sem dúvida um dos maiores e mais amplos na história da medicina. O fruto deste esforço pode ser verificado avaliando-se o número e qualidade de publicações a respeito deste tema. O resultado mais visível, no aspecto do mecanismo do desenvolvimento da aterosclerose, é a determinação dos fatores de risco clássicos desta patologia.

Estes fatores clássicos estão divididos academicamente em mutáveis e não mutáveis (6). Dentre os mutáveis, estão tabagismo, dislipidemia, diabetes melito (DM), pressão arterial elevada, obesidade, fatores dietéticos, fatores trombogênicos, sedentarismo e consumo excessivo de álcool. Já os fatores não mutáveis seriam o gênero, idade, antecedente pessoal de doença arterial coronariana (DAC) e antecedente familiar de DAC.

Outro avanço importante na elucidação do mecanismo aterogênico vem do entendimento do componente inflamatório na formação da placa do ateroma. Neste contexto, existe uma compreensão das células envolvidas no processo, sua quimiotaxia e cinética da produção de muitas das citocinas e proteínas inflamatórias. Em termos da prática clínica, já é possível aplicar este conhecimento com o uso de exames laboratoriais para acessar a inflamação, (7) dentre eles amiloíde sérico A, contagem de células brancas no sangue periférico, fibrinogênio (8,9) e proteína C reativa de alta sensibilidade (PCR-as), sendo esta última a que melhor apresenta características para a utilização prática; ressalta-se que para este exame já existe um consenso para o seu uso, formulado pela American Heart Association (AHA) e pelo Center for Disease Control (CDC) (7). Outros potenciais alvos para checar a inflamação que podem tornar-se úteis na prática clínica seriam fatores pró-

inflamatórios, como a lipoproteína de baixa densidade oxidada (LDL-ox), citocinas (interleucina-1, fator de necrose tumoral alfa) e moléculas de adesão celular (molécula de adesão celular-1 e selectinas) (10).

Outro mecanismo, que vem sendo bastante estudado nos últimos anos, e que sem dúvida apresenta suma importância na aterogênese é a disfunção endotelial, provocada diretamente pelo estresse oxidativo nas células das paredes dos vasos, levando a um aumento de expressão de certas moléculas e diminuição de outras. Podem-se citar dentre outras alterações uma diminuição da produção de óxido nítrico, um potente vasodilatador, aumento da produção de angiotensina II, um vasoconstritor, além do aumento dos níveis séricos do inibidor do ativador do plasminogênio-1 (PAI-1), um fator trombogênico. Todas estas alterações listadas corroboram para a formação do ateroma e seu desfecho clínico (11).

Neste contexto, o estresse oxidativo é responsável por efeitos deletérios, principalmente, devido à formação de ROS, as quais desempenham papel central na promoção da inflamação *in situ* e na disfunção celular (1).

ESTRESSE OXIDATIVO

A mitocôndria, além de ser a organela celular na qual ocorre a maior parte das reações oxidativas, visando à produção de moléculas com a capacidade de armazenar energia e cedê-la posteriormente em outros processos celulares dependentes de energia, é também fonte celular de formação das ROS. A formação das moléculas energéticas se dá em um processo de transferência de elétrons para um receptor final, o oxigênio, formando

água, e durante este processo ocorrem várias reações intermediárias, nas quais elétrons podem ser perdidos, gerando ROS (12-14).

A formação de ROS se dá dentro da cadeia de transporte de elétrons, que reduz continuamente oxigênio para formar o potencial eletroquímico transmembrana de prótons, necessário para a síntese de ATP. Neste processo, porém, pode haver um escape de elétrons que formam ROS. Apesar de a mitocôndria ser a maior fonte celular de ROS (13,14), é surpreendente sua eficácia energética, pois apenas 1-5% destes elétrons são perdidos durante o processo.

A maior parte destes elétrons que escapam da cadeia respiratória liga-se monoeletronicamente com o oxigênio, em etapas intermediárias da cadeia, gerando assim o radical superóxido (13,14). Os principais sítios mitocondriais de formação de superóxido são o complexo I (NADH desidrogenase) e o complexo III (Ubiquinona – citocromo C oxiredutase) pela coenzima Q (12,15,16).

As ROS formadas no compartimento intra-celular, em especial nas mitocôndrias, têm forte correlação com inúmeros processos degenerativos, dentre eles se destacam o envelhecimento celular e a morte celular apoptótica e necrótica.

A geração de radicais livres é um processo contínuo no compartimento mitocondrial, entretanto existe na mitocôndria um sistema antioxidante que defende a organela e, por fim, a célula deste femônemo. Dentre as formas pelas quais a célula defende-se da formação destes radicais livres podem-se citar: Mn-superóxido dismutase, glutationa peroxidase e glutationa redutase. Estas enzimas trabalham em seqüência visando à eliminação das ROS (17). Neste contexto, a NADPH é a maior fonte de equivalentes

redutores para os sistemas antioxidantes sistemas glutationa peroxidase e glutationa redutase e tioredoxina redutase/peroxidase.

Antioxidantes lípidicos solúveis, como o α-tocoferol (vitamina E) e a ubiquinona reduzida, impedem a peroxidação lipídica (18); o citocromo c mitocondrial pode devolver o elétron à cadeia respiratória (19); o complexo IV (citocromo oxidase) previne a formação de espécies reativas de nitrogênio (NRS) (20); e, por fim, os resíduos de metionina das proteínas mitocondriais que, quando oxidados, não comprometem a funcionalidade proteíca e evitam a oxidação da cistina, que resultaria em alteração estrutural comprometendo a sua funcionalidade (21).

De uma forma geral, os antioxidantes mitocôndriais protegem a organela da maioria das lesões oxidativas em condições fisiológicas. Quando a geração de ROS está aumentada e/ou a capacidade das defesas antioxidantes está abaixo do normal, as mitocôndrias acumularão ROS e sofrerão conseqüências do estresse oxidativo como a permeabilização da membrana interna e a formação do estado de transição de permeabilidade mitocondrial com inchamento da organela, o qual poderá levar a célula à morte quer por apoptose e/ou por necrose.

LIPEMIA PÓS-PRANDIAL E FUNCIONALIDADE DO HDL

O estado pós-prandial, fenômeno fisiológico que ocorre diversas vezes ao dia, iniciado após a absorção intestinal de uma dieta contendo lipídeos, proteínas e carboidratos, tem despertado o interesse crescente da comunidade científica há aproximadamente 30 anos, desde a sua primeira correlação com o processo de aterosclerose (22).

Neste sentido, muitos dos mecanismos aterogênicos podem apresentar-se no período pós-prandial devido à lipemia aguda e/ou sub-aguda, que, de acordo com os hábitos dietéticos, representa grande parte do dia dos indivíduos, principalmente nas sociedades ocidentais, onde as refeições seqüenciais são uma rotina.

O período pós-alimentar é sem dúvida um dos pontos cruciais no metabolismo de lipoproteínas. O primeiro fenômeno que pode ser identificado é a formação, e posterior liberação na corrente sanguínea de moléculas lipoprotéicas ricas em triglicérides pelos enterócitos, denominadas de quilomícrons, que se diferenciam de outras lipoproteínas ricas em triglicérides pela presença da apolipoproteína B-48.

O quilomícron gerado pelo enterócito pode seguir algumas vias metabólicas conhecidas. A primeira a ser citada seria a absorção destas partículas por células em processo ativo de divisão, como fibroblastos, miócitos e células da linhagem hematopoiéticas, além dos hepatócitos, os quais são a única linhagem de células quiescentes que expressam número considerável de receptores para as lipoproteínas, sendo que este processo de internalização dependende de alguns receptores celulares (23, 24). Nas células em processo ativo de divisão as partículas lipoprotéicas podem ser usadas como componentes estruturais e/ou fonte energética, já no hepatócito a capitação aumentada de lipotroteínas parece estar relacionada ao processo de exportação de colesterol via bile, a produção de lipoproteínas de densidade muito baixa.

O segundo mecanismo pelo qual a partícula rica em triglicérides gerada pelo intestino, pode ser metabolizada é via lípase periférica. Esta enzima, localizada na superfície das células endoteliais age preferencialmente sobre os triglicerídeos dos quilomícrons levando à hidrólise de triglicérides com liberação de ácidos graxos,

monoglicerídios e glicerol, os quais são internalizados por diversos tipos celulares (25). A ação da lípase conduz à redução do volume global das lipoproteínas ricas em triglicérides, e também a sua concentração de triglicérides (26).

Neste contexto de redução de volume dos quilomícrons, uma outra partícula pode ser formada a partir da sobra de componentes de superfície (colesterol livre, fosfolípides e apolipoproteínas), denominada pré-βHDL (27). Esta, por sua vez, é a precursora da HDL, cuja formação estaria na dependência de proteínas de transferência de fosfolípides e da enzima plasmática lecitina-colesterol-acil-transferase (LCAT) (28). Logo, estes componentes que restaram da superfície dos quilomícrons transformar-se-iam em cilindros e depois em esferas, dependendo do acréscimo de seu conteúdo (29).

Dois tipos diferentes de HDL estão presentes no plasma, o primeiro a ser formado no processo metabólico pós-prandial seria o HDL_3, do qual colesterol esterificado é transferido via CETP para lipoproteínas contendo apolipoproteína-B (30). Esta troca de colestrol por TG faz emergir uma partícula com características diferentes, a HDL_2, que pode novamente ser convertida a HDL_3 após a ação da lípase hepática (31, 29). A composição desta lipoproteína em relação a sua fração protéica pode ser modificada durante os processos plasmáticos, determinando tanto sua função quanto a remoção periférica de colesterol. As HDLs com presença exclusiva de apoA-I são eficientes na remoção de colesterol da periferia, enquanto a presença concomitante de apolipoproteína-AII inibe esta capacidade (29).

A concentração plasmática das HDL é reconhecidamente um dos fatores protetores contra a aterosclerose. Desde o estudo de Framingham, associam-se estas partículas a uma proteção ao desenvolvimento de DCV (32,33).

As HDLs formam um grupo heterogênio de partículas esféricas com diferentes propriedades físico-químicas, metabolismo intravascular e atividade biológica, sua densidade varia entre 1,063 a 1,21 g/mL (34). Este grupo de partículas é ainda subcategorizado em HDL_2 (subdividido em HDL_{2b}, HDL_{2a}) e HDL_3 (subdividido em HDL_{3a}, HDL_{3b}, HDL_{3c}), a depender de sua densidade.

Estudos indicam várias propriedades antiaterogênicas das HDLs, são elas: transporte reverso de colesterol para o fígado, atividade antiinflamatória, atividade antioxidante, atividade antiapoptótica, atividade antiinfecciosa, atividade antitrombótica e ação vasodilatadora (33).

O transporte reverso de colesterol inicia-se com o efluxo de colesterol das células periféricas, principalmente dos macrófagos. A apoA-I, apolipoproteína mais comum das HDLs, cataliza o efluxo celular de colesterol e fosfolípides via *ATP-binding cassete transpoters* A1, G1 e G4 (ABCA1, ABCG1 e ABCG4) (35, 36). O efluxo de colesterol pode ainda ocorrrer via *scavenger receptor B type I* (SR-BI) por difusão aquosa (35). A capacidade de transporte reverso de colesterol pelas HDLs é inversamente proporcional a sua concentração de lipídios, logo as partículas pequenas e com pouca concentração lipídica (HDL_{3b} e HDL_{3c}) são as mais eficientes aceptoras de colesterol para transporte reverso.

A atividade antioxidante das HDLs é a sua mais importante função ateroprotetora e é comumente mensurada em ensaios *in vitro* como inbição da oxidação da LDL (37). Esta atividade depende da presença das apolipoproteínas e enzimas com capacidade antioxidante. A apoA-I é a principal responsável por este processo removendo lípides oxidados das LDLs (38). As outras enzimas que corroboram com esta propriedade das HDLs são a paroxonase 1 (PON 1), fator ativador de plaqueta – acetil hidrolase (PAF-AH,

também conhecida como fosfolipase A_2) e a LCAT, todas estas enzimas contidas nas HDLs podem hidrolisar fosfolípides oxidados derivados de LDLs. Também como ocorre com o transporte reverso de colesterol, as partículas pequenas e densas de HDL (HDL_{3b} e HDL_{3c}) são mais aptas para exercer a função anti-oxitante, tendo em vista a maior concentração destas enzimas (38).

Já a atividade antiinflamatória endotelial das HDLs decorre da diminuição da expressão de moléculas de adesão e conseqüente redução de células inflamatórias no espaço subendotelial nos estágios iniciais da aterosclerose (34, 39). Esta propriedade das HDLs é atribuída à hidrólise de lípides oxidados, um mecanismo, portanto, semelhante ao da atividade antioxidante. Consistente com este fato a propriedade antiinflamatória das HDLs também é mais efetiva nas subfrações HDL_{3b} e HDL_{3c} (40).

O mecanismo aterogênico no período pós-prandial, proposto por Zilversmit há 30 anos (22), postulava a hipótese de que a geração de remanecentes de partículas ricas em triglicérides (TRL) deste período estaria envolvida na formação do ateroma.

Naquela época havia problemas para o estudo do estado pós-prandial, dentre eles, podem-se citar: a falta de uma metodologia para estudar os remanecentes de TRL, e como dissociar estatisticamente as TRL remanecentes das TRL de jejum na promoção da DAC, tendo em vista as similaridades destas partículas. Pelo menos para o primeiro problema há soluções práticas já disponíveis e amplamente aceitas. O primeiro passo, dado nesta direção, foi a identificação da apolipoproteína B-48 (41), marcadora específica de quilomícrons de origem intestinal. Outro avanço neste aspecto, alcançado mais recentemente, foi o desenvolvimento de um teste imunológico para identificar partículas de colesterol similares às remanecentes (RLP) em plasma humano (42), que tem sido

empregado em vários estudos recentes para estimar as partículas remanecentes de TRL potencialmente aterogênicas(43-45).

Primariamente, o potencial patológico do estado pós-prandial seria devido à penetração dos remanecentes TRL no endotélio, apesar de apenas os pequenos quilomícrons apresentarem esta habilidade, outra forma pela qual as TRL promovem a aterosclerose é pela geração de partículas pequenas e densas de LDL altamente aterogênicas (46).

Em vários estudos, já foi demonstrada a associação entre retardo na remoção de triglicérides e doença coronoriana (47) e doença vascular cerebral em idosos (48,49). A elevação transitória de triglicérides em indivíduos aparentemente saudáveis leva à formação de partículas de LDL mais densas e menores com grande potencial aterogênico e mais suceptíveis a oxidação. Estas partículas, quando no espaço sub-endotelial podem ser reconhecidas primariamente pelos macrófagos, os quais podem dar início ao processo de aterogênese. Vale resaltar que, ao contrário das partículas de LDL, as partículas ricas em triglicérides são captadas pelo macrófago sem necessidade de oxidação prévia (46).

Outros mecanismos, já estudados no período de lipemia pós-prandial, que também levariam à doença arterial coronariana, seriam os efeitos pró-trombóticos (50-53) deste estado e o efeito deletério na função endotelial (54,55). A lipemia leva a um aumento de fator VII da coagulação e do fibrinogênio no plasma, evento que associado à instabilidade das placas ateroscleróticas, promoveria a obstrução arterial. Além do mais, neste estado, há um incremento na produção do PAI-1, que diminuiria a possibilidade de fibrinólise caso um trombo se forme nas artérias (52,56). Ao mesmo tempo, em que a função endotelial está

diminuída (57-60), e que a hipertrigliceridemia crônica induzida em modelos animais associa-se a modificações deletérias na respiração mitocondrial destas celulas (61).

Evidências recentes parecem apontar para diferentes respostas individuais à sobrecarga de lipídios provinientes de uma dieta padronizada, com a presença de momentos distintos do pico de triglicérides no período pós alimentar, variando entre 2-8h, sendo os indivíduos com pico tardio, aparentemente, mais susceptíveis a desenvolvimento de aterosclerose (62).

Na tentativa de modular a resposta deletéria da lipemia pós-prandial, diversas drogas já foram testadas nesta situação, em especial os fibratos (63-66) e as estatinas (67, 68), apesar de outras classes de medicamentos também já terem sido testadas, como os derivados do ácido nicotínico (69) e a colestiramina (70). Os fibratos por atuarem diretamente na via exógena do metabolismo das lipoproteínas, estimulando a produção da enzima lipoproteína lípase, apresentam melhores resultados do que as estatinas na redução dos níveis de triglicérides no período em questão, porém algumas estatinas mais potentes, como a atorvastatina e rosuvastatina, apresentem resultado mais parecido com o dos fibratos. Associações de fibratos com estatinas também foram testadas neste período (71,72) com boa resposta dos níveis de triglicérides. Estudos prospectivos evidenciam ganho em sobrevida ou diminuição de eventos adversos nos pacientes tratados com estas drogas (73-75).

