University of São Paulo
School of Public Health

Associations of blood biomarkers and structural changes in atherosclerosis in the Longitudinal Study of Adult Health (ELSA-Brasil): emphasis on pre- and postmenopausal women

Marília Izar Helfenstein Fonseca

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DEDICATION

To my beloved mother Cristina, and my father Francisco, both eternal inspirations for my carreer and life;

To my boyfriend George, for his patience and love;

To my dear sisters, Luísa and Amanda, and my brother Rodrigo, for their incentive to go on;

To my sweet grandmother Niryan, who I miss talking to;

To my family, specially my aunts, Isabel, Sílvia and Lúcia, for their advices and special friendship;

To my patients, for being the reason I chose medicine;

To life itself.

SPECIAL DEDICATION

To my dear teacher, counselor and friend, Professor Sandra, for her attention, experience, guidance, vision and unconditional partnership enabling my personal and professional development throughout these years. My special admiration not only for her care, skills, wisdom and intelligence, but also as person; I trully wish I can become more like her in the future;

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To Renilda, for all the suport to every single thing that I asked (and I did ask a lot from her...);

To my patients, without whom, this road would not make any sense.

Resumo

Introdução: A doença cardiovascular se constitui na principal causa de morbimortalidade em mulheres globalmente. A predição de evento cardiovascular é incompleta e novos marcadores de risco cardiometabólico podem auxiliar na identificação precoce da aterosclerose. Dados epidemiológicos brasileiros no sexo feminino são mais escassos. **Objetivos**: Revisar o impacto da menopausa e diabetes nas lipoproteínas, subfrações e risco cardiovascular; avaliar o perfil de risco cardiometabólico de mulheres do ELSA-Brasil, bem como associações de marcadores sanguíneos [subfrações de lipoproteínas circulantes, índice de resistência à insulina (HOMA-IR) e aminoácidos de cadeia ramificada (BCAA)] e estruturais [cálcio nas artérias coronárias (CAC)] de aterosclerose segundo sua idade e estado menopausal. **Métodos**: Análise transversal de dados basais de 2258 mulheres acompanhadas no centro de São Paulo do ELSA-Brasil, estratificadas por idade e estado menopausal, respeitando características amostrais estabelecidas para cada artigo. Estatística descritiva, testes de comparação entre grupos e análises de regressão múltipla foram realizadas conforme natureza e distribuição das variáveis para cada artigo. **Resultados**: Artigo 1: A revisão da literatura permitiu concluir sobre a associação de menopausa e diabetes com pior perfil lipídico, consistindo de hipertrigliceridemia, baixos níveis de HDL-c e HDL_2-c e elevados de HDL_3-c e LDL-c pequena e densa. Mulheres menopausadas com diabetes apresentam o maior risco cardiovascular. Artigo 2: Comparando-se mulheres pré-menopausadas com as menopausadas, categorizadas segundo tempo de menopausa [duração <2 anos; 2-5,9 anos; 6-9,9 anos e ≥10 anos (n=1916)], aquelas na pós-menopausa apresentaram perfil de lipoproteínas e suas subfrações mais aterogênico e a duração da menopausa <2 anos associou-se independentemente com remanescentes de lipoproteínas ricas em triglicérides (TRL-c) [7,21 mg/dL (IC95% 3,59–10,84)] e com a partícula pequena e densa de $VLDL_3$-c [2,43 mg/dL (IC95% 1,02–3,83)], mas não foram encontradas associações de categorias de menopausa com as subfrações de HDL-c ou LDL-c, considerando-se as pré-menopausadas como referência. Artigo 3: Comparando mulheres pré-menopausadas com idade ≤ ou >45 anos e as menopausadas (n=2047), pior perfil de risco cardiometabólico foi encontrado em mulheres na pós-

menopausa. Observou-se associação entre CAC>0 com TRL-c e LDL-c densa, mas não com HOMA-IR e BCAA. Mulheres menopausadas tiveram cerca de 2 vezes mais chance de apresentar CAC>0 quando comparadas com mulheres mais jovens na pré-menopausa [OR 2,37 (IC95% 1,17-4,81)]. **Discussão**: Nossos achados sugerem que a menopausa natural está associada a alterações no perfil lipídico tradicional e subfrações (especialmente nos primeiros 2 anos pós-menopausa) e ao depósito de cálcio nas artérias coronárias independentemente da idade e de outros fatores de risco, mas não com BCAA e HOMA-IR. Investigação aprofundada do perfil lipídico e outros marcadores de risco cardiovascular em mulheres que se aproximam da menopausa pode melhorar a identificação de risco, prevenção de desfechos cardiovasculares e proporcionar melhores condições de saúde.

