GIULIANO GENEROSO

Associação entre as subfrações de colesterol da lipoproteína de alta densidade mensuradas pelo método de Perfil Vertical Automático e síndrome metabólica, inflamação, resistência à insulina e risco de doença vascular subclínica: Estudo Longitudinal de Saúde do Adulto

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências
Programa de Cardiologia
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Association between high-density lipoprotein-cholesterol subfractions measured by Vertical Auto Profile method and metabolic syndrome, insulin resistance and risk of subclinical atherosclerosis: The Brazilian Longitudinal Study of Adult Health

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DEDICATÓRIA

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À minha esposa **Mariély Trigo Tumasz Generoso**, que me motivou e apoiou diariamente desde o início do processo, além do companheirismo e amor incondicional compartilhado, me fazendo pessoa melhor dia a dia.

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LIST OF ABBREVIATIONS AND SYMBOLS

ApoA-I – apolipoprotein A-I

ASCVD – atherosclerotic cardiovascular disease

CAC – coronary artery calcium

CETP – cholesteryl ester transfer protein

CHD – coronary heart disease

cIMT – carotid intima-media thickness

DATASUS – Departamento de Informática do Sistema Único de Saúde

ELSA-Brasil – Brazilian Longitudinal Study of Adult Health

HOMA-IR - homeostasis model assessment-estimated insulin resistance

LCAT – lecithin-cholesterol acyltransferase

LDL – low-density lipoprotein

LDL-c - low-density lipoprotein cholesterol

Lp(a) – lipoprotein a

HDL – high-density lipoprotein

HDL-c – high-density lipoprotein cholesterol

PON-1 – paraoxonase 1

RCT – reverse cholesterol transport

CRP – c-reactive protein

TG - triglycerides

VLDL – very-low density lipoprotein

T2D – type 2 diabetes mellitus

RESUMO

RESUMO

Generoso G. *Associação entre as subfrações de colesterol da lipoproteína de alta densidade mensuradas pelo método de Perfil Vertical Automático e síndrome metabólica, inflamação, resistência à insulina e risco de doença vascular subclínica: Estudo Longitudinal de Saúde do Adulto* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2020.

Apesar de dados epidemiológicos sugerirem associação inversa entre os níveis de colesterol das lipoproteínas de alta densidade (HDL-c) e doença aterosclerótica, estudos recentes de randomização mendeliana não provaram efeito causal protetor do HDL-c. Visto que o grupo das lipoproteínas de alta densidade (HDL) é composto de partículas diferentes em tamanho, forma, densidade, lipidoma e proteoma, essas subfrações podem ter diferentes papéis na aterogênese. O objetivo do presente estudo foi verificar a relação entre estas subfrações do HDL-c e a razão dessas subfrações (HDL_2-c, HDL_3-c e HDL_2-c-/HDL_3-c, respectivamente) com (1) síndrome metabólica, cada um dos seus componentes e alterações metabólicas associadas (como a inflamação subclínica e resistência à insulina); (2) espessura médio-intimal das carótidas (EMIC), um marcador de doença vascular subclínica; (3) escore de cálcio coronariano (ECC), importante marcador de doença arterial coronariana subclínica. Os dados foram obtidos dos participantes do Estudo Longitudinal de Saúde do Adulto – ELSA-Brasil, sem doença cardiovascular prévia e que não estavam em uso de hipolipemiantes (um total de 4.532 participantes com analisados no contexto da síndrome metabólica; 3.930 submetidos a EMIC e 3.674 submetidos a análise do ECC). O estudo levou às seguintes conclusões

principais: (1) os níveis de HDL-c, HDL_2-c e HDL_3-c foram inversamente associados a todos os critérios diagnósticos para a síndrome metabólica, inflamação, resistência à insulina após ajuste para fatores de confusão; houve associação inversa da razão das subfrações com cada critério para síndrome metabólica, inflamação e resistência à insulina mesmo após ajuste para o HDL-c total; (2) os níveis de HDL-c, HDL_2-c e HDL_3-c foram inversamente associados à EMIC mesmo após ajuste para os fatores de risco tradicionais; o diagnóstico de diabetes intensifica a associação negativa entre a razão das subfrações (HDL_2-c/HDL_3-c) e EMIC; (3) não houve associação do ECC com nenhum perfil do HDL-c estudado (seja o HDL-c total, seja suas subfrações ou a razão das subfrações), principalmente após o ajuste para o nível dos triglicérides. Nossos resultados indicam o papel protetor das subfrações do HDL nas alterações metabólicas iniciais da aterogênese e na doença vascular subclínica. Ainda nesse contexto, a diferente composição do HDL pelas subfrações conferem papel protetor adicional. No entanto, pela importante interação com o metabolismo dos triglicérides, não é possível atribuir um fator anti-aterogênico independente às subfrações do HDL na DAC.

Descritores: Colesterol; HDL-colesterol; Aterosclerose; Espessura íntima-média carotídea; Síndrome metabólica; Lipoproteínas HDL2; Lipoproteínas HDL3.

ABSTRACT

ABSTRACT

Generoso G. *Association between high-density lipoprotein-cholesterol subfractions measured by Vertical Auto Profile method and metabolic syndrome, insulin resistance and risk of subclinical atherosclerosis: The Brazilian Longitudinal Study of Adult Health* [thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”;2020.

Although epidemiological data suggests an inverse association between high-density lipoprotein cholesterol (HDL-c) levels and atherosclerotic disease, recent Mendelian randomization studies have not proven causality in the HDL-c protective effects. Since the high-density lipoprotein (HDL) group is composed of different particles in size, shape, density, lipidome and proteome, these subfractions may play different roles in atherogenesis. The aim of the present study was to assess the relationship between these HDL-c subfractions and the subfractions ratio (HDL_2-c, HDL_3-c and HDL_2-c/HDL_3-c, respectively) with (1) metabolic syndrome, each one of its defining components and associated metabolic abnormalities (such as low-grade inflammation and insulin resistance); (2) carotid intima-media thickness (IMT), a marker of subclinical vascular disease; and (3) coronary artery calcium (CAC) score, an important marker of subclinical coronary artery disease. Data were obtained from participants in the Brazilian Longitudinal Study of Adult Health - ELSA-Brasil, without prior cardiovascular disease and who were not taking lipid-lowering drugs, 4,532 participants for the context of the metabolic syndrome; 3,930 underwent cIMT exam and 3,674 for CAC score analysis. The study led to the follow main conclusions: (1) total HDL-c, HDL_2-c and HDL_3-c levels were inversely associated with all diagnostic criteria

for metabolic syndrome, low-grade inflammation and insulin resistance after adjustment for confounders; there was an inverse association of the HDL_2-c/HDL_3-c ratio with each criterion for metabolic syndrome, low-grade inflammation and insulin resistance even after adjusting for total HDL-c; (2) total HDL-c, HDL_2-c and HDL_3-c levels were inversely associated with cIMT even after adjusting for traditional risk factors; the diagnosis of diabetes intensifies the negative association between HDL_2-c/HDL_3-c ratio and cIMT; (3) there was no association of CAC with any HDL-c profile studied (either total HDL-c, its subfractions or HDL_2-c/HDL_3-c ratio), especially after adjusting for triglycerides levels. Our results indicate the protective role of HDL subfractions in the initial metabolic changes linked to atherogenesis and subclinical vascular disease. Still in this context, the different composition of HDL by subfractions gives an additional protective role. However, due to the critical interaction with triglyceride metabolism, it is not possible to attribute an independent anti-atherogenic factor to HDL subfractions in coronary artery disease.

Descriptors: Cholesterol; HDL-cholesterol; Atherosclerosis; Carotid intima-media thickness; Metabolic syndrome; Lipoproteins, HDL2; Lipoproteins HDL3.

INTRODUCTION

Cardiovascular disease is the leading cause of death in the world. Although there was a decline in its rates in the last decades, cardiac issues are responsible for one-third of the total fatal events in the United States of America, amounting to approximately 600,000 deaths in 2014(1). The scenario is similar in Brazil, according to DATASUS. The main clinical syndromes composing this fatal cardiovascular outcome are atherosclerotic coronary artery disease, ischemic stroke, and peripheral artery disease (PAD). In 2019, Brazilian data revealed about 330,000 deaths related to ASCVD - defined as acute coronary syndromes (ACSs), a history of myocardial infarction (MI), stable or unstable angina, coronary or other arterial revascularization, stroke, transient ischemic attack or peripheral arterial disease with a presumed atherosclerotic origin. There were approximately 200,000 ischemic stroke events that resulted in large expenses to the Brazilian public health system, estimated to be as high as US$ 150,000,000.00 each year(2). Moreover, epidemiologic studies detected a 12% ASCVD prevalence in the world population between 65 and 84 years old(3).

These aforementioned clinical manifestations have atherosclerosis as a common pathophysiological mechanism, a chronic inflammatory disease whose pathway comprises irritative insults resulting in endothelial dysfunction and structural changes. These abnormalities allow the low-density lipoproteins (LDL) particles to the subendothelial space and LDL-cholesterol to deposit in the arterial wall, followed by immune system cells infiltration, macrophages, and smooth muscle cells activation, inflammation and, thus, development of the atherosclerotic plaque (4–6).

Cholesterol and Lipoproteins

Although the first reports by Fallopius date from the 16th century as "degeneration into bones" of the arterial wall, it was not until 1843 that Vogel discovered cholesterol in arteries with atherosclerosis (7). More recently, the role of lipid metabolism in atherogenesis came to be explored in the early twentieth century, when Anitschkow tested the “atherogenic diet” theory. The Russian pathologist showed that rabbits on a cholesterol-laden diet developed atherosclerotic lesions similar to those found in humans, including by a drawing representation of the foam cells(8).

In 1929, Macheboeuf reported for the first time that large amounts of lipids in serum were present in association with proteins (9). In the next 20 years, lipoproteins were better characterized, divided into two major fractions (9,10), but without clarifying their origin and biological significance. Then, in 1951, Russ, Eder and Barr, studying pre-menopausal women, observed a negative correlation between serum α-lipoproteins (that we now recognize as HDL) and the incidence of coronary artery disease (11). It was perhaps the first study that suspected, even if superficially, that the different fractions of lipoproteins would have different effects on atherosclerosis (12).

After the mid-20th century, large epidemiological studies corroborated the relation between cholesterol and lipoproteins with atherogenesis and its clinical outcomes (13–18). First, the Seven Country Study observed that cholesterol levels were directly correlated with fat intake and cardiovascular death, including substantial differences between populations. But the Framingham Heart Study was the milestone in the concept of dyslipidemia as a risk factor for

cardiovascular disease, through hypercholesterolemia(16) and low HDL-c levels(19).

High-Density Lipoprotein

The high-density lipoproteins (HDL) are set up in the liver and small bowel and composed by 40-60% of lipids (cholesterol, cholesterol esters, phospholipids and triglycerides) and by a protein portion, mostly by Apolipoprotein A-I (ApoA-I – 70%) and A2 (ApoA2 – 20%). Also, HDL presents in its composition high levels of antioxidants molecules, as PON1 and LCAT (20,21). This class of lipoproteins plays two significant roles in the metabolism: reverse cholesterol transport and modulation of inflammation, as detailed below.

1. Reverse Cholesterol Transport (RCT)

The RCT is a process involved in the removal of excess cholesterol from the peripheral tissues by HDL. The cholesterol accumulated, ingested through diet or produced in the extrahepatic tissues is transported to the liver and excreted as feces via the bile (converted to bile acid). The HDL RCT contributes to lipidic homeostasis, promoting downregulation in the biosynthesis, esterification and cholesterol ingestion(20) (Figure 1).

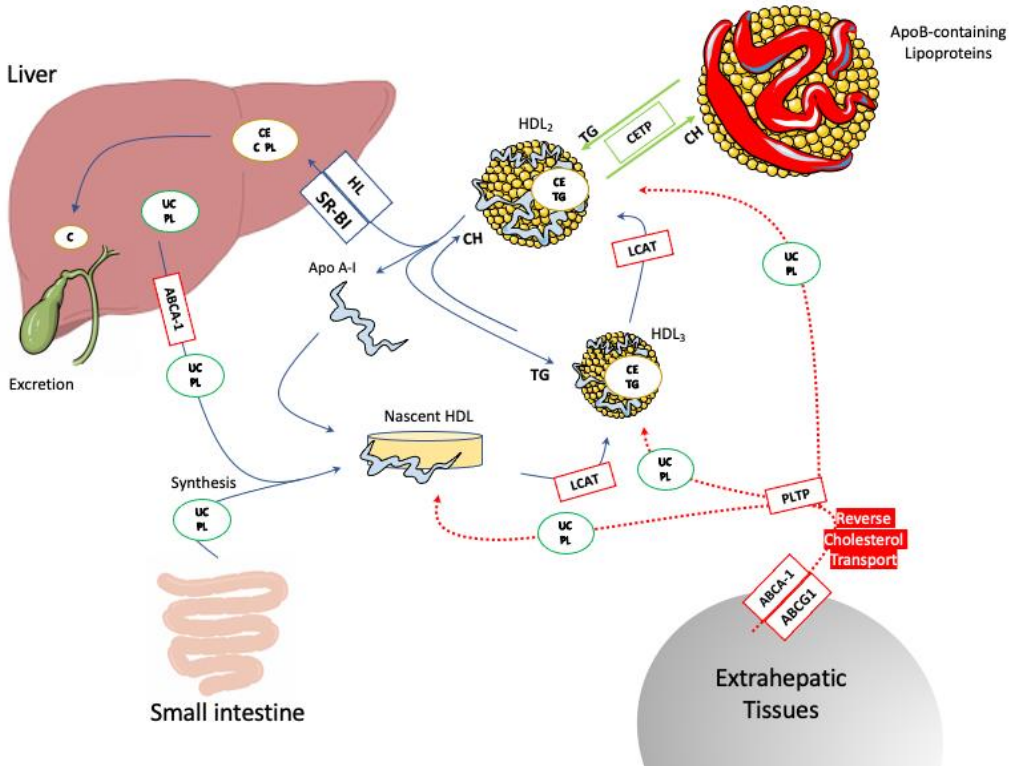


Figure1 - HDL Metabolism. The Apo A-I is synthesized by the intestine and the liver. Once in the bloodstream, Apo A-I deposits phospholipid (PL) into the cells. Also, unesterified cholesterol (UC) and PL efflux from the peripheral cell, via ABCA1, and are captured by the Apo A-I nascent HDL particle. Therefore, the LCAT activity in this particle alters its shape, generating spherical HDL particles: the denser and small HDL_3, and the larger and less dense HDL_2. These HDL particles can receive additional lipids, via both efflux (mediated by ABCA1) and exchange with ApoB-containing lipoproteins (mediated by CETP). The CETP also facilitates the lipid interchange between HDL subfractions. The phospholipids efflux to HDL is mediated by phospholipid transfer protein (PLTP). UC and cholesterol of mature HDL is carried to the liver by the hepatic scavenger receptor class B member 1 (SR-BI). Finally, the cholesterol delivered to the liver is excreted in the bile acids.

2. Modulation of inflammation

The HDL works effectively in the antioxidant and anti-inflammatory mechanism: for example, PON1 prevents the generation of products from lipidic peroxidation, which stimulates the inflammatory mediators' production. Also, ApoA-I reduces the pro-inflammatory impact of the oxidized lipids. An *in vitro* reduction of

adhesion molecules expression (ICAM-1 and VCAM-1) and a higher nitric oxide (NO) production by endothelial cells has also been reported (22).

However, oxidized and enzymatically modified HDL molecules decrease the anti-inflammatory functions, which may be associated with a higher risk of coronary heart disease(CHD) (23). Also, over 40% of the Framingham Heart Study participants showed normal HDL-c levels (≥40 mg/dl in men and ≥50 mg/dl in women)(24), suggesting that the best translation of the HDL cardiovascular protective effect would not be the cholesterol measurement in this lipoprotein. Considering the heterogeneous composition of the HDL particle pool (detailed below), the assumption is that some HDL subfractions may have protective effects, while others may not. Likewise, some studies suggest that the proportion of each subclass in the total HDL pool could also result in different impacts on atherogenesis(25,26).

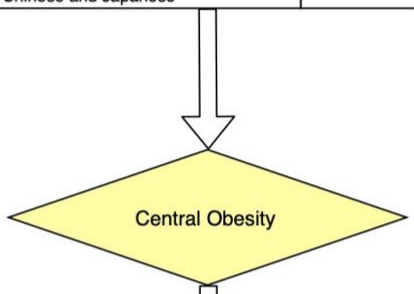
Metabolic Syndrome

Closely involved in the atherogenic process, the metabolic syndrome (MetS) is a chronic inflammatory state of low activity resulting from a complex interaction between genetic and environmental factors(27,28). A MetS diagnosis increases the risk of incident diabetes mellitus by 3-fold; the risk of cardiovascular disease(29) by 2-fold, including a significant increase in the risk of stroke, myocardial infarction; and increase in the risk of death by 2-fold when in comparison with the healthy population(30). Insulin resistance, visceral obesity, dyslipidemia, endothelial dysfunction, genetic factors, hypertension, hypercoagulability and chronic stress are components of the metabolic syndrome(31), as currently defined by the International Diabetes Federation-IDF.

The classification recognizes central obesity as the pivotal determinant for the metabolic syndrome, making it an essential component in the diagnostic criteria (Table 1)(32). Once the diagnosis is performed, the treatment is based on lifestyle changes including caloric restriction, regular physical activity, control of blood pressure, hyperglycemia and dyslipidemia(33).

Table 1 - The new International Diabetes Federation (IDF) definition

BMI > 30 kg/m^2 **or** waist circumference (cm)		
Country/Ethnic group	Man	Woman
United States of America	≥ 102	≥ 88
Europids. Sub-Saharan Africans, Eastern Mediterranean and Middle East	≥ 94	≥ 80
South Asians and Ethnic South and Central Americans, Chinese and Japanese	≥ 90	≥ 80



plus any two of the following four factors:

Hypertriglyceridemia	Triglycerides ≥ 150 mg/dL (1.7 mmol/L) or specific treatment for this lipid abnormality
Low HDL-cholesterol	HDL-c < 40 mg/dL (1.03 mmol/L) in men HDL-c < 50 mg/dL (1.29 mmol/L) in women or specific treatment for this lipid abnormality
Hypertension	systolic blood pressure ≥130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension
Hyperglycemia	fasting plasma glucose ≥100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes

Assessment of Subclinical Atherosclerosis and Vascular Disease

In the decades leading up to ASCVD, preclinical changes resulting from the initial stages of the atherogenic process are present long before the advanced lesions detected in vascular events(34). The first morphological abnormalities can be detected with non-invasive, high-resolution and easily performed imaging methods. Two of these methods are detailed below:

a. *Intima-media carotid thickness (cIMT)* is an inexpensive and straightforward method achieved by applying a B-mode ultrasound technique that has a good correlation with histological findings(34). It is measured by calculating the distance between the vascular / intimate lumen and the media-adventitia layers. The cIMT reflects not only early atherosclerosis but also pre-atherosclerotic findings, such as the compensatory increase with medial hypertrophy deriving from smooth muscle cell hyperplasia and fibrocellular hypertrophy. The method is widely used in population studies, with several extensive analyses observing a direct association between cIMT and both myocardial infarction and stroke (35).

b. *Coronary Artery Calcium (CAC)* is determined by cardiac computed tomography obtained in synchrony with the R-R interval of the electrocardiogram. The CAC score quantified by the Agatston method provides an absolute score based on the calcification area and density(36). The CAC score is a marker of coronary atherosclerosis and large prospective studies have shown a strong positive association with

cardiovascular outcomes(37). In addition, the CAC score increases the accuracy of models that calculate cardiovascular risk(38–40). Conversely, the absence of CAC implies a very low risk of future events.