Sendo assim, apesar de muitos aspectos já terem sido abordados sobre o período pós alimentar, há ainda muitos aspectos a serem estudados, em especial sobre o estresse oxidativo em grupos de indivíduos que apresentem estados patológicos durante este período

como cardiopatas, diabéticos, hipertensos, dislipidêmicos e outros para uma melhor compreensão do processo de aterogênese.

Objetivos

GERAL

Caracterizar a composição química da HDL por sexo e por idade em população normolipidêmica no jejum e no período pós-prandial com ênfase no estresse oxidativo plasmático e celular.

ARTIGO 1

Os objetivos do artigo foram estabelecer os valores de normalidade da concentração de colesterol e triglicerídeos contidos nas subfrações de HDL (HDL_2 e HDL_3) em adultos normolipidêmicos, estratificando estes valores em subgrupos relativos ao sexo e idade dos indivíduos; bem como comparar a concentração de colesterol e triglicerídeos contidos nas subfrações de HDL nestes subgrupos.

ARTIGO 2

Os objetivos do artigo foram avaliar o impacto de uma refeição padronizada na composição química das HDL e na sua atividade antioxidante em indivíduos adultos saudáveis; bem como caracterizar a atividade das proteínas tranferidoras de lipídios e sua influência na composição química das HDL e na sua atividade antioxidante no período pós-prandial; e ainda avaliar o impacto do enriquecimento em triglicerídeos das HDL sobre a conformação da apolipoproteína A-I.

ARTIGO 3

Os objetivos do artigo foram caracterizar as mudanças na geração intracelular de espécies reativas de oxigênio, consumo intracelular de oxigênio e na função endotelial no período pós-prandial em adultos saudáveis após uma refeição padronizada; bem como correlacionar estes achados com as mudanças no perfil lipídico e glicídico.

Capítulo 1

TITLE

HIGH-DENSITY LIPOPROTEIN SUBFRACTIONS IN NORMOLIPIDEMIC INDIVIDUALS WITHOUT CLINICAL ATHEROSCLEROSIS

RUNNING TITLE

High-density lipoprotein subfractions in an adult population.

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Abstract

Background: For a better understanding of the metabolism of high-density lipoproteins's (HDL) subfractions we analyzed the distribution of lipids in HDL_2 and HDL_3 in an adult normolipidemic population without coronary atherosclerosis.

Methods: Ninety-three males and 92 females, healthy and normolipidemic, volunteered to be submitted to a clinical examination, a blood collection and to answer a questionnaire aimed at determining signs and symptoms of atherosclerotic disease. Their fasting plasma lipid, lipoproteins, apolipoproteins and the cholesterol and triglyceride concentrations in HDL_2 and HDL_3, isolated by microultracentrifugation, were determined by enzymatic-colorimetric methods.

Results: The interpercentile intervals (2.5 to 97.5) for the population were established as being 5 to 18 mg/dL in men and 4 to 28 mg/dL in women for HDL_2chol and 1 to 57 mg/dL in men and 2 to 61 mg/dL in women for HDL_3chol. HDL_2Tg in men was 1 to 26 mg/dL and in women 2 to 28 mg/dL; moreover, the HDL_3Tg intervals were established as 4 to 46 mg/dL for both sexes.

Conclusions: The determination of reference ranges for lipids in HDL subfractions in populations without overt atherosclerosis, is an useful tool for metabolic, diagnostic and therapeutic approaches. We determined the intervals for HDL_2chol, HDL_3chol, HDL_2Tg and HDL_3Tg. There were variations with sex and/ or age for HDL_2chol, HDL_3chol and HDL_2Tg in the studied population.

Key words: HDL subfractions reference ranges; HDL_2chol; HDL_3chol; HDL_2Tg; HDL_3Tg.

1. Introduction

As in economically developed countries worldwide, Brazil has a very high frequency of cardiovascular disease, although a decrease in the mortality rate from CHD may now be observed, as shown in a recent study [1]. In Campinas, an urban area in the state of São Paulo, the age-classified mortality rates for ischemic disease are similar to those obtained for the North-American population. The most frequent cause of death is cardiovascular disease, representing 30.5% of the deaths notified in 2002 and 2003 [2].

The great clinical interest in the evaluation of plasma lipoproteins is generated by its importance in the determination of CHD risk, the therapeutic follow-up of dyslipidemia and the prevention of CHD [3].

Prospective studies demonstrate that the risk for coronary disease is directly related to low-density lipoprotein cholesterol (LDLchol) and inversely related to high-density lipoprotein cholesterol (HDLchol) levels in plasma [4, 5]. An increase of 10 mg/dL in HDLchol levels is associated with a 42% reduction in the risk for CHD [4-7].

Using multivariate analysis, studies carried out by Lipid Research Clinics Program Follow-Up Study demonstrated that the HDLchol is a variable with strong predictive value for mortality by CHD. Independently of cholesterol levels (including values below 200mg/dL), the CHD risk was inversely associated with the level of HDLchol and the total-chol/HDLchol ratio identified people at high risk. According to the same analysis, HDLchol was shown to be a factor having twice the predictive power than LDLchol for CHD risk [8, 9].

The mechanisms responsible for the antiatherogenic property of HDL are still not completely elucidated and are certainly not restricted to its plasmatic concentration. The heterogeneity of HDL particles, in terms of density (HDL_2, HDL_3), size (HDL_{2b}, HDL_{2a},

HDL_{3a}, HDL_{3b} and HDL_{3c}, and protein content of apolipoprotein (apo AI or apo AI plus apo AII) should be considered specially in patients with increased risk for CHD. Therefore, the analysis of the composition of HDL subfractions could be a better predictor of risk of coronary disease than the total HDL quantification. Recent studies correlate concentrations of different HDL subfractions with the risk of coronary and extra-coronary disease [10-18] but this issue is not clear yet.

Until now there have been very few reports in the literature on the determination of the reference intervals for lipids in HDL subfractions in adults. Therefore the objective of this work was to determine the reference ranges of cholesterol and triglycerides in HDL subfractions, by sex and age, from an adult population without evidence of established cardiovascular disease.

2. Materials and methods

2.1. Subjects and lipoprotein profile evaluation

A total of healthy individuals, selected by consecutive sampling, 93 men and 92 women, aged from 20 to 86 years participated in this study. They were as well normolipidemic according to NCEP III's (19) criteria and presented serum levels up to borderline for total cholesterol (Tchol), up to near or above optimal for LDLchol, and desirable for triglyceride (Tg).

The individuals were examined at the Dyslipidemia Clinic of the University Hospital - UNICAMP for anthropometric measurements and for information on the presence of cardiovascular disease. Established cardiovascular disease (CVD) was defined as the occurrence of one or more of myocardial infarction, coronary artery bypass grafting and percutaneous

transluminal coronary angioplasty, stroke, transient ischemic attack and peripheral arterial disease and these individuals were excluded from the study.

Blood samples were collected in EDTA-containing tubes (following a 12-hour fast) for the lipoprotein profile evaluation (total cholesterol, LDLchol, HDLchol, triglycerides, apolipoprotein AI and B100) and to obtain HDL (HDL_2 and HDL_3) subfractions. Total-cholesterol (Tchol) and triglyceride (Tg) serum levels were quantified by enzymatic-colorimetric methods in the analyzer Hitachi (Roche). HDLchol was enzymatically determined in the supernatant after sodium phosphotungstate/$MgCl_2$ ApoB100-containing lipoprotein precipitation and LDL-chol by the Friedewald's equation. Apolipoprotein AI and B100 (Apo AI and B100) were quantified by nephelometry Array 360 (Beckman, Palo Alto, EUA).

HDL subfraction isolation was achieved by the microultracentrifugation [20] technique. Following Apo B100-containing lipoprotein precipitation, 90μL of the supernatant containing total HDL was transferred to polyethylene tubes, with 45μL of KBr solution (d=1.346 g/mL) added for density adjustment to 1.125 g/mL. HDL_2 was isolated after ultracentrifugation for 3 hours and thirty minutes at 4ºC, 20 psi, 100,000g, in an Airfuge microcentrifuge rotor A100-30 (Beckman, Palo Alto, EUA). Thirty microliters of the supernatant (corresponding to the HDL_2) were removed for cholesterol and triglyceride quantification. The remaining infranatant (105μL), corresponding to the HDL_3 fraction, was submitted to the same measurements (Tchol and Tg).

The study protocol was approved by the Ethics Committee of the School of Medical Sciences, UNICAMP.

2.2. Statistical Analysis

Data normal distribution was tested by the Kolmogorov-Smirnov test. Logarithmic transformation was applied to Tchol, HDL_3Tg and ApoB100. For the comparison tests for sex and age groups (≤39, 40 to 59 and ≥60years old), the analysis of variance (ANOVA) monofactorial was used as well as Mann-Whitney or Kruskal-Wallis tests. Correlation between the HDL subfractions and anthropometric variables were verified by the Spearman test. All statistical analyses were considered significant when p values were ≤ 0.05.

3. Results

According to NCEP III's criteria [19] serum lipids, lipoproteins, and body mass index (BMI) were within optimal and/or desirable limits. The Apo AI and B100 values (Table 1) are in agreement with the manufacturer's reference ranges: 73 to 169 mg/dL and 58 to 138 mgdL, respectively.

When the population was analyzed by age, significant higher values for Tchol, LDLchol, HDLchol, ApoAI and Apo B100 serum levels were found in individuals older than 40 years of age, in agreement with the literature (Table 1).

Surprisingly, when the data were analyzed by sex, Tchol, LDLchol were higher in women than in men, as well as ApoAI and Apo B100, but no age or BMI differences were found (Table 1). The subgroup with BMI ≥25kg/m^2 presented higher concentrations of total cholesterol, LDLchol, Lp(a), HDLchol, HDL_3chol, as compared to individuals with BMI < 25kg/m^2.

HDLchol concentrations were significantly increased amongst women and increased with age reaching a *plateau* by their fourties (Table 1). The older women did not present higher Tchol or LDLchol values as compared to men in the same age group (data not shown).

Cholesterol and triglyceride contents of HDL subfractions (HDL_2 and HDL_3) are represented in Table 2. A significant difference was observed in HDL_2chol, HDL_2Tg and HDL_3chol, which were greater in women as compared to men; HDL_3chol was over two times higher than HDL_2chol in both sexes. There were no significant differences in HDL_3Tg concentrations irrespective of sex or age. Age differences were observed only for HDL_3chol, with lower values in the middle aged group (Tables 2).

The interpercentile intervals (2.5 to 97.5) for HDL_2chol was 5 to 18 mg/dL in men and 4 to 28 mg/dL in women. The same intervals for HDL_3chol of the studied population were established as being from 1 to 57 mg/dL for men and 2 to 61 mg/dL for women. For HDL_2Tg in men was 1 to 26 mg/dL and in women 2 to 28mg/dL; moreover the HDL_3Tg intervals were established as 4 to 46 mg/dL for both sexes.

Age correlated positively with HDL_2chol (r=0.156), HDL_3chol (r=0.275) and with HDL_3 Tg (r=0.147). The BMI did not correlate significantly with any one of the variables tested.

4. Discussion

In the present study cholesterol concentrations in HDL subfractions are similar to some population-control studies that evaluated cardiovascular risk [11,21-23], although they differ from others [13, 24-27]. There is a lot of controversy in the literature concerning the role of

HDL subfractions against atherosclerosis. Bakogianni et al [28] consider HDL_2 more protective but others describe increased protection by HDL_3 including protection against LDL oxidation [29-31].

Our data are in agreement with Hartung et al. [23], which showed an HDL_3chol mean concentration of 39 mg/dL in sedentary women with a mean age of 41 years. Similar values were described by Gardner et al. [32] that demonstrated values significantly higher in women when compared to men in the same age group. Moreover, coherent results were obtained in Meilahn's study [21], carried out in women with an average age of 50.6 years, mean BMI of 25.9 Kg/m^2, whose HDL_3chol value was 39.5 mg/dL. We did not find statistically significant sex differences in serum HDL_2chol concentrations, as described by Gardner [32].

However, Shepherd et al. [33] showed that HDL_2 and HDL_3 are substantially different in lipid and protein composition and, that HDL_2chol concentrations are higher in women than in men. The increased HDL_3chol in women, demonstrated in the present work, could be a protective factor against atherosclerosis in this sex. In contrast with our study, HDL_3chol concentrations was not different between the sexes in Shepherd et al.'s [34] study.

The variability of results found in the literature regarding cholesterol concentrations in HDL subfractions may be due to small sample sizes, differences in methodologies (ultracentrifugation, electrophoresis or selective precipitation) or to characteristics variations in the studied populations, such as BMI, age range, sex or lifestyle.

Evidence from controlled clinical trials indicates that modifications in lifestyle are determinant for concentrations of HDL, HDL_2chol, HDL_3chol and Apo AI [10, 21, 33]. Physical activity and alcohol consumption increase the two subfractions; physical activity affects predominantly HDL_2chol [10, 21, 24, 34], while alcohol consumption interferes with

HDL_3chol [14, 35, 36]. Obesity and insulin resistance are associated with low HDLchol and HDL_2chol levels and a high rate of HDL_3chol [32]. The exogenous estrogen predominantly increases HDL_2chol levels, while testosterone may diminish them [22, 32].

In climacterium, when cardiovascular diseases are important cause of death, women present a decline in HDLchol and an increase in LDLchol levels with consequent increased risk [27]. Moreover, it is known that HDLchol levels present a greater impact on the mortality rate and on cardiovascular disease evaluation risk in women than in men [37]. Therefore, in this period, cholesterol evaluation in HDL subfractions could contribute to define lifestyle alterations, precocious use of medication or more rigorous control of already existing pathologies.

This study introduces cholesterol and triglyceride reference ranges for HDL subfractions in a normolipidemic adult population, without evidence of established CVD. Moreover, these reference limits in a population without overt atherosclerosis, are important guides in the evaluation of patients' CHD risk. Further studies on the chemical composition of HDL in populations presenting atherosclerosis are necessary to fully understand the clinical meaning of these parameters in atherosclerosis.

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Table 1 - CLINICAL AND BIOCHEMICAL PARAMETERS BY SEX AND AGE[a]

	SEX		AGE		
Parameters (n)	Male (93)	Female (92)	≤39 years (122)	≥40 ≤59 years (45)	≥ 60 years (18)
AGE (years)	35±18	39±14	27±5	49±6	74±8
BMI[b] (Kg/m^2)	23± 2	23± 3	23±2	24±3	24±3
Tchol (mg/dL)	162±28	178±35*	159±29**	191±31	194±29
LDLchol (mg/dL)	100±23	107±27*	97±24**	119±24	115±23
HDLchol (mg/dL)	42±11	51±11*	44±11**	51±12	55±12
Tg (mg/dL)	104±46	109±51	103±51	110±48	117±37
Apo AI (mg/dL)	135±23	150±25*	137±24**	151±24**	151±29
Apo B100 (mg/dL)	77±20	102±34*	86±33**	101±27	91±21

[a] Data expressed as mean ± SD; n=185; [b] BMI= body mass index; Mann-Whitney and ANOVA tests,*and ** $p<0.05$, respectively to sex and age comparison

Table 2- CHOLESTEROL AND TRIGLYCERIDES IN HDL SUBFRACTIONS BY SEX AND AGE[a]

	SEX		AGE		
Parameters (n)	Male (93)	Female (92)	≤39 years years (122)	≥40 ≤59 years (45)	≥60 years (18)
HDL_2 chol (mg/dL)	11±3	14±7*	13±5	14±10	11±4
HDL_2Tg (mg/dL)	9±6	12±8*	14±7	12±8	13±5
HDL_3chol (mg/dL)	28±16	33±18*	42±10	35±15*	43±10
HDL_3Tg (mg/dL)	16±8	19±12	16±6	20±12	18±8

[a] Data expressed as mean ± SD; n=185; Mann-Whitney and ANOVA tests: * $p<0.05$; differences defined by age groups

Capítulo 2

Postprandial lipemia induces impaired antioxidative activity of dense HDL3 particles: Implication of core lipid composition and cholesteryl ester transfer protein activity

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Running title: Impaired functionality of postprandial HDL

Background and Objective The postprandial phase features perturbed intravascular lipid metabolism of apolipoprotein B-containing (VLDL, IDL, LDL) and apolipoprotein A-I-containing (HDL) lipoproteins, inflammation and oxidative stress, key proatherogenic factors. Dense HDL3 exert potent antioxidative activity (AOX) which is inversely related to oxidative stress. Is HDL antioxidative function therefore defective postprandially?