Palavras-chave: risco cardiovascular, aterosclerose, menopausa, biomarcadores, escore de cálcio coronário, subfrações de lipoproteínas, mulher, aminoácidos de cadeia ramificada.

Abstract

Background: Cardiovascular disease is the leading cause of morbidity and mortality in women worldwide. Cardiovascular risk prediction is incomplete and new markers may help in the early identification of atherosclerosis. Brazilian epidemiological data in women are scarce. **Objectives**: To review the impact of menopause and diabetes on lipids, lipoprotein subfractions and cardiovascular risk; evaluate cardiometabolic risk in women from the ELSA-Brasil, as well as associations of blood biomarkers [lipoprotein subfractions, insulin resistance index (HOMA-IR) and branched-chain amino acids (BCAA)] and structural changes of atherosclerosis [presence of calcium in the coronary arteries (CAC)] according to age and menopausal status. **Methods**: Cross-sectional baseline analyzes of 2,258 female participants from the São Paulo site of the ELSA-Brasil, stratified by age and menopausal status, with specific sample and eligibility criteria for each paper. Descriptive statistics, between-group comparisons and multiple regression were performed according to the nature and distribution of the variables for each paper. **Results**: Paper 1: Literature revision enabled conclusions regarding the association of menopause and diabetes with a worse lipid profile, including hypertriglyceridemia, lower levels of HDL-c and HDL_2-c, higher levels of HDL_3-c and small dense LDL-c. Postmenopausal diabetic women consist of the highest cardiovascular risk level. Paper 2: Comparing pre- and postmenopausal women categorized according to time since menopause [menopausal duration <2 years, 2-5.9 years, 6-9.9 years or ≥10 years (n=1916)], postmenopausal women had a worse lipid and lipoprotein subfraction profile and duration of menopause <2 years was independently associated with remnant lipoprotein cholesterol (TRL-c) [7.21 mg/dL (95% CI 3.59–10.84)] and smaller denser $VLDL_3$-c [2.43 mg/dL (95%CI 1.02–3.83)], but no associations of menopausal categories with HDL-c or LDL-c subfractions were found, when taking premenopausal women as reference. Paper 3: Comparing premenopausal ≤ or >45 years and postmenopausal women (n=2047), postmenopausal ones had the worst cardiometabolic risk profile. CAC>0 was found to be associated with TRL-c and dense LDL-c, but not with BCAA levels nor HOMA-IR. Postmenopausal women were about twice as likely to have CAC>0 than younger premenopausal ones [OR 2.37 (95%CI 1.17-4.81)]. **Discussion**: Our findings suggest

that natural menopause is associated with changes in lipoprotein fractions and subfractions (especially in the first 2 years post-menopause) and with calcium deposition in the coronary arteries independently of age and other risk factors, but not with BCAA nor HOMA-IR. Deep investigation on lipid profile and other biomarkers in women approaching to menopause is needed in order to identify cardiovascular risk, prevent cardiovascular outcomes and provide better health conditions.

Keywords: cardiovascular risk, atherosclerosis, menopause, biomarkers, coronary calcium score, lipoprotein subfractions, women, branched-chain amino acids.

CONTENTS

1 INTRODUCTION

1.1 Cardiovascular disease: a major health problem in women

Atherosclerotic cardiovascular disease (CVD) is the leading cause of death in both sexes in Brazil[1] and worldwide[2-4]. According to the World Health Organization (WHO), 18.6 million people died due to CVD in 2019, representing approximately 35% of all deaths globally, including 8.9 million women[5-8]. In Brazil, 381 thousand people (180 thousand women) died in the same time-period by CVD[9]. In Europe, the proportion of cardiovascular deaths reaches 51% of all causes in women, higher than in men[10] and this sex-difference becomes more pronounced after menopause[11,12]. Among younger women, CVD is still leader[13] with coronary artery disease (CAD) reaching the number one cause of death in both sexes in Brazil[14].

Although CVD occurs about 10 years later in women when compared to men, CAD is largely responsible for morbidity and mortality before and after menopause, being the main fatal event in women under 75 years of age[15]. Also, women are more susceptible than men to die after the first myocardial infarction (MI), particularly younger ones[16-19]. However, improvement in the in-hospital treatment of acute coronary syndrome has contributed to a 30% reduction in the lethality[20].