HDL-Cholesterol and subclinical atherosclerosis

Several population studies have shown that HDL-c levels are negatively associated with coronary calcification(41–43), cIMT (44,45) and with ankle-brachial index (46). However, the researchers cannot demonstrate whether HDL-c has a causal role in the atherogenesis or if it is only a marker of the pathophysiology as meticulous adjusts for ethnicity, genre and other risk factors results in consistent attenuation of its protective effect for cardiovascular events and mortality (47,48). Also, this independent protective role of the HDL particles has been disputed by Mendelian randomization studies(49,50), that could not demonstrate a causal association. Similarly, no clinical benefit of pharmacologic interventions designed to raise HDL-c through the CETP inhibitors have been noted (51–55). This context led to alternative methods to test HDL-c and its subcomponents (in addition to the HDL heterogeneity method mentioned above) in the quest to define its relevance for cardiovascular risk.

HDL subfractions

The HDL group comprises several molecules which are distinct in size (from 7nm to 17 nm diameter), density, shape (spherical or discoid), lipidome and proteome (56,57).Thus, the separation of the molecule can be achieved by different methods:

- Precipitation: after separation by ApoB containing serum lipoproteins, the HDL-c mass is directly quantified in the supernatant. The reagents applied in this process are polyanions such as heparin, dextran sulfate, and sodium phosphotungstate, which are used with a divalent cation, such as magnesium, heparin–manganese, or calcium. The main limitation is that proteins and the apoE fraction may confound the HDL-c measurement.
- Chromatography: This technique separates lipoproteins by size, using permeation columns. The separation of lipoproteins can be performed by applying two principal methods: with a gel filtration column or with an anion exchange column.
- Electrophoresis: Lipoproteins can be separated by electrophoresis, receiving classifications according to their mobility. This can be done by one-dimensional gel electrophoresis (ND-PAGGE) or by combining ND-PAGGE with agarose gel electrophoresis, separating by charge density in the first dimension and particle size in the second dimension.
- Ultracentrifugation: The density gradient ultracentrifugation is the classic method for separating lipoprotein subfractions. In the past decades, methods such as preparative ultra-centrifugation or short sequence steps based on ultracentrifugation have improved the process. The VAP method uses an inverted rate zonal, single vertical spin, density gradient ultracentrifugation technique that simultaneously measures cholesterol concentrations of all five lipoprotein classes (HDL, “real” LDL [LDL-R; the LDL without Lp(a) and IDL], VLDL, IDL, and Lp(a)) and their subclasses (HDL2, HDL3, LDL1, LDL2, LDL3, LDL4, VLDL1, VLDL2 and VLDL3) - Figure 2.

Concerning the HDL subfractions, the HDL_2 is larger, less dense, and more strongly associated with ApoA-I. This particle carries most of the HDL-c measured (58). HDL_3 is smaller, denser, and carries enzymes involved in preventing oxidative stress. Also, it receives cholesterol from peripheral tissues in reverse transport mechanism (59,60).

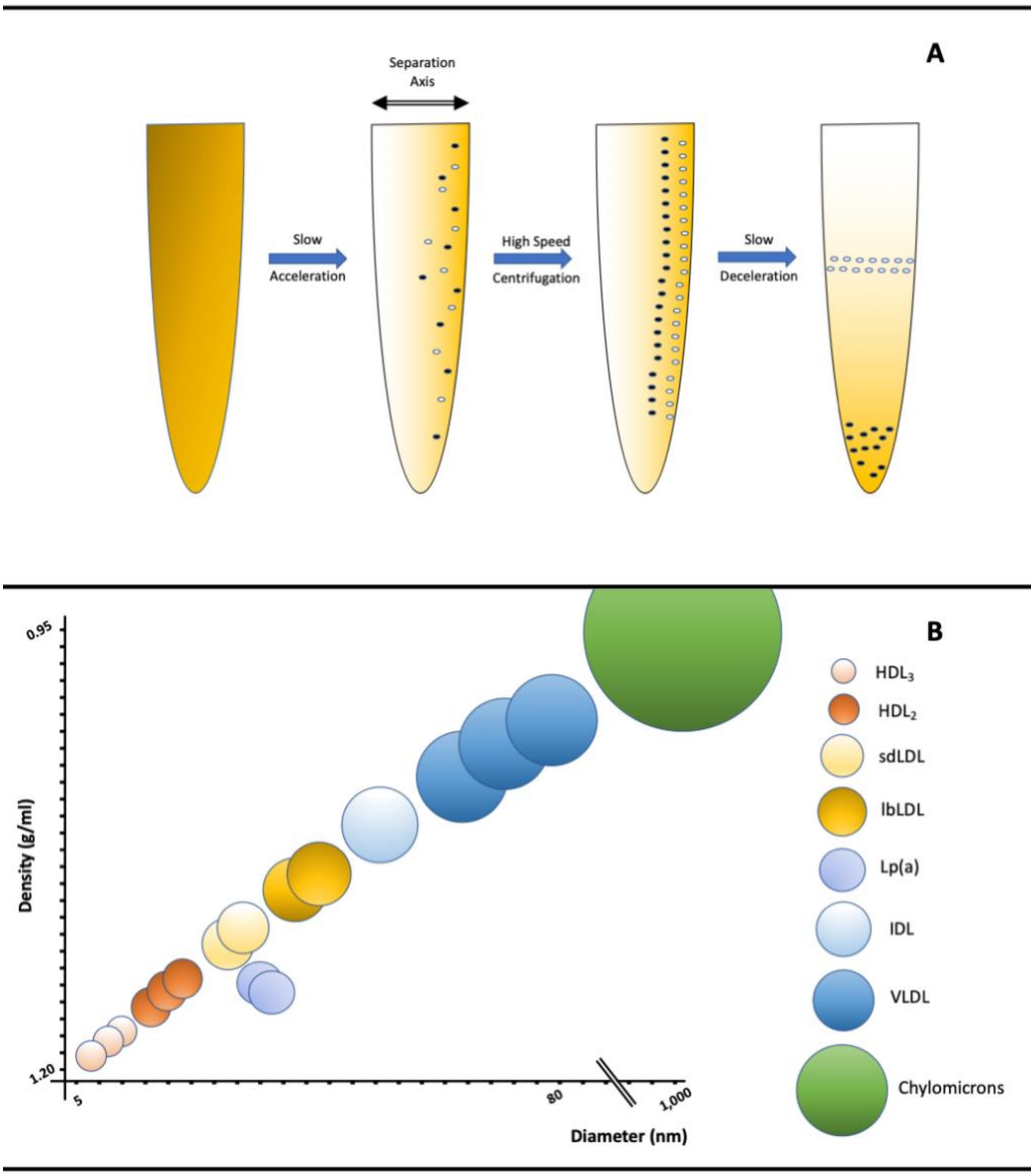


Figure 2. A: Zonal density gradient separation using vertical rotor in a Vertical Auto Profile method; **B:** Schematic representation of lipoproteins according to its diameter and density (decreasing). HDL means high-density lipoprotein; sdLDL, small-dense low-density lipoprotein; lbLDL, large-buoyant low-density

lipoprotein; Lp (a), lipoprotein (a); IDL, intermediate density lipoprotein; and VLDL, very low-density lipoprotein.

HDL-c subfractions and atherosclerosis

Some studies have observed controversial results in the relationship between both HDL-c subfractions and subclinical or manifested atherosclerosis. In pre-clinical atherosclerosis studies some investigators observed an inverse association between HDL_2-c and cIMT(61) or CAC (62–64), while others found no link (65–67) or even direct correlation(68) with cIMT. Likewise, HDL_3-c has been reported to be negatively associated with cIMT(68) and CAC(64) or not(61,62,65–67,69).

Similarly, the association with ASCVD events has been controversial. Several studies observed a negative association with HDL_3-c in both primary(70–73) and secondary(74) prevention, but other authors found neutral results(75–77). Also, conflicting findings were observed concerning HDL_2-c, which has been reported as protective(75–77) or not(70–72,74). Finally a review involving 80 studies showed no difference in cardioprotective effect of either subfractions(78).

When analyzing the proportion of HDL-c subfractions as aforementioned, the subfraction ratio (HDL_2-c/HDL_3-c ratio) was used. Thus, although there are no studies addressing subclinical atherosclerosis, researchers found an association of the HDL_2-c/HDL_3-c ratio with insulin resistance and the number of criteria for metabolic syndrome.

Some limitations may explain the lack of consensus regarding the HDL-c subfractions, including different populations and divergent statistical adjusting models (60). In addition, there is no data regarding HDL-c subfractions and vascular disease markers, and the correlation with other populations is unknown. Moreover, because of the smaller sample size of most studies, there are

restraints in the HDL-c subfractions evaluation in sub-groups, like those with diabetes, metabolic syndrome, renal failure, among others.

In light of the above, the aim of our study was assessing the HDL-c subfractions and their impact on the atherogenic process, observing their correlation from the known ASCVD involved factors (metabolic syndrome, inflammation, insulin resistance) until established non-invasive vascular disease markers (cIMT and CAC).

OBJECTIVES

1. Evaluate the association between HDL-c subfractions (HDL_2-c and HDL_3-c, isolated and combined as HDL_2-c/HDL_3-c ratio) and the following metabolic factors:

    1a. Metabolic Syndrome diagnosis and each of its components

    1b. Inflammation, assessed by c-reactive protein

    1c. Insulin resistance, assessed by the HOMA-IR

2. Evaluate the association between HDL-c subfractions (HDL_2-c and HDL_3-c, isolated and combined as HDL_2-c/HDL_3-c ratio) and the following vascular disease markers:

    2a. Carotid intima-media thickness (cIMT)

    2b. CAC score

MATERIAL AND METHODS

Sample – The ELSA-Brasil Study

The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) is the largest Brazilian population cohort, composed by 15,015 civil servants between 35 and 74 years of age from six institutions (the federal universities of Bahia, Espirito Santo, Minas Gerais, and Rio Grande do Sul; the University of Sao Paulo; and the Oswaldo Cruz Foundation), retired or active. The study excluded servants with severe cognitive impairment or communication difficulties; pregnancy or recent childbirth (less than four months before the first interview); and, if retired, living outside of a study center's metropolitan area (45,79,80).

All first examinations were conducted between August 2008 and December 2010. The volunteers attended the study centers for clinical examination. In addition, they were visited in their respective workplaces for the collection of data about sociodemographic reports, medical history, occupational exposure, familial medical history, reproductive health, medical assistance, psychosocial factors, body weight history, food and alcohol consumption, smoking status, physical activity, medicine use, cognitive function and mental health. In all centers, the participants were attended by trained professionals under strict quality control.

Methods

From the total ELSA-Brasil sample, we included those participants who had plasma cholesterol analyzed through the Vertical Auto Profile (VAP) method; intima-media thickness measurement by ultrasound technique; and CAC score

acquired by cardiac computed tomography, following all methods detailed above. Also, in order to observe only participants in the primary prevention scenario, we excluded those with manifested cardiovascular disease: any history of myocardial infarction, stroke, heart failure or coronary revascularization.

Blood Sample Analysis

In the ELSA-Brasil study, the blood collection of the participants was performed by venipuncture following nocturnal fasting. Further, the samples were centrifuged in each research center and stored in tubes at -80ºC to be transported to the São Paulo center.

HDL-c and subfractions: Conventional HDL-c concentrations were determined by a non-precipitated colorimetric method using ADVIA 1200 Siemens® equipment(81). Also, the sample was processed by Vertical Auto Profile (VAP Diagnostics, Birmingham, AL). Due to the strong correlation between methods (r_s=0.94), we considered the HDL-c values measured by the VAP rather than conventional testing.

C-reactive protein levels – the CRP level was measured using a high-sensitivity assay by immunochemistry–nephelometry (BN II; Siemens).

Insulin resistance - Glucose was determined by the hexokinase enzymatic method and insulin levels were quantified by chemiluminescence (sandwich immunoassay). We obtained the model assessment-estimated insulin resistance (HOMA-IR) value by the formula: fasting blood glucose*0.0555*fast plasma insulin/22.5.

Carotid Intima-media Thickness Measurement

Carotid intima-media thickness was measured in all participants under a standardized protocol in ultrasound equipment (Aplio XG™, Toshiba) with a 7,5 MHz linear transducer. To analyze plaques, carotid bifurcation was observed over 3 cm in addition to 1 cm of the common carotid arteries, about 1 cm below the bifurcation.

All collected images at the research centers were recorded and sent to the ultrasound reading center (Sao Paulo)(82). The exam analysis was centralized and automated by the use of the software MIA™ with images from three cardiac cycles. The cIMT value was calculated by the mean between both carotid arteries. Also, the analysis took into consideration race, sex, age, presence of ASCVD risk factors and use of medication (83,84).

Coronary Artery Calcium Score Measurement

The participants underwent an ECG-gated acquired coronary artery calcium score in a 64-detector CT scanner (Phillips 64; Philips Healthcare, Best, Netherlands). The default settings included 120 kV, mA adjusted to body mass index (100 to 150 mA), 1-phase prospective acquisition at 70% of the cardiac cycle (retrospective acquisition was used if heart rate > 65 bpm) and collimation of 2.5 mm, gantry rotation of 400ms, and reconstruction with a standard filter. The CAC score measurement was calculated using a threshold of 130 Hounsfield Units. All the images were analyzed using dedicated software (Brilliance Workspace).

The CAC score was calculated by Agatston method (36), multiplying the area of calcification in mm^2 by a factor 1 = 130-199, 2 = 200-299, 3 = 300-399 or 4 = >399 Hounsfield Units. A score for each region of interest was calculated by multiplying the density score and the area. A total coronary calcium score was determined by adding up each of these scores for all intervals.

Statistical Analysis

The continuous variables were analyzed and presented as descriptive form, in mean and standard deviation if the distribution was normal and, for non-normal distributions, the data was presented in median and interquartile range. For categoric variables, the descriptive analysis was presented in absolute and relative frequencies. The data was displayed according to the metabolic syndrome diagnosis in the first study, HDL-c quartiles in the c-IMT analysis and CAC classification (0; 0 - <100 and ≥100 Agatston Units) in the last research.

For comparison between groups of numeric variables, we used the parametric tests Student t-test (non-paired) or analysis of variance (ANOVA) with a Bonferroni *post hoc* test for multiple comparisons, and the non-parametric tests Mann-Whitney U-test or Kruskal-Wallis with Dunn *post hoc* test for multiple comparisons. For categorical data, we used the Chi-squared test (χ^2) or Fisher's exact test. Finally, Pearson and Spearman's coefficients were adopted to calculate the correlation and linear regression analysis.

We used linear regression models to examine the association of the HDL-c, as well as each subfraction and HDL_2-c/HDL_3-c ratio with several variables of this study, as detailed below. Also, to predict categorical variables, we

constructed logistic regression models for a 1-standard deviation change of HDL-c, each subfraction and its ratio.

Regarding metabolic syndrome components, c-reactive protein and HOMA-IR, we performed linear regression models. Further, multiple linear regression models were cumulatively adjusted for age, sex and ethnicity (model 1); smoking status (current smoker, former smoker and nonsmoker), physical activity and alcohol use (model 2). In the HDL_2-C/HDL_3-c ratio analysis, there was a third model, also adjusted for HDL-c.

For the carotid intima-media thickness, we also constructed linear regression models that were cumulatively adjusted for age, sex and ethnicity (model 1); smoking status (current smoker, former smoker and nonsmoker), physical activity and alcohol use (model 2); LDL-C, systolic blood pressure, waist circumference, fasting plasma glucose, log-transformed triglycerides, estimated glomerular filtration rate and antihypertensive use – yes/no (model 3). Once again, the HDL_2-C/HDL_3-c ratio was additionally adjusted for HDL-c in the model 4. As we observed a significant interaction between HDL_2-C/HDL_3-c ratio and diabetes, additional analyses were performed, stratified by diabetes status.

Finally, the association between HDL-c subfractions and variables was determined according to CAC classification. For the comparison between CAC=0 and CAC>0, as well as between CAC<100 and CAC≥100, multiple logistic regression models were performed. For continuous values of CAC score two multiple linear regression models were constructed - ln(CAC+1) as the dependent variable considering the entire sample and, for the participants who presented CAC>0, we used ln(CAC) as the variable. The multiple regression analysis models were also adjusted for age, sex and ethnicity (model 1); smoking, alcohol

use, physical activity and LDL-cholesterol (model 2); and log-transformed triglycerides (model 3).

In order to compare the HDL-c-related variables in these association analyses, we standardized the variables HDL-C, HDL_2-C, HDL_3-c and HDL_2-C/HDL_3-c ratio. The statistical significance was defined as $p < 0.05$. All the statistical analyses were performed with Stata 14.0 (StataCorp, USA).

Ethical Aspects

The ELSA-Brasil protocol complied with the main Resolution 196/96, Resolution CNS 346/05 (multicentric projects) and Resolution CNS 347/05 (biologic material storage). In addition, the protocol was approved by each institution's committee of ethical research involved and by the National Committee of Ethics in Research of the National Health Council (CONEP).

The Ethical Committee, Recruitment and Social Communication helps in the coordination of accomplishments of ethical aspects and communication with the study involved institutions and with their participants(85).

All the volunteers were oriented about the longitudinal study design during Phase 1 of the cohort and subscribed to the consent form. The consent also allows access to their medical records, in both health institutions and under their doctor's control. A decision to refuse to remain in the study is always sovereign and respected(86).

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RESULTS

RESULTS

Article 01: Association between high-density lipoprotein subfractions and low-grade inflammation, insulin resistance and metabolic syndrome components: The ELSA-Brasil Study

In the original research *"Association between high-density lipoprotein subfractions and low-grade inflammation, insulin resistance, and metabolic syndrome components: The ELSA-Brasil study"* published in the Journal of Clinical Lipidology, September–October 2018, Volume 12, Issue 5, Pages 1290–1297.e1, we enrolled 4,532 participants of the ELSA-Brasil study (51±9 years, 45.2% men) with no manifested cardiovascular disease and who were not using fibrates. We observed an inverse association between the HDL-c, as well as HDL_2-c and HDL_3-c, with all metabolic syndrome defining features and inflammation (assessed by c-reactive protein levels) and insulin resistance (assessed by HOMA-IR score). Also, the HDL_2-C/HDL_3-c ratio was an independent predictor of metabolic syndrome, inflammation and insulin resistance even after adjustment for HDL-c.

Running Title: HDL subfractions and metabolic syndrome

Association between high-density lipoprotein subfractions and low-grade inflammation, insulin resistance and metabolic syndrome components: The ELSA-Brasil Study

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Abstract

***Background*:** HDL cholesterol (HDL-C) can be divided into subfractions, which may have variable effects in atherogenesis. The results regarding the association between HDL-C subfractions and risk factors for cardiovascular disease are mixed.

Objective: The objective of this study was to analyze the association between HDL-C subfractions and each metabolic syndrome component, HOMA-IR and C-reactive protein.

Methods: 4,532 individuals between 35 and 74 years without previous manifest cardiovascular disease not using fibrates were enrolled. HDL-C subfractions were separated by vertical ultracentrifugation (Vertical Auto Profile - in mg/dl) into HDL_2-C and HDL_3-C. HDL_2-C/HDL_3-C ratio, insulin resistance (HOMA-IR), and high-sensitivity C-reactive protein (CRP) were also included in the analysis.

Results: Mean age of participants was 51 ± 9 years, and 54.8% were women. In the univariate analysis, HDL-C, HDL_2-C, and HDL_3-C were all inversely associated with each of the MetS defining factors, HOMA-IR values, and serum CRP. We also observed a negative association between HDL_2-C/HDL_3-C ratio and the variables mentioned above even after adjusting for smoking, alcohol use, physical activity, and HDL-C levels ($p < 0.01$).

Conclusion: HDL-C and its subfractions (HDL_2-C and HDL_3-C) are inversely associated with the defining features of MetS, insulin resistance, and systemic inflammation. Additionally, the HDL_2-C/HDL_3-C ratio measured by VAP is significantly associated with the former factors even after comprehensive adjustment for HDL-C and other confounding variables.

Introduction

High-density lipoproteins (HDL) comprise a group of heterogeneous subfractions which vary in size, density, shape, lipidome, and proteome(56). Epidemiological studies have shown an independent inverse association between HDL-cholesterol (HDL-C) concentrations and the risk of atherosclerotic cardiovascular disease (ASCVD)(87). However, the independent protective role of the HDL particles has been disputed by Mendelian randomization studies as well as the lack of clinical efficacy of pharmacologic interventions designed to raise HDL-C. (50–55)

Low HDL-C concentrations are a component of atherogenic dyslipidemia that is associated with visceral obesity, insulin resistance, and heightened inflammation. These findings define the MetS, a constellation of risk factors associated with an increased risk of type 2 diabetes, ASCVD, and death(28,88,89). Nevertheless, the role of HDL in atherogenesis is not entirely clear, though it might protect against atherosclerosis by antagonizing oxidation, thrombosis, and inflammation, and potentiating reverse cholesterol transport (20,22).