Design and Results Healthy normolipidemic males (n=10) consumed a liquid meal (25% fat) providing 40g fat/m^2 body surface. Postprandial hypertriglyceridemia (plasma triglyceride (TG) concentration, +78% at 4h; $p<0.01$) was associated with increased cholesteryl ester transfer protein (CETP) activity and diminution of HDL-cholesterol concentration (-6%; $p<0.05$) and HDL cholesteryl ester (CE) content (-13%; $p<0.05$). AOX of postprandial dense HDL3c was attenuated (up to -22%; $p<0.05$) as determined by protection of reference LDL from oxidation, was negatively correlated with plasma TG concentrations and HDL TG content and positively correlated with HDL CE content. When dense HDL particles were enriched in TG in vitro, their AOX was abrogated and the accessibility of apolipoprotein A-I to monoclonal antibodies reduced.

Conclusions Transient postprandial elevation in CETP activity drives reduction of HDL CE core content, resulting in impaired HDL AOX, potentially via conformational alterations in apolipoprotein A-I. Such phenomenon may contribute to enhanced atherogenicity of the postprandial phase.

Keywords: CETP; triglycerides; oxidative stress; HDL subfractions; core lipids; apolipoprotein A-I

Elevated nonfasting concentrations of triglyceride (TG) have been recently reported to represent a novel risk factor for cardiovascular disease.[1, 2] The postprandial phase is characterised by transient alterations in lipid and carbohydrate metabolism including hypertriglyceridemia, reduced concentrations of HDL-cholesterol (HDL-C) and hyperglycemia.[3, 4] Postprandial TG-rich, apolipoprotein B (apoB)-containing lipoproteins, such as chylomicron remnants, VLDL and VLDL remnants, may penetrate the arterial intima and possess multiple proatherogenic properties including induction of inflammatory response, oxidative stress and endothelial dysfunction. These factors, acting in a synergistic manner, play an important role in the initiation, progression and clinical complications of atherosclerosis.[5]

Atherogenic dyslipidemia, an imbalance between circulating concentrations of atherogenic apoB-containing lipoproteins relative to those of anti-atherogenic, apoA-I-containing lipoproteins, is associated with arterial retention of LDL, oxidative stress, inflammation and endothelial dysfunction.[6] Oxidative stress, an emerging cardiovascular risk factor,[7, 8] acts directly to modify LDL in the arterial intima into highly pro-atherogenic particles.[6] In contrast, apoA-I-containing HDL exerts a spectrum of antiatherogenic and vasculoprotective activities principal among which are cellular cholesterol efflux, together with antioxidative, anti-inflammatory, and anti-thrombotic actions.[9]

HDL particles are however highly heterogeneous in structure, intravascular metabolism and antiatherogenic activity.[10, 11] Thus, small, dense, protein-rich HDL particles possess elevated capacities to accept cellular cholesterol,[12] to inhibit expression of adhesion molecules[13] and to protect LDL from oxidation[14] as compared to large, light, lipid-rich HDL. Such potent anti-atherogenic activities of small, dense HDL can however be compromised in the atherogenic dyslipidemias characteristic of two common insulin-resistant states, i.e. Metabolic Syndrome

(MetS) and Type 2 diabetes.[15, 16] Alterations in HDL metabolism and composition, which include attenuated plasma residence time, accelerated renal catabolism and core lipid enrichment in TG and depletion of cholesteryl ester (CE) as a result of enhanced cholesteryl ester transfer protein (CETP) activity, are characteristic of such atherogenic lipid phenotypes.[15, 16] Dense HDL3 particles in these dyslipidemias are deficient in antioxidative activity (AOX); furthermore, AOX of dense HDL is inversely related both to core lipid replacement of CE by TG, and to elevated levels of oxidative stress in vivo, as measured by plasma 8-isoprostanes, an integrative biomarker.[15, 16].

In the postprandial phase, CETP activity increases concomitant with increase in plasma concentrations of TG-rich particles which act as acceptors of CE from HDL, but equally as donors of TG to HDL.[17, 18] As a consequence, HDL become CE depleted and TG enriched, suggestive of impaired AOX.[19] A link between postprandial lipid metabolism and HDL functionality has been equally suggested by recent studies of the influence of fatty acid composition of a meal on the anti-inflammatory potential of HDL.[20] We therefore evaluated the impact of meal intake on HDL AOX in normolipidemic subjects over the postprandial period and provide evidence for impaired AOX in postprandial dense HDL.

Subjects and Methods

Subjects. Ten healthy, non-obese (body mass index (BMI, 23.1±1.7 kg/m^2), normotensive (systolic blood pressure, 119±7 mm Hg; diastolic blood pressure, 72±8 mm Hg), normolipidemic (**Table 1**), normoglycemic, male volunteers were recruited at the Campinas State University (Campinas, Brazil). All subjects were aged between 20 and 39 years (27.6±3.7 years), non-smokers, and either abstainers or moderate alcohol consumers (<25 g/d). Blood pressure and

physical data were determined during a complete clinical examination. None of the subjects presented renal, hepatic, gastrointestinal, pulmonary, endocrine, or oncological disease or were receiving a specific diet, antioxidative vitamin supplementation or drugs known to affect lipoprotein metabolism for at least 2 months before the study. All subjects gave written informed consent; the protocol was in accordance with local institutional guidelines and was approved by the local Ethical Committee for Clinical Research.

Meal. After a 12h overnight fast, a lactose-free, high-fat, milkshake liquid meal was consumed at 8 a.m. over a period of up to 10 min. The meal contained 25% fat, 55% dextromaltose and 14% protein providing 40g fat/m^2 of body surface. The meal contained 43% saturated, 38% monounsaturated and 19% polyunsaturated fat.

Blood samples. Venous blood samples were collected on ice in the fasting state at baseline and 4 and 8h later in the postprandial phase. Serum and EDTA plasma (final EDTA concentration, 1 mg/mL) were immediately separated by centrifugation at 4°C, aliquoted and frozen at -80°C; each aliquot was thawed only once immediately before analysis.

Plasma total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglycerides (TG) and lipoprotein (a) concentrations were measured using commercially available kits (coefficients of variation <7%).[14-16]

Isolation of lipoproteins. Five physicochemically-defined HDL subfractions corresponding to light HDL2b (d 1.063-1.087 g/mL) and 2a (d 1.088-1.110 g/mL), and dense HDL3a (d 1.110-1.129 g/mL), 3b (d 1.129-1.154 g/mL) and 3c (d 1.154-1.170 g/mL), were isolated by isopycnic density gradient ultracentrifugation.[21] The resolution and reproducibility of this preparative density fractionation of highly purified HDL particle subspecies have been extensively documented.[14, 21, 22] Total HDL was prepared by mixing all five HDL subfractions at their

equivalent serum concentrations. Reference LDL (1.018-1.065 g/mL), VLDL (d 1.000-1.018 g/mL) and centrifugal bottom fraction (d >1.21 g/mL) was isolated by the same procedure from one healthy normolipidemic control subject. All subfractions were extensively dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4°C in the dark, stored at 4°C and used within 2 days.

Enrichment of dense HDL in TG in vitro. Dense HDL3b subfraction was chosen for these experiments as on the one hand, the yield of HDL3c subfraction, which displays the most potent AOX, is not sufficient for such approach and on the other hand, AOX of HDL3b is still high enough as compared to less dense HDL subfractions characterised by a higher yield. Freshly isolated HDL3b (650 μL) was enriched in TG upon incubation with freshly isolated VLDL (570 μL) and centrifugal bottom fraction (80 μL) as a source of CETP for 3h at 37°C. At the end of the incubation, HDL3b particles were re-isolated using density gradient ultracentrifugation as above. As a control, HDL3b was incubated in parallel in the absence of VLDL and the bottom fraction.

Chemical composition of lipoproteins. TC, free cholesterol (FC), phospholipid (PL) and TG contents of isolated lipoprotein subfractions were measured using commercially available kits (CHOP-PAP, Biomerieux, France); cholesteryl ester (CE) content was calculated by multiplying the difference between TC and FC by 1.67.[21] Total protein was measured using the BCA assay. Total lipoprotein mass was calculated as the sum of total protein, CE, FC, PL and TG. Concentrations of apoA-I and apoA-II were determined by immunonephelometry.[14]

Oxidation of lipoproteins. We have previously established that the dense HDL3c subfraction displays potent antioxidative activity (AOX).[14] In the present study, we therefore measured AOX of total HDL and of the HDL3c subfraction. The AOX was assessed towards normolipidemic reference LDL.[16, 23] LDL (10 mg TC /dL) was oxidised in the absence or presence of HDL particles at 37°C in Dulbecco's PBS (pH 7.4) by 1 mmol/L 2,2´-azobis-(2-amidinopropane)

hydrochloride (AAPH); HDL subfractions were added to LDL immediately before oxidation. Antioxidative activity of the HDL3c subfraction was determined at a final concentration of 10 mg total mass/dL.[16, 23] Total HDL was used at a final concentration of 40 mg total mass/dL.

Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm.[16, 23] Absorbance kinetics were corrected for the absorbance of AAPH itself run in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the lag and propagation phases. For each curve, the duration of each phase, average oxidation rates within the propagation phase and amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated.

Activities of plasma lipid transfer proteins. Plasma cholesteryl ester transfer protein (CETP) activity was measured using an exogenous fluorescent substrate (Roar Biomedical, New York, NY, USA). Lecithin-cholesterol acyltransferase (LCAT) activity was measured as a phospholipase activity using a fluorescent LCAT activity kit (Roar Biomedical, New York, NY, USA). Inter- and intraassay coefficients of variation were 5.1 and 6.5% for LCAT activity[16] and <3% for CETP activity[24] measurements, respectively.

Biomarkers of oxidative stress and inflammation. Systemic levels of oxidative stress were characterised as plasma concentrations of oxidised LDL (oxLDL) measured using an ELISA kit (Mercodia, Uppsala, Sweden). This assay employs antibody 4E6 directed against a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of aldehyde substitution of the lysine residues of apoB-100.

The level of systemic inflammation was assessed as plasma concentrations of high-sensitive C-reactive protein (hsCRP) measured with an immunoassay.[15, 16]

ApoA-I conformation in HDL. Set of previously characterised epitope-defined monoclonal antibodies to apoA-I was used to probe the influence of TG enrichment on the conformation of apoA-I in HDL. The association and dissociation rate constants of each antibody were measured using surface plasmon resonance analysis as described elsewhere.[25] All measurements were obtained on a BIAcore 2000 system (Pharmacia Biosensor AB, Uppsala, Sweden).

Statistical analysis. Differences between paired variables were analyzed by Wilcoxon's matched pairs test or by Student's t-test when appropriate. Differences between unpaired variables were analyzed using Mann-Whitney U-test or Student's t-test when appropriate. Spearman's correlation coefficients were calculated to evaluate relationships between variables. All results are expressed as means ±SD unless otherwise indicated. Calculations were performed using STATISTICA 6.1 (StatSoft Inc., www.statsoft.com) software package.

Results

Time course of changes in plasma lipids, apolipoproteins, lipid transfer protein activity and biomarkers of oxidative stress and inflammation over the postprandial period. As expected, circulating concentrations of TG significantly increased after consumption of a fat-rich meal (+78% and +58% at 4 and 8h respectively; Table 1). The TG area-under-the-curve values were typical for those reported for normolipidemic controls[18] (data not shown).

Small but significant reductions (-6%, $p < 0.05$ vs. baseline) in plasma concentrations of both HDL-C and LDL-C were observed 4h after the meal; these concentrations rebounded to baseline after 8h. By contrast, no postprandial changes were detected in circulating concentrations of TC, apoB, apoA-I, lipoprotein (a) and glucose (Table 1).

CETP activity measured against an exogenous substrate significantly increased in the postprandial phase (up to +7.4% at 4h, $p < 0.05$ vs. baseline), whereas LCAT activity was not modified significantly (Table 1). Similarly, systemic biomarkers of oxidative stress (oxLDL) and inflammation (hsCRP), which were normal at baseline,[16, 23] remained unchanged after meal intake (Table 1). In the whole dataset, CETP activity was positively correlated with plasma concentrations of hsCRP ($r=0.56$, $p<0.01$). LCAT activity was positively related to plasma apoA-I concentrations ($r=0.45$, $p<0.05$), consistent with the role of apoA-I as an activator for LCAT.[26]. Circulating concentrations of oxLDL were strongly correlated with those of TC, LDL-C and apoB (data not shown).

Circulating concentrations, chemical composition and size of HDL particles. As seen above for HDL-C concentrations, total HDL mass was also significantly reduced after 4h (-9.5%, $p < 0.05$ vs. baseline), but partially rebounded at 8h (**Table 2**). The postprandial decrease in total HDL mass was accompanied by significant decreases in HDL concentrations of CE (26.5±7.5 vs. 30.5±5.2 mg/dL at baseline, $p<0.01$), FC and total protein (data not shown). The relative % chemical composition of total HDL was affected to a lesser degree (Table 2). Thus, the content of PL increased significantly (+4%) after 4h, while that of CE tended to fall and that of TG to rise ($p=0.09$), potentially reflecting enhanced CE/TG heteroexchange by CETP (Table 2).

Compositional changes observed in total HDL were confirmed in individual HDL subfractions. Small, dense HDL particles were affected to the highest extent; HDL3a, 3b and 3c subfractions presented significant diminution in mass 4h after the meal (-16%, $p<0.01$; - 16%, $p<0.05$; and -19%, $p<0.01$, respectively; Table 2). The decrease in the mass of dense HDL particles reflected significant reductions in absolute concentrations of CE, FC, apoA-I, apoA-II, total protein (except HDL3c) and PL (only in HDL3c) after 4h; in addition, CE concentrations were significantly

reduced in HDL3b after 8h (data not shown). The postprandial weight % composition of HDL subfractions was characterised by diminished content of CE, FC and total protein as well as by elevated content of PL (Table 2, or data not shown). By contrast, no significant alteration in the molar content of apoA-I and apoA-II in HDL subfractions, or in the apoA-I/apoA-II ratio, was observed during the postprandial phase (data not shown). Similarly, particle size was not significantly altered during the postprandial phase in any HDL subfraction (data not shown).

In the whole dataset, plasma CETP activity was negatively correlated with HDL content of CE (total HDL, $r=-0.37$, $p<0.05$; HDL2b, $r=-0.44$, $p<0.05$; HDL3a, $r=-0.46$, $p<0.05$) and positively with HDL content of PL (total HDL, $r=0.38$, $p<0.05$; HDL2b, $r=0.54$, $p<0.01$; HDL3a, $r=0.39$, $p<0.05$). Plasma LCAT activity was positively correlated with HDL concentrations of CE (HDL3a, $r=0.53$, $p<0.01$; HDL3b, $r=0.50$, $p<0.01$; HDL3c, $r=0.51$, $p<0.01$), total protein and apoA-I (data not shown). Plasma hsCRP showed positive correlations with lipoprotein content of TG (e.g. total HDL, $r=0.52$, $p<0.01$; HDL3c, $r=0.63$, $p<0.001$) and negative correlations with the content of CE (e.g. total HDL, $r=-0.59$, $p<0.01$; HDL3c, $r=-0.57$, $p<0.01$) in all five HDL subfractions.

AOX of HDL. Previous studies have presented evidence that small, dense HDL subfractions potently protect LDL from oxidation at late stages of oxidation and are less active at early stages such as the lag phase.[14] Therefore, we evaluated the AOX of dense HDL3c during the first (lag) and second (propagation) phases of LDL oxidation. Consistent with published data,[14] both the dense HDL3c subfraction and total HDL fraction delayed AAPH-induced oxidation of reference LDL at a physiological HDL to LDL ratio of 2-6 mol/mol. Thus, at baseline, both dense HDL3c and total HDL significantly prolonged the propagation phase (+75%, $p<0.001$, and +101%, $p<0.01$, respectively), decreased the oxidation rate of LDL in this phase (-51%, $p<0.001$, and -

75%, p<0.001, respectively) and reduced maximal formation of conjugated dienes (-9%, p<0.05, and -34%, p<0.001, respectively); in addition, HDL3c prolonged the lag phase (+60%, p<0.05; **Figure 1**).

Potent AOX of total HDL was significantly attenuated in the postprandial phase as documented by a shorter propagation phase of oxidation (-35%) measured in the presence of HDL isolated from postprandial sera of 8h as compared to baseline samples (148±46 vs. 201±57% of values measured with LDL alone respectively, p <0.01; Figure 1A). The oxidation lag phase was equally shorter (114±27% vs. 128±46 respectively); this difference did not however reach significance (p=0.25).

Consistent with these data, the duration of the propagation phase measured in the presence of dense HDL3c was significantly shorter (-12%) at the 8h time-point (156±43 vs. 175±47% at baseline, p<0.05; Figure 1B), thereby demonstrating that AOX of HDL3c was significantly diminished in the postprandial phase. Furthermore, the duration of the lag phase determined in the presence of HDL3c was significantly reduced at 8h postprandially (-22%; 131±28 vs. 160±61% at baseline, p<0.05).