The symptomatology of acute coronary syndrome differs between sexes and atypical chest pain is more frequent in women. An explanation may lie in the different presentation and distribution of atherosclerosis: women have more diffuse atherosclerotic disease, affecting smaller arteries and have a predominance of less vulnerable atherosclerotic plaques. Before menopause, acute coronary events result from erosions in atherosclerotic plaques, while in men and postmenopausal women, it is observed a tendency of rupture of the atheroma with subsequent thrombosis[21,22].

Traditional cardiovascular risk factors are of great relevance for atherogenesis in both sexes[23-25], accounting for more than 75% of CVD. Sedentary lifestyle, inadequate diet, hypertension (HT) and smoking have the same impact in both sexes; however, the deleterious effect of type 2 diabetes mellitus (DM) is significantly higher in women. Evidence has indicated that cardiovascular risk of the diabetic woman is equal to that of non-diabetic men at the same age[26]. Dyslipidemia is one of the main risk factors regardless of gender, and it is worth highlighting that after menopause there

is a deleterious impact of the decrease in oestrogen levels on the circulating lipoproteins profile. High prevalence rates of dyslipidemia have been described in a Brazilian population[27,28], with hypercholesterolemia achieving ~50% of men and 42% of women of middle-older age, but low rates of awareness, treatment and control were reported in both sexes, confirming that we are still far from desirable recognition and control of a true risk factor for atherosclerosis and death.

Women are less evaluated in clinical studies, which often have higher male representativeness[29,30]. In addition, women compared to men of the same age group, are often under investigated, underdiagnosed and undertreated from a cardiovascular point of view due to the myth of being protected by oestrogen[31-33].

The contribution of Framingham studies to the identification of cardiovascular risk in populations is unquestionable; however, it is known that traditional risk factors do not explain the totality of cardiovascular events[24,25]. Thus, the search for novel factors that can improve CVD prediction, therapy and prognosis remains important, particularly exploring possible differences between sexes.

1.2 Women life cycle and cardiovascular risk

Since birth, several factors are present in a woman's life that contribute to increase cardiovascular risk. Birth weight – as a proxy for intrauterine conditions – seems to be an important health marker in the short- and long-terms in both sexes. Low birth weight has been associated with a higher risk of manifestations of metabolic syndrome (MS) in adulthood[34,35] and increased cardiovascular and all-cause mortality[36,37].

Menarche is a milestone of female sexual development. Association between age at menarche and cardiovascular risk factors in adult women was reported[38], being early menarche associated with higher occurrence of overweight, HT, DM and MS, including in Brazilians[39,40]. There is some evidence of an association between early menarche and cardiovascular events, cardiovascular mortality or any-cause of death in adulthood[41-43], although it is under debate how much this would be dependent of the body adiposity.

Polycystic ovary syndrome (PCOS) is the most frequent endocrine disease during reproductive ages. Since insulin resistance is involved in its pathophysiology[44,45], this syndrome is associated with several cardiovascular risk

factors[46,47]. Thus, women with manifestations of ovarian dysfunction (menstrual irregularity, anovulation, hirsutism, infertility) should be investigated for the presence of HT and metabolic disturbances. Meta-analyses found an association of polycystic ovary syndrome with inflammatory markers, atherosclerosis and increased risk for cardiovascular events[48-51].

Gestational intercurrences, such as pre-eclampsia and gestational diabetes are associated not only with both maternal and fetal complications during pregnancy, but also with risk of HT and type 2 DM. Recent meta-analysis has confirmed the association of pre-eclampsia with elevated risk for HT and stroke[52]. Regarding gestational diabetes, another meta-analysis found a seven-fold increase in the risk of DM compared to normoglycemic women during pregnancy[53]. Such disturbances of glucose metabolism in women's lives should contribute to increase the risk for CVD.

Menopause causes a great impact on cardiovascular risk in women, especially when it occurs early[54-56] as it deteriorates insulin sensitivity and induces metabolic abnormalities. Postmenopausal women tend to gain weight, become obese and are more predisposed to have central deposition of fat, including more visceral fat[57-60]. Several studies have proven an increase in the prevalence of cardiometabolic diseases in this stage of life, attributed in part to hypoestrogenism[61-63]. Oestrogen has recognized actions in the control of food intake, energy expenditure, and white adipose tissue distribution and contributes to energy balance and glucose homeostasis, partly explaining the obesity after menopause[64,65]. Also, follicle-stimulating hormone (FSH) seems to play a role in obesity, as emerging evidence shows that blocking FSH action reduces body fat, activates brown adipose tissue and increases thermogenesis[66]. Meanwhile, endogenous oestrogen may protect against atherosclerosis through facilitation of nitric oxide-mediated vasodilation, reduction of inflammatory activation and cell-adhesion, improvement in endothelial function, as well as decreasing low-density lipoprotein cholesterol (LDL-c) and increasing high-density lipoprotein cholesterol (HDL-c). Therefore, its gradual decline during the peri-menopause together with a relative hyperandrogenemia may influence negatively the risk for cardiovascular events[67]. Increased risk of CVD in women with shortened oestrogenic exposure during the life course was reported[68]. A systematic review and meta-analysis indicated a higher risk of CVD and mortality in women who experienced premature or early menopause[69], consisting in a risk factor for CVD[70], which could be at least partly due to oestrogen decline. Meanwhile, aging is also associated with increased risk. As in

physiological terms aging and menopause occur concomitantly, the individual impact of age and menopause in CVD is not completely understood.