Since HDL comprises a heterogeneous group of particles, some of these effects may be related to specific subfractions. One method to analyze these subfractions is to measure their cholesterol by separating the HDL-C into two groups: HDL_2-C (carried in larger and less dense particles) and HDL_3-C (carried in smaller and denser particles) (57). There is substantial controversy surrounding the capacity of different HDL subfractions to protect against atherogenesis, with the evidence being decidedly mixed: HDL_3-C was positively(74) and inversely (72) associated with cardiovascular outcomes, as well as HDL_2-C, established as a predictor of ASCVD reduction in some analyses

(75,77) while this finding did not appear in other studies(72,74). No clear, consistent picture has emerged. Moreover, the exact interplay between such subfractions and parameters of the MetS has not been adequately investigated. Thus, the objective of the present study was to evaluate the association of HDL-C and its subfractions (HDL_2-C and HDL_3-C) with the defining features of MetS as well as serum markers of inflammation and insulin resistance.

Methods

Sample. Between August 2008 and December 2010, 15,015 men and women were enrolled in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil), a prospective longitudinal cohort composed of civil servants aged 35 to 74 years from six Brazilian cities, which has previously been described in detail (79,80). We included those who underwent HDL-C measurement by a conventional method and by the vertical auto profile (VAP) method. All participants were from the São Paulo center. Exclusion criteria for the present analysis were lack of serum measurement of any component of the lipid profile, CRP, and insulin; individuals with prior cardiovascular disease (myocardial infarction, stroke, heart failure and coronary revascularization); and participants using fibrates. We did not exclude persons receiving statin therapy since there was a sub-group analysis excluding this population (Appendix 1) and it showed similar results.

HDL-C and subfractions analysis. Blood collection was obtained from participants after nocturnal fasting. The samples were centrifuged at the sites and stored in tubes at -80°C. Conventional HDL-C concentrations were determined by a non-precipitated colorimetric method using ADVIA 1200 Siemens™ equipment(81). HDL-C and the HDL_2-C and HDL_3-C subfractions were measured by the VAP method (Atherotech®), an inverted rate zonal, single vertical spin, density gradient ultracentrifugation (UC) technique that simultaneously measures cholesterol concentrations after fraction separation(57). After separation, cholesterol in subfractions was measured by enzymatic methods. Because of the strong correlation between methods (ρ=0.95), we considered HDL-C those values measured by the VAP rather than conventional testing.

CRP levels. Blood was collected from overnight fasting and CRP was measured using a high-sensitivity assay by immunochemistry – nephelometry (BN II; Siemens).

Insulin Resistance. Glucose was determined by the hexokinase enzymatic method and insulin was quantified by chemiluminescence (sandwich immunoassay)(90). The HOMA-IR value was obtained by fasting blood glucose*0.0555*fast blood insulin/22.5 (91).

Metabolic Syndrome and study variables. The definition for MetS adopted by the International Diabetes Federation (IDF) was used: central obesity (waist circumference ≥ 94cm for men and ≥ 80cm for women, with ethnicity-specific values for other groups) plus any two of the following four factors: raised TG level: ≥ 150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality; reduced HDL cholesterol: < 40 mg/dL (1.03 mmol/L) in males and < 50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality; elevated blood pressure defined as systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, or treatment of previously diagnosed hypertension; raised fasting plasma glucose (FPG) ≥ 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes(92).

Diabetes. Diabetes was defined as a reported history of diabetes mellitus, oral antidiabetic drugs or insulin use, fasting serum glucose ≥ 126 mg/dl; HbA1c levels ≥ 6.5% or a 2-hour oral glucose tolerance test ≥ 200 mg/dl.

Hypercholesterolemia. Hypercholesterolemia was defined as low-density lipoprotein cholesterol (LDL-C) level ≥130 mg/dl or lipid-lowering drug use.

Smoking. Participants were divided into never nsmokers, current smokers and former smokers (If the individual stopped tobacco but smoked more than 100 cigarettes throughout life).

Alcohol consumption. The study considered as non-users, former consumers, and current consumers. One drink of alcohol was defined as 1 can/bottle of beer (350ml), 1 glass of wine (120–150 ml) or 1 shot of spirits (40 ml). Participants who abstained or consumed less than 1 drink per week were considered as non-users. Since our previous cohort study has observed lower low-HDL-C as higher alcohol consumption per week(93), we also have performed a sensitivity analysis constructing a multiple regression analysis using the alcohol consumption in grams per week as an adjustment variable. Thereby, both final results are similar.

Physical Activity: The participants answered the International Physical Activity Questionnaire (IPAQ) on leisure-time and going to work physical activity and were classified in sedentarism, physical activity < 150 minutes per week and ≥ 150 minutes per week.

Statistical analysis: Continuous variables were analyzed and presented as descriptive statistics with mean and standard deviation for normal distribution and, for non-normal distributions, with median (quartiles). We showed categorical variables in absolute and relative frequency. As triglyceride, HOMA-IR and CRP did not present normal distributions they were transformed using the natural logarithm in regression models.

Comparison of quantitative variables across groups was performed using analysis of variance (ANOVA) for variables with normal distribution and the Kruskal-Wallis test for those with non-normal distributions. Categorical variables were analyzed by chi-square test (χ^2) and Fisher's exact test. As correlation measurement, we used Pearson in continuous variables analysis and partial correlation coefficient in multivariate analysis.

To examine the association between HDL-C subfractions and MetS components or other variables, we constructed bivariate and multivariate linear regression models. For these analyses, we standardized HDL-C, HDL_2-C, HDL_3-C and HDL_2-C/HDL_3-C ratio. Multiple linear regression models were adjusted for smoking status (current smoker, former smoker, and nonsmoker), physical activity and alcohol use. The HDL_2-C/HDL_3-C ratio was also adjusted for total HDL-C. Statistical significance was defined as $p < 0.05$. Analyses were performed with Stata version 14.0 (StataCorp, USA).

Results

We included 4,532 participants with a mean age of 51±9 years, 2,052 (45.2%) were males and 1,526 (33.7%) met criteria for MetS. When stratified by the diagnosis of MetS, positive criteria were associated with worse risk profiles, with a higher prevalence of hypertension, smoking status, diabetes, and dyslipidemia, including greater total cholesterol and triglycerides and lower HDL-C levels in this group, as expected (Table 1).

HDL-C and subfractions and metabolic syndrome: Individuals with MetS presented (Table 2) with higher total cholesterol and lower levels of HDL-C, HDL_2-C and HDL_3-C (all p <0.001), as well higher insulin resistance levels (HOMA-IR 3.9 vs. 1.6 - p<0.001). As expected, there was a strong correlation of HDL-C from VAP and HDL-C measured by the conventional method (ρ =0.95).

Both HDL-C and subfractions (HDL_2-C and HDL_3-C) were inversely correlated with each diagnostic criterion for MetS. There was a higher correlation of HDL subfraction values with waist circumference, HOMA-IR, and triglycerides and lower correlation with systolic blood pressure, HbA1C, and CRP (all p<0.001 - Table 3). HDL-C, HDL_2-C, and HDL_3-C levels were also inversely associated with the prevalence of abnormal criteria for MetS (Figure 1).

HDL_2-C/HDL_3-C ratio: A positive association was observed between HDL_2-C/HDL_3-C ratio and HDL-C (ρ= 0.73). In bivariate linear regression, the HDL_2-C/HDL_3-C ratio was inversely associated with each of the MetS criterion, as well as with CRP, HOMA-IR, and BMI. This finding persisted even after adjustment for covariates (p<0.001 – Table 4).

Discussion

In the present study, we have demonstrated that total HDL-C measurement by the VAP method, as well as its subfractions HDL_2-C and HDL_3-C, are inversely associated with each other component of the MetS, as well as with hemoglobin A1C, insulin resistance, and systemic inflammation. Interestingly, the ratio of HDL_2-C/HDL_3-C is not constant across the spectrum of HDL-C levels. This occurs due to the fact that HDL_2-C increases more than HDL_3-C as total HDL-C increases. Also, lower ratio values are directly associated with each of the individual components of the MetS as well as with insulin resistance (determined by HOMA-IR) and subclinical inflammation (assessed by serum c-reactive protein levels) even after adjustment for total HDL-C values. This finding suggests the cluster of metabolic abnormalities is not only associated with lower HDL-C values, but it also leads to a change in its particle size and distribution. This fact was particularly prominent in its association with triglycerides, waist circumference, CRP, and insulin resistance, even after controlling for known confounders such as smoking, physical activity, and alcohol use.

Since the 1960s, HDL has been divided into subfractions for the analysis of its relationship to cardiovascular disease (72,94). The HDL3 subfraction, smaller and denser, plays an important role in reverse cholesterol transport. Nascent discoidal and small, less lipidated HDL particles interact with macrophages (foam cells) and promote the mobilization and externalization of cholesterol via ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1) and scavenger receptor class B type I (SR-BI). The released cholesterol is esterified by lecithin-cholesteryl acyltransferase (LCAT) and used to lipidate, HDL3 to larger HDL2. Finally, CETP transfers cholesterol ester out of

HDL particles to ApoB-containing lipoproteins in exchange for triglycerides, which are then rapidly hydrolyzed by hepatic lipase (HL), promoting the conversion of large HDLs into smaller species(95,96).

Several studies assessing the association between both HDL_2-C and HDL_3-C subfractions with coronary heart disease (CHD) yielded mixed results(70,72,73,75,78,97–99). The HDL_2-C subfraction was negatively associated with CHD in some analyses(73,75,98), but not in others (78). The same occurs with HDL_3-C results (70,73,78,99). Two studies using VAP, one cohort study(72) and one in secondary prevention(74) showed a negative association of CHD with HDL_3-C but not with HDL_2-C. In MetS, our findings exhibit inverse relationship and similar intensity of both HDL_2-C and HDL_3-C with MetS components, insulin resistance, and inflammation and agree with previous studies (100) that have used other HDL-C subfractions measuring techniques.

Our findings are observational and do not provide information about whether or not HDL3 or HDL2 influence atherogenesis or correlate with cardiovascular events. Our observations are, however, biologically plausible. In the setting of insulin resistance, HDL metabolism undergoes multiple alterations. First, because of inhibition of lipoprotein lipase, less Apo AI is liberated from chylomicrons(101,102), reducing HDL formation in serum. Second, ABCA1 is down-regulated on the surface of visceral adipocytes(103,104), also reducing HDL formation. Third, CETP is activated (105) and leads to more significant enrichment of HDL with triglycerides, rendering this lipoprotein a better substrate for lipolysis by hepatic lipase, leading to the formation of smaller HDLs. Fourth, there is less Apo AI production and HDL formation by hepatocytes (106).

Our study must be read within the constraints of its design. It is not possible to infer causation in a cross-sectional study. Additionally, although these results add data in a different population showing similar issues, they need to be validated in other centers and with other ethnic and racial distributions. Finally, the potential clinical impact of the use of HDL subfractions cannot be defined from the current analysis.

Conclusions

In conclusion, HDL-C, as well as HDL_2-C and HDL_3-C subfractions, are inversely associated with all the factors that define MetS, insulin resistance, and inflammation. Additionally, the HDL_2-C/HDL_3-C subfractions ratio assessed by VAP is also significantly associated with subclinical inflammation, insulin resistance, and MetS components even after further adjustment for total HDL-C and other confounding variables.

Tables and Figures

Table 1 -Characteristics of the Participants at Baseline and by studied groups

Metabolic syndrome	All (n = 4,532)	Yes (n =1,526)	No (n= 3,006)	p
Age, years	51 (±9)	52.8 (±8.9)	50.2 (±8.7)	<.0001
Race %)				NS
White	2640 (58.2%)	889 (58.2%)	1751 (58.2%)	
Brown	962 (21.2%)	327 (21.4%)	635 (21.1%)	
Black	622 (13.7%)	223 (14.6%)	399 (13.3%)	
Asian	193 (4.3%)	47 (3.1%)	146 (4.8%)	
Indigenous	53 (1.2%)	18 (1.2%)	35 (1.2%)	
Other	62 (1.4%)	22 (1.4%)	40 (1.3%)	
Men, %	2052 (45.2%)	702 (46.2%)	1350 (44.8%)	NS
Hypertension, %	1336 (29.5%)	770 (50.5%)	566 (18.8%)	<.0001
BMI, kg/m2	27,2 (±4.2)	30.6 (±4.6)	25.5 (±4)	<.0001
Waist Circumference, cm	89.7 (±12.4)	99.3 (±10.3)	84.8 (±10.4)	<.0001
Smoking, %				<.0001
Current	746 (16.4%)	265 (17.3%)	481 (16%)	
Former Smokers	1367 (30.1%)	519 (34%)	848 (28.2%)	
Never	2419 (53.4%)	742 (48.6%)	1677 (55.8%)	
Systolic blood pressure, mmHg	119.3 (±16.2)	127 (±17)	115.4 (±14.3)	<.0001
Diastolic blood pressure, mmHg	75.1 (±10.7)	80.3 (±10.8)	72.4 (±9.6)	<.0001
Antihypertensive drug use, %	1021 (22.5%)	583 (38.2%)	438 (14.6%)	<.0001
Diabetes, %	874 (19.3%)	564 (37%)	310 (10.1%)	<.0001
Hypercholesterolemia, %	2538 (56%)	959 (62.8%)	1579 (52.5%)	<.0001
Statin use, %	486 (10.7%)	252 (16.5%)	234 (7.8%)	<.0001
Fasting blood glucose, mmol/l	6.16 (±1.6)	6.85 (±2.21)	5.81 (±1)	<.0001
Total cholesterol, mmol/l	5.64 (±1.1)	5.71 (±1.17)	5.6 (±1.04)	<.0001
LDL-C, mmol/l	3.38 (±0.89)	3.44 (±0.97)	3.34 (±0.84)	<.01
HDL-C, mmol/l	1.46 (±0.37)	1.53 (±0.38)	1.31 (±0.3)	<.0001
Triglycerides, mmol/l	1.28 (0.90-1.83)	1.83 (1.33-2.41)	1.09 (0.81-1.46)	0.0001
Antidiabetic drug use, %	362 (8%)	298 (19.5%)	64 (2.1%)	<.0001

*Values are presented by mean (standard deviation), absolute frequency (relative frequency) or median (interquartile range). LDL-C indicates low-density lipoprotein cholesterol; and HDL-C, high-density lipoprotein cholesterol. For total, HDL, and LDL cholesterol, we divided mg/dL values by 38.67. For triglycerides, we divided mg/dL values by 88.57. †The lipid profile was measured by enzymatic method.

Table 2 - Total Cholesterol, total HDL-cholesterol, HDL subfractions plasma concentration, insulin resistance and HDL_2-C/HDL_3-C ratio in the studied groups.

	All	Metabolic syndrome		p
		Yes	No	
TCHOL, mmol/l	5.64 (±1.09)	5.71 (±1.17)	5.60 (±1.04)	<.01
HDL-C, mmol/l	1.42 (±0.37)	1.26 (±0.30)	1.50 (±0.37)	<.0001
HDL_2-C, mmol/l	0.38 (±0.17)	0.31 (±0.13)	0.48 (±0.18)	<.0001
HDL_3-C, mmol/l	1.04 (±0.21)	0.95 (±0.19)	1.08 (±0.21)	<.0001
HDL_2-C/HDL_3-C ratio	0.36 (±0.1)	0.32 (±0.08)	0.38 (±0.1)	<.0001
C-reactive protein, mg/dL	2.85 (0.71-3.34)	3.91 (1.15-4.79)	2.3 (0.59-2.59)	.0001
HOMA-IR	2.4 (±3.1)	3.9 (±4.6)	1.6 (±1.4)	<.0001

Values are presented by mean (standard deviation), absolute frequency (relative frequency) or median (interquartile range). TCHOL means total cholesterol and HDL-C, high-density lipoprotein cholesterol. *The lipid profile was measured by VAP method.

Table 3 – HDL-C, HDL subfractions and HDL_2-C/HDL_3-C ratio and their Pearson's correlation with metabolic syndrome components, HOMA-IR and CRP*

	HDL-C	HDL_2-C	HDL_3-C	HDL_2-C/ HDL_3-C ratio
Waist circumference	-0.344	-0.379	-0.290	-0.376
SBP	-0.121	-0.140	-0.096	-0.158
Fast blood glucose	-0.145	-0.153	-0.129	-0.146
BMI	-0.224	-0.247	-0.188	-0.241
HOMA_IR	-0.208	-0.208	-0.192	-0.192
Triglycerides	-0.368	-0.350	-0.353	-0.298
CRP	-0. 059	-0.060	-0.054	-0.054

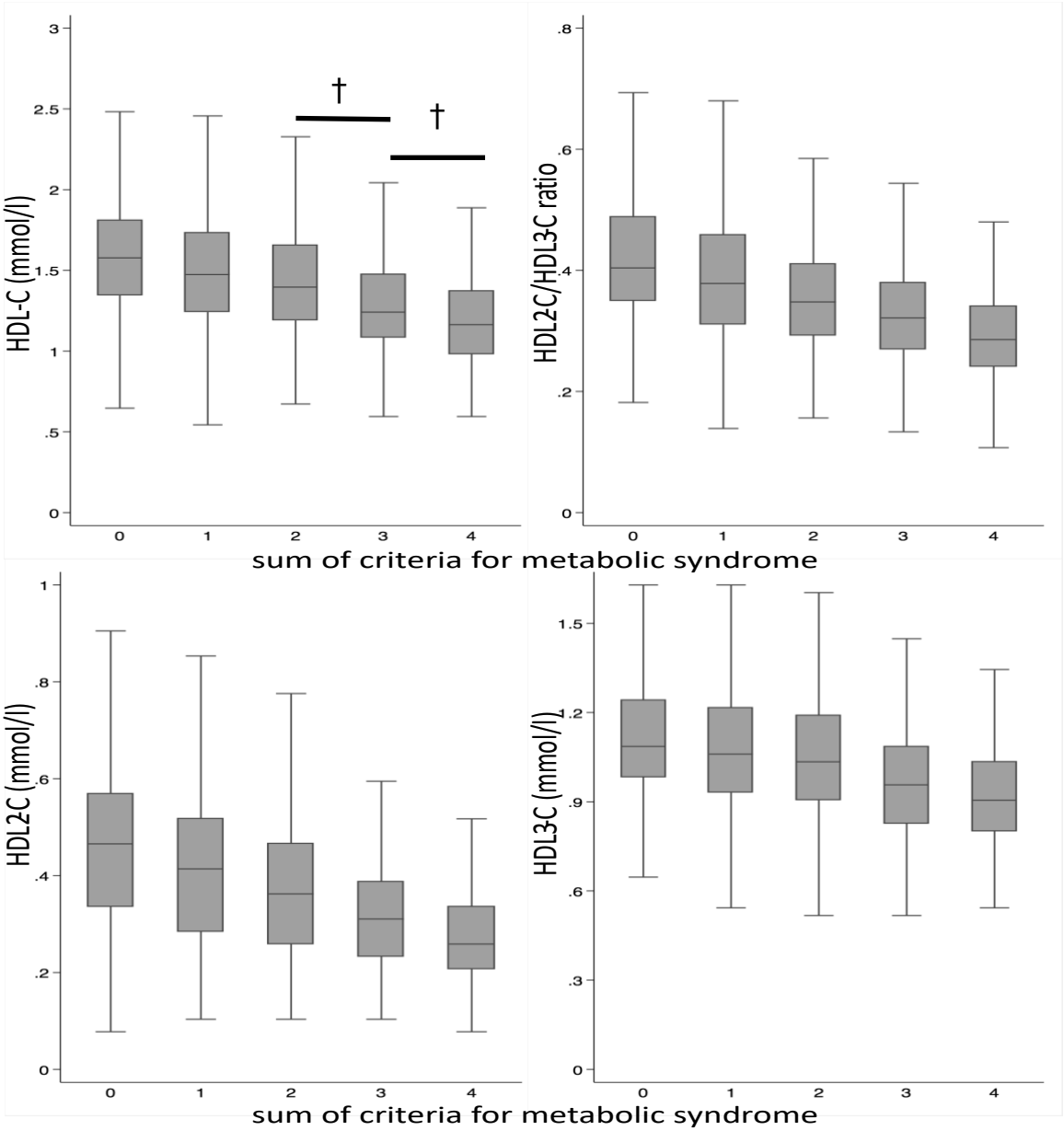
BMI indicates body mass index; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; and SBP, systolic blood pressure. *All p<0.001

Table 4 – Bivariate and Multivariate linear regression analysis between HDL_2-C/HDL_3-C ratio, insulin resistance, CRP, and diagnostic factors of metabolic syndrome*.