In the whole dataset, circulating concentrations of TG were inversely associated with AOX of total HDL and dense HDL3c particles, consistent with positive correlations between plasma TG concentrations and oxidation rates, and with negative correlations between TG concentrations and the duration of the propagation phase measured in the presence of total HDL and HDL3c (**Table 3**; **Figure 2A**). Furthermore, changes in plasma TG concentrations were correlated with changes in HDL AOX as exemplified by positive correlations between the increase in plasma TG and the increases in the oxidation rate of total HDL (r=0.89, n=10, p=0.001) and of HDL3c (r=0.75, n=10, p=0.01) after 8h, indicating that the observed correlations reflected metabolic alterations in

the postprandial phase rather than interindividual variability. By contrast, plasma concentrations of HDL-C were directly related to AOX of total HDL, consistent with the negative correlation between HDL-C concentration and maximal amounts of dienes (Table 3; **Fig; 2B**).

Interestingly, concentrations of hsCRP were inversely related to AOX of HDL particles, consistent with negative correlations between hsCRP concentrations on the one hand and duration of lag phase and propagation phase on the other (Table 3); by contrast, no significant relationship was observed between AOX of HDL and concentrations of oxLDL (data not shown).

The AOX of HDL particles was intimately related to their chemical composition. Thus, the AOX of both total HDL and dense HDL3c was inversely related to their TG content, as documented by negative correlations between %TG content and duration of the oxidation phase (Table 3; **Figure 3A**); furthermore, correlations of %TG content in HDL with oxidation rates and maximal amount of dienes were positive. By contrast, the content of CE in HDL was directly associated with AOX of both total HDL and dense HDL3c (positive correlations of %CE with duration of the oxidation phase; negative correlations between %CE content and oxidation rates and maximal amount of dienes; Table 3; **Figure 3B**). As a consequence, the CE/TG ratio was directly related to the AOX of total HDL (negative correlation with maximal amount of dienes; Table 3). Furthermore, changes in the HDL content of core lipids were correlated with changes in the AOX as exemplified by correlations between the increase in the oxidation rate and the decreases in the CE weight% ($r=0.75$, $n=10$, $p=0.01$) and in the CE/TG ratio ($r=0.64$, $n=10$, $p<0.05$) in HDL3c after 8h. In addition, HDL content of PL was negatively associated with AOX of HDL (Table 3).

Finally, strong intercorrelations were observed between antioxidative activities of HDL3c and total HDL particles. For example, significant positive correlations were observed between oxidation rates in the propagation phase ($r=0.73$, $p<0.001$), phase duration ($r=0.60$, $p<0.001$) and

maximal diene concentrations ($r=0.70$, $p<0.001$) measured in the presence of total HDL and HDL3c, suggesting that HDL3c can account for up to 50% of the AOX of total HDL. Correlations between oxidation rates in the propagation phase with the duration of this phase were equally significant but negative (e.g. $r=-0.51$, $p<0.01$, for the correlation between oxidation rate in the presence of total HDL and the phase duration in the presence of HDL3c, or $r=-0.61$, $p<0.001$, for the correlation between oxidation rate in the presence of HDL3c and the phase duration in the presence of total HDL).

Enrichment of dense HDL in TG in vitro. In order to directly address the role of HDL core lipids for the AOX, HDL3b subfraction freshly isolated from a healthy normolipidemic donor was enriched in TG in vitro upon incubation with freshly isolated VLDL and centrifugal bottom fraction as a source of CETP (modified from [27]). As a result, HDL3b content of TG was increased by +88% (on the basis of the TG/protein ratio; $p<0.01$, $n=5$) as compared to control HDL3b incubated in parallel in the absence of VLDL and the bottom fraction. Whereas control HDL3b inhibited oxidation of reference LDL (decrease in the oxidation rate in the propagation phase of -22% and prolongation of this phase of +33%), HDL3b enriched in TG completely lost its AOX (**Figure 4**).

The influence of TG enrichment on the conformation of apoA-I in dense HDL was characterised using a set of previously characterised epitope-defined monoclonal antibodies to apoA-I.[25] The association rate constants of these antibodies with reconstituted HDL are differentially influenced by TG enrichment, distinguishing between antibodies that are sensitive (such as AI-115.1 and AI-141.7) and insensitive (such as AI-11) to the conformational changes in apoA-I.[25] Using surface plasmon resonance analysis, we observed that the association rate constants of the antibodies AI-115.1 and AI-141.7 were decreased by -13 and -23% respectively as a result of in vitro TG

enrichment of dense HDL3c (**Figure 5**). By contrast, the association rate constant of the antibody AI-11 remained unchanged.

Discussion

Our studies provide evidence that AOX of dense HDL particles is significantly impaired in the postprandial phase in healthy normolipidemic subjects following intake of a fat-rich meal. Indeed, the diminished core content of CE and elevated TG of postprandial HDL particles, which reflected enhanced activity of CETP over the postprandial period, correlated with impairment in the AOX of dense HDL. As dense HDL particles were major determinants of the AOX of the total HDL fraction, accounting for up to 50% of the latter, total HDL equally displayed diminished AOX in the postprandial phase paralleled by CE depletion and TG enrichment.

Our previous studies have established that deficient AOX is a key feature of dense HDL in the atherogenic dyslipidemias of MetS and Type 2 diabetes.[15, 16] Mechanistically, this deficiency may involve CETP-mediated replacement of CE by TG in the HDL lipid core, resulting in the altered conformation of apoA-I,[25, 28] whose capacities to remove oxidised lipids from LDL[29] and/or to degrade lipid hydroperoxides[30] may be compromised. More precisely, enrichment in TG reduces exposure of apoA-I to the aqueous phase, because of its increased penetration into the lipid core of HDL,[25] whose fluidity increases.[31] In the present studies, we observed that in vitro enrichment of dense HDL particles in TG abrogated their AOX and reduced the accessibility of apolipoprotein A-I to monoclonal antibodies, further supporting the link between altered conformation of apoA-I and functional deficiency of dense HDL. These data are highly consistent with our present findings concerning the relationship of core CE and TG content to AOX of postprandial HDL.

The postprandial phase features marked elevation in plasma concentrations of TG-rich particles maintained over the 8h period, indicative of accelerated CETP-mediated heterotransfer of CE from HDL to TG-rich lipoproteins and accelerated transfer of TG from TG-rich lipoproteins to HDL.[17, 18] Such accelerated transfer rates primarily result from increase in donor and acceptor particle numbers rather than in CETP mass,[18] ultimately leading to altered chemical composition (TG enrichment, CE depletion) of HDL particles[19] and reduced plasma concentrations of HDL-C and of total HDL mass.[32] All these major metabolic alterations were observed in our study, being particularly pronounced in dense HDL3. The key role of CETP in CE depletion of HDL is emphasised by negative correlations between CETP activity and %CE content in HDL. By contrast, LCAT produces CE from FC in postprandial HDL; this activity is primarily associated with dense HDL,[33] consistent with positive correlations between LCAT activity and %CE content in HDL3. In addition, transfer of lipolytically-derived surface remnants of TG-rich lipoproteins to HDL produces HDL acceptor particles enriched in PL,[19] a feature of the postprandial phase equally confirmed in our studies.

Similar mechanisms may thus account for the impairment of AOX of dense HDL3 and total HDL in atherogenic dyslipidemia of MetS and Type 2 diabetes and in the postprandial phase, as both MetS and Type 2 diabetes on the one hand and the postprandial state on the other, are characterised by elevated concentrations of TG, reduced concentrations of HDL-C and elevated CETP activity.[34] Negative correlations between AOX of HDL and plasma concentrations and HDL content of TG, positive correlations between AOX of HDL and plasma concentrations of HDL-C, and positive correlation between the AOX and HDL content of CE strongly support this mechanistic link. Moreover, the postprandial replacement of CE by TG in the lipid core of dense HDL3 and resulting deficiency in the AOX of HDL may also play a pro-inflammatory role, as

suggested by negative correlations between circulating concentrations of hsCRP and AOX, positive correlations between hsCRP concentrations and HDL content of TG and negative correlations between hsCRP concentrations and HDL content of CE. Importantly, potent vasodilatory capacity of HDL particles has been recently reported to be deficient and to negatively correlate with HDL content of TG in patients with Type 2 diabetes[35] and in obese subjects.[36] Furthermore, AOX of total HDL is compromised in postmenopausal as compared to premenopausal women and is negatively correlated to HDL TG,[37] lending further support to our postulate that alterations of HDL core lipid composition are a key determinant of antiatherogenic activities of HDL particles.

Postprandial lipemia represents a prooxidative and pro-inflammatory state. [3, 4] Mechanisms potentially involved in the postprandial induction of oxidative stress include increased generation of superoxide anion and peroxynitrite, reduced bioavailability of nitric oxide, and dietary consumption of lipid or carbohydrate peroxidation products.[3, 4] We did not however observe any significant increase in concentrations of oxLDL, a biomarker of systemic oxidative stress, in this postprandial study involving 10 normolipidemic subjects. As expected, concentrations of oxLDL strongly correlated with those of LDL-C; small but significant diminution in LDL-C concentrations might therefore have contributed to the absence of a significant postprandial increase in oxLDL concentrations. Moreover, we observed significantly elevated concentrations of oxLDL (+21%, p=0.016 vs. baseline) 8h postprandially in a larger study which involved 41 healthy normolipidemic subjects (E. de Faria et al., unpublished data). The lack of significance of the elevation in oxLDL concentrations in the present study can therefore be regarded as a consequence of the small sample size. Our data on the deficient AOX of postprandial HDL are thus consistent with elevated oxidative stress; deficient AOX of HDL, primarily of dense HDL3,

might contribute to enhanced oxidative stress in the postprandial state. This mechanism is further supported by the detection of both deficient AOX of dense HDL3[23] and elevated concentrations of oxLDL[38] and 8-isoprostanes[23] in hypertriglyceridemic MetS subjects. It is of note that oxidative stress may in turn impact on HDL particles, resulting in the oxidation of specific amino acid residues of apoA-I, such as Tyr[39] and Met,[40] with potentially deleterious effects on HDL functionality. However, taking into account that postpandial changes in HDL core lipids are much greater than those in biomarkers of oxidative stress, we consider this possibility unlikely.

We hypothesise that postprandial deficiency in the AOX of HDL particles may result in the accumulation of proinflammatory oxidised lipids, primarily oxidised PL,[41] and in chronic low-grade oxidative stress and inflammation in the arterial wall. Endothelial dysfunction may ensue, contributing to postprandial atherogenesis. [3, 4] Indeed, in hyperlipidemic and hyperglycemic subjects at high cardiovascular risk, endothelium-dependent vasodilation is impaired and biomarkers of oxidative stress elevated in the postprandial state, suggesting an important pathological role of postprandial oxidative stress.[3, 4] A significant period during the day is spent in the postprandial state in Western societies,[42] further emphasising the importance of the postprandial deficiency in HDL AOX.

Consistent with our data, consumption of a meal rich in saturated fat significantly reduced the anti-inflammatory potential of HDL and impaired endothelial function in healthy subjects.[20] In contrast, the anti-inflammatory activity of HDL improved after consumption of polyunsaturated fat,[20] raising the possibility that postprandial proinflammatory and prooxidative response may be reduced via modulation of meal composition. The fatty acid composition of our test meal was intermediate between the two meals, rich in either saturated or polyunsaturated fat, employed by

Nicholls et al.,[20] consistent with the moderate but significant deficiency in the AOX of HDL observed in our studies.

The potential importance of dietary intervention in the postprandial phase is further supported by the beneficial effects on postprandial oxidative stress and endothelial dysfunction observed upon co-supplementation with antioxidants.[4] Therapeutic reduction of hypertriglyceridemic and proinflammatory responses in the postprandial phase, such as that provided by statins,[43] represents another promising approach. Our present studies thus suggest that small, dense HDL particles constitute a novel therapeutic target in the atherogenic postprandial phase, which can of special interest in subjects displaying an abnormally elevated postprandial response, such as in insulin-resistant states.[44]

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Table 1. Plasma lipid and apolipoprotein concentrations, lipid transfer protein activities and biomarkers of oxidative stress and inflammation at baseline and during the postprandial phase in normolipidemic healthy subjects (n=10)

	Baseline (0h)	Postprandial	
		4h after the meal	8h after the meal
TC (mg/dL)	147±27	147±27	148±25
TG (mg/dl)	90±42	160±89**	142±93*
HDL-C (mg/dl)	50±6	47±7*	49±8
LDL-C (mg/dl)	95±24	89±24*	93±22
ApoB (mg/dl)	70±19	71±15	74±15
ApoA-I (mg/dl)	132±12	133±12	134±10
Lipoprotein (a) (mg/dl)	19±22	18±20	18±20
Glucose (mg/dl)	87±7	95±20	83±5
CETP activity[1]	45±13	49±15*	47±15
LCAT activity[2]	25±8	25±6	26±7

oxLDL (U/L)	54±14	56±14	56±19
hsCRP (mg/L)	1.2±1.2	1.1±1.2	1.0±1.1

**p <0.01, *p <0.05 vs. corresponding value at baseline (in a fasting state); [1] % transferred substrate; [2] % hydrolysed substrate.

Table 2. Circulating concentrations and % chemical composition of total HDL and of dense HDL3c at baseline and during the postprandial phase in normolipidemic healthy subjects (n=10)

	Baseline (0h)	Postprandial	
		4h after the meal	8h after the meal
Total HDL			
Total mass (mg/dl)	177±33	160±27*	166±30
Chemical composition (weight %)			
FC	3.3±0.3	3.3±0.4	3.5±0.4
PL	28.4±1.8	29.4±2.0*	31.0±5.3
CE	17.2±2.2	16.7±2.5	17.2±4.0
TG	4.8±1.9	5.2 ±2.3	4.9±2.1
Protein	45.8±2.5	45.5±1.7	48.2±7.9
HDL3c			
Total mass (mg/dl)	47±9	38±7**	43±10

Chemical composition (weight %)			
FC	2.3±0.8	1.8±0.5*	2.0±0.5
PL	18.7±1.7	16.4±5.8	19.6±1.5*
CE	10.7±2.6	9.6±3.9	10.2±4.4
TG	4.4±3.2	4.9±4.8	4.9±4.7
Protein	64.0±2.5	65.9±3.1	63.4±3.4

**p <0.01, *p <0.05 vs. corresponding baseline value.

Table 3. Correlations between plasma parameters, chemical composition of total HDL and the HDL3c subfraction and parameters of LDL oxidation at baseline and during the postprandial phase in normolipidemic healthy subjects (n=10)

	Correlation coefficients							
	Duration of the lag phase		Oxidation rate in the propagation phase		Duration of the propagation phase		Maximal diene concentration	
	HDL3c	total HDL	HDL3c	total HDL	HDL3c	total HDL	HDL3c	total HDL
HDL-C	-0.08	-0.08	-0.28	-0.31	0.03	-0.16	-0.13	-0.41*
TG	-0.31	0.12	0.64***	0.61***	-0.53**	-0.45*	0.10	0.33
hsCRP	-0.41*	-0.26	0.34	0.31	-0.46**	-0.44*	-0.07	0.01
CE% in HDL	0.42**	0.14	-0.64***	-0.60***	0.59***	0.42*	0.34	-0.47**
CE% in HDL3c	0.49**	-0.001	-0.51**	-0.49**	0.42*	0.16	-0.34	-0.58***
TG% in HDL	-0.42**	-0.09	0.57***	0.53**	-0.43*	-0.35	0.37*	0.49**

TG% in HDL3c	-0.42*	-0.41*	0.10	0.40*	-0.19	-0.20	0.13	0.32
PL% in HDL	-0.40*	-0.10	0.31	0.15	-0.31	-0.44*	0.17	-0.09
PL% in HDL3c	-0.04	0.15	0.20	0.11	-0.24	-0.11	-0.13	0.12
CE/TG in HDL	-0.25	-0.02	0.09	-0.33	0.03	0.16	0.28	-0.41*
TG/TP in HDL	-0.35	-0.08*	0.11	0.58***	-0.07	-0.36	0.22	0.56***

***p <0.001, **p <0.01, *p <0.05.

Figure Legends

Figure 1. Antioxidative activity of total HDL (A) and HDL3c (B) expressed as duration of the lag phase of LDL oxidation (squares), oxidation rate in the propagation phase (diamonds), duration of the propagation phase (triangles) and maximal concentration of conjugated dienes (circles) before and after meal intake. Numbers denote % differences relative to corresponding values at 0h and are only shown if these differences are significant; ** $p<0.01$, * $p<0.05$ vs 0h (Wilcoxon's matched pairs test).