In view of these considerations, there is still room to deepen the investigation of non-traditional cardiovascular risk factors and sex-related differences and to improve the understanding of peculiarities regarding the reproductive life of women in order to intervene more effectively[71].

1.3 Menopause: deleterious effects on circulating lipids

The loss of cardioprotection in postmenopausal women has been attributed, at least in part, to the decline in sex hormones[61,72], while the impact of FSH and androgens are yet to be determined[73,74]. Among the metabolic abnormalities in this phase of life, a deterioration of the lipid profile occurs becoming more atherogenic[75-80]. Usually, there is an increase in total cholesterol (TC), very low-density lipoprotein cholesterol (VLDL-c), LDL-c and triglycerides (TG), with a concomitant reduction in HDL-c. However, the HDL-c reduction after menopause has been recently challenged due to conflicting data[81-83]. Higher levels of apolipoprotein B (Apo-B) have also been described after menopause compared to women of reproductive age, but these changes in the lipid profile may not be explained only by aging[84,85]. Despite controversies, some studies have reported reductions in HDL_2, the large and buoyant HDL subfraction, which greatly contributes to the reverse cholesterol transport to the liver. Meanwhile, some authors equate menopause to MS since it manifests with insulin resistance, hypertriglyceridemia, low HDL-c and predominance of small dense LDL particles, which increase the risk of CVD in postmenopausal women[86].

Metabolic consequences of menopause led to the hypothesis that hormone replacement therapy (HRT) with oestrogen could bring benefits on the lipid profile and other factors, reducing cardiovascular risk in women. Several studies have shown changes in HRT-induced cholesterolemia, particularly reductions in TC and LDL-c and increased HDL-c, as well as other metabolic benefits[87-91]. The effects of HRT on lipoprotein subfractions were less investigated with some reports of increased HDL-c, mostly HDL_2, and reduction of lipoprotein (a), without changes in LDL subfractions[92-99]. A Brazilian study showed an increase in large VLDL particles in response to HRT[97], while reductions in lipoprotein (a) were observed by others[91]. There may be an increase in TG with this therapy, deserving particular attention to previously

hypertriglyceridemic women. It is known that the route of administration, choice and dose of medications influence the lipid pattern as well as the risk of adverse events[90,100].

Relevant clinical studies, such as the HERS (Heart and Estrogen/Progestin Replacement Study) in secondary prevention and WHI (Women's Health Initiative) in primary prevention failed to demonstrate cardiovascular benefits of HRT, although significant changes in lipids occurred, including a 10% increase in HDL-c and an 11% reduction in LDL-c[101-103]. It is noteworthy that these studies included elderly women, one decade after the beginning of menopause, when it may be too late to observe oestrogen-related cardioprotective effects. More recent meta-analyses provided conflicting data on the effect of HRT on cardiovascular outcomes and mortality[104-106]. Some authors suggest that initiating HRT right after menopause in younger women may reduce the incidence of CAD and all-cause mortality[104,105,107]. In this case, benefits should be attributed to oestrogen-induced vasodilation and decreases in platelet and inflammatory activation in a "healthy" endothelium. However, once endothelial dysfunction and atherosclerosis are present, the same oestrogen seems to have opposite effects, impairing vasodilation and promoting inflammation and instability of the atherosclerotic plaque[30,63]. This supports the theory of a "window of opportunity", which suggests a possible benefit of HRT initiated right after menopause due to clinical symptoms, and no advantage when initiated many years post-menopause or with stablished CVD, conditions in which oestrogen would do "more harm than good"[63].

Despite the potential of HRT in ameliorating the dyslipidemia of postmenopausal women, literature is conflicting and may depend in part on the type and route of administration[86-89]. To date, HRT has been shown to be ineffective in reducing the incidence of cardiovascular events and has not been indicated for primary and secondary prevention[103,108]. Reasons for the inconsistencies are not fully clarified. Currently, HRT is indicated for perimenopausal or recent menopausal women, aged < 60 years with vasomotor symptoms[109-111]. The risk-benefit of this therapy should be discussed individually, considering the risk for cancer and thromboembolic events[112].