	HDL_2-C/ HDL_3-C ratio (per 1SD)					
	Bivariate			Multivariate		
	r	β (95% CI).		*R*	β (95% CI).	
Waist circumference (cm)	-0.394	-4.68	(-5.017; -4.345)	-0.18	-3.152	(-3.646; -2.656)
SBP (mmHg)	-0.199	-2.58	(-3.051; -2.115)	-0.10	-2.437	(-3.139; -1.734)
Fasting serum glucose (mg/dl)	-0.252	-4.19	(-5.020; -3.364)	-0.06	-2.446	(-3.696; -1.196)
BMI (kg/m2)	-0.261	-1.16	(-1.302; -1.029)	-0.12	-0.805	(-1.008; -0.603)
HOMA_IR	-0.327	-0.60	(-0.688; -0.509)	-0.06	-0.256	(-0.380; -0.132)
Triglycerides – Ln(TG)	-0.468	-0.22	(-0.237; -0.210)	-0.14	-0.095	(-0.115; -0.075)
CPR – Ln(CPR)	-0.12	-0.14	(-0.179; -0.114)	-0.05	-0.084	(-0.133; -0.036)

*Linear regression and partial correlation coefficient (*r*) are adjusted for smoking, alcohol, physical activity, and high-density lipoprotein-cholesterol. BMI indicates body mass index; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; and SBP, systolic blood pressure.

Figure 1 - High-density lipoprotein-cholesterol, HDL-c subfraction levels, and HDL2-c/HDL3-c ratio according to the criteria sum for the metabolic syndrome*. In both subfractions, as well as HDL2-c/HDL3-c ratio, the values found were progressively smaller as diagnostic criteria for metabolic syndrome were added.



*All p<0.05 between groups, except as shown, †p=NS.

SUPPLEMENTARY MATERIAL

Supplemental Table

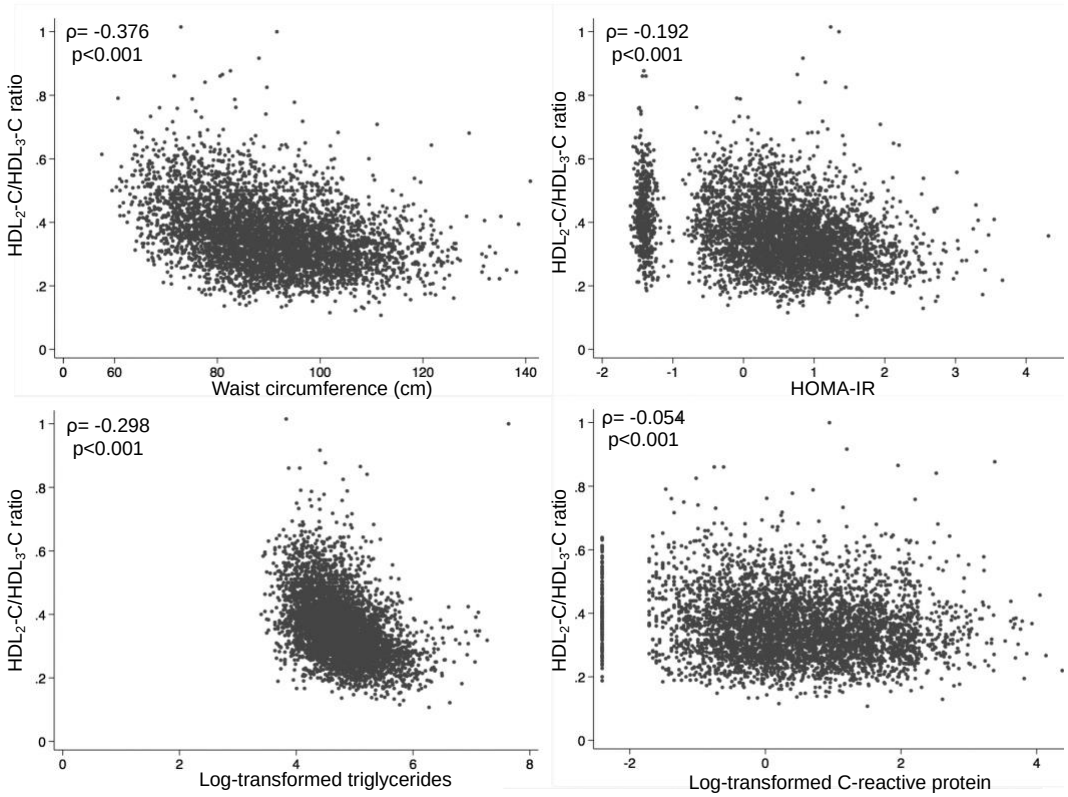
Table I - Bivariate and Multivariate linear regression analysis between HDL_2-C/HDL_3-C ratio and risk and diagnostic factor of metabolic syndrome after excluding participants using statins (n = 4,046) *

	Univariate		Multivariate	
HDL_2-C/ HDL_3-C ratio (per 1SD)	β	CI (95%)	β	CI (95%)
Waist circumference	-4.704	-5.057; -4.351	-3.183	-3.705; -2.662
SBP	-2.481	-2.976; -1.986	-2.292	-3.037; -1.548
Fasting blood glucose	-3.816	-4.643; -2.989	-2.210	-3.460; -0.960
BMI	-1.179	-1.323; -1.034	-0.825	-1.039; -0.610
HOMA_IR	-0.533	-0.612; -0.453	-0.224	-0.330; -0.119
Triglycerides - Ln(TG)	-0.223	-0.238; -0.209	-0.098	-0.119; -0.077
CRP - Ln(CRP)	-0.145	-0.179; -0.110	-0.092	-0.144; -0.041

*Linear regression adjusted for smoking, alcohol, physical activity, and high-density lipoprotein-cholesterol. BMI indicates body mass index; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; and SBP, systolic blood pressure.

Supplemental Figure

Figure I - Correlation between HDL_2-C/HDL_3–C ratio and metabolic syndrome components, HOMA-IR and CRP



Article 02: Diabetes Alters the Association Between High-Density Lipoprotein Subfractions and Carotid Intima-Media Thickness: The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil)

In this original research *"Diabetes alters the association between high-density lipoprotein subfractions and carotid intima-media thickness: The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil)"*, published in the Diabetes and Vascular Disease Research, November 2018, Volume 15, Issue 6, pages 541-547, we evaluated 3,930 civil servants of the ELSA-Brasil study (50±8 years, 45.6% men) without manifested cardiovascular disease and not using any lipid-lowering drug. We found an inverse association between both HDL-c subfractions even when adjusted for cardiovascular risk factors, but they are not independent predictors since the adjustment for HDL-c resulted in lack of association. Further, the presence of diabetes modified the association between the HDL_2-C/HDL_3-c ratio and c-IMT: the negative association of the HDL_2-c/HDL_3-c ratio with c-IMT was more robust in the population diagnosed with diabetes.

Diabetes Alters the Association Between High-Density Lipoprotein Subfractions and Carotid Intima-Media Thickness: The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil)

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Abstract

Introduction: HDL-C comprises a group of heterogeneous subfractions that might have differential effects on atherosclerosis. Moreover, prior investigations suggest the presence of diabetes (T2D) modifies the impact of some subfractions on atherosclerosis. In the present study, we aimed to evaluate the association between HDL-C subfractions and carotid intima-media thickness (cIMT) in the baseline assessment of ELSA-Brasil participants from the São Paulo investigation center.

Methods: We evaluated 3,930 individuals between 35 and 74 years without previous cardiovascular disease not using lipid-lowering drugs. HDL-C subfractions (HDL_2-C and HDL_3-C) were measured by vertical ultracentrifugation (Vertical Auto Profile). The relationship between each HDL-C subfraction and cIMT was analyzed by multiple linear regression models.

Results: Total HDL-C, as well as HDL_2-C and HDL_3-C, were negatively associated with cIMT after adjustment for demographic data (all $p<0.001$) and traditional risk factors (all $p <0.05$). When stratified by T2D status, the HDL_2-C/HDL_3-C ratio showed a negative association with cIMT in participants with T2D ($p=0.032$), even after fully controlling for confounding variables, including total HDL-C.

Conclusion: HDL_2-C, HDL_3-C, and HDL_2/HDL_3-C ratio are inversely associated with cIMT after adjustment for traditional risk factors. Association of the HDL_2-C/HDL_3-C ratio is modified by the presence of diabetes, being more pronounced in diabetic individuals.

Introduction

High-density lipoproteins (HDL) comprise a group of heterogeneous particles, and HDL-cholesterol (HDL-C) has a well-established inverse association with the risk of cardiovascular events in prospective longitudinal cohorts followed throughout the world (107,108). However, genetic polymorphisms that increase HDL-C do not correlate with cardiovascular risk when excluded variants additionally linked to LDL-C or triglycerides(45,50,109). Also, pharmacologic interventions with cholesteryl ester transfer protein (CETP) inhibitors to raise HDL-C levels did not reduce cardiovascular outcomes and even might be harmful (52,55,110,111). This lack of association has motivated studies of HDL-C subfractions to more comprehensively evaluate the mechanisms linking HDL-related pathways with atherogenesis. Among the methods used to separate those subfractions, the Vertical Auto Profile (VAP) separates HDL-C into two subfractions: HDL_2-C (larger and less dense) and HDL_3-C (smaller and denser)(57).

Carotid intima-media thickness (cIMT) is an important marker for subclinical vascular disease and is considered an atherosclerosis surrogate (112). Increases in cIMT are accompanied by a higher incidence of cardiovascular morbidity including coronary artery disease, myocardial infarction(35,113,114) and stroke (115). Although low-HDL-C has been associated with increased cIMT(116), this relationship is not always observed(65), suggesting that total HDL may not entirely explain HDL's anti-atherogenic properties. Also, the association of HDL-C subfractions and cIMT remains disputed since different results are obtained with use of different separation methods (66–68). This relationship is even more intricate because diabetic individuals have lower HDL-C levels and a lower HDL_2-

C/HDL_3-C ratio, which seems to modify the association of HDL-C with cIMT in comparison with the nondiabetic population. It is also well documented that the functionality of HDL species from patients with T2D is impaired and even dysfunctional(117,118).

In this study, we evaluated the association between HDL-C subfractions and cIMT in a Brazilian sample without clinical manifestations of any cerebrovascular disease. Additionally, we evaluated whether the presence of diabetes modifies this association.

Methods

Sample. The ELSA-Brasil design has been previously described(79,80). Briefly, we enrolled 15,015 civil servants from six Brazilian cities, between August 2008 and December 2010. We included those who underwent HDL-C measurement by the VAP method and cIMT assessment - that were all participants from São Paulo center. Exclusion criteria for the present analysis were the cohort restrictions (pregnancy or recent childbirth; cognitive deficit or impaired communication; in retired servants, living outside of a study center's area) in addition to individuals with prior cardiovascular disease (myocardial infarction, stroke, heart failure and coronary revascularization) and participants using lipid-lowering drugs.

HDL-C and subfractions. Blood collection was performed in patients after nocturnal fasting. The samples were centrifuged at the sites and stored in tubes at -80°C (81). The HDL-C, HDL_2-C and HDL_3-C subfractions were obtained by the Vertical Auto Profile (VAP) method through an apparatus whose vertical rotor holds the tube perpendicular to the base of the equipment(57).

Carotid Intima-Media Thickness Measurement. The measurement protocol was published previously(82,84,119) CIMT measurements were performed using a Toshiba (Aplio XG™) with a 7.5 MHz linear transducer. IMT was measured in the outer wall of a pre-defined carotid segment of 1 cm in length from 1 cm below carotid bifurcation, during three cardiac cycles. All participating centers obtained the carotid images, and images were forwarded to the centralized core reading center in São Paulo. Images were classified as valid if the following was obtained for the left and right sides: [1] the anatomic guides for the common carotid arteries, [2] interfaces between the lumen and the far vessel wall, and [3]

interfaces between the media and the adventitia layers of the far vessel wall. We used MIA™ software to standardize the reading and interpretation of carotid scans.

Statistical Analysis. We presented continuous variables as descriptive statistics with mean and standard deviation for normal distribution and, for non-normal distributions, with medians and quartiles. Categorical variables were shown in absolute and relative frequency. Baseline characteristics table was exposed according to total HDL-C quartiles to show different HDL-C subfractions levels across total HDL-C groups. As correlation measurement, we used Pearson. The association between HDL-C subfractions and variables was determined by linear regression. We standardized HDL-C, HDL_2-C, HDL_3-C and HDL_2-C/HDL_3-C ratio, which were included as the main explanatory variables in separated multiple linear models. Models including HDL_2-C, HDL_3-C or HDL_2-C/HDL_3-C ratio were also further adjusted for total HDL-C (as exposed in Table 2 – model 4 and Table 3 – Model 2).

As we observed significant interaction terms between HDL_2-C/HDL_3-C ratio and T2D, we performed additional analyses stratified by diabetes status. The models were adjusted for race/ethnicity, age and sex, smoking, alcohol, physical activity, low-density lipoprotein-cholesterol, systolic blood pressure, waist circumference, fasting glucose, log-transformed triglycerides, estimated glomerular filtration rate, and antihypertensive use variables. Statistical significance was defined as $p < 0.05$. Analyses were performed with Stata version 14.0 (StataCorp, USA).

Results

We included 3,930 participants (1,048 exclusions from São Paulo center) with a mean age of 50.1±8.4 years and 1,793 (45.6%) males. The mean HDL-C was 1.42±0.37 mmol/l and mean cIMT 0.796±0.195 mm (Table 1).

As previous publications analyzing HDL-C subfractions(68,76,120), we observed, we observed a high correlation between total HDL-C and both subfractions HDL_2-C (ρ=0.94) and HDL_3-C (ρ=0.96) and a lower correlation with HDL_2-C/HDL_3-C ratio (ρ=0.73). In crude models, an inverse association between HDL-C, HDL_2-C, HDL_3-C and HDL_2-C/HDL_3-C ratio with mean cIMT was noted. Those results remained essentially unchanged after adjusting for demographic characteristics (Model 1) and cardiovascular risk factors (except in HDL_2-C/HDL_3-C ratio - Model 3). However, when subfractions were adjusted for HDL-C, they were no longer associated with lower mean cIMT (Table 2).

We found an interaction between T2D status and HDL_2-C/HDL_3-C ratio. Further, when stratified by the presence of T2D, HDL_2-C/HDL_3-C ratio was inversely associated with cIMT in participants with diabetes in full models (figure 1), but not in participants without diabetes. We also found that HDL_3-C was negatively associated with cIMT in non-T2D individuals and not in participants with T2D. In this case, a p-value of borderline significance (p=0.051) was observed. After further adjustment for HDL-C levels, only the association between HDL_2-C/HDL_3-C ratio and cIMT in individuals with diabetes remained significant (p=0.032) (Table 3).

Discussion

Our results demonstrate that both HDL_2-C and HDL_3-C are inversely associated with cIMT even after controlling for demographic and CV risk factors, but they are not independent predictors. This association is particularly robust with HDL_2-C/HDL_3-C ratio for diabetics, whereas such findings were noted in non-diabetics only with HDL_3-C.

Previous studies have observed controversial results in the relationship between HDL-C subfractions and cIMT. While some studies have found no association of HDL_2-C or HDL_3-C with cIMT(66,67), a recent publication has shown that, in addition to HDL-C, the HDL_2-C subfraction is associated with a lower cIMT, suggesting that this subfraction is atheroprotective (68). In contrast to these data, a Finnish study(61) demonstrated that HDL-C, HDL_2-C, and HDL_3-C subfractions and also the HDL_2-C/HDL_3-C ratio are negatively associated with cIMT, a finding that was reproduced in our study. These discordant results might be explained by ethnic differences in the population, differences in baseline characteristics (younger, more obese people), and participants not being treated with lipid-lowering therapies.

The lipid panel is known to be altered in about 50% of the individuals with diabetes, either due to high triglycerides or LDL-C levels or by low HDL-C, (121). Diabetic dyslipidemia is related to the impact of insulin resistance on metabolic pathways and systemic changes related to diabetes, such as renal disease. HDL-C was the only lipid parameter to be independently associated with carotid IMT in a cohort of young adults with type 2 diabetes(122) and, in another trial, low-HDL-C elevation secondary to statin use was related to the IMT decrease(123,124).

Diabetes leads to impaired reverse cholesterol transport through both reduced HDL concentrations and HDL dysfunction. Thus, a more pronounced inverse association of HDL_2-C/HDL_3-C ratio with cIMT could be explained in some pathways: inhibition of lipoprotein lipase, where less Apo AI is liberated from chylomicrons, decreasing HDL formation in serum; ABCA1 down-regulation on the surface of visceral adipocytes, also reducing HDL formation(125); lecithin-cholesterol acyltransferase (LCAT) dysfunction(126), which lowers cholesterol esterification - decreases the maturation of HDL_3 to HDL_2; finally, increases in CETP activity promotes triglyceride enrichment of HDL particles, rendering them better substrates for lipolysis and catabolism by hepatic lipase.

Indeed, studies have shown that in patients with diabetes, raising HDL-C levels by drugs other than statin is not accompanied by improvement or reverse cholesterol transport(127).

Thus, considering that infusion of reconstituted HDL in patients with CHD led to changes in inflammatory markers, cholesterol efflux and plaque conformation(128), perhaps this fact calls attention to need to clinically evaluate the functionality of HDL instead of its level individually, especially in the diabetic milieu. Moreover, although CETP inhibitors are known to increase asymmetrically HDL-C subfractions(129), as well as statin use, which alters the HDL composition and HDL_3-C levels(130), it is unclear whether the variability of the HDL_2-C/HDL_3-C ratio may result in cardiovascular benefit.

Strengths of the present study include a large cohort, a method of direct subfraction measurement, and the use of a computer-aided protocol for cIMT measurements that provided reliable and accurate cIMT data. Our study also has some limitations. As a cross-sectional study, the described associations do not

confirm causation. Also, cIMT was used instead of plaque burden, and total plaque area may be more representative of atherosclerosis than cIMT. However, the cIMT may be more reversible, which may explain the weaker association with cardiovascular disease events than the plaque(119). We are also unable to evaluate the antiatherogenic effects of HDL and its subfractions directly mechanistically.

In conclusion, in a unicentric, cross-sectional study of a Brazilian sample, HDL-C and its subfractions HDL_2-C and HDL_3-C are inversely associated with cIMT. In participants with diabetes, the negative association is limited to the HDL_2-C/HDL_3-C ratio, which has a statistically significant association even when adjusted for HDL-C.