Figure 2. Correlations between oxidative parameters and plasma lipid concentrations. (A) Correlation between LDL oxidation rate in the propagation phase and plasma TG concentrations. (B) Correlation between maximal concentration of conjugated dienes and plasma HDL-C concentrations. Open circles, baseline; crosses, 4h after meal consumption; filled circles, 8h after meal consumption.

Figure 3. Correlations between LDL oxidation rate in the propagation phase and HDL contents of TG (A) and CE (B). Open circles, baseline; crosses, 4h after meal consumption; filled circles, 8h after meal consumption.

Figure 4. Antioxidative activity of dense HDL3b enriched in TG in vitro. (A) Kinetics of LDL oxidation in the absence (open circles) and presence of control HDL3b (filled circles) and HDL3b enriched in TG (crosses). Results of one representative experiment are shown. (B) Oxidation rate in the propagation phase and duration of the propagation phase measured in presence of control HDL3b (open bars) and HDL3b enriched in TG (filled bars). The data are means±SD of 5 independent experiments with HDL3b obtained from 2 healthy normolipidemic donors; * $p<0.05$ vs control HDL3b (Wilcoxon's matched pairs test).

Figure 5. Influence of TG enrichment of dense HDL3c on the association rate constants of epitope-defined monoclonal antibodies AI-115.1, AI-141.7 and AI-11 raised against human apoA-I.[25] The association rate constants measured using surface plasmon resonance analysis are shown for control HDL3c (open bars) and HDL3c enriched in TG (filled bars); the numbers at the bars represent % change vs. control HDL3c. The data are means±SEM of 3 to 6 independent experiments with HDL obtained from 3 healthy normolipidemic donors.

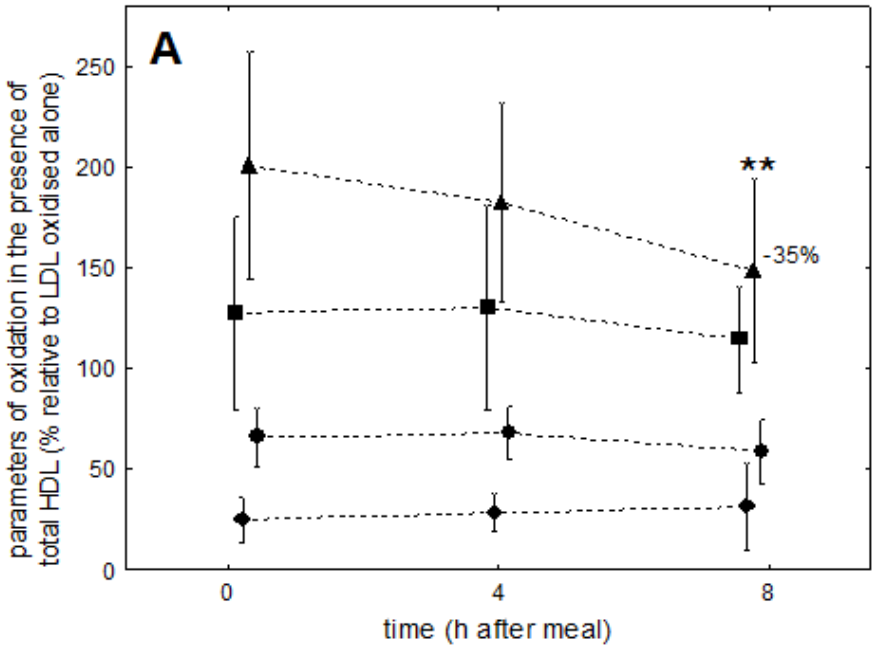

Figure 1A

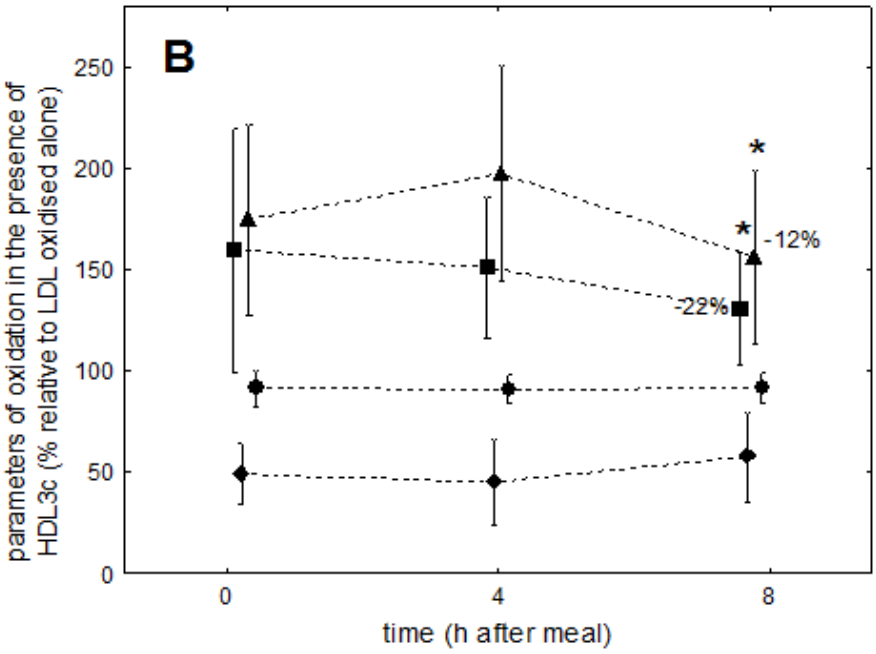

Figure 1B

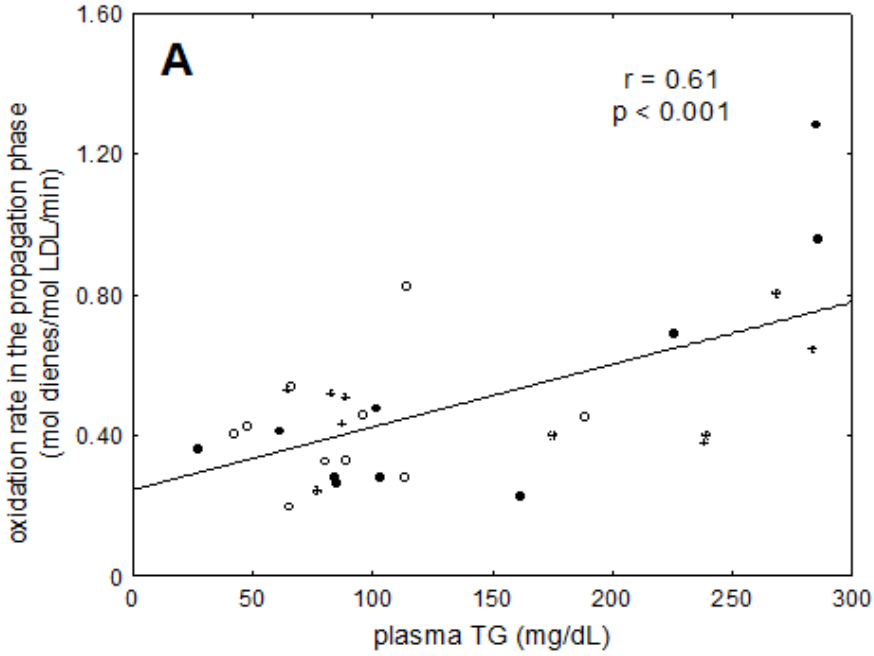

Figure 2A

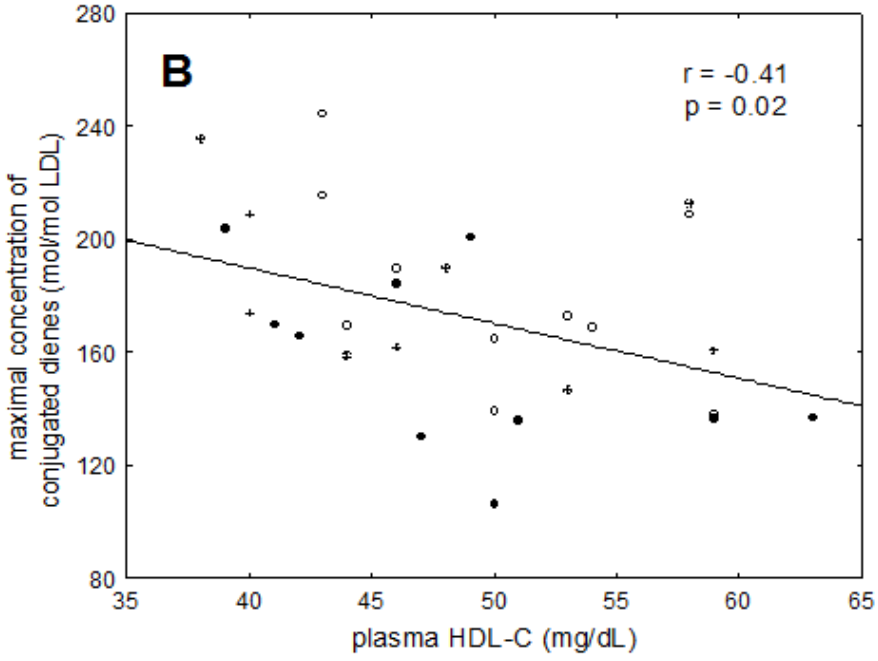

Figure 2B

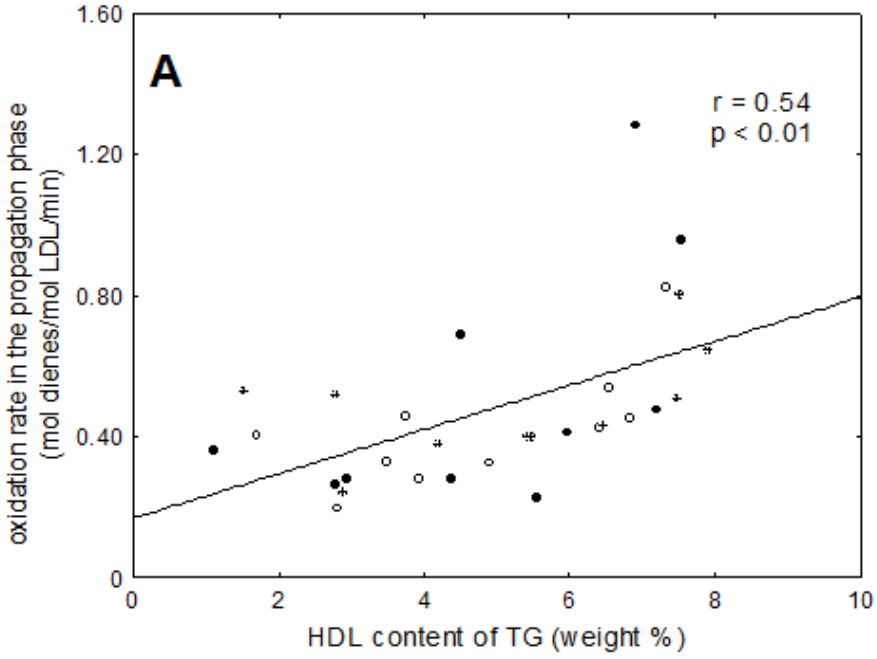

Figure 3A

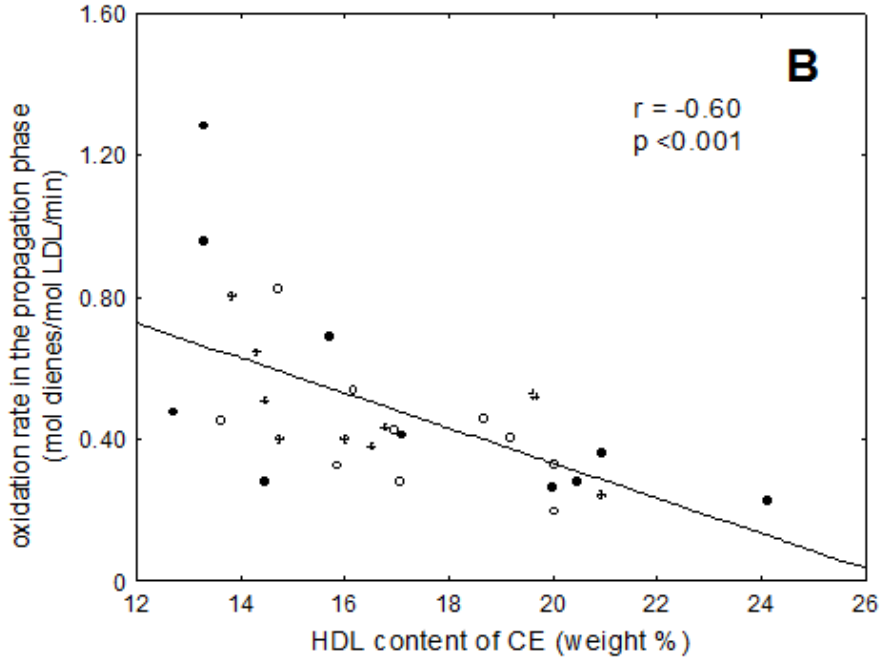

Figure 3B

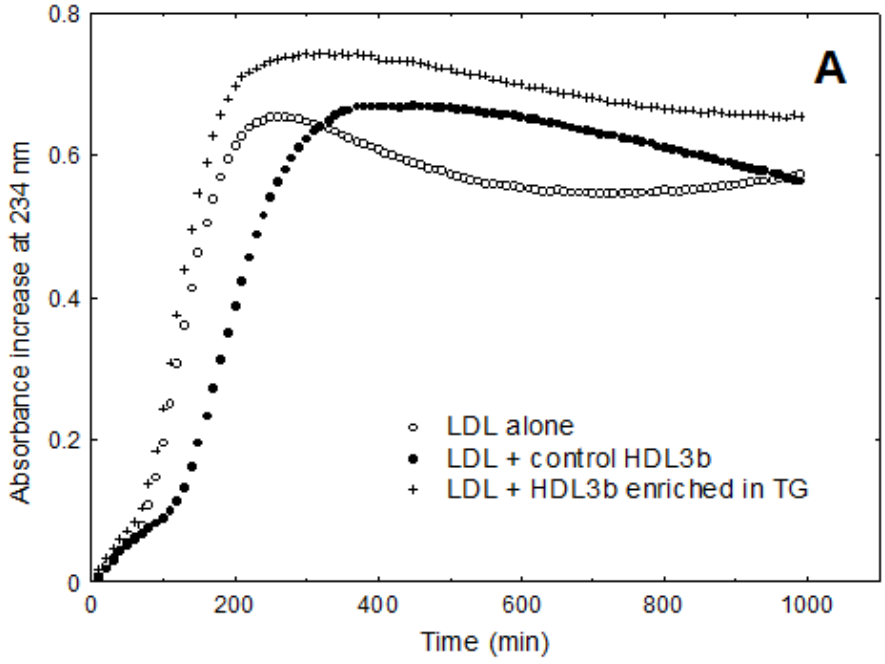

Figure 4A

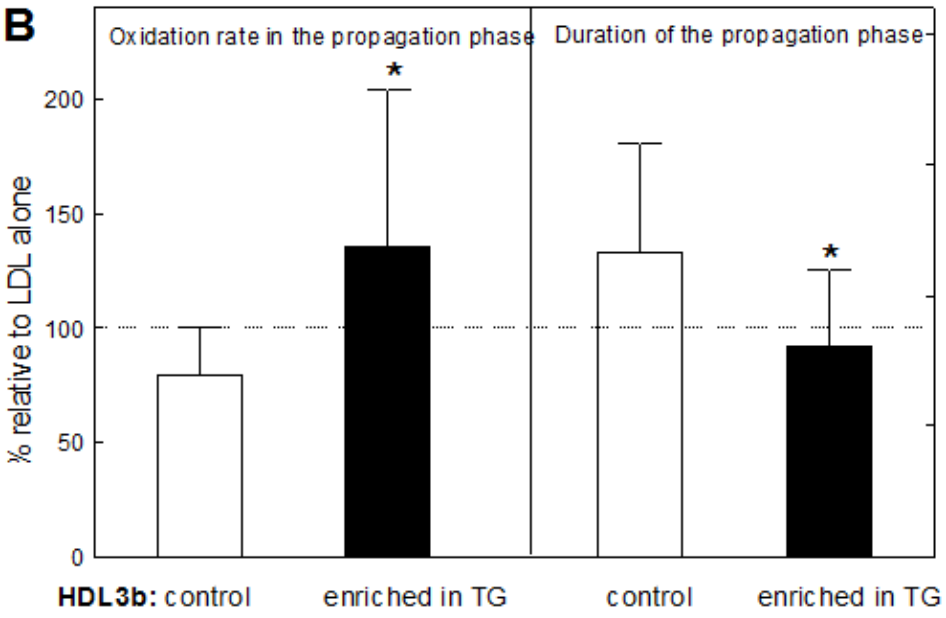

Figure 4B

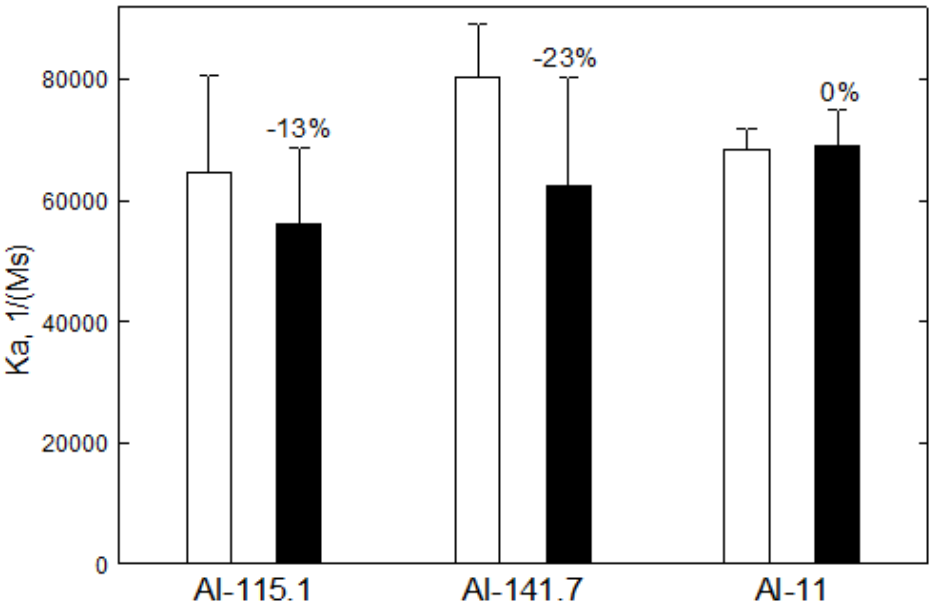

Figure 5

Capítulo 3

Reduction in generation of reactive oxygen species and endothelial dysfunction during postprandial state

Running title: A paradoxical postprandial phenomenon

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Keywords: postprandial period; reactive oxygen species; lipids; endothelial dysfunction.