Despite recognizing that the gradual decline in oestrogen levels during menopause period is accompanied by lipid abnormalities, few studies have investigated the association of menopause duration with lipid variables[82] or lipoprotein subfractions[113]. Also, the lipid profile under physiological conditions or interventions may differ across populations, and it is desirable to know this picture in different regions

of the globe. The existence of a large epidemiological study – the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) – has allowed the collection of data related to the cardiometabolic risk profile for the Brazilian population[114]. The baseline of ELSA-Brasil included 3,281 postmenopausal women, thus offering great opportunity to deepen knowledge in this stratus of the population[115].

1.4 Novel blood biomarkers of cardiovascular risk: lipoprotein subfractions, insulin resistance index and branched-chain amino acids

A considerable proportion of individuals with acute coronary syndrome has normal cholesterol levels determined by routine methods. The determination of the traditional lipid profile allows assessing the cholesterol content of TG and HDL, and estimating LDL-c by Friedewald formula, while VLDL-c is calculated by dividing TG by five. However, indirect LDL-c analysis has limitations since it suffers significant impact of TG levels. The VLDL-c calculation also estimates that all VLDL particles contain five times more TG than cholesterol, which is not always true. Other atherogenic lipoproteins, such as intermediate density lipoprotein (IDL) and lipoprotein (a) are not measured by those methods[116]. Thus, a direct quantification of lipoproteins and subfractions could offer advantages in refining risk stratification and therapeutic decision[117,118].

Several methods for assessing lipoprotein subfractions are available such as ultracentrifugation, lipoprotein electrophoresis and magnetic resonance imaging[118]. These methods are expensive and time-consuming, and mainly used for research purposes. An exception is the Vertical Auto Profile (VAP-II) technique, which is fast and accurate, being of particular interest for this study.

Although the predictive value of traditional lipid profile (TC, HDL-c, LDL-c and TG) for outcomes and utility for therapeutic monitoring, there is evidence that in-depth investigation of lipoprotein subfractions can improve identification of at-risk individuals. For instance, small dense LDL is more susceptible to oxidation, penetrates the endothelium more easily and form foam cells initiating atherogenesis[119-122]; among HDL particles, protective effect of large buoyant HDL_2 is greater than the others[123-125].

Small dense LDL has been consistently associated with cardiovascular risk[126-130] and CAD in both sexes[127,131,132]. Decreased HDL_2 values were reported in individuals with CVD of the same population[133-135], but in others HDL_3 was the main

responsible for the inverse relationship between HDL-c and CAD[136]. Different methods of subfractionation of lipoproteins may partially explain the contrasting results.

Remnant lipoprotein cholesterol (TRL-c) is an emerging cardiovascular risk factor[137,138] which is the sum of the small VLDL ($VLDL_3$-c) and IDL-c, triglyceride-enriched precursors to LDL. TRL-c levels have been associated with endothelial dysfunction, unstable carotid plaques, carotid intima-media thickness (cIMT), and CAD in several studies[139-141]. It is raised that TRL-c levels could be increased after menopause. Recently, in a cross-sectional analysis of the ATENA Project regarding 228 postmenopausal women, associations between VLDL-c and cIMT values and between high concentrations of IDL-c and TRL-c with the presence of carotid plaques were detected[138]. An analysis from the Framingham Heart Study confirmed an association of TRL-c with CVD in women, suggesting that TRL-c is an independent risk factor in this gender, and may be more informative than TG[142].

Apo-B is the main apolipoprotein present in atherogenic particles such as VLDL, IDL and LDL[143]. Its determination provides a good estimate of the number of these particles in blood[144]. This is particularly important when the proportion of small dense LDL is elevated. Apo-B target was not established for statin intervention studies, but several analyses suggested being not only risk marker but also a promising therapeutic target[145,146].

Insulin resistance mediates common metabolic abnormalities present in woman life, such as MS and PCOS[147-149]. Prevalence of MS increases after menopause, in part due to loss of protective role of oestrogens and relative increase in androgens, which results in changes to body fat distribution favoring abdominal obesity[150]. Visceral adipose tissue secretes several bioactive substances, as proinflammatory cytokines, reactive oxygen species, prothrombotic, and vasoconstrictor factors[150,151]. Some studies found independent associations of insulin resistance with coronary artery calcium (CAC)[152-154], but these findings are controversial[155,156]. These associations may not be independent of traditional risk factors, which makes it questionable if evaluation of insulin resistance could improve risk prediction[156]. How much the increase in body fat, its central deposition and insulin resistance contribute to lipid changes and cardiovascular risk in postmenopausal women is unknown.