Tables and Figures

Table 1 – Demographic and Clinical Characteristics of the Study Sample

	Mean (±SD or IQR) or *n* (%)		
	n = 3,930	HDL-C p≤50	HDL-C p>50
Age, years	50.1 (±8.4)	49.6 (±8.4)	50.8 (±8.5)
Race, %			
White	2,269 (57.7%)	1,151 (57.5%)	1,118 (58%)
Brown	860 (21.9%)	486 (24.3%)	374 (19.4%)
Black	554 (14.1%)	254 (12.7%)	300 (15.6%)
Asian	152 (3.9%)	55 (2.7%)	97 (5%)
Indigenous	46 (1.2%)	33 (1.65%)	13 (0,7%)
Other	49 (1.3%)	23 (1.15%)	26 (1.35%)
Men, %	1,793 (45.6%)	1,225 (61.2%)	568 (29.5%)
Hypertension, %	1,018 (25.9%)	586 (29.3%)	432 (22.4%)
BMI, kg/m2	27 (±4.75)	27.9 (±4.7)	26.1 (±4.7)
Waist Circumference, cm	89 (±12.3)	92.7 (±11.5)	85.1 (±11.9)
Smoking, %			
Current	657 (16.7%)	371 (18.5%)	286 (14.8%)
Former Smokers	1,155 (29.4%)	612 (30.6%)	543 (28.2%)
Never	2,118 (53.9%)	1,019 (50.9%)	1,099 (57%)
SBP, mmHg	118.9 (±16.2)	120.8 (±16.2)	116.9 (±16)
DBP, mmHg	75 (±10.8)	76.5 (±10.7)	73.5 (±10.7)
Diabetes, %	641 (16.3%)	394 (19.7%)	247 (12.8%)
Dyslipidemia, % *	1,989 (50.6%)	1,046 (52.3%)	943 (48.9%)
Triglycerides, mmol/l	1.24 (0.88-1.78)	1.56 (1.11-2.16)	1 (0.77-1.37)
Fasting blood glucose, mmol/l	6.06 (±1.48)	6.24 (±1.7)	5.9 (±1.2)
Total cholesterol, mmol/l	5.68 (±1.08)	5.49 (±1.07)	5.87 (±1.07)
LDL-C, mmol/l †	3.42 (±0.88)	3.46 (±0.89)	3.38 (±0.87)
HDL-C, mmol/l	1.42(±0.37)	1.14 (±0.16)	1.72 (±0.29)
HDL_2-C, mmol/l	0.39 (±0.17)	0.26 (±0.07)	0.51 (±0.16)
HDL_3-C, mmol/l	1.04 (±0.22)	0.88 (±0.11)	1.21 (±0.17)
Mean cIMT (mm)	0.796 (±0.195)	0.809 (±0.207)	0.773 (±0.177)

cIMT indicates carotid artery intima-media-thickness; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; and BMI, body mass index. Dyslipidemia was defined as high triglycerides (TG) (≥150 mg/dL), low HDL-C (<40 [men] and <50 [women] mg/dL), or high LDL-C (≥130 mg/dL or ever taking lipid-lowering agents). All p<0.001, except: † p=0.006 and *p=0.03

Table 2 - Association of total HDL-C, HDL_2–C and HDL_3–C subfractions and HDL_2–C /HDL_3–C ratio with cIMT

	Model	β	IC (95%)	p
HDL-C	Univariate	-0.021	(-.0276; -.0155)	<0,001
	Model 1	-0.026	(-.0320; -.0204)	<0,001
	Model 2	-0.024	(-.0298; -.0180)	<0,001
	Model 3	-0.008	(-.0142; -.0016)	0.014
HDL_2-C	Univariate	-0.024	(-.0301; -.0180)	<0,001
	Model 1	-0.028	(-.0336; -.02205)	<0,001
	Model 2	-0.025	(-.0316; -.0198)	<0,001
	Model 3	-0.006	(-.0128; -.0001)	0.04
	Model 4	0.005	(-.0107; +.0215)	0.51
HDL_3-C	Univariate	-0.018	(-.024; -.0118)	<0,001
	Model 1	-0.022	(-.0282; -.0168)	<0,001
	Model 2	-0.02	(-.0260; -.0143)	<0,001
	Model 3	-0.01	(-.0151; -.0032)	0.003
	Model 4	-0.008	(-.0281; +.0120)	0.43
HDL_2-C/HDL_3-C Ratio	Univariate	-0.026	(-.0325; -.0204)	<0,001
	Model 1	-0.027	(-.0328; -.0213)	<0,001
	Model 2	-0.026	(-.0315; -.0198)	<0,001
	Model 3	-0.006	(-.0119; +.0004)	0.069
	Model 4	-0.014	(-.0093; +.0065)	0.728

Linear regression model: model 1; adjusted for race/ethnicity, age and sex; model 2: adjusted for model 1 and smoking, alcohol and physical activity; model 3: adjusted for model 2 and low density lipoprotein-cholesterol, systolic blood pressure, waist circumference, fasting glucose, body mass index, log-transformed triglycerides, EGFR, and antihypertensive use; and model 4: adjusted for model 3 and total HDL-C. cIMT indicates carotid intima-media thickness and HDL-C, high-density lipoprotein-cholesterol

Table 3 - Association of total HDL-C, HDL_2–C and HDL_3-C subfractions and HDL_2–C /HDL_3-C ratio with cIMT when stratified by Diabetes Mellitus status

		Non-diabetes			Diabetes			Interaction
		β	IC	p	β	IC	p	p
Model 1	HDL-C	-0.007	(-.0138; -.0005)	0.03	-0.011	(-.0302; +.0071)	0.22	0.77
	HDL_2-C	-0.005	(-.0116; +.0017)	0.11	-0.016	(-.0355; +.0034)	0.11	0.35
	HDL_3-C	-0.008	(-.0144; -.0015)	-0.01	-0.007	(-.0248; +.0103)	0.41	0.051
	HDL_2-C/HDL_3-C Ratio	-0.003	(-.0091; +.0039)	0.44	-0.023	(-.0409; -.0046)	0.01	**0.036**
Model 2	HDL_2-C	0.011	(-.0063; +.0274)	0.22	-0.032	(-.0814; +.0176)	0.21	0.38
	HDL_3-C	-0.015	(-.0360; +.0059)	0.16	0.038	(-.0230; +.0992)	0.22	0.77
	HDL_2-C/HDL_3-C Ratio	0.003	(-.0052; +.0115)	0.46	-.025	(-.0474; -.0022)	0.03	**0.032**

Linear regression adjusted for race/ethnicity, age and sex, smoking, alcohol, physical activity, low-density lipoprotein-cholesterol, systolic blood pressure, waist circumference, fasting glucose, body mass index, log-transformed triglycerides, antihypertensive use, EGFR, and HDL-C (except the own variable HDL-C). cIMT indicates carotid intima-media thickness and HDL-C, high-density lipoprotein-cholesterol

Figure 1 – Detailed enrollment flow diagram.

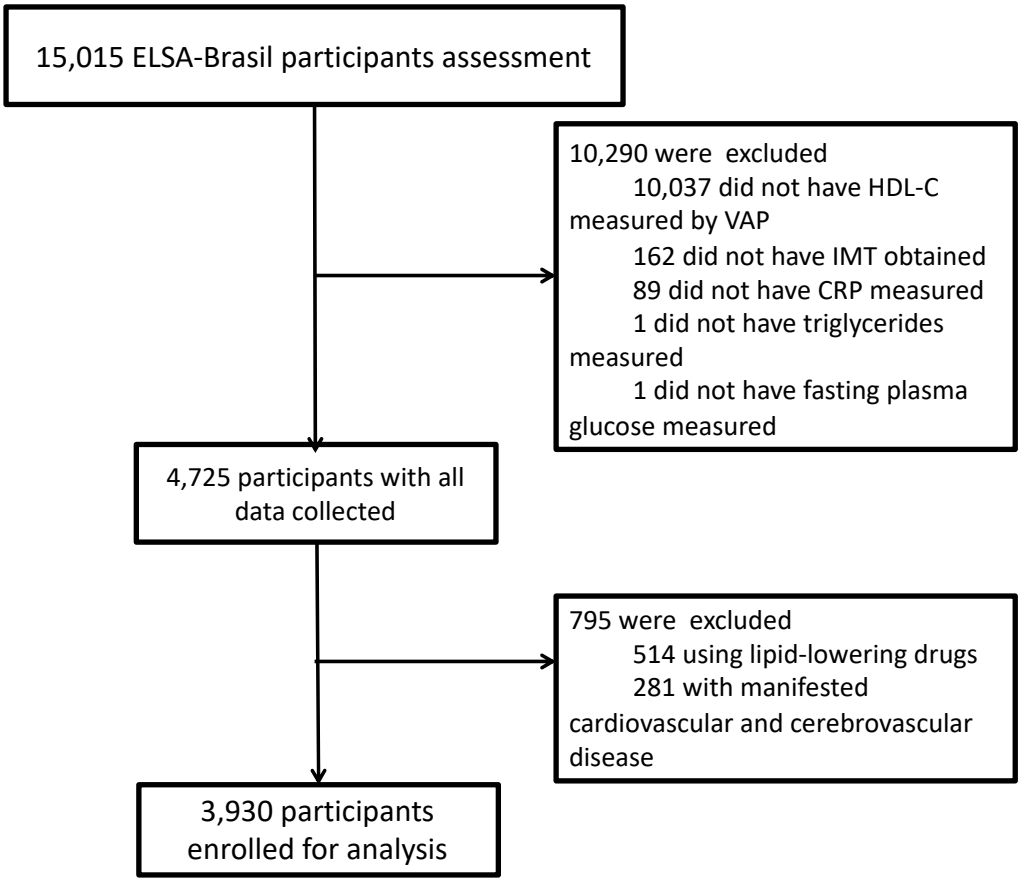
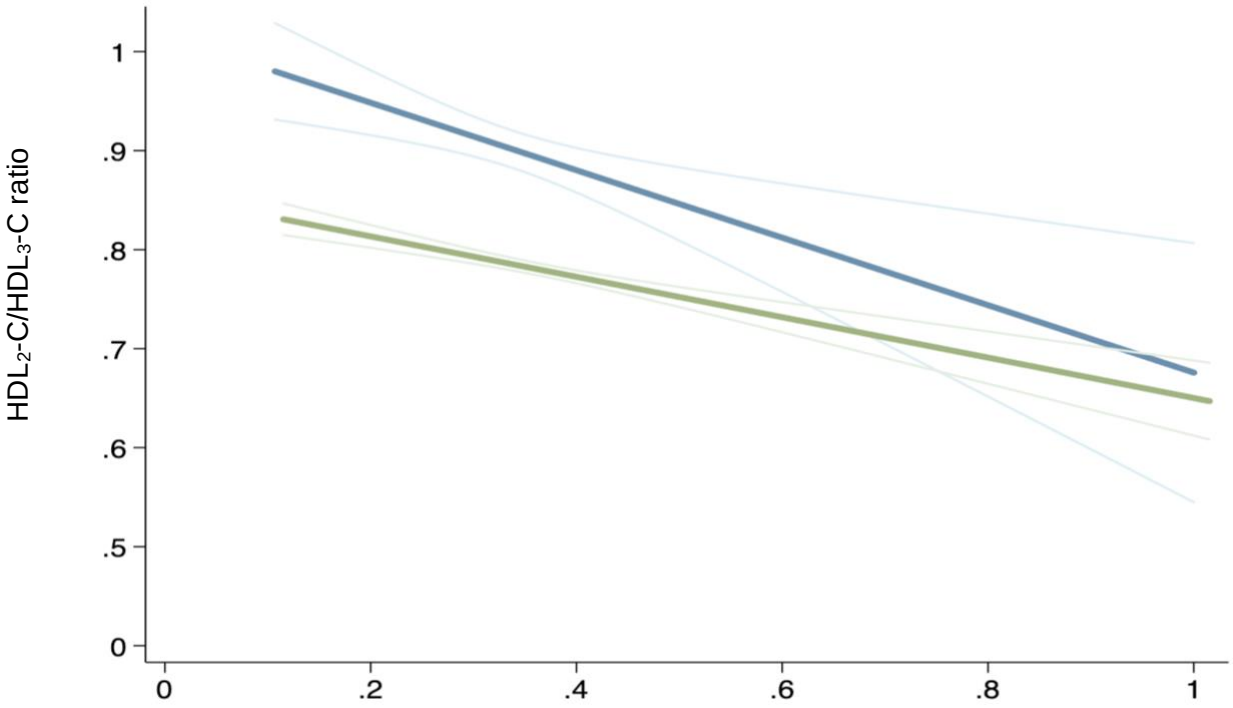


Figure 2 – Association between HDL_2-C/HDL_3-C ratio and cIMT in univariate analysis



The dak-blue line corresponds to HDL2-C/HDL3-C ratio in non-T2D according to cIMT and light-blue lines, 95% IC; while the dark-green line means HDL2-C/HDL3-C ratio in T2D according to cIMT and light-green lines, 95% IC. $p < 0.001$

Article 03: High-density lipoprotein-cholesterol subfractions and coronary artery calcium: the ELSA-Brasil study

In the original research *"High-density lipoprotein subfractions and coronary artery calcium: the ELSA-Brasil study"*, published in the Archives of Medical Research, 2019, Volume 50, pages 362-367, we selected 3,674 participants of ELSA-Brasil study (49.8±8 years, 46% men) without manifested cardiovascular disease and not using any lipid-lowering drug and observed that HDL-c and both HDL-c subfractions were negative predictors of CAC presence after adjustment for age, gender, smoking, hypertension, alcohol use, physical activity and LDL-c ($p<.05$). However, after controlling for triglycerides, all analyses were no longer statistically significant.

Running Title: HDL subfractions and subclinical atherosclerosis

High-density lipoprotein-cholesterol subfractions and coronary artery calcium: the ELSA-Brasil study

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Abstract

Background: Although elevated high-density lipoprotein cholesterol (HDL-C) is considered protective against atherosclerotic cardiovascular disease, no causal relationship has been demonstrated. HDL-C comprises a group of different subfractions that might have different effects on atherosclerosis. Our objective was to investigate the association between HDL-C subfractions with the coronary artery calcium (CAC) score.

Methods: We included 3,674 (49.8±8.3 years, 54% women) participants from the ELSA-Brasil study who had no prior history of CVD and were not currently using lipid-lowering medications. We measured the fasting lipoprotein cholesterol fractions (in mmol/l) by a zonal ultracentrifugation method (VAP). We analyzed the independent predictive values of total HDL-C, HDL_2-C, and HDL_3-C subfractions and in the HDL_2-C/HDL_3-C ratio using linear regression to predict Ln(CAC+1) and logistic regression to predict the presence of CAC.

Results: Overall 912 (24.8%) of the participants had CAC>0, and 294 (7.7%) had CAC>100. The mean total HDL-C, HDL_2-C, and HDL_3-C were: 1.42±0.37, 0.38±0.17 and 1.03±0.21 mmol/l, respectively. Individuals with CAC>0 had lower levels of total HDL-C as well as of each subfraction ($p<0.001$). When adjusted for age, gender, smoking, hypertension, alcohol use, physical activity, and LDL-C, we observed an inverse association between HDL-C and its subfractions and CAC ($p<.05$). However, by adding triglycerides in the adjustment, neither total HDL-C nor its subfractions remained independently associated with the presence or extent of CAC.

Conclusion: In this cross-sectional analysis, neither the total HDL-C nor its subfractions (HDL_2-C and HDL_3-C, as well as HDL_2-C/HDL_3-C ratio) measured

by VAP are independently associated with the presence or extent of coronary calcification.

Introduction

Dyslipidemia is an independent marker of higher atherosclerotic risk. Although the reduction of circulating pro-atherogenic apolipoprotein B containing lipoproteins decreases cardiovascular (CV) risk in both primary and secondary prevention, about two-thirds of these events are not prevented (131–133). To reduce this residual risk, trials to raise high-density cholesterol (HDL-C) levels failed to decrease outcomes(51–54). Also, Mendelian randomization studies showed no association between genetically decreased HDL-C and CV disease(49,134) despite evidence that low-HDL-C is an independent predictor of coronary artery disease(19,107).

Those results led some authors to suggest that HDL might only be a bystander of atherogenesis(135). However, since HDL is composed of several subfractions that can vary in size, shape, density, and function (136), some authors have proposed that additional characterization of HDL might be useful in defining its role in the development and progression of atherosclerosis. Among the various assays available for the quantification of HDL subfractions, the Vertical Auto Profile (Atherotech®) is a zonal ultracentrifugation method that measures total HDL, expressed as its cholesterol content, and its subfractions: HDL_2-C (larger and more buoyant) and HDL_3-C (smaller and denser)(57). These subfractions have been intensively studied, showing controversial results: while some studies showed an association between HDL_2-C and CV disease(75,77), others founded this association with HDL_3-C subfraction(72,74).

In the pre-clinical atherosclerosis scenario, coronary artery calcium (CAC) scoring with cardiac computed tomography is a widely accepted marker and risk predictor for CV events. Thus, the CAC score plays an essential role as a non-

invasive tool to investigate associations between presumed CVD risk factors and subclinical atherosclerosis.

Few studies investigated the association between different HDL particles and CAC score through HDL-C subfractions measurement. Together, these studies included less than 800 individuals with CAC>0 (62,63,69,137). Although HDL_2-C is the subfraction linked to less coronary calcification, the study that enrolled the larger sample (62) did not demonstrate this association.

The objective of our study was to examine cross-sectionally if HDL-C subfractions are associated with the presence of subclinical atherosclerosis detected by coronary artery calcium scores using baseline data from a cohort study of apparently healthy individuals.

Methods

Sample. Briefly, ELSA-Brasil is a cohort study composed of 15,105 civil servants, recruited between August 2008 and December 2010, from six Brazilian cities(79,80). In this present analysis, we included only participants from São Paulo center - those who underwent HDL-C measurement by VAP method and underwent CAC score as part of the baseline examination. Women less than four months after childbirth; civil servants with cognitive impairment or impaired communication; and those living outside the metropolitan area of the city were not eligible for the cohort. We also excluded in our study the participants with previous manifested CVD (myocardial infarction, stroke, heart failure, and coronary revascularization) and or using lipid-lowering drugs.

Plasma measurements. After nocturnal fasting, blood samples were collected from the participants. The samples were centrifuged and stored in tubes at -80°C(81). Total HDL-C, HDL_2-C, and HDL_3-C subfractions were obtained by the Vertical Auto Profile (VAP) ultracentrifugation method(57). Total cholesterol was measured by ADVIA 1200 Siemens™ equipment. The LDL-C was calculated by the Friedewald equation if triglycerides were < 400 mg/dL and by an enzymatic assay if triglycerides were > 400 mg/dL(138). CRP was determined using a high-sensitivity test by immunochemistry - nephelometry (BN II; Siemens). Glucose was measured by the hexokinase enzymatic method.

Coronary Artery Calcium measurement. All participants from São Paulo center underwent non-contrast tomography cardiac tomography scan for CAC acquisition (Brilliance 64, Philips Healthcare, Best, Netherlands)(139). The images were acquired with 120 kV, and mA adjusted to body mass index and reconstructed with standard filtered back projection. Images were analyzed in a

dedicated workstation (Brilliance Workspace) where the CAC was measured using the Agatston score.

Other Variables: Race/ethnicity was self-reported and categorized as white, brown, black and other (including Asian and indigenous). Smoking status was defined as current, former, and never smoking. Alcohol use was divided into non-users, former users, and current users and used the total alcohol ingested by week (in g). We classified the physical activity as sedentarism, physical activity < 150 minutes per week and ≥ 150 minutes per week on leisure-time and going to work according to the International Physical Activity Questionnaire (IPAQ).

Statistical Analysis. We presented continuous variables as descriptive statistics with mean and standard deviation for normal distribution and, for non-normal distributions, with medians and interquartile range and compared using analysis of variance (ANOVA) or Mann-Whitney U-test after assessing normality assumptions. Categorical variables were shown in absolute and relative frequency and compared using chi-squared or Fisher exact test as appropriate. The association between HDL-C subfractions and variables was determined according to CAC classification. For comparison between CAC=0 and CAC>0, as well as between CAC<100 and CAC≥100, we performed multiple logistic regression analyses. For continuous values of CAC score, we used ln(CAC+1) as the dependent variable in multiple linear regression models for all sample. When we assessed only those with CAC>0, we used ln(CAC) as the variable.

The multiple regression analyses models were constructed adjusting for age, gender, race/ethnicity (model 1), smoking, alcohol use, physical activity, and LDL-cholesterol (model 2), and log-transformed triglycerides (model 3). We

standardized the variables HDL-C, HDL_2-C, HDL_3-C and HDL_2-C/HDL_3-C ratio for these analyses.

Statistical significance was defined as $p<0.05$. We performed all analyses with Stata version 14.0 (StataCorp, USA).