Acronyms: reactive oxygen species (ROS); Peripheral blood mononuclear cells (PBMC); Artery flow-mediated vasodilation (FMD); low-density lipoprotein (LDL); HDL-cholesterol (HDL-chol); remnant-like particles (RLPs); total cholesterol (Tchol); triglyceride (TG); LDL-chololesterol (LDL-chol); Lipoprotein (a) [Lp (a)]; apolipoprotein A-I (apo A-I); apolipoprotein B100 (apo B100); Oxidized LDL (Ox-LDL); Carotid intima-media thickness (IMT); ethidium (ETH); dichlorofluorescein (DCF).

Abstract

***Background and aims*:** To characterize changes in generation of cellular reactive oxygen species (ROS) in healthy males during postprandial state, and to analyze the influence of postprandial state on endothelial ROS generation and endothelial dysfunction. ***Methods and Results*:** Seventeen healthy subjects were recruited. Blood samples were collected before and 2, 4, 6 and 8 hours (hrs) after liquid meal intake (25% fat, 55% dextromaltose, 14% protein), providing 40g fat/m^2 body surface. Plasma lipids, apolipoproteins, glucose and insulin were measured during this period. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. The influence of postprandial state on intracellular ROS generation was measured by two different methods in PBMC and in a human immortalized endothelial cell line (ECV 304). Artery flow-mediated vasodilation (FMD) was used to evaluate the endothelial function and oxygen consumption by PBMC was measured. Reduced ROS generation was observed in all methods and cells during the postprandial period. FMD was impaired 8 hrs after meal intake (23±6 vs. 13±2, $p<0.05$, vs. baseline). The consumption of oxygen was reduced in PBMC (-14% into 2 hrs, $p<0.05$ vs. baseline and -27% after 4 hrs, $p<0.01$ vs. baseline). ROS generation was correlated with plasma lipids, insulin, apolipoproteins and oxygen consumption. ***Conclusions*:** In contrast to previously reported elevation of postprandial oxidative stress, this study shows reduced ROS generation in PBMC and in ECV 304. Data obtained in both cellular models suggest the existence of a protective response against plasma postprandial oxidative stress.

Introduction

Atherosclerosis is a chronic degenerative disease of the arteries characterized by an accumulation of lipids in arterial walls (1, 2). Oxidation of lipoproteins has been postulated as one of the initial events in the development of atherosclerosis (3, 4). The oxidants responsible for changes in lipoprotein structure have been intensively studied, and several studies consider superoxide generation as the major agent that promotes low-density lipoprotein (LDL) oxidation by macrophages and endothelial cells (5). Normally, this ROS generation occurs due to an electron "leakage" from mitochondrial electron transport chains. As well, the cell could be damaged by free radicals in the presence of hydrogen peroxide. In spite of being not a radical, this molecule is readily converted to the highly reactive hydroxyl radical and leads to lipid peroxidation.

Although genetic factors strongly predispose individuals to an increased risk of atherogenesis, environment and unhealthy lifestyle also play a fundamental role. In 1970s, Zilversmit postulated that postprandial phenomenon was a promoter of atherosclerosis (6). The mechanisms that link the postprandial state with cardiovascular disease include endothelial dysfunction (7), oxidative stress (8) and inflammation (2, 9).

The postprandial state is characterized by alteration in lipid and carbohydrate metabolism, including hypertriglyceridemia, hyperglycemia and reduced concentration of HDL-cholesterol (HDL-chol) (10, 11). The triglyceride-rich particles become progressively smaller by action of lipoprotein lipase generating remnant-like particles (RLPs). The elevation in RLPs is related to impairment of endothelium-dependent vasodilation (12); moreover, it may penetrate in the arterial intima and directly initiate the atherogenic process. Increased triglyceride and glucose into plasma change the energetic metabolism and promote imbalance in oxidative status;

postprandial hypertriglyceridemia leads to a decrease in LDL size, which is more prone to oxidation; in addition, oxidized lipids can be directly absorbed by the intestine and released into plasma by intestinal cells (13). Finally, the observed meal-related increases of proinflammatory cytokines are indicative of low-grade inflammation commonly observed in proatherogenic states (14).

Therefore, we carried out this study to characterize changes in cellular ROS generation in healthy male subjects after a standard meal, by measuring ROS generation in freshly isolated peripheral blood mononuclear cells (PBMC), using different intracellular markers and the influence of postprandial sera in ROS generation on endothelial cell line. We related these results to lipid and carbohydrate changes during the postprandial state, and we also evaluated endothelial function by measuring the endothelial-mediated vasodilation during this period.

Materials and Methods

Subjects. Seventeen adult male non-smokers aged 20 to 39 years were recruited for this study. Blood pressure and physical data were determined during a complete clinical examination. None of the subjects presented signs of diseases by laboratory data or medical examination or was receiving medicaments for at least two months prior to the study. All subjects were normolipidemic, according to the National Cholesterol Education Program III (15), and fasting blood glucose was ≤100mg/dl, systolic blood pressure ≤130mm Hg, diastolic blood pressure ≤85mm Hg, and waist circumference ≤102cm. All participants gave written informed consent; the procedures followed were in accordance with local institutional guidelines and approved by the local Research Ethics Committee.

Postprandial protocol. In a second day (an average of two days later), after a 12-hr overnight fast, the subjects ingested a lactose-free milkshake liquid meal at 8 a.m. This liquid meal contained 25% of fat, 55% of dextromaltose and 14% of protein, providing 40g fat/m^2 of body surface for a period up to 10min. Blood samples were collected at fast state and after 2, 4, 6, 8 hrs. Plasma and serum were immediately separated by centrifugation at 4°C; aliquoted and frozen at -80°C; each aliquot was thawed only once directly before experiments. Twenty ml of whole blood was collected in heparin for PBMC isolation.

Chemical characterization of serum parameters. Glucose, total cholesterol (Tchol), triglyceride (TG), HDL-chol and LDL-chololesterol (LDL-chol) sera levels were quantified by enzymatic-colorimetric methods using commercially available kits (Roche Diagnostic, Mannheim, Germany). Lipoprotein (a) [Lp (a)], apolipoprotein A-I (apo A-I) and apolipoprotein B100 (apo B100) were quantified by nephelometry (Beckman, Brea, USA), and insulin was measured by enzymatic chemiluminescence (DPC, Los Angeles, USA).

Oxidized LDL (Ox-LDL). Ox-LDL was measured by ELISA (Mercodia AB, Uppsala, Sweden). In this assay, a monoclonal antibody is directed against antigenic determinants in the Ox-LDL molecule (mAB-4E6). Ox-LDL concentrations are strongly correlated with plasma LDL concentrations, and the latter is thus a key factor in determining absolute plasma Ox-LDL concentration. To overcome this, several researchers in the field have considered the ratio Ox-LDL/LDL-chol concentration. The results are expressed as the ratio.

Carotid intima-media thickness (IMT). The IMT was measured by ultrasonography, using HDI 1500 ultrasound system equipment (ATL Ultrasound, Bothell, USA), with a 7-to 12-MHz

color Doppler probe. The carotid IMT was calculated according to a standardized method and individual results were expressed in millimeters as an average of the left and right carotid IMT.

Artery flow-mediated vasodilation (FMD): The measurement of brachial FMD was first described by Anderson and Mark in 1989 (16). In order to assess non-invasively endothelial function, brachial arteries are scanned using high resolution ultrasound imaging, under baseline conditions (at rest) and during hyperemia induced by inflation and deflation of a sphygmomanometer cuff mostly around the forearm distal to the site scanned with ultrasound. The induced shear stress caused by the increased blood flow following transient ischemia leads to nitric oxide release, which in turn causes local arterial vasodilation. Endothelial function, defined as FMD, is estimated as the percent increase in vessel diameter, from baseline conditions, to maximum vessel diameter during hyperemia. Impaired endothelial function of the brachial artery assessed in this manner has been reported in asymptomatic adults with elevated cardiovascular risk factors, such as smoking, hypercholesterolaemia, hypertension, diabetes mellitus, and hyperhomocysteinaemia (17). This procedure was performed before, and 4 and 8 hrs after diet intake.

Peripheral blood mononuclear cell (PBMC) isolation. The blood sample collected in heparin was used for PBMC isolation. The whole blood was layered on the Ficoll-Paque Plus® solution and centrifuged at 1.350g for 25min. Differential migration during centrifugation results in layers containing different cell types. The PBMC are found at the interface between plasma and Ficoll-Paque Plus®. The cells were removed with a Pasteur pipette, transferred to a new tube and washed twice with RPMI medium at 500g for 10min. Cellular viability was

tested using the Trypan Blue exclusion method, and it was considered satisfactory when over 95%.

Endothelial cell model. ECV 304 cells are an immortalized endothelial cell line of human umbilical vein origin (18). The cells were cultured in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100mg/mL streptomycin, and 10% fetal calf serum. The cells were grown at 37ºC, 5% CO_2, in fully humidified air and subcultured twice weekly. The viability of the ECV 304 cells was assessed by the Trypan Blue exclusion method.

Flow cytometric determination of intracellular ROS and light scatter of cell subpopulations. Intracellular ROS generation was assessed by flow cytometry (FACS-Calibur, Becton–Dickinson, San Jose, USA) as the ethidium (ETH) fluorescence, which is the oxidation product of dihydroethidium. Dihydroethidium is known as a probe that monitors the ROS generation in living cells (19). The PBMC ($1x10^6$ cells/ml) were incubated in RPMI supplemented with 1% fetal calf serum and 5 μM dihydroethidium for 70 minutes at 37ºC in an environment with 5% CO_2. Lymphocytes and monocytes were identified in PBMC by their light-scattering characteristics, enclosed with electronic gates, and analyzed for the intensity of the fluorescent probe signal with CellQuest software.

ECV-304 cells (1.5×10^6 cells/ml) were seeded into 12-well plates and incubated in RPMI medium supplemented with sera of the participants (25%) during the fasting and postprandial periods for 50 minutes. After this time, we used the same environment described above. The results were expressed as ETH fluorescence units.

Fluorimetric intracellular ROS measurement. The net ROS generation by PBMC (6×10^6 cells/ml) was monitored using the membrane-permeable fluorescent dye 2′,7′-

dichlorodihydrofluorescein diacetate (1μm), according to Garcia Ruiz et al. (20). Fluorescence was determined at 488 nm for excitation and at 525 nm for emission, with a slit width of 3 nm in a spectrofluorometer (Hitachi f-4010, Tokyo, Japan). A calibration curve was obtained with known concentrations of dichlorofluorescein (DCF), the product of dichlorodihydrofluorescein diacetate oxidation.

Determination of cell oxygen consumption. Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments Limited, Norfolk, UK) in standard reaction medium (125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, and HEPES buffer, pH 7.2) at 37◦, in a 0.5 mL (15×10^6 cells/mL) thermostated sealed glass cuvette equipped with a magnetic stirrer. Other additions are indicated in the figure legends.

Statistical analysis. Differences between paired variables were analyzed by Student's t-test. Spearman's correlation coefficients were calculated to evaluate relationships between variables. All results are expressed as means ±SD.

Results

Baseline parameters. Participants were young adults (26±4 years), and all biochemical parameters, anthropometric data, metabolic characteristics and IMT were within the reference limits (data not shown).

Postprandial changes in plasma lipids, apoproteins, glucose, insulin, ox-LDL, ROS generation and oxygen consumption. Two hours after diet intake, these subjects presented, as expected, an increased concentration of plasma TG; the TG value was 80% higher than at baseline ($p < 0.001$), significant increments were found into 4 and 6 hrs $p<0.001$, and into 8

hrs, the TG values returned to baseline levels (table 1). By contrast, no postprandial changes were seen in plasma HDL-chol and LDL-chol, Tchol, apo AI, apo B100, Lp(a) (table 1).

Carbohydrate metabolism was analyzed by sequential measurements of glucose and insulin. Glucose presented no changes during the postprandial period; high concentration of carbohydrates in the diet elicited a great increase in insulin concentrations observed 2, 4 and 6 hrs into the postprandial period ($p<0.001$), elevation that returned to baseline values 8 hrs after diet intake (table 1).

An increase of 4% in circulating levels of Ox-LDL/LDL-chol ratio was observed during the postprandial period, but no significant change was detected (table 1).

ROS generation presented significant reduction in all probes and models tested. ROS generation measured by DCF in human PBMC showed a progressive reduction until 8 hrs into postprandial period (-13% into 2 hrs, $p<0.05$ vs. baseline, -16% after 4 hrs, $p<0.01$ vs. baseline and -29% after 8 hrs, $p<0.001$ vs. baseline) (figure 1A). Significant reduction of ROS generation measured by ETH in the same cellular model described above was observed (data not shown). The probe ETH detected an important decrease of ROS generation in human lymphocytes (-21% into 2 hrs, $p<0.001$ vs. baseline, -19% after 4 hrs, $p<0.01$ vs. baseline and -15% after 8 hrs, $p<0.01$ vs. baseline) and monocytes (-17% into 2 hrs, $p<0.05$ vs. baseline, -21% after 4 hrs, $p<0,05$ vs. baseline and -18% after 8 hrs, $p<0.01$ vs. baseline) (figure 1B and 1C). The same phenomenon was observed in endothelial cellular model after stimuli with fasting and postprandial sera (-8% into 2 hrs, $p<0.05$ vs. baseline) (figure 1D).

In parallel, the consumption of oxygen was reduced in human PBMC (-14% into 2 hrs, $p<0.05$ vs. baseline and -27% after 4 hrs, $p<0.01$ vs. baseline) (figure 1E).

Correlations between plasma parameters and intracellular ROS generation and oxygen consumption. Significant negative correlations were observed between ROS generation measured by ETH and TG ($r=-0.35$, $p<0.01$ in lymphocytes, and $r=-0.25$, $p<0.05$ in monocytes). ROS generation measured by ETH in lymphocytes also showed significant negative correlations with LDL-C ($r=-0.25$, $p<0.05$), apo B100 ($r=-0.32$, $p<0.01$), and insulin ($r=-0.34$, $p<0.01$). ROS generation measured by DCF was negatively correlated with HDL-C ($r=-0.29$, $p<0.05$), and showed a strong negative correlation with apo AI ($r=-0.36$, $p<0.01$); an interesting positive correlation with Lp(a) was observed ($r=0.24$, $p<0.05$). In the endothelial cellular model, the ROS generation measured by ETH was negatively correlated with apo AI ($r=-0.30$, $p<0.05$); no other significant correlations were found in this experimental model with plasmatic parameters (table 2).