Valine, leucine and isoleucine are branched-chain amino acids (BCAA) obtained from diet, that play a role in energy balance, insulin and glucose

homeostasis[157,158]. BCAA levels have been associated with several cardiovascular risk factors, such as obesity[159,160], insulin resistance[161], HT[158], DM[161-163], dyslipidemia[164] and non-alcoholic fatty liver disease[165]. Recent studies described effects of BCAA on endothelial function, inflammation and oxidative stress[166] and that they may predict DM[161,162,165,167,168] and CVD[169,170]. Whether BCAAs cause such metabolic disorders or just represent an insulin resistance marker requires investigation as well as their association with menopause.

Exploring gaps in the literature related to implications of BCAA, insulin resistance and lipoprotein subfractions in women is needed to evaluate their impact on the prediction of cardiovascular outcomes.

1.5 Structural markers of atherosclerosis: the role of coronary artery calcium score

Atherosclerosis is a multifactorial chronic inflammatory disease, which occurs in response to endothelial injury, affecting mainly the intima of medium and large caliber arteries. Main territories affected are coronary, carotid, femoral, popliteal and mesenteric arteries. Endothelial dysfunction leads to a disturbance in intimal permeability to plasma lipoproteins (mainly small dense LDL), which penetrate the subendothelial space[171-173]. LDL particles undergo oxidation, activate the immune system and adhesion molecules which result in monocyte attraction to subendothelial space. Monocytes differentiate into macrophages, capturing oxidized-LDL to form foam cells. They produce inflammatory cytokines and proteolytic enzymes, capable of degrading collagen and other local tissue components, promote migration and proliferation of smooth muscle cells to the intima, contributing to the formation of extracellular matrix and atheroma plaque[171-173]. During the damage and recovery occurs calcium deposition in the arterial walls. Depending on the inflammatory, lipid, cellular component and its fibrous cover, these plaques become more or less stable and susceptible to rupture, exposing highly thrombogenic lipid material, leading to thrombosis and clinical manifestations of atherosclerosis[171-173].

Invasive and non-invasive tests are available for an anatomical evaluation of atherosclerosis. Asymptomatic individuals are commonly investigated through non-invasive tests, with particular relevance to the coronary calcium score (coronary artery calcium scoring or CAC). This measurement is obtained using computed tomography,

being able to quantify calcium in the coronary arteries. Through the quantification of calcium, it is possible to infer risk of CAD, since coronary calcification occurs almost exclusively due to atherosclerotic disease. It is noteworthy that not every plaque is calcified, and calcification can correspond to up to 20% of the total volume of the atheroma[174]. Even so, there is a good correlation between lesions detected in coronary angiography or autopsy and CAC[175-179]. Since traditional cardiovascular risk scores, such as Framingham score[180] often underestimate the real individual risk of women, CAC may be an important tool that allows the assessment and reclassification of asymptomatic individuals, previously considered at low-to-moderate-risk and may impact therapeutic decision[174]. A previous study showed that individuals at intermediate level of Framingham risk score with CAC > 300 Agatston units had a cardiovascular event rate of 2.8% per year (or 28% in 10 years), which placed them in the high-risk group (> 20% in 10 years) according to risk scores[181].

CAC is an excellent method for the evaluation of subclinical atherosclerosis, particularly in asymptomatic individuals, and is considered an independent predictor of cardiovascular events and mortality[182-184]. The higher the amount of calcium in the coronary artery, the greater the chance of a cardiovascular event. Epidemiological studies have confirmed its prognostic value[185-189], based on the occurrence of major cardiovascular events in medium and long-term follow-up.

There is still no formal recommendation for the assessment and quantification of coronary calcium. Although guidelines of the Brazilian Society of Cardiology in 2006 suggested the determination of CAC in individuals at intermediate risk, the exam is not available on a large scale[178]. According to the Brazilian guidelines for dyslipidemias and prevention of atherosclerosis[173] and for cardiovascular prevention[190], in primary prevention level, values of CAC > 10 Agatston units suggest presence of subclinical atherosclerotic disease in DM, while CAC > 100 Agatston units identify individual at high risk[190], with individualized lipid targets for the prevention of complications. In diabetic individuals, CAC has also been associated with events and death[191-193]. Joint guidelines of the Brazilian Society of Cardiology, Brazilian Society of Diabetes and the Brazilian Society of Endocrinology and Metabology indicate chest tomography with CAC evaluation in individuals with DM at low-to-moderate cardiovascular risk for improvement in risk stratification[194]. None of the guidelines distinguish between these values (normal or altered) of CAC according to gender and age. Traditionally, men,

Caucasians and elderly have higher amounts of CAC, while lower values are expected for females in the same age group[195,196].