Results

We enrolled 3,674 participants with a mean age of 49.7 ±8.3 years and including 45.9% males. The mean HDL-C was 1.42±0.37 mmol/l; 25% had hypertension, and 16% had diabetes. When observed by CAC score categories (0, 1-100 and >100), higher calcification was associated with older age, hypertension, diabetes, and higher BMI, and total and LDL-C (Table 1).

Observing all participants, we first performed a linear regression analysis to evaluate the association between ln(CAC+1) and HDL-C subfractions (Table 2). In the bivariate analysis and when adjusted for demographic (Model 1) and behavioral factors + LDL-C (Model 2), total HDL-C, both subfractions, and HDL_2-C/HDL_3-C ratio were inversely associated with ln(CAC+1). However, there was no statistically significant association when added ln(triglycerides) as covariate (Model 3). Also considering the entire sample, in a logistic regression analysis to predict CAC presence, total HDL-C and its subfractions, as well as HDL_2-C/HDL_3-C ratio, were inversely associated with risk of CAC>0 in bivariate analysis and when adjusted for both demographic (Model 1) and behavioral variables + LDL-C (Model 2). However, when further adjusted for ln(triglycerides), the association was no longer significant (Model 3 - Table 3). Besides, there was no difference in the HDL-C, HDL_2-C, or HDL_3-C levels of the individuals when stratified by the CAC≤100 and CAC> 100 (p= NS, Table 4).

A total of 912(24.8%) participants had CAC>0. Assessing only this sample, we constructed multiple linear regression models with ln(CAC), and we found no association between HDL-C, HDL_2-C, HDL_3-C or HDL_2-C/HDL_3-C ratio and ln(CAC) in any model (Table 5).

Discussion

The results of this cross-sectional analysis using baseline data from ELSA-Brasil demonstrate that neither HDL-C nor its subfractions, including HDL_2-C/HDL_3-C ratio, show any association with CAC after adjustment for epidemiological variables and traditional CV risk factors. These results were consistent using different analytical strategies.

While some studies demonstrated a strong association between the presence of CAC score and low-HDL-C values, making HDL-C a useful predictor for coronary calcification (41,43), our findings are in agreement with other analyses which do not support this conclusion(140). Different statistical analyses approach might, at least partially, explain these conflicting results: one study did not adjust for triglycerides and other(43), when TG levels were included in the model, there was no association with CAC in the population without T2D(41). Moreover, as previously mentioned, large Mendelian randomization studies that HDL-c levels did not show any causal association with ischemic heart disease(49,50,134) or diabetes mellitus(141). It is noteworthy that adjustment for TG is vital in the multivariate analysis as recent findings showing a strong association of triglycerides and triglyceride-rich lipoproteins with CAC (142–144) and cardiovascular events(145,146), as well as robust positive results after focused treatment(147).

Concerning HDL-C subfractions, some analyses observed an increased prevalence and extent of CAC across decreasing HDL_2-C levels(63,69). In contrast, we did not find any association between this subfraction and CAC score, in agreement with other researches(62,137). We also found no correlation between coronary calcification and HDL_3-C. Again, the difference in results might

be justified by statistical analysis construction, since the findings showed neutral association when the authors included triglycerides as a covariate adjustment in the multivariate analysis.

Lastly, our study was the only one that evaluated the correlation between the HDL_2-C/HDL_3-C ratio and CAC, and we found no association with the presence of coronary calcification. We tested HDL_2-C/HDL_3-C ratio because previous studies observed a direct association with other subclinical atherosclerosis surrogates(148) and, thus, it might represent the HDL-C functionality. However, we found no association with CAC in our analysis.

The HDL anti-atherogenic properties have been studied, and it is known several metabolic pathways, based on reverse cholesterol transport (RCT) and anti-oxidative functions, both closely linked to metabolic syndrome cluster – inflammation(149), insulin resistance(150) and pro-atherogenic lipoproteins(151). Previous studies from our group corroborated these findings since both subfractions are negatively associated with metabolic syndrome, inflammation, and insulin resistance(152) and, also, inversely correlated to vascular disease assessed by the carotid intima-media thickness(148), even after adjustment for CV risk factors. However, this lack of association between HDL-C (and its subfractions) and CAC after triglycerides adjustment shows a different scenario regarding subclinical atherosclerosis.

It is known there is a direct LDL and triglycerides interplay in HDL metabolism, through the continuous cholesterol and TG exchange with Apo-B lipoprotein particles by the activate CETP, and the inhibition of expression(153) and proteasomal degradation(154) of adenosine triphosphate-binding cassette transporter A1 (ABCA1) by unsaturated free fatty acids in the liver, leading to less

HDL generation. Therefore, the strong attenuation of the association of HDL-c (and also of its subfractions) with coronary calcification might be explained by this close interaction in the metabolic pathway. Despite this relationship, we did not find multicollinearity in any of our analyses.

Strengths of our study consist of a large sample; although less than 25% have CAC>0, it is the largest sample in the literature that directly measured HDL-C subfractions and their association with coronary calcification. However, as a cross-sectional study, we cannot observe CAC progression over time nor evaluate causality.

In conclusion, in a large population without known cardiovascular disease, there was no association between HDL-C, its subfractions HDL_2-C and HDL_3-C, as well as HDL_2-C/HDL_3-C ratio and coronary calcification presence or extension by CT-assessed CAC score.

Tables

Table 1 - Demographic baseline characteristics

Demographic baseline characteristics

CAC	All	0	0-100	>100	p
n	3,674	2,762	628	284	
Men	1,686 (45.9%)	1,074 (38.9%)	390 (62.1%)	222 (78.1%)	
Mean Age (years)	49.7 (±8.3)	47.9 (±7.4)	54.4 (±8)	57.7 (±8.5)	
Hypertension	952 (25.9%)	580 (21%)	216 (34.4%)	156 (54.9%)	
Smoking					
Current	1,090 (29.7%)	773 (28%)	212 (33.8%)	105 (37%)	
Former Smokers	604 (16.4%)	410 (14.8%)	127 (20.2%)	67 (23.6%)	
Body Mass Index (kg/m2)	27.1(±4.8)	26.9 (±4.9)	27.6 (±4.8)	27.5 (±4.3)	
Waist Circumference (cm)	89.2 (±12.3)	87.9 (±12.2)	92.3 (±12)	94.4 (±11.8)	
Diabetes Mellitus	598 (16.3%)	357 (12.9%)	152 (24.2%)	89 (31.3%)	<0.001
Fasting Plasma Glucose (mg/dL)	109 (±26.5)	106.2 (±21)	115.8 (±35.2)	122.6 (±41.1)	
c-Reactive Protein	2.91 (0.72-3.4)	2.84 (0.79-3.51)	3.14 (0.8-3.8)	2.18 (0.77-2.88)	
Total Cholesterol (mg/dL)	291.2 (±42)	215.5 (±40.7)	228.7 (±43.6)	233.6 (±44.1)	
LDL-C (mg/dL)	132.3 (±34.2)	129.5 (±33.1)	140 (±35.9)	142.4 (±36.2)	
Triglycerides (mg/dL)	133.6 (±91.7)	126.3 (76-151)	154.1 (90.5-185.5)	159.8 (102-187)	
Total HDL-C	54.8 (±14.4)	55.4 (±14.2)	52.9 (±14.6)	52.7 (±15.4)	
HDL_2-C	14.8 (±6.7)	15.1 (±6.7)	13.8 (±6.6)	13.7 (±7)	
HDL_3-C	40 (±8.3)	40.3 (±8.2)	39.1 (±8.6)	39.1 (±9)	
HDL_2-C/HDL_3-C ratio	0.36 (±0.1)	0.365 (±0.1)	0.34 (±0.1)	0.34 (±0.1)	

*Values are presented by the mean (standard deviation), absolute frequency (relative frequency) or median (interquartile range). LDL-C indicates low-density lipoprotein cholesterol; and HDL-C, high-density lipoprotein cholesterol. For total, HDL, and LDL cholesterol, we divided mg/dL values by 38.67. For triglycerides, we divided mg/dL values by 88.57

Table 2 - Multiple regression analysis between HDL-C and its subfractions and ln(CAC+1) (per 1SD **decrease**)

	Bivariate				**Model 1**				**Model 2**				**Model 3**			
	B	CI		p	β	CI		p	β	CI		p	β	CI		p
Total HDL-C	0.135	0.076	0.193	<0.001	0.070	0.014	0.126	0.01	0.078	0.020	0.135	0.008	0.044	-0.020	0.108	NS
HDL_2-C	0.155	0.097	0.214		0.071	0.015	0.127	0.01	0.064	0.006	0.122	0.03	0.029	-0.035	0.093	
HDL_3-C	0.107	0.107	0.165		0.062	0.007	0.117	0.03	0.081	0.024	0.138	0.005	0.051	-0.011	0.113	
HDL_2-C/HDL_3-C ratio	0.178	0.120	0.237		0.061	0.006	0.117	0.03	0.041	-0.170	0.099	NS	0.007	-0.055	0.069	

Model 1: Adjusted for gender, race and age. Model 2: Model 1 + smoking (current/former/never smoker), alcohol use (g/week), physical activity (sedentarism, less than 150 minutes/week, more than 150 minutes/week), and LDL-C. Model 3: Model 2 + log-transformed triglycerides. n= 3,674

Table 3 – Logistic regression to determine the OR for CAC>0 vs. CAC= 0 according to HDL-C and its subfractions (per 1SD **decrease**)

	Bivariate				**Model 1**				**Model 2**				**Model 3**			
	OR	CI		p	OR	CI		p	OR	CI		p	OR	CI		p
Total HDL-C	1.209	1.117	1.309	<0.001	1.122	1.023	1.230	0.015	1.136	1.031	1.251	0.010	1.041	0.933	1.161	NS
HDL_2-C	1.253	1.155	1.361		1.143	1.039	1.257	0.006	1.123	1.017	1.241	0.022	1.027	0.919	1.147	
HDL_3-C	1.160	1.074	1.253		1.095	1.001	1.197	0.047	1.135	1.033	1.247	0.008	1.049	0.944	1.165	
HDL_2-C/HDL_3-C ratio	1.292	1.192	1.401		1.154	1.050	1.269	0.003	1.104	1.000	1.219	0.051	1.020	0.916	1.135	

Model 1: Adjusted for gender, race and age. Model 2: Model 1 + smoking (current/former/never smoker), alcohol use (g/week), physical activity (sedentarism, less than 150 minutes/week, more than 150 minutes/week), and LDL-C. Model 3: Model 2 + log-transformed triglycerides. n=912

Table 4 – Logistic regression to determine the OR for CAC≥100 vs. CAC<100 according to HDL-C and its subfractions (per 1SD **decrease**)

	Bivariate				**Model 1**				**Model 2**				**Model 3**			
	OR	CI		p	OR	CI		p	OR	CI		p	OR	CI		p
Total HDL-C	1.188	1.044	1.350	0.009	1.030	0.896	1.184	NS	1.042	0.903	1.204	NS	0.940	0.800	1.105	NS
HDL_2-C	1.243	1.085	1.424	0.002	1.062	0.918	1.229		1.046	0.902	1.214		0.943	0.800	1.112	
HDL_3-C	1.137	1.003	1.287	0.044	1.008	0.881	1.154		1.041	0.905	1.197		0.951	0.814	1.110	
HDL_2-C/HDL_3-C ratio	1.300	1.138	1.484	<0.001	1.110	0.957	1.286		1.065	0.915	1.239		0.977	0.828	1.152	

Model 1: Adjusted for gender, race and age. Model 2: Model 1 + smoking (current/former/never smoker), alcohol use (g/week), physical activity (sedentarism, less than 150 minutes/week, more than 150 minutes/week), and LDL-C. Model 3: Model 2 + log-transformed triglycerides.

Table 5 – Multiple regression analysis between HDL-C and its subfractions and log-transformed CAC in individuals with score>0 (per 1SD **decrease**)

	Bivariate				Model 1				Model 2				Model 3			
	β	CI		p	β	CI		p	β	CI		p	β	CI		p
Total HDL-C	0.035	-0.082	0.153	NS	0.025	-0.094	0.143	NS	0.028	-0.093	0.150	NS	0.006	-0.139	0.135	NS
HDL_2-C	0.048	-0.073	0.170		0.035	-0.087	0.158		0.038	-0.087	0.163		0.025	-0.080	0.151	
HDL_3-C	0.023	-0.093	0.138		0.015	-0.100	0.131		0.199	-0.099	0.139		0.015	-0.152	0.122	
HDL_2-C/HDL_3-C ratio	0.056	-0.069	0.180		0.041	-0.085	0.167		0.041	-0.086	0.169		0.014	-0.127	0.156	

Model 1: Adjusted for gender, race and age. Model 2: Model 1 + smoking (current/former/never smoker), alcohol use (g/week), physical activity (sedentarism, less than 150 minutes/week, more than 150 minutes/week), and LDL-C. Model 3: Model 2 + log-transformed triglycerides.

DISCUSSION

These current analyses sought to widely examine the role of HDL subfractions on atherosclerosis, from metabolic pathways associated with atherogenesis, advancing through a study with cIMT as a marker of vascular disease, and ultimately assessing the coronary artery calcification

In the first analysis, we found a strong negative association of both subfractions and with all metabolic syndrome components, insulin resistance and low-grade inflammation. Also, we observed the HDL_2-c/HDL_3-c ratio as an independent predictive variable for all MetS diagnostic elements. This occurs because the ratio is not constant across the HDL-c levels: the HDL_2-c increases more than HDL_3-c as total HDL-c increases. Therefore, lower ratio values have direct association with all covariates studied even after adjustment for total HDL-c, particularly with triglycerides, waist circumference, CRP and insulin resistance, suggesting that the cluster of metabolic abnormalities is also associated with HDL size and distribution and not only with a fixed low HDL-c value.

Our second study also demonstrated that both HDL_2-c and HDL_3-c are inversely associated with cIMT even after controlling for demographic and CV risk factors. However, they are not independent predictors for all participants regarding the presence of subclinical vascular disease. Further, when we stratified the analyses by the presence of diabetes, we observed that the presence of diabetes modifies these associations. In participants without diabetes, the lower HDL_3-c is independently associated with higher cIMT, while in the participant with diabetes the HDL_2-c/HDL_3-c ratio is lower as cIMT increases, even after controlling for HDL-c. As discussed in the article, this robust inverse association between HDL_2-c/HDL_3-c ratio and cIMT might be explained by several abnormalities in HDL

metabolism and functionality in the insulin resistance scenario. Regarding the HDL_3-c as a protector predictor, the different findings from other researchers may be explained, at least partially, by the sample size (Alagona *et a*l (61) enrolled 89 participants) and some significant differences in the baseline populations studied: Maeda *et al* (66) and Notsu *et a*l (67) enrolled only the Japanese ethnicity and an older population (mean age 61 and 72 years-old, respectively).

Our last article addressed subclinical coronary artery disease and consistently showed that, after using different analytical strategies, none of HDL-c subfractions, analyzed individually or by HDL_2-c/HDL_3-c ratio, are associated with CAC presence or extent after controlling for epidemiological variables and traditional CV risk factors. Once again, our findings are in disagreement with other studies that demonstrated strong association between the presence of CAC score and low-HDL-c (41,43). This difference is likely to be driven by different statistical analyses approach: in the Alison *et al* paper the model was not adjusted for triglycerides (43) and, in the Martin *et al* research, there was no association with CAC in the population without T2D when TG levels were included in the model (41).

Our study is the first to analyze HDL-c subfractions in the Brazilian population and also is the largest sample studied in the literature about a direct HDL-c subfraction measurement method with metabolic syndrome, inflammation, insulin resistance, carotid intima-media thickness and coronary artery calcium. However, we have some limitations: 1) as a cross-sectional study, we cannot infer causality from the associations found; 2) although the sample is ethnically diverse, all the participants included in the analyses are from the Sao Paulo

research center; 3) we used cIMT instead of plaque burden, that might be more representative of atherosclerosis; and 4) we have not data about CAC progression over time.

CONCLUSIONS

In conclusion, in the participants without known cardiovascular disease from ELSA-Brasil study, HDL-c, as well as HDL_2-c and HDL_3-c, are inversely associated with all the factors that define MetS, insulin resistance and inflammation. Additionally, the HDL_2-C/HDL_3-C ratio is also associated with subclinical inflammation, insulin resistance and MetS components even after adjustment for total HDL-C and other confounding variables.

Total HDL-c, HDL_2-c and HDL_3-c are inversely associated with cIMT. In participants with diabetes, the negative association is limited to the HDL_2-c/HDL_3-c ratio when adjusted for HDL-c. In the non-diabetic sample, the HDL_3-c was a protector factor for cIMT.

Finally, there is no association of the HDL-C, HDL_2-C, or HDL_3-C and coronary calcification presence or extension by CT-assessed CAC score. Further, the HDL_2-C/HDL_3-C ratio is not correlated with CAC presence or extent.

SUPPLEMENTAL MATERIAL

USP - HOSPITAL UNIVERSITÁRIO DA UNIVERSIDADE DE SÃO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Subfrações das lipoproteínas de alta densidade mensuradas pelos métodos de Perfil Vertical Automático e Ressonância Nuclear Magnética e sua relação com medidas de aterosclerose subclínica no Estudo Longitudinal de Saúde do Adulto (ELSA-Brasil)

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Instituição Proponente: Hospital Universitário da Universidade de São Paulo
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.929.916

Apresentação do Projeto:
A despeito do HDL colesterol ser considerado um fatore de proteção importante para gênese e progressão da aterosclerose, estudos recentes têm demonstrado que o HDL-colesterol não é uma partícula única, mas sim um pool de partículas de colesterol de diferentes densidades. No entanto, o papel de cada subfração de HDL-colesterol no desenvolvimento da doença aterosclerótica ainda não é claro.
No presente estudo iremos avaliar a associação entre as subfrações do HDL-colesterol e marcadores de aterosclerose subclínica, incluindo o escore de cálcio, a espessura médio-intimal de carótidas e a presença e extensão de placas de aterosclerose em artérias coronárias. O estudo será realizado utilizando os dados da população do estudo ELSA-Brasil, que incluiu aproximadamente 5000 participantes em que as subfrações de HDL e as medidas de aterosclerose subclínica foram avaliadas.
Hipóteses:
1. Indivíduos com menor HDL-C e HDL3 apresentam maior prevalência de aterosclerose.
2. HDL-P apresenta relação inversa com aterosclerose.
3. Quanto maior a relação HDL-C/P, maior o risco de aterosclerose.

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4. MS-HDL-P apresenta relação inversa com aterosclerose

5. A proteína C reativa é uma mediadora da associação entre HDL-C, suas subfrações e os marcadores de aterosclerose subclínica.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a relação entre as Subfrações de HDL mensuradas pelas técnicas VPA e RNM e os marcadores de aterosclerose escore de cálcio coronariano, espessura médio-intimal das carótidas e índice tornozelo-braquial.

Objetivo Secundário:

1. Avaliar a relação entre a razão HDL2/HDL3 mensurados no método VPA e os marcadores de aterosclerose subclínica.

2. Avaliar a relação entre a razão HDL-C/HDL-P, sendo a última mensurada no método de RNM e os marcadores de aterosclerose subclínica.

3. Avaliar a relação entre a soma das partículas pequenas e médias de HDL (MS-HDL-P) mensurada pelo método de RNM e os marcadores de aterosclerose subclínica.

4. Avaliar se os níveis de proteína C reativa de alta sensibilidade, um marcador inflamatório modifica a relação entre as subfrações de HDL-C e os marcadores de aterosclerose subclínica.

Metodologia Proposta:

Foram incluídos nesse estudo os participantes de São Paulo que foram submetidos a:

1. Análise das subfrações de HDL pelos métodos PVA e RNM.

2. Tomografia das Artérias Coronárias (TAC) para realização do Escore de Cálcio Coronariano (ECC).

3. Ultrassonografia das carótidas para medida da espessura médio-intimal das carótidas (cIMT).

4. Aferição do Índice tornozelo-braquial (ITB)

Foram excluídos:

1. Indivíduos com doença cardiovascular manifesta: antecedente de infarto do miocárdio, acidente vascular encefálico, insuficiência cardíaca e revascularização coronariana.