The reduction of oxygen consumption was negatively correlated with insulin. Although no other correlation was observed with plasmatic parameters, the oxygen consumption was correlated with the ROS generation measured by ETH in lymphocytes ($r=0.28$, $p<0.05$) and endothelial cellular model ($r=0.32$, $p<0.05$). In addition, oxygen consumption was also correlated with ROS generation measured by DCF ($r=0.32$, $p<0.05$) (figure 2).

Finally, strong correlations between ROS generation measured by ETH were found in different cellular models; between lymphocytes and monocytes ($r=0.68$, $p<0.001$), lymphocytes and endothelial cells ($r=0.26$, $p<0.05$), and a trend between monocytes and endothelial cellular model ($r=0.21$, $p=0.08$). ROS generation measured by DCF in PBMC was also positively correlated with ROS generation measured by ETH in endothelial cell line ($r=0.45$, $p<0.001$) (table 3).

Endothelial function. FMD estimated as the percent increase in vessel diameter from baseline conditions to the maximum vessel diameter during hyperemia was impaired 4 hrs after diet intake (23±6 vs. 13±5, p=0.1), and significantly reduced 8 hrs after meal intake (23±6 vs. 13±2, $p<0.05$) (figure 1F).

Discussion

Several studies have shown that plasma environment is prone to oxidation during the postprandial state (21, 22); the consumption of a meal containing oxidized and oxidizable lipids leads to an increased concentration of lipid hydroperoxides in plasma (23). Moreover, during postprandial lipemia, which is well-established to represent a prooxidative and pro-inflammatory state, fat meal consumption is associated with elevated plasma biomarkers of oxidative stress, including increased circulation levels of oxidation products (malondialdehyde, nitrotyrosine), decrease of antioxidants (vitamin E, vitamin C, thiols, glutathione peroxidase), increase susceptibility of LDL oxidation and elevated levels of CRP, interleukin and TNF-α and adhesion molecules (2, 16, 24, 25).

Paradoxically, this study provides the first evidence that intracellular ROS generation is reduced during the postprandial period in healthy subjects. We demonstrated a reduced ROS generation, detected by two different probes in PBMC of these individuals. Consistent with this data, the results were confirmed in an immortalized human endothelial cell line stimulated with postprandial sera of these subjects.

The strong correlations among all the ROS generation models point in the direction that this is a closely related phenomenon, and at least in healthy subjects these cellular models could represent the same mechanism of protection due a prooxidative plasma environment. The

reduction of oxygen consumption suggests that mitochondria plays a pivotal role in the regulation of this cellular mechanism of protection against plasmatic oxidative stress during the postprandial period; in addition, oxygen consumption was correlated with ROS generation.

Plasma TG and insulin were two markers that presented more pronounced modifications, reflecting the extension of alteration in lipid and carbohydrate metabolism during the postprandial phase; both of them were negatively correlated with at least one of the markers of ROS generation. The lipoproteins or their apoproteins were correlated with models of ROS generation; in case of proatherogenic ones, such as Ox-LDL, apo B100 and LDL-chol, the negative correlation found may suggest a chronic prooxidative state, leading to an indirect cellular defence mechanism in healthy subjects. In contrast, negative correlations between HDL-chol and its apolipoprotein (apo AI) reflect the capacity of this lipoprotein to function as a lipid acceptor to remove oxidized lipids from circulation, as demonstrated by several authors (26, 27). The antioxidative activity of HDL decreases the oxidative stress commonly observed during this period and, consequently, the cellular protective response.

Lipoproteins seem to be the most important inductors of this response against postprandial oxidative stress, and it could be observed in an immortalized human endothelial cell line stimulated with postprandial sera after a pretreatment using protein extracts. None of the effects described in ROS generation was observed (data not shown).

In hyperlipidemic and hyperglycemic subjects, as type 2 diabetics, endothelium-dependent dilation is impaired during the postprandial state, and biomarkers of oxidative stress are elevated (10, 11, 28). Consistent with these data, the metabolic changes during the postprandial state of healthy subjects confirmed these findings with reduction in ~50% of

FMD, indicating the great reduction in generation of endothelial cell nitric oxide *in vivo*, providing us an estimate of the endothelial dysfunction, and suggesting an important pathological role of oxidative stress during postprandial state and cellular metabolic changes.

In western societies, a significant part of the day is spent in the postprandial state, further emphasising the importance of this period in the development of atherosclerosis, pathology that is closely related to unhealthy life styles and inappropriate dietary habits. The complete elucidation of the involved mechanisms may improve the appropriateness of such dietary intervention supported by beneficial effects on postprandial oxidative stress and endothelial dysfunction (29), and the use of therapeutic strategies to reduce hypertriglyceridemia, oxidative stress and proinflammatory responses during the postprandial phase (25, 30).

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hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment. Circulation 2002; 106: 1211-1218

Table 1. Plasma lipid, apolipoproteins, glucose and insulin at baseline and during the postprandial phase in normolipidemic healthy subjects (n=17)

	Baseline (0h)	Postprandial			
		2 hrs	4 hrs	6 hrs	8 hrs
Tchol (mmol/L)	4.07±0.73	4.04±0.62	4.04±0.62	4.07±0.62	4.12±0.65
TG (mmol/L)	0.89±0.54	**1.56±0.70*** **	**1.41±0.66*** **	**1.13±0.72** **	0.98±0.27
HDL-chol (mmol/L)	1.19±0.21	*1.14±0.23†*	1.14±0.21	1.17±0.21	1.22±0.21
LDL-chol (mmol/L)	2.75±0.70	*2.64±0.65†*	2.69±0.65	2.72±0.62	2.82±0.67
OxLDL/LDL-chol (U/mmol)	14.1±2.1	-----	14,7±2.9	----	14.4±3.6
Apo B100 (g/L)	0.80±0.20	0.81±0.16	0.80±0.17	0.81±0.16	0.82±0.16
Apo A-I (g/L)	1.25±0.15	1.24±0.17	1.26±0.17	1.26±0.16	1.28±0.15
Lp (a) (µmol/L)	0.89±0.61	0.89±0.57	0.89±0.57	0.89±0.61	0.93±0.61
Glucose (mmol/L)	4.8±0.4	4.8±1.1	4.7±1.1	4.7±0.6	4.7±0.3
Insulin (pmol/L)	42±31	**329±182*** **	**273±28*** **	**196±98** **	42±28

*** p <0.001, **p <0.01, *p <0.05, †p<0.10 vs. corresponding value at baseline

Table 2. Correlation coefficients for plasma lipids, apolipoproteins, oxLDL, glucose, and insulin with ROS generation in normolipidemic healthy subjects *(n=17)*

	ETH generation in lymphocytes	ETH generation in monocytes	ETH generation in ECV-304	DCF generation in PBMC
Tchol	-0.22	-0.11	-0.14	-0.08
TG	**-0.35****	**-0.25***	0.01	-0.16
HDL-chol	-0.04	0.11	-0.15	**-0.29***
LDL-chol	**-0.26***	0.08	-0.19	-0.04
Apo A-I	0.12	-0.11	**-0.30***	**-0.36****
Apo B100	**-0.32***	-0.18	-0.09	-0.03
oxLDL/LDL-chol	**-0.32***	0.04	**-0.38****	-0.18
Lp(a)	-0.16	-0.16	0.11	**0.24***
Glucose	-0.08	-0.08	0.20	0.22
Insulin	**-0.34****	-0.11	-0.04	-0.02

*** p <0.001, **p <0.01, *p <0.05

Table 3. Correlation coefficients between plasma ROS generation in different cellular models in normolipidemic healthy subjects *(n=17)*

Parameters	ETH generation in lymphocytes	ETH generation in monocytes	ETH generation in ECV-304	DCF generation in PBMC
ETH in lymphocytes	**---**	**0.68*** **	**0.26***	*0.21†*
ETH in monocytes	**0.68*** **	---	*0.21†*	0.08
ETH generation in ECV-304	**0.26***	*0.21†*	---	**0.45*** **
DCF generation in PBMC	*0.21†*	0.08	**0.45*** **	---

*** p <0.001, *p <0.05, †p<0.10

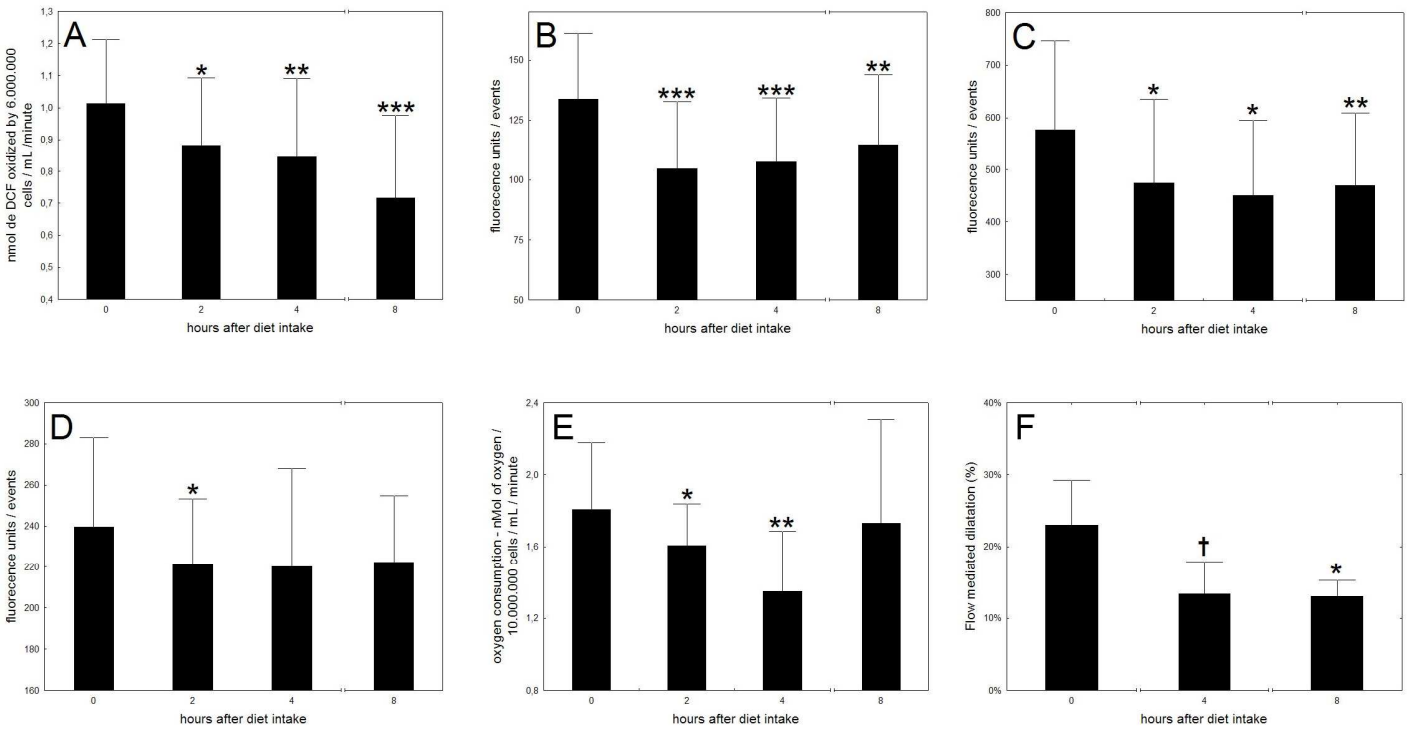
A
nmol de DCF oxidized by 6.000.000 cells / mL /minute
hours after diet intake
B
fluorecence units / events
hours after diet intake
C
fluorecence units / events
hours after diet intake
D
fluorecence units / events
hours after diet intake
E
oxygen consumption - nMol of oxygen / 10.000.000 cells / mL / minute
hours after diet intake
F
Flow mediated dilatation (%)
hours after diet intake

Figure 1

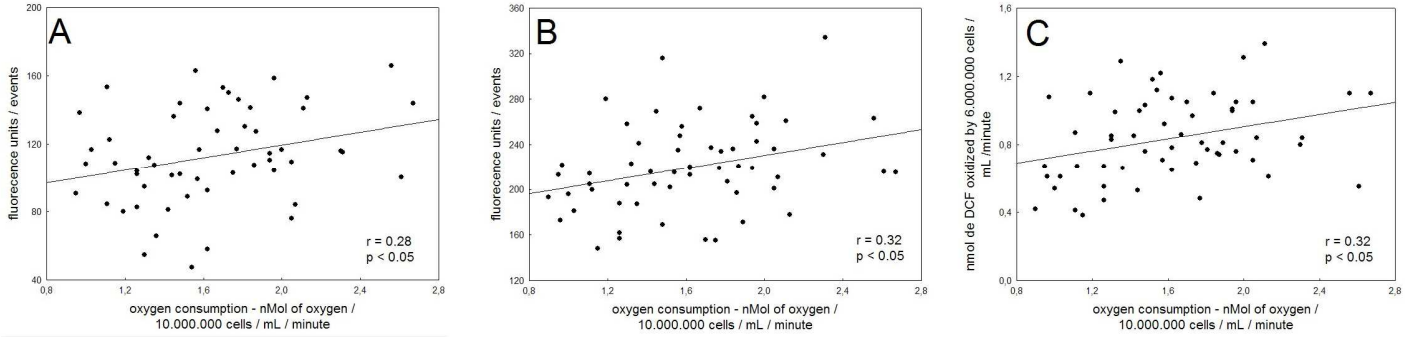
A
fluorecence units / events
200
160
120
80
40
0,8
1,2
1,6
2,0
2,4
2,8
r = 0.28
p < 0.05
oxygen consumption - nMol of oxygen /
10.000.000 cells / mL / minute
B
fluorecence units / events
360
320
280
240
200
160
120
r = 0.32
p < 0.05
oxygen consumption - nMol of oxygen /
10.000.000 cells / mL / minute
C
nmol de DCF oxidized by 6.000.000 cells /
mL /minute
1,6
1,2
0,8
0,4
0,0
r = 0.32
p < 0.05
oxygen consumption - nMol of oxygen /
10.000.000 cells / mL / minute

Figure 2

Figure Legends

Fig. 1. ROS generation measured by DCF in human PBMC (A), ROS generation measured by DHE in human lymphocytes (B), monocytes (C), and in an immortalized human endothelial cell line (D), oxygen consumption in human PBMC (E); Flow-mediated dilation (FMD) estimated as the percent increase (F). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, † $p < 0.10$ vs. corresponding value at baseline.

Fig. 2. Correlation between oxygen consumption and ROS generation measured by DHE in lymphocytes (A). Correlation between oxygen consumption and ROS generation measured by DHE in endothelial cellular model (B). Correlation between oxygen consumption and ROS generation measured by DCF in PBMC (C).

Discussão geral

Diversos estudos demonstram uma clara associação entre concentração plasmática de colesterol contido nas HDL e proteção contra doença cardiovascular (32,33). Esta proteção se deve a algumas características físico-químicas desta lipoproteína que age no transporte reverso de colesterol para o fígado e possui atividades antiinflamatória, antioxidante, antiapoptótica, antiinfecciosa, antitrombótica e ação vasodilatadora (33). Nesta tese foram estabelecidos, pela primeira vez os valores de normalidade da concentração de colesterol e triglicérides contidos nas subfrações de HDL (HDL_2 e HDL_3) em adultos normolipidêmicos brasileiros; estratificando estes valores em subgrupos relativos ao sexo e idade dos indivíduos; e comparando as concentrações de colesterol e triglicerídeos contidos nas subfrações de HDL nestes subgrupos.

Os dados, obtidos nesta tese em relação aos valores de normalidade do conteúdo de colesterol e triglicérides nas HDL_2 e HDL_3, conferem os obtidos por outros pesquisadores (76-78). Além deste achado, os dados apresentados neste artigo relativos às comparações entre as concentraçôes de triglicérides e colesterol nas HDL por sexo também estão em consonância com os disponíveis na literatura para outras populações (77). A presença de concentrações mais elevadas de colesterol nas HDL_2 e HDL_3 pode ser um dos fatores que contribuem para uma menor incidência de DCV nos pacientes de sexo feminino, principalmente na subfração HDL_3, na qual a concentração de triglicérides não apresenta diferença entre os sexos.

Variações de resultados em relação aos valores da normalidade para o conteúdo de colesterol e triglicérides nas HDL_2 e HDL_3 podem ser encontradas na literatura e isto se deve principalmente à metodologia utilizada. Os métodos mais usuais para a separação das

lipoproteínas são a ultracentrifugação, eletroforese e precipitação seletiva. Nesta tese foi escolhida a ultracentrifugação. Este método foi selecionado pois é o método que mais preserva as características físicas e químicas da HDL, esta metodologia permite a separação em meio aquoso das subfrações e a análise da atividade antioxidante desta partícula, como foi feito em outro experimento desta mesma tese. Outro fator que deve ser levado em consideração é a seleção da população estudada, principalmente o estilo de vida, dieta, IMC, idade e sexo. Este estudo dos valores da normalidade para a população saudável serviu de base para a avaliação do período pós-prandial no compartimento plasmático neste grupo de indivíduos.