Studies have published normality and cutoff values for this examination in different populations, highlighting the relevant difference between ethnic groups and sexes[197-199]. More recently, in the ELSA-Brasil, a nomogram of CAC values was proposed for the Brazilian population without DM and at low risk[200], but there is no consensus regarding the optimal cutoff.

Since CAD is an important cause of morbidity and mortality in postmenopausal women, CAC could help in early detection of atherosclerosis. Novel risk biomarkers might have the potential to improve prediction of CVD in women. Furthermore, the detection of structural changes associated with these circulating markers would contribute to reinforce the value of these determinations. There are few studies with this approach, particularly in large population samples, such as the ELSA-Brasil.

1.6 The ELSA-Brasil

The ELSA-Brasil is the largest multicenter cohort study ever conducted in the country that aims to evaluate the incidence of CVD and metabolic diseases and their biological, behavioral, environmental, occupational, psychological and social risk factors in employees of six universities or research institutions in three geographic regions of the country (Federal University of Bahia, Federal University of Espírito Santo, Federal University of Minas Gerais, Federal University of Rio Grande do Sul, University of São Paulo and the Oswaldo Cruz Foundation). Details about design, sample and methods were previously described[201]. Its sample size has made possible to investigate differences between subgroups of individuals not only related to cardiometabolic risk, but also to other morbidities[201,202].

Research protocol and biobanks for storage of biological material were carefully developed[203] and logistical structuring of the participating centers was prepared with the creation of local laboratories[204] and reading centers for standardization of study measures (carotid ultrasound, retinography, echocardiography and other tests), as well as training of multidisciplinary teams, for homogeneous trial conduction in all centers.

A total of 15,105 public servants aged 35 to 74 years were included between 2008 and 2010, which composes the baseline of the ELSA-Brasil. After signing an

informed consent form[205] and participating in interviews to answer questionnaires, participants visited the research centers for clinical and complementary exams. Biological samples were collected and imaging tests performed. Interviews and annual telephone contacts are still being conducted, with calls for new interviews, clinical evaluation and collection of face-to-face exams every three years. The second wave of ELSA-Brasil interviews and exams was concluded in 2016.

The ELSA-Brasil data, although non-representative of the Brazilian population, provide opportunities to investigate morbidity and mortality of several conditions. The follow-up has allowed the evaluation of changes in health conditions over time[206]. Particularly for this thesis, this offers the unique opportunity to investigate novel aspects of the cardiometabolic risk in a big sample of women.

1.7 Rationale

CVD is the leading cause of mortality in both sexes, but the real risk to which women are exposed has been underestimated[2,31]. Women are often under investigated, underdiagnosed and undertreated for cardiovascular risk factors as they are presumed to be "protected" by the effect of oestrogen[29,30,72,207]. In addition, traditional risk scores also underestimate the risk of events in women[15]. It is possible that additional cardiometabolic markers, such as circulating lipoprotein subfractions, insulin resistance index and BCAA may improve the identification of cardiovascular risk, particularly in women in the transition to menopause.

There is insufficient data in literature addressing the impact of menopause and DM on lipoprotein subfractions and cardiovascular risk[75-80,208,209]. It is not fully clear how menopause, independent of age, contributes to the lipid and lipoprotein subfractions abnormalities found in postmenopausal women, nor if menopause itself has impact on arterial structural changes, such as calcium deposition in coronary arteries[81,82,207].

Brazilian data on cardiovascular risk in large samples of women are scarce[210]. ELSA-Brasil allows the examination of novel risk markers in a considerable portion of our population, under different levels of cardiovascular risk, as well as their association with arterial structural lesions. The longitudinal design also allows evaluating the ability of these markers in predicting cardiometabolic outcomes.

Given the relevance of CVD, gaps in knowledge about risk factors and markers especially in women and the unique opportunity of this investigation in the ELSA-Brasil, this thesis was developed.

1.8 Hypotheses

- Postmenopausal women have a worse cardiometabolic profile, including a more atherogenic lipid panel, insulin resistance and higher BCAA levels;
- Major lipid alterations occur right after menopause due to a decline in oestrogen levels, and after that the deterioration of lipid profile is attenuated;
- Menopause is associated with lipoprotein subfractions and BCAA, independently of traditional and non-traditional cardiometabolic risk factors;
- Menopause is associated with calcium deposition in the coronary arteries, independently of traditional and non-traditional cardiometabolic risk factors;
- There is an association between novel cardiometabolic markers (BCAA, insulin resistance index and circulating lipoprotein subfractions) and subclinical atherosclerosis (coronary artery calcium deposition).

2 OBJECTIVES

2.1 General objective

To analyze associations of blood biomarkers and subclinical atherosclerosis in pre- and postmenopausal women from the ELSA-Brasil.

2.2 Specific objectives

Considering that this thesis was designed to present papers for publications, specific objectives corresponded to the aim of each.

- Paper 1: To review the state of the art related to the impact of menopause and DM on traditional lipid profile, lipoprotein subfractions and cardiovascular risk;

- Paper 2: To examine whether the status and duration of menopause are associated with lipoprotein subfractions abnormalities in women from the ELSA-Brasil;

- Paper 3: To evaluate a broad cardiometabolic risk profile – including lipoprotein subfractions, insulin resistance index and BCAA – in women stratified by menopausal status and to investigate if menopause *per se* is associated with the presence of calcium in the coronary arteries.

In addition, the content of a panel presented in a scientific meeting was included, aiming at evaluating if menopause is independently associated with BCAA in women from the ELSA-Brasil.

3 METHODS

3.1 ELSA-Brasil

The ELSA-Brasil objectives and methodological features were previously reported. Briefly, its main interest regards to the development and progression of clinical and subclinical chronic diseases, particularly cardiovascular diseases and diabetes. At baseline (2008-2010), the study enrolled 15,105 civil servants from 5 universities and 1 research institute. Participants were submitted to interviews, clinical examination, biological samples' collection and several other procedures. The ELSA-Brasil protocol complied with Resolution 196/96ª, CNS Resolution 346/05 of multicenter projects and CNS Resolution 347/05 for the storage of biological materials. It was approved by institutional research ethics committees and by the National Research Ethics Commission of the National Health Council[205]. All participants signed an informed consent form (Attachment 1).

Exclusion criteria were pregnancy or recent puerperium (< 4 months), intention to stop working in the institution in the near future, severe cognitive or communication dysfunction and, if retired, living outside the metropolitan region[201]. The current study was based on data from the center located in the University of Sao Paulo which included 5,061 individuals (2,726 women).

After questionnaires were applied by trained interviewers, participants were invited to attend the centers for baseline evaluation, which consisted of anthropometry, clinical examination, blood and urine collections and imaging tests[211]. Numerous biochemical and hormonal determinations were performed, and aliquots were frozen in liquid nitrogen for later determinations. Participants underwent electrocardiogram, carotid ultrasound with measurement of the intima-media thickness, echocardiogram, measurement of pulse wave velocity, analysis of heart rate variability, hepatic ultrasonography and retinography[201]. A subsample from the Sao Paulo center had also novel inflammatory markers, circulating lipoprotein subfractions and branched-chain amino acids determined, and was submitted to computed tomography of the chest for quantification of calcium in coronary arteries.

The present study is a cross-sectional analysis of ELSA-Brasil baseline data.

3.2 Variables of interest

3.2.1 Questionnaires

Standardized questionnaires included questions about sociodemographic characteristics, previous personal and family medical history, occupational and reproductive history, mental health and medication use, dietary and lifestyle habits, among others[205]. Skin color (self-reported race), smoking, alcohol use, leisure physical activity and education level were self-reported.

For the purposes of this study, variables of interest included aspects related to women's health, such as previous or current use of hormone replacement, age at and duration of menopause, type of menopause (Attachment 2).

Self-reported use of medications was checked by the medical team at the visit to the research center. Medications taken in the last two weeks prior to the interview were recorded, particularly regarding the use of medications to control blood pressure, glucose and lipid levels[212]. These variables and female hormone replacement agents were of interest for the present analysis.

3.2.2 Clinical examination

Anthropometric measurements included weight, height, seated height and waist, hip and neck circumferences, collected according to standard equipment and techniques[211]. Electronic scales were used to obtain body weight and fixed rigid stadiometer was used to assess height. Individuals were weighed in light clothing, without shoes. Body mass index (BMI) was calculated by weight (kg) divided by squared height (m). Circumference measurements were obtained using an inelastic tape. Waist circumference was measured with the individual standing still, at the point of the upper edge of the iliac crest perpendicular to the axial axis of the body[213].

Blood pressure (BP) was measured with the individual in a sitting position after a five-minute rest, in a quiet environment with controlled temperature, three consecutive times, at intervals of one-minute each, using a validated oscilometric device (Omron HEM 705CPINT)[214]. Means of the latest