2. Diagnóstico prévio de Diabetes Mellitus

Sendo assim, serão utilizadas análises de 3.616 indivíduos sadios que não apresentaram critérios de exclusão para participar do estudo.

Avaliação dos Riscos e Benefícios:

Riscos:Nenhum, visto o processo de coleta de sangue já ter ocorrido, bem como os outros exame subsidiários presenciais.

Benefícios: -Melhor compreensão do papel das subfrações de HDL no risco cardiovascular.

Comentários e Considerações sobre a Pesquisa:

O Projeto ELSA continua avançando na investigação da gênese multifatorial da aterosclerose, um conhecimento inestimável a ser alcançado.

Considerações sobre os Termos de apresentação obrigatória:

TCLE do estudo ELSA já assinado previamente. O estudo atual não acrescenta nenhuma nova intervenção distinta, e a possibilidade de ampliação do estudo no sentido de esmiuçar os caminhos da aterogênese já fora previsto no TCLE.

Recomendações:

Não existem recomendações para o projeto em tela.

Conclusões ou Pendências e Lista de Inadequações:

Não existem.

Considerações Finais a critério do CEP:

O projeto foi apresentado e aprovado na reunião de hoje.Lembramos que cabe ao pesquisador elaborar e apresentar a este Comitê, relatórios parciais e final, de acordo com a Resolução nº 466/2012 do Conselho Nacional de Saúde, inciso XI.2, letra "d".

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_830196.pdf	27/12/2016 07:25:59		Aceito
Outros	Anuencia.pdf	23/12/2016 11:56:22	Roberta Ferreira de Mello	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_Infraestrutura.pdf	02/12/2016 08:59:33	Roberta Ferreira de Mello	Aceito
Folha de Rosto	FR.pdf	02/12/2016 08:59:08	Roberta Ferreira de Mello	Aceito
Outros	beneficios.pdf	23/11/2016 18:59:42	Marcio Sommer Bittencourt	Aceito
Declaração de Pesquisadores	declaracao.pdf	23/11/2016 18:57:01	Marcio Sommer Bittencourt	Aceito
Outros	cadastroHU.pdf	23/11/2016 18:55:21	Marcio Sommer Bittencourt	Aceito
Cronograma	cronograma.pdf	23/11/2016 18:54:46	Marcio Sommer Bittencourt	Aceito

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Continuação do Parecer: 1.929.916

Orçamento	orcamento.pdf	23/11/2016 18:50:17	Marcio Sommer Bittencourt	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	23/11/2016 18:39:11	Marcio Sommer Bittencourt	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 17 de Fevereiro de 2017

Assinado por:
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REFERENCES:

1. Institute for Health Metrics and Evaluation (IHME). Causes of Death (COD) Data Visualization. [Internet]. IHME, University of Washington, Seattle, WA; 2017. Available from: http://vizhub.healthdata.org/cod

2. Ministério da Saúde D. Estatísticas Vitais - Mortalidade e Nascidos Vivos [Internet]. Ministério da Saúde; [cited 2016 Oct 28]. Available from: http://www2.datasus.gov.br/DATASUS/index.php?area=02

3. Cesar L, Ferreira J, Armaganijan D, Gowdak L, Mansur A, Bodanese L, et al. Guideline For Stable Coronary Artery Disease. Arq Bras Cardiol [Internet]. 2014 [cited 2016 Jul 10];103(2). Available from: http://www.gnresearch.org/doi/10.5935/abc.2014S004

4. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011 May 18;473(7347):317–25.

5. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011 Nov 7;17(11):1410–22.

6. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis. Nat Rev Dis Primer. 2019 Dec;5(1):56.

7. Mackay J, Mensah GA. The atlas of heart disease and stroke. Geneva: World Health Organization; 2004. 112 p.

8. Anitschkow N. Ueber experimentelle Cholester-insteatose und ihre Bedeutung fuer die Entstehung einiger pathologischer Prozesse. Zentrbl Allg Pathol Pathol Anat. 1913;24:1–9.

9. Macheboeuf M. Recherches sur les phosphoaminolipides et les sterides du serum et du plasma sanguins. Bull Soc Chim Biol. 1929;11:268–93.

10. Cohn EJ, Strong LE, Hughes Wl, Mulford D, Ashworth J, Melin M ea, et

al. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids1a, b, c, d. J Am Chem Soc. 1946;68(3):459–75.

11. Barr DP, Russ EM, Eder HA. Protein-lipid relationships in human plasma: II. In atherosclerosis and related conditions. Am J Med. 1951;11(4):480–93.

12. Steinberg D. *Thematic review series: The Pathogenesis of Atherosclerosis.* An interpretive history of the cholesterol controversy: part I. J Lipid Res. 2004 Sep;45(9):1583–93.

13. Rosengren A, Hagman M, Wedel H, Wilhelmsen L. Serum cholesterol and long-term prognosis in middle-aged men with myocardial infarction and angina pectoris. A 16-year follow-up of the Primary Prevention Study in Göteborg, Sweden. Eur Heart J. 1997 May;18(5):754–61.

14. Pekkanen J, Linn S, Heiss G, Suchindran CM, Leon A, Rifkind BM, et al. Ten-Year Mortality from Cardiovascular Disease in Relation to Cholesterol Level among Men with and without Preexisting Cardiovascular Disease. N Engl J Med. 1990 Jun 14;322(24):1700–7.

15. Stamler J. Is Relationship Between Serum Cholesterol and Risk of Premature Death From Coronary Heart Disease Continuous and Graded?: Findings in 356 222 Primary Screenees of the Multiple Risk Factor Intervention Trial (MRFIT). JAMA. 1986 Nov 28;256(20):2823.

16. Anderson KM. Cholesterol and Mortality: 30 Years of Follow-up From the Framingham Study. JAMA. 1987 Apr 24;257(16):2176.

17. O'Donnell CJ, Elosua R. Cardiovascular Risk Factors. Insights From Framingham Heart Study. Rev Esp Cardiol Engl Ed. 2008 Jan;61(3):299–310.

18. Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M, Alzola C.

Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. Am J Epidemiol. 1991 Aug 1;134(3):250–6.

19. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med. 1977 May;62(5):707–14.

20. Navab M, Reddy ST, Van Lenten BJ, Fogelman AM. HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. Nat Rev Cardiol. 2011 Apr;8(4):222–32.

21. Xavier HT, Izar MC, Faria Neto JR, Assad MH, Rocha VZ, Sposito AC, et al. V Diretriz Brasileira de Dislipidemias e Prevenção da Aterosclerose. Arq Bras Cardiol. 2013;101(4):01–22.

22. Ashby DT, Rye KA, Clay MA, Vadas MA, Gamble JR, Barter PJ. Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells. Arterioscler Thromb Vasc Biol. 1998 Sep;18(9):1450–5.

23. Fisher EA, Feig JE, Hewing B, Hazen SL, Smith JD. High-Density Lipoprotein Function, Dysfunction, and Reverse Cholesterol Transport. Arterioscler Thromb Vasc Biol. 2012 Dec;32(12):2813–20.

24. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. Am J Med. 1977 May;62(5):707–14.

25. Moriyama K, Takahashi E. HDL_2/HDL_3 Ratio Changes, Metabolic Syndrome Markers, and Other Factors in a Japanese Population. J Atheroscler Thromb. 2016;23(6):704–12.

26. Moriyama K, Negami M, Takahashi E. HDL2-cholesterol/HDL3-cholesterol ratio was associated with insulin resistance, high-molecular-weight adiponectin, and components for metabolic syndrome in Japanese. Diabetes Res Clin Pract. 2014 Nov;106(2):360–5.

27. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. The Lancet. 2005 Apr;365(9468):1415–28.

28. Kaur J. A Comprehensive Review on Metabolic Syndrome. Cardiol Res Pract. 2014;2014:1–21.

29. Ford ES. Risks for All-Cause Mortality, Cardiovascular Disease, and Diabetes Associated With the Metabolic Syndrome: A summary of the evidence. Diabetes Care. 2005 Jul 1;28(7):1769–78.

30. Katzmarzyk PT, Church TS, Janssen I, Ross R, Blair SN. Metabolic Syndrome, Obesity, and Mortality: Impact of cardiorespiratory fitness. Diabetes Care. 2005 Feb 1;28(2):391–7.

31. Klancic T, Woodward L, Hofmann SM, Fisher EA. High density lipoprotein and metabolic disease: Potential benefits of restoring its functional properties. Mol Metab. 2016 May;5(5):321–7.

32. Zimmet P, Magliano D, Matsuzawa Y, Alberti G, Shaw J. The metabolic syndrome: a global public health problem and a new definition. J Atheroscler Thromb. 2005;12(6):295–300.

33. Grundy SM. Metabolic syndrome update. Trends Cardiovasc Med. 2016 May;26(4):364–73.

34. Touboul P-J, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, et al. Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th

and 5th watching the risk symposia, at the 13th, 15th and 20th European Stroke Conferences, Mannheim, Germany, 2004, Brussels, Belgium, 2006, and Hamburg, Germany, 2011. Cerebrovasc Dis Basel Switz. 2012;34(4):290–6.

35. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of Clinical Cardiovascular Events With Carotid Intima-Media Thickness: A Systematic Review and Meta-Analysis. Circulation. 2007 Jan 30;115(4):459–67.

36. Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol. 1990 Mar;15(4):827–32.

37. Raggi P, Gongora MC, Gopal A, Callister TQ, Budoff M, Shaw LJ. Coronary Artery Calcium to Predict All-Cause Mortality in Elderly Men and Women. J Am Coll Cardiol. 2008 Jul;52(1):17–23.

38. Detrano R, Guerci AD, Carr JJ, Bild DE, Burke G, Folsom AR, et al. Coronary Calcium as a Predictor of Coronary Events in Four Racial or Ethnic Groups. N Engl J Med. 2008 Mar 27;358(13):1336–45.

39. Polonsky TS. Coronary Artery Calcium Score and Risk Classification for Coronary Heart Disease Prediction. JAMA. 2010 Apr 28;303(16):1610.

40. Yeboah J, McClelland RL, Polonsky TS, Burke GL, Sibley CT, O'Leary D, et al. Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate-risk individuals. JAMA. 2012 Aug 22;308(8):788–95.

41. Martin SS, Qasim AN, Wolfe M, St. Clair C, Schwartz S, Iqbal N, et al. Comparison of High-Density Lipoprotein Cholesterol to Apolipoprotein A-I and A-II to Predict Coronary Calcium and the Effect of Insulin Resistance. Am J Cardiol. 2011 Feb;107(3):393–8.

42. Orakzai SH, Nasir K, Blaha M, Blumenthal RS, Raggi P. Non-HDL cholesterol is strongly associated with coronary artery calcification in asymptomatic individuals. Atherosclerosis. 2009 Jan;202(1):289–95.

43. Allison MA, Wright CM. A comparison of HDL and LDL cholesterol for prevalent coronary calcification. Int J Cardiol. 2004 May;95(1):55–60.

44. Okumura K, Tsukamoto H, Tsuboi H, Hirayama H, Kamiya H, Watarai M, et al. High HDL cholesterol level after treatment with pitavastatin is an important factor for regression in carotid intima–media thickness. Heart Vessels. 2015 Mar;30(2):154–61.

45. Laurinavicius AG, Santos IS, Santos RD, Bensenor IM, Conceição RD, Lotufo PA. Extremely elevated HDL-cholesterol levels are not associated with increased carotid intima-media thickness: data from ELSA Brasil. J Clin Lipidol. 2016 Jul;10(4):898-904.e1.

46. Sanna G, Alesso D, Mediati M, Cimminiello C, Borghi C, Fazzari AL, et al. Prevalence of peripheral arterial disease in subjects with moderate cardiovascular risk: Italian results from the PANDORA study Data from PANDORA (Prevalence of peripheral Arterial disease in subjects with moderate CVD risk, with No overt vascular Diseases nor Diabetes mellitus). BMC Cardiovasc Disord. 2011 Oct 7;11:59.

47. Sung K-C, Wild SH, Byrne CD. Controlling for apolipoprotein A-I concentrations changes the inverse direction of the relationship between high HDL-C concentration and a measure of pre-clinical atherosclerosis. Atherosclerosis. 2013 Dec;231(2):181–6.

48. Sung K-C, Ryu S, Wild SH, Byrne CD. An increased high-density lipoprotein cholesterol/apolipoprotein A-I ratio is associated with increased

cardiovascular and all-cause mortality. Heart Br Card Soc. 2015 Apr;101(7):553–8.

49. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. The Lancet. 2012 Aug;380(9841):572–80.

50. Holmes MV, Asselbergs FW, Palmer TM, Drenos F, Lanktree MB, Nelson CP, et al. Mendelian randomization of blood lipids for coronary heart disease. Eur Heart J. 2015 Mar 1;36(9):539–50.

51. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, et al. Effects of Dalcetrapib in Patients with a Recent Acute Coronary Syndrome. N Engl J Med. 2012 Nov 29;367(22):2089–99.

52. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJP, Komajda M, et al. Effects of Torcetrapib in Patients at High Risk for Coronary Events. N Engl J Med. 2007 Nov 22;357(21):2109–22.

53. Nicholls SJ, Brewer HB, Kastelein JJP, Krueger KA, Wang M-D, Shao M, et al. Effects of the CETP Inhibitor Evacetrapib Administered as Monotherapy or in Combination With Statins on HDL and LDL Cholesterol: A Randomized Controlled Trial. JAMA [Internet]. 2011 Nov 16 [cited 2017 Aug 30];306(19). Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2011.1649

54. The HPS3/TIMI55-REVEAL Collaborative Group. Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. N Engl J Med [Internet]. 2017 Aug 28 [cited 2017 Sep 6]; Available from: http://www.nejm.org/doi/10.1056/NEJMoa1706444

55. The HPS2-THRIVE Collaborative Group. Effects of Extended-Release

Niacin with Laropiprant in High-Risk Patients. N Engl J Med. 2014 Jul 17;371(3):203–12.

56. Hafiane A, Genest J. High density lipoproteins: Measurement techniques and potential biomarkers of cardiovascular risk. BBA Clin. 2015 Jun;3:175–88.

57. Kulkarni KR. Cholesterol Profile Measurement by Vertical Auto Profile Method. Clin Lab Med. 2006 Dec;26(4):787–802.

58. Asztalos BF, Tani M, Schaefer EJ. Metabolic and functional relevance of HDL subspecies. Curr Opin Lipidol. 2011 Jun;22(3):176–85.

59. Kontush A, Chantepie S, Chapman MJ. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. Arterioscler Thromb Vasc Biol. 2003 Oct 1;23(10):1881–8.

60. Kim DS, Li YK, Bell GA, Burt AA, Vaisar T, Hutchins PM, et al. Concentration of Smaller High-Density Lipoprotein Particle (HDL-P) Is Inversely Correlated With Carotid Intima Media Thickening After Confounder Adjustment: The Multi Ethnic Study of Atherosclerosis (MESA). J Am Heart Assoc. 2016 May;5(5):e002977.

61. Alagona C, Soro A, Ylitalo K, Salonen R, Salonen JT, Taskinen M-R. A low high density lipoprotein (HDL) level is associated with carotid artery intima-media thickness in asymptomatic members of low HDL families. Atherosclerosis. 2002 Dec;165(2):309–16.

62. Jug B, Papazian J, Lee R, Budoff MJ. Association of Lipoprotein Subfractions and Coronary Artery Calcium In Patient at Intermediate Cardiovascular Risk. Am J Cardiol. 2013 Jan;111(2):213–8.

63. Sailam V, Karalis DG, Agarwal A, Alani F, Galardi S, Covalesky V, et al. Prevalence of Emerging Cardiovascular Risk Factors in Younger Individuals

with a Family History of Premature Coronary Heart Disease and Low Framingham Risk Score. Clin Cardiol. 2008 Nov;31(11):542–5.

64. Aneni EC, Osondu CU, De La Cruz J, Martin SS, Blaha MJ, Younus A, et al. Lipoprotein Sub-Fractions by Ion-Mobility Analysis and Its Association with Subclinical Coronary Atherosclerosis in High-Risk Individuals. J Atheroscler Thromb [Internet]. 2018 [cited 2018 Nov 17]; Available from: https://www.jstage.jst.go.jp/article/jat/advpub/0/advpub_40741/_article

65. Yan F, Tian L, Yu H, Baskota A, Gao Y, Li S, et al. Association of lipoprotein subclasses and carotid intima-media thickness in the Chinese population. Clin Lipidol. 2014 Aug;9(4):407–15.

66. Maeda S, Nakanishi S, Yoneda M, Awaya T, Yamane K, Hirano T, et al. Associations between Small Dense LDL, HDL Subfractions (HDL2, HDL3) and Risk of Atherosclerosis in Japanese-Americans. J Atheroscler Thromb. 2012;19(5):444–52.

67. Notsu Y, Yano S, Takeda M, Yamasaki M, Isomura M, Nabika T, et al. Association of High-Density Lipoprotein Subclasses with Carotid Intima-Media Thickness: Shimane CoHRE Study. J Atheroscler Thromb [Internet]. 2017 [cited 2017 Jul 4]; Available from: https://www.jstage.jst.go.jp/article/jat/advpub/0/advpub_38844/_article

68. Tiozzo E, Gardener H, Hudson BI, Dong C, Della-Morte D, Crisby M, et al. High-density lipoprotein subfractions and carotid plaque: The Northern Manhattan Study. Atherosclerosis. 2014 Nov;237(1):163–8.

69. Joshi PH, Xu H, LeStrange R, Flockhart N, Kirkland B, Vazquez G, et al. The M235T single nucleotide polymorphism in the angiotensinogen gene is associated with coronary artery calcium in patients with a family history of

coronary artery disease. Atherosclerosis. 2013 Feb;226(2):433–9.

70. Albers JJ, Slee A, Fleg JL, O'Brien KD, Marcovina SM. Relationship of baseline HDL subclasses, small dense LDL and LDL triglyceride to cardiovascular events in the AIM-HIGH clinical trial. Atherosclerosis. 2016 Aug;251:454–9.

71. Yu S, Yarnell JWG, Sweetnam P, Bolton CH. High density lipoprotein subfractions and the risk of coronary heart disease: 9-years follow-up in the Caerphilly Study. Atherosclerosis. 2003 Feb;166(2):331–8.

72. Joshi PH, Toth PP, Lirette ST, Griswold ME, Massaro JM, Martin SS, et al. Association of high-density lipoprotein subclasses and incident coronary heart disease: The Jackson Heart and Framingham Offspring Cohort Studies. Eur J Prev Cardiol. 2016 Jan 1;23(1):41–9.

73. Stampfer MJ, Sacks FM, Salvini S, Willett WC, Hennekens CH. A Prospective Study of Cholesterol, Apolipoproteins, and the Risk of Myocardial Infarction. N Engl J Med. 1991 Aug 8;325(6):373–81.

74. Martin SS, Khokhar AA, May HT, Kulkarni KR, Blaha MJ, Joshi PH, et al. HDL cholesterol subclasses, myocardial infarction, and mortality in secondary prevention: the lipoprotein investigators collaborative. Eur Heart J. 2015 Jan 1;36(1):22–30.

75. Salonen JT, Salonen R, Seppanen K, Rauramaa R, Tuomilehto J. HDL, HDL2, and HDL3 subfractions, and the risk of acute myocardial infarction. A prospective population study in eastern Finnish men. Circulation. 1991 Jul 1;84(1):129–39.

76. Lamarche B, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Despres J-P. Associations of HDL2 and HDL3 Subfractions With Ischemic Heart Disease

in Men: Prospective Results From the Quebec Cardiovascular Study. Arterioscler Thromb Vasc Biol. 1997 Jun 1;17(6):1098–105.

77. Drexel H, Amann FW, Rentsch K, Neuenschwander C, Luethy A, Khan SI, et al. Relation of the level of high-density lipoprotein subfractions to the presence and extent of coronary artery disease. Am J Cardiol. 1992 Aug 15;70(4):436–40.

78. Superko HR, Pendyala L, Williams PT, Momary KM, King SB, Garrett BC. High-density lipoprotein subclasses and their relationship to cardiovascular disease. J Clin Lipidol. 2012 Nov;6(6):496–523.

79. Aquino EML, Barreto SM, Bensenor IM, Carvalho MS, Chor D, Duncan BB, et al. Brazilian Longitudinal Study of Adult Health (ELSA-Brasil): objectives and design. Am J Epidemiol. 2012 Feb 15;175(4):315–24.

80. Schmidt MI, Duncan BB, Mill JG, Lotufo PA, Chor D, Barreto SM, et al. Cohort Profile: Longitudinal Study of Adult Health (ELSA-Brasil). Int J Epidemiol. 2015 Feb 1;44(1):68–75.

81. Fedeli LG, Vidigal PG, Leite CM, Castilhos CD, Pimentel RA, Maniero VC, et al. Logistica de coleta e transporte de material biologico e organizacao do laboratorio central no ELSA-Brasil. Rev Saúde Pública. 2013 Jun;47:63–71.

82. Mill JG, Pinto K, Griep RH, Goulart A, Foppa M, Lotufo PA, et al. Afericoes e exames clinicos realizados nos participantes do ELSA-Brasil. Rev Saúde Pública. 2013 Jun;47:54–62.

83. Freire CMV, Alcantara ML de, Santos SN dos, Amaral SI do, Veloso O, Porto CLL, et al. Recomendação para a Quantificação pelo Ultrassom da Doença Aterosclerótica das Artérias Carótidas e Vertebrais: Grupo de Trabalho do Departamento de Imagem Cardiovascular da Sociedade Brasileira de

Cardiologia – DIC - SBC. Arq Bras Cardiol - IMAGEM Cardiovasc [Internet]. 2015 [cited 2016 Nov 9];28. Available from: http://www.gnresearch.org/doi/10.5935/2318-8219.20150018

84. Santos IS, Bittencourt MS, Oliveira IRS, Souza AG, Meireles DP, Rundek T, et al. Carotid intima–media thickness value distributions in The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil). Atherosclerosis. 2014 Nov;237(1):227–35.

85. Aquino EML, Vasconcellos-Silva PR, Coeli CM, Araujo MJ, Santos SM, Figueiredo RC de, et al. Aspectos eticos em estudos longitudinais: o caso do ELSA-Brasil. Rev Saúde Pública. 2013 Jun;47:19–26.

86. Barreto SM, Ladeira RM, Bastos M do SCB de, Diniz M de FHS, Jesus EA de, Kelles SMB, et al. Estrategias de identificacao, investigacao e classificacao de desfechos incidentes no ELSA-Brasil. Rev Saúde Pública. 2013 Jun;47:79–86.

87. Wilson PW, Abbott RD, Castelli WP. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arterioscler Dallas Tex. 1988 Dec;8(6):737–41.

88. Lakka H-M, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA. 2002 Dec 4;288(21):2709–16.

89. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, et al. Cardiovascular Morbidity and Mortality Associated With the Metabolic Syndrome. Diabetes Care. 2001 Apr 1;24(4):683–9.

90. Pereira AC, Bensenor IM, Fedeli LM, Castilhos C, Vidigal PG, Maniero V, et al. Delineamento e implementacao do biobanco do ELSA-Brasil: estudo

prospectivo na populacao brasileira. Rev Saúde Pública. 2013 Jun;47:72–8.

91. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985 Jul;28(7):412–9.

92. Alberti KGMM, Zimmet P, Shaw J. Metabolic syndrome-a new world-wide definition. A Consensus Statement from the International Diabetes Federation. Diabet Med. 2006 May;23(5):469–80.

93. Vieira BA, Luft VC, Schmidt MI, Chambless LE, Chor D, Barreto SM, et al. Timing and Type of Alcohol Consumption and the Metabolic Syndrome - ELSA-Brasil. Abulseoud OA, editor. PLOS ONE. 2016 Sep 19;11(9):e0163044.

94. Williams PT, Krauss RM, Vranizan KM, Stefanick ML, Wood PD, Lindgren FT. Associations of lipoproteins and apolipoproteins with gradient gel electrophoresis estimates of high density lipoprotein subfractions in men and women. Arterioscler Thromb J Vasc Biol. 1992 Mar;12(3):332–40.

95. von Eckardstein A, Kardassis D, editors. High Density Lipoproteins [Internet]. Cham: Springer International Publishing; 2015 [cited 2017 May 30]. (Handbook of Experimental Pharmacology; vol. 224). Available from: http://link.springer.com/10.1007/978-3-319-09665-0

96. Mann DL, Zipes DP, Libby P, Bonow RO, Braunwald E, editors. Braunwald's heart disease: a textbook of cardiovascular medicine. Tenth edition. Philadelphia, PA: Elsevier/Saunders; 2015. 980–989 p.

97. Berthold HK, Rizzo M, Spenrath N, Montalto G, Krone W, Gouni-Berthold I. Effects of Lipid-Lowering Drugs on High-Density Lipoprotein Subclasses in Healthy Men—A Randomized Trial. Gaunt TR, editor. PLoS ONE. 2014 Mar

24;9(3):e91565.

98. Rizzo M, Corrado E, Coppola G, Muratori I, Novo G, Novo S. Prediction of cardio- and cerebro-vascular events in patients with subclinical carotid atherosclerosis and low HDL-cholesterol. Atherosclerosis. 2008 Oct;200(2):389–95.

99. Kim DS, Burt AA, Rosenthal EA, Ranchalis JE, Eintracht JF, Hatsukami TS, et al. HDL-3 is a Superior Predictor of Carotid Artery Disease in a Case-Control Cohort of 1725 Participants. J Am Heart Assoc. 2014 Jun 25;3(3):e000902–e000902.

100. Lagos KG, Filippatos TD, Tsimihodimos V, Gazi IF, Rizos C, Tselepis AD, et al. Alterations in the High Density Lipoprotein Phenotype and HDL-Associated Enzymes in Subjects with Metabolic Syndrome. Lipids. 2009 Jan;44(1):9–16.

101. Yost TJ, Froyd KK, Jensen DR, Eckel RH. Change in skeletal muscle lipoprotein lipase activity in response to insulin/glucose in non-insulin-dependent diabetes mellitus. Metabolism. 1995 Jun;44(6):786–90.

102. Rashid S, Watanabe T, Sakaue T, Lewis GF. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. Clin Biochem. 2003 Sep;36(6):421–9.

103. Tang C, Kanter JE, Bornfeldt KE, Leboeuf RC, Oram JF. Diabetes reduces the cholesterol exporter ABCA1 in mouse macrophages and kidneys. J Lipid Res. 2010 Jul;51(7):1719–28.

104. Patel DC, Albrecht C, Pavitt D, Paul V, Pourreyron C, Newman SP, et al. Type 2 Diabetes Is Associated with Reduced ATP-Binding Cassette Transporter

A1 Gene Expression, Protein and Function. Uversky VN, editor. PLoS ONE. 2011 Jul 27;6(7):e22142.

105. Bagdade JD, Lane JT, Subbaiah PV, Otto ME, Ritter MC. Accelerated cholesteryl ester transfer in noninsulin-dependent diabetes mellitus. Atherosclerosis. 1993 Dec;104(1–2):69–77.

106. Mooradian AD, Haas MJ, Wong NCW. Transcriptional Control of Apolipoprotein A-I Gene Expression in Diabetes. Diabetes. 2004 Mar 1;53(3):513–20.

107. Lewington S, Whitlock G, Clarke R, et al. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55?000 vascular deaths. The Lancet. 2007 Dec;370(9602):1829–39.

108. Di Angelantonio E, Sarwar N, Perry P, et al. Major Lipids, Apolipoproteins, and Risk of Vascular Disease. JAMA. 2009 Nov 11;302(18):1993.

109. Nordestgaard BG, Tybjærg-Hansen A. Genetic determinants of LDL, lipoprotein(a), triglyceride-rich lipoproteins and HDL: concordance and discordance with cardiovascular disease risk. Curr Opin Lipidol. 2011 Apr;22(2):113–22.

110. The AIM-HIGH Investigators. Niacin in Patients with Low HDL Cholesterol Levels Receiving Intensive Statin Therapy. N Engl J Med. 2011 Dec 15;365(24):2255–67.

111. Kaur N, Pandey A, Negi H, Shafiq N, Reddy S, Kaur H, et al. Effect of HDL-Raising Drugs on Cardiovascular Outcomes: A Systematic Review and Meta-Regression. Bursill C, editor. PLoS ONE. 2014 Apr 11;9(4):e94585.

112. Peters SAE, den Ruijter HM, Bots ML, Moons KGM. Improvements in risk stratification for the occurrence of cardiovascular disease by imaging subclinical atherosclerosis: a systematic review. Heart. 2012 Feb 1;98(3):177–84.

113. Plichart M, Celermajer DS, Zureik M, Helmer C, Jouven X, Ritchie K, et al. Carotid intima-media thickness in plaque-free site, carotid plaques and coronary heart disease risk prediction in older adults. The Three-City Study. Atherosclerosis. 2011 Dec;219(2):917–24.

114. Lorenz MW, Polak JF, Kavousi M, Mathiesen EB, V?lzke H, Tuomainen T-P, et al. Carotid intima-media thickness progression to predict cardiovascular events in the general population (the PROG-IMT collaborative project): a meta-analysis of individual participant data. The Lancet. 2012 Jun;379(9831):2053–62.

115. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med. 1999 Jan 7;340(1):14–22.

116. Touboul P-J, Labreuche J, Bruckert E, Schargrodsky H, Prati P, Tosetto A, et al. HDL-C, triglycerides and carotid IMT: A meta-analysis of 21,000 patients with automated edge detection IMT measurement. Atherosclerosis. 2014 Jan;232(1):65–71.

117. Srivastava RAK. Dysfunctional HDL in diabetes mellitus and its role in the pathogenesis of cardiovascular disease. Mol Cell Biochem [Internet]. 2017 Aug 21 [cited 2018 Jan 29]; Available from: http://link.springer.com/10.1007/s11010-017-3165-z

118. Farbstein D, Levy AP. HDL dysfunction in diabetes: causes and possible treatments. Expert Rev Cardiovasc Ther. 2012 Mar;10(3):353–61.

119. Santos IS, Alencar AP, Rundek T, Goulart AC, Barreto SM, Pereira AC, et al. Low Impact of Traditional Risk Factors on Carotid Intima-Media Thickness: The ELSA-Brasil Cohort. Arterioscler Thromb Vasc Biol. 2015 Sep;35(9):2054–9.

120. Kim DS, Burt AA, Rosenthal EA, Ranchalis JE, Eintracht JF, Hatsukami TS, et al. HDL-3 is a Superior Predictor of Carotid Artery Disease in a Case-Control Cohort of 1725 Participants. J Am Heart Assoc. 2014 Jun 25;3(3):e000902–e000902.

121. Schofield JD, Liu Y, Rao-Balakrishna P, Malik RA, Soran H. Diabetes Dyslipidemia. Diabetes Ther. 2016 Jun;7(2):203–19.

122. Shah AS, Urbina EM, Khoury PR, Kimball TR, Dolan LM. Lipids and lipoprotein ratios: Contribution to carotid intima media thickness in adolescents and young adults with type 2 diabetes mellitus. J Clin Lipidol. 2013 Sep;7(5):441–5.

123. Ishigaki Y, Kono S, Katagiri H, Oka Y, Oikawa S, NTTP investigators. Elevation of HDL-C in Response to Statin Treatment is Involved in the Regression of Carotid Atherosclerosis. J Atheroscler Thromb. 2014;21(10):1055–65.

124. Davidson M, Meyer PM, Haffner S, Feinstein S, D'Agostino R, Kondos GT, et al. Increased High-Density Lipoprotein Cholesterol Predicts the Pioglitazone-Mediated Reduction of Carotid Intima-Media Thickness Progression in Patients With Type 2 Diabetes Mellitus. Circulation. 2008 Mar 31;117(16):2123–30.

125. McGillicuddy FC, Reilly MP, Rader DJ. Adipose Modulation of High-Density Lipoprotein Cholesterol: Implications for Obesity, High-Density Lipoprotein Metabolism, and Cardiovascular Disease. Circulation. 2011 Oct 11;124(15):1602–5.

126. Nakhjavani M, Esteghamati A, Esfahanian F, Ghanei A, Rashidi A, Hashemi S. HbA1c negatively correlates with LCAT activity in type 2 diabetes. Diabetes Res Clin Pract. 2008 Jul;81(1):38–41.

127. Fadini GP, Iori E, Marescotti MC, Vigili de Kreutzenberg S, Avogaro A. Insulin-induced glucose control improves HDL cholesterol levels but not reverse cholesterol transport in type 2 diabetic patients. Atherosclerosis. 2014 Aug;235(2):415–7.

128. Shaw JA, Bobik A, Murphy A, Kanellakis P, Blombery P, Mukhamedova N, et al. Infusion of Reconstituted High-Density Lipoprotein Leads to Acute Changes in Human Atherosclerotic Plaque. Circ Res. 2008 Nov 7;103(10):1084–91.

129. Mabuchi H, Nohara A, Inazu A. Cholesteryl ester transfer protein (CETP) deficiency and CETP inhibitors. Mol Cells. 2014 Nov;37(11):777–84.

130. de Souza Zago VH, Tanus-Santos JE, Gardin Danelon MR, Parra ES, Alexandre F, Vieira IC, et al. Chemical modification of high density lipoprotein subfractions - HDL2 and HDL3 - after use of atorvastatin. Int J Clin Pharmacol Ther. 2014 Apr;52(4):277–83.

131. Mora S, Glynn RJ, Ridker PM. High-density lipoprotein cholesterol, size, particle number, and residual vascular risk after potent statin therapy. Circulation. 2013 Sep 10;128(11):1189–97.

132. Sampson UK, Fazio S, Linton MF. Residual Cardiovascular Risk Despite

Optimal LDL Cholesterol Reduction with Statins: The Evidence, Etiology, and Therapeutic Challenges. Curr Atheroscler Rep. 2012 Feb;14(1):1–10.

133. Naci H, Brugts JJ, Fleurence R, Tsoi B, Toor H, Ades A. Comparative benefits of statins in the primary and secondary prevention of major coronary events and all-cause mortality: a network meta-analysis of placebo-controlled and active-comparator trials. Eur J Prev Cardiol. 2013 Aug;20(4):641–57.

134. Haase CL, Tybjærg-Hansen A, Qayyum AA, Schou J, Nordestgaard BG, Frikke-Schmidt R. LCAT, HDL cholesterol and ischemic cardiovascular disease: a Mendelian randomization study of HDL cholesterol in 54,500 individuals. J Clin Endocrinol Metab. 2012 Feb;97(2):E248-256.

135. Ko DT, Alter DA, Guo H, Koh M, Lau G, Austin PC, et al. High-Density Lipoprotein Cholesterol and Cause-Specific Mortality in Individuals Without Previous Cardiovascular Conditions. J Am Coll Cardiol. 2016 Nov;68(19):2073–83.

136. Kontush A, Lindahl M, Lhomme M, Calabresi L, Chapman MJ, Davidson WS. Structure of HDL: Particle Subclasses and Molecular Components. In: von Eckardstein A, Kardassis D, editors. High Density Lipoproteins [Internet]. Cham: Springer International Publishing; 2015 [cited 2018 Mar 1]. p. 3–51. Available from: http://link.springer.com/10.1007/978-3-319-09665-0_1

137. García-Sánchez C, Posadas-Romero C, Posadas-Sánchez R, Carreón-Torres E, Rodríguez-Pérez JM, Juárez-Rojas JG, et al. Low concentrations of phospholipids and plasma HDL cholesterol subclasses in asymptomatic subjects with high coronary calcium scores. Atherosclerosis. 2015 Feb;238(2):250–5.

138. Lotufo PA, Santos RD, Figueiredo RM, Pereira AC, Mill JG, Alvim SM, et

al. Prevalence, awareness, treatment, and control of high low-density lipoprotein cholesterol in Brazil: Baseline of the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil). J Clin Lipidol. 2016 Jun;10(3):568–76.

139. Pereira AC, Gomez LM, Bittencourt MS, Staniak HL, Sharovsky R, Foppa M, et al. Age, Gender, and Race-Based Coronary Artery Calcium Score Percentiles in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil): Age, Gender, and Race-Based CAC Score. Clin Cardiol. 2016 Jun;39(6):352–9.

140. Loria CM, Liu K, Lewis CE, Hulley SB, Sidney S, Schreiner PJ, et al. Early Adult Risk Factor Levels and Subsequent Coronary Artery Calcification. J Am Coll Cardiol. 2007 May;49(20):2013–20.

141. Haase CL, Tybjærg-Hansen A, Nordestgaard BG, Frikke-Schmidt R. HDL Cholesterol and Risk of Type 2 Diabetes: A Mendelian Randomization Study. Diabetes. 2015 Sep;64(9):3328–33.

142. Bittencourt MS, Santos RD, Staniak H, Sharovsky R, Kondapally R, Vallejo-Vaz AJ, et al. Relation of Fasting Triglyceride-Rich Lipoprotein Cholesterol to Coronary Artery Calcium Score (from the ELSA-Brasil Study). Am J Cardiol. 2017 May;119(9):1352–8.

143. Tsao CW, Preis SR, Peloso GM, Hwang S-J, Kathiresan S, Fox CS, et al. Relations of Long-Term and Contemporary Lipid Levels and Lipid Genetic Risk Scores With Coronary Artery Calcium in the Framingham Heart Study. J Am Coll Cardiol. 2012 Dec;60(23):2364–71.

144. Paramsothy P, Knopp RH, Bertoni AG, Blumenthal RS, Wasserman BA, Tsai MY, et al. Association of Combinations of Lipid Parameters With Carotid Intima-Media Thickness and Coronary Artery Calcium in the MESA (Multi-Ethnic Study of Atherosclerosis). J Am Coll Cardiol. 2010 Sep;56(13):1034–41.

145. Sarwar N, Danesh J, Eiriksdottir G, Sigurdsson G, Wareham N, Bingham S, et al. Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation. 2007 Jan 30;115(4):450–8.

146. Thomsen M, Varbo A, Tybjaerg-Hansen A, Nordestgaard BG. Low Nonfasting Triglycerides and Reduced All-Cause Mortality: A Mendelian Randomization Study. Clin Chem. 2014 May 1;60(5):737–46.

147. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, et al. Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. N Engl J Med [Internet]. 2018 Nov 10 [cited 2018 Nov 19]; Available from: http://www.nejm.org/doi/10.1056/NEJMoa1812792

148. Generoso G, Bensenor IM, Santos IS, Santos RD, Goulart AC, Jones SR, et al. Diabetes alters the association between high-density lipoprotein subfractions and carotid intima-media thickness: The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil). Diab Vasc Dis Res. 2018 Jul 19;147916411878808.

149. Aharoni S, Aviram M, Fuhrman B. Paraoxonase 1 (PON1) reduces macrophage inflammatory responses. Atherosclerosis. 2013 Jun;228(2):353–61.

150. Borggreve SE, De Vries R, Dullaart RPF. Alterations in high-density lipoprotein metabolism and reverse cholesterol transport in insulin resistance and type 2 diabetes mellitus: role of lipolytic enzymes, lecithin:cholesterol acyltransferase and lipid transfer proteins. Eur J Clin Invest. 2003 Dec;33(12):1051–69.

151. Lee DY, Kim JH, Park SE, Park C-Y, Oh K-W, Park S-W, et al. Effects of

Low-density Lipoprotein Cholesterol on Coronary Artery Calcification Progression According to High-density Lipoprotein Cholesterol Levels. Arch Med Res. 2017 Apr;48(3):284–91.

152. Generoso G, Bensenor IM, Santos RD, Santos IS, Goulart AC, Jones SR, et al. Association between high-density lipoprotein subfractions and low-grade inflammation, insulin resistance, and metabolic syndrome components: The ELSA-Brasil study. J Clin Lipidol. 2018 Sep;12(5):1290-1297.e1.

153. Uehara Y, Engel T, Li Z, Goepfert C, Rust S, Zhou X, et al. Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1. Diabetes. 2002 Oct;51(10):2922–8.

154. Wang Y, Oram JF. Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C delta pathway. J Lipid Res. 2007 May;48(5):1062–8.