Desde a primeira publicação, no final da década de setenta, que associava o período pós-prandial com aterosclerose (22), esta relação tem sido estudada em diversos aspectos (26,29,31). Os lipídios ingeridos na dieta são absorvidos pelos enterócitos e rapidamente liberados na corrente sangüínea sob a forma de uma lipoproteína rica em triglicérides e de densidade reduzida, os quilomícrons. A presença dos quilomícrons na circulação sanguínea altera o metabolismo das lipoproteínas e é uma das razões pelas quais o período pós-prandial é potencialmente aterogênico.

A mais consistente hipótese para o desenvolvimento da aterosclerose indica que a geração de ROS e a subseqüente oxidação da LDL, e sua deposição no espaço subendotelial, são os eventos iniciais desta doença (79,80). Dados obtidos pelo Laboratório de Lípides da Universidade Estadual de Campinas indicam que há um aumento da concentração de LDL oxidada neste período em indivíduos saudáveis (81). Outros grupos de pesquisadores já identificaram que este período está intimamente relacionado ao estresse oxidativo plasmático, principalmente em indivíduos portadores de diabetes e/ou síndrome metabólica (82).

Na presente tese, a ingestão da dieta padronizada levou a uma alteração significativa nas concentrações de triglicérides e insulina, resposta esperada à absorção de nutrientes pelo tubo digestivo. Acompanhando o aumento de triglicérides foi observada uma elevação da atividade da CETP, enzima responsável pela troca de éster de colesterol e triglicérides nas lipoproteínas. Como as ações antiateroscleróticas da HDL são intimamente ligadas a sua composição química (34,38), o aumento da atividade da CETP provocou uma redução da massa total e do conteúdo de ésteres de colesterol da HDL, principalmente na sua subfração pequena e densa e com mais potencial protetor contra a aterogênese, a HDL_3.

Neste contexto, foi demonstrado pela primeira vez que indivíduos saudáveis apresentam uma redução da atividade antioxidante da HDL e de suas sub-frações após ingestão de uma dieta padronizada. A avaliação da atividade antioxidante das HDLs foi feita *in vitro* baseada na duração da fase inicial (*lag phase*) de oxidação, na duração e na taxa de oxidação da fase de propagação (*propagation phase*) e pela concentração final de produtos da oxidação ou concentração total de dienos conjugados (*conjugated dienes*).

A perda de atividade antioxidante foi observada no período pós-prandial nestes indivíduos devido a significativas reduções na duração da fase inicial e na fase de propagação bem como aumento na taxa de oxidação.

Três causas básicas levaram a estes achados. Em primeiro lugar, a diminuição da massa total do HDL e de suas subfrações, evidenciadas no artigo, reduzem diretamente suas atividades. Ademais, o aumento da atividade da CETP provocou uma modificação da composição química da HDL, levando a um aumento da concentração de triglicérides e redução da concentração de ésteres de colesterol. A ação desta enzima tornou a HDL destes indivíduos saudáveis muito semelhante quimicamente à HDL de indivíduos portadores de

dislipidemias relacionadas ao diabetes e a síndrome metabólica. É sabido que nestas patologias há uma redução da concentração total de colesterol nas HDL e diminuição das atividades antioxidante, antiinflamatória, antiapoptótica e vasodilatadora (83-86). Por fim, a deficiência funcional da apoA-I, observada pela diminuição das constantes de associação desta proteína com anticorpos monoclonais específicos, leva a uma redução da sua capacidade de remover lípides oxidados da LDL e de degradar hidroperóxidos lipídicos (29,87,88). Todas estas modificações descritas acima foram mais pronunciadas na subfração pequena e densa da HDL.

Vale ressaltar que o papel da CETP na redução da concentração de ésteres de colesterol contidos nas HDL fica evidente com a correlação negativa entre estes dois parâmetros. O artigo também confirma a correlação positiva entre LCAT e a concentração ésteres de colesterol na HDL e ainda apresenta um aumento de fosfolípides na HDL provenientes de remanescentes das lipoproteínas ricas em TG.

O mecanismo desta redução da capacidade antioxidante das HDLs fica ainda mais evidente pelas correlações positivas desta atividade com a concentração total de HDL e conteúdo de ésteres de colesterol, bem como a correlação negativa com o conteúdo de TG nas HDLs.

Além da atividade antioxidante, o estudo sugere que estas modificações na massa total, na composição química e conseqüentemente na atividade antioxidante estão associadas com o estado pró-inflamatório devido à correlação positiva entre PCR-as e conteúdo de TG nas HDLs e negativa entre esta proteína de fase aguda e o conteúdo de CE nas HDLs. Outro dado importante que pode ocorrer em especial neste período em indivíduos saudáveis é a perda da capacidade vasodilatadora em portadores de HDLs com este perfil de composição química já relatados para indivíduos diabéticos (89,90) e obesos (91).

Diante do exposto, a lipemia pós-prandial pode ser entendida como um estado pró-oxidativo e pró-inflamatório no compartimento plasmático de indivíduos saudáveis, logo esta tese evoluiu para a análise do compartimento intracelular. Nesta fase foi avaliada a geração de ROS neste compartimento.

Estudos apontam que animais dislipidêmicos apresentam uma elevação da geração de ROS principalmente nas mitocôndrias (92). Apesar de seu eficiente sistema de defesa antioxidante esta organela também pode ser alvo de seus próprios radicais livres. Quando lesada a mitocôndria apresenta uma permeabilização não específica de sua membrana interna, fenômeno conhecido como transição de permeabilidade mitocondrial (20,93). A conseqüência deste fato é o edemaciamento da organela e sua ruptura que pode levar à apoptose e à necrose celular (94.). Em humanos dislipidêmicos o excesso de produção de ROS já foi demonstrado (95).

Nesta tese, ao contrário do observado em cobaias e seres humanos dislipidêmicos, foi demonstrada uma redução da geração de ROS no compartimento intracelular nas duas sondas e nos modelos celulares testados de indivíduos saudáveis no período pós-prandial.

Foram testadas duas sondas a dihidroetídio (DHE) e diclorofluoresceína (DCF) na avaliação de células sanguíneas periféricas mononucleares (CSPM) e o DHE em uma linhagem. O DHE mostrou uma redução mais expressiva nas primeiras horas após a ingestão da dieta, enquanto a DCF apresentou redução mais expressiva oito horas após a ingestão da dieta. A diferente cinética de produção de ROS nestas duas sondas provavelmente deve-se ao fato de elas possuírem características físico-químicas e metodológicas diferentes (96,97).

Independente destas diferenças entre as sondas descritas acima, a redução da geração das ROS foi consistente em ambas, indicando que, tanto no citoplasma como no compartimento

mitocondrial, a redução de formação e acumulo de radicais livres está reduzida. Mais do que isto, foram encontradas correlações significantes na geração de ROS mensurada pelo DCF e pelo DHE nos modelos celulares testados mesmo com um número limitado de indivíduos utilizados neste estudo, sinalizando uma associação entre os fenômenos descritos.

O modelo endotelial celular testado também apresentou uma redução na geração de ROS quando avaliado pela sonda DHE. Este último modelo apresentou queda significativa na geração de radicais livres apenas quando estimulada com soro da segunda hora após a ingestão da dieta. Esta resposta menos expressiva do fenômeno pode ser atribuída ao grau do estímulo, pois estas células ficaram expostas pouco mais de uma hora a uma concentração de 25% de soro humano. Apesar disto apresentou forte correlação com as outras duas sondas testadas, ratificando tratar-se de um evento reprodutível em outros modelos celulares com uma origem plasmática. Neste contexto, durante o jejum a célula tende a utilizar como fonte energética os ácidos graxos livres estimulando a via metabólica da beta oxidação dos ácidos graxos e inibindo o ciclo do ácido tricarboxílico (98). A ativação da beta oxidação dos ácidos graxos é funcionalmente associada ao *peroxisome proliferator-activated receptor alfa* (PPARα) (99), enquanto a inibição do ciclo do ácido tricarboxílico é realizada pela isoforma 4 da piruvato desidrogenase quinase (100). A alternância da fonte energética celular entre o período de jejum e pós-prandial pode estar associada ao decréscimo da produção intracelular de ROS pela mudança das vias celulares geradoras de energia como descrito acima.

Em sendo a mitocôndria a maior geradora de ROS dentro da célula (13,14), foi avaliado o consumo de oxigênio das CSPM. Esta medida também apresentou significativa redução no período pós-prandial, principalmente, nas primeiras horas deste período. Quando este achado foi correlacionado com a produção de ROS, foram demonstradas significativas correlações

positivas em todos os modelos e sondas apresentadas, levando à conclusão de que o metabolismo mitocondrial desempenhou papel fundamental nesta modificação do comportamento celular.

Neste estudo, foi avaliada também a função endotelial baseada na vasodilatação mediada pelo fluxo arterial (101). A redução da funcionalidade do endotélio *in vivo* encontrada denota uma incapacidade de responder e/ou de produzir óxido nítrico. Este achado é compatível com o aumento do estresse oxidativo plasmático no período pós-prandial, tanto pela presença de substâncias oxidadas ou indutoras de oxidação (102) como pela perda da funcionalidade das HDLs já discutidas nesta tese.

O grande aumento observado na concentração sérica de insulina e de triglicérides reflete a extensão da alteração do metabolismo lipídico e glicídico no período pós-prandial. As correlações negativas entre a geração de ROS e estes dois parâmetros foram encontradas, mais uma vez associando-se o fenômeno pós-prandial com mudança do comportamento celular.

As lipoproteínas e apoproteínas aterogênicas, como a LDL-ox, apolipoproteína B100 e LDLs, apresentaram correlações negativas com a geração de ROS. Estas correlações foram interpretadas como uma resposta celular protetora ao estresse oxidativo em indivíduos saudáveis. Em oposição a este fato as correlações negativas com as HDLs e apoA-I refletem a capacidade de estas partículas removerem lípides oxidados da circulação (29,87,88), reduzindo o estresse oxidativo plasmático e em conseqüência a resposta intracelular de redução da geração de ROS. A importância das proteínas, em especial das lipoproteínas, foi ratificada na ausência de resposta protetora intracelular (redução da geração de ROS) quando as células endoteliais imortalizadas foram estimuladas com o mesmo soro pós-prandial pré-tratado para

extração das proteínas. Este achado indica mais uma vez a importância do ambiente plasmático em relação à resposta intracelular.

Todos estes achados descritos acima levam à conclusão de que indivíduos saudáveis apresentam uma redução da geração intracelular de ROS em resposta às modificações metabólicas pró-oxidativas plasmática encontradas durante o período pós-prandial. A completa elucidação do fenômeno e caracterização de indivíduos com modificações no metabolismo, que os tornem susceptíveis ao desenvolvimento de doença aterosclerótica poderão indicar intervenções dietéticas e/ou terapêuticas que reduzam o estresse oxidativo e melhorem a função endotelial durante a fase pós-prandial.

Conclusões

ARTIGO 1

- ✓ As concentrações de triglicérides e colesterol nas subfrações de HDL em indivíduos saudáveis e normolipidêmicos brasileiros não diferem de outros grupos populacionais já estudados. Nesta população, os indivíduos de sexo feminino apresentaram concentrações mais elevadas de colesterol e triglicérides na sub-fração HDL_2 e de colesterol na HDL_3. Indivíduos com idade entre quarenta e sessenta anos apresentaram níveis reduzidos de colesterol na HDL_3.

ARTIGO 2

- ✓ As modificações no metabolismo lipídico no período pós-prandial de indivíduos normolipidêmicos produzem alteração da composição química das HDLs reduzindo a capacidade antioxidante desta lipoproteína, principalmente da sua subfração pequena e densa. Esta ação é mediada pela CETP, que diminui a concentração de ésteres de colesterol, aumenta a concentração de triglicérides e diminui a funcionalidade da apoAI nesta lipoproteína, tornando-a muito semelhante quimicamente às HDLs de indivíduos portadores de síndrome metabólica e/ou diabetes tipo 2, caracterizando este período como pró-oxidativo e pró-inflamatório no compartimento plasmático.

ARTIGO 3

- ✓ O período pós-prandial, caracterizado como um fenômeno plasmático pró-oxidativo e pró-inflamatório, reduz a geração intracelular de ROS das células sangüíneas periféricas em indivíduos normolipidêmicos. Este fenômeno também é reprodutível em células endoteliais *in vitro* e o endotélio apresenta-se disfuncional *in vivo.* A redução da geração das ROS ocorre tanto no citoplasma como na mitocôndria e está

correlacionada com a redução de consumo intracelular de oxigênio. Esta redução caracteriza uma defesa celular contra o ambiente plasmático pró-oxidativo.

GERAL

- ✓ A composição química da HDL em uma população brasileira normolipidêmica no jejum não difere de outras populações. No período pós-prandial, indivíduos normolipidêmicos apresentaram aumento do estresse oxidativo e inflamatório no compartimento plasmático. Foram detectadas modificações na composição química das HDLs compatíveis com sua perda de função. Reativamente, foi demonstrada, pela primeira vez na literatura, a redução da geração de ROS intracelular neste período. Experimentos seguintes deverão estudar os mecanismos bioquímicos desta resposta protetora.

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Anexo 1 – Parecer do Comitê de Ética em Pesquisa

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA
Caixa Postal 6111, 13083-970 Campinas, SP
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CEP, 15/03/05.
(Grupo I)

PARECER PROJETO: N° 672/2004

I-IDENTIFICAÇÃO:

PROJETO: **"BIOMARCADORES PÓS-PRANDIAIS DE OXIDABILIDADE PLASMÁTICA EM ADULTOS SAUDÁVEIS: ASSOCIAÇÃO ENTRE DIETA, ESTRESSE OXIDATIVO MITOCONDRIAL E ATEROSCLEROSE"**
PESQUISADOR RESPONSÁVEL: Fábio Lima Sodré
INSTITUIÇÃO: HC/Unicamp
APRESENTAÇÃO AO CEP: 26/01/2004
APRESENTAR RELATÓRIO EM: 15/03/06

II - OBJETIVOS

Caracterizar, em adultos saudáveis, se o estresse oxidativo intraplasmático e(ou) a hipertrigliceridemia dieta-induzidos têm efeitos agudos sobre os parâmetros de função mitocondrial e de função antioxidante das lipoproteinas de densidade alta, além de correlacioná-los com medidas bioquímicas, moleculares e com marcadores de aterosclerose.

III - SUMÁRIO

Serão avaliados de 10 a 15 indivíduos do sexo masculino, com idades entre 20 e 40 anos, sem sinais de doença cardiovascular, dislipidemia e sobrepeso/obesidade, sendo excluidos os fumantes, alcoólatras ou portadores de condições clinicamente detectáveis e em uso de qualquer medicamento. Além disso, serão descontinuados os indivíduos nos quais houver suspeita de prejuízo à saúde em qualquer etapa. Os sujeitos serão submetidos a avaliação clínica incluindo anamnese e exame físico completo. Será realizada ultrassonografia das carótidas, para medida da espessura da íntima-média. Serão coletadas amostras de sangue periférico para as análises bioquímicas, extração de DNA e separação de linfomonócitos/monócitos, após jejum, uma, duas, quatro, oito e 10 horas pós-prandial, com dieta padrão. Os exames bioquímicos compreendem dosagens plasmáticas de glicose, insulina, colesterol total e frações, triglicérides e lipoproteína lipase, além de proteína transferidora do éster de colesterol, produtos de peroxidação de lípides insaturados, capacidade antioxidante plasmática, atividade da óxido nítrico sintetase, produtos de lesão oxidativa de DNA, produtos de oxidação de colesterol e inibidor da NOS. Amostras de células serão analisadas em citômetro de fluxo para identificação das populações celulares equanto a características de tamanho e granulosidade. Outros estudos incluem a medida do potencial elétrico de transmembrana mitocondrial, determinação do consumo de oxigênio,

estimativa da produção de EROs, medida da concentração de cálcio intracelular e medida da atividade da superóxido dismutase mitocondrial. Por fim, haverá análise do gene da proteína UCP-2 usando-se um software de análise molecular.

IV - COMENTÁRIOS DOS RELATORES

Foram realizadas, de forma adequada, as modificações solicitadas na folha de rosto, TCLE e "aspectos éticos" do projeto.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar o Protocolo de Pesquisa supracitado, com a restrição de que seja acrescentada no Termo de Consentimento Livre e Esclarecido a opção de o sujeito de pesquisa doador do material biológico consentir ou não com o armazenamento para estudos futuros, bem como o nome do profissional habilitado que irá realizar o aconselhamento genético.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na III Reunião Ordinária do CEP/FCM, em 15 de março de 2005.

Profa. Dra. Carmen Sílvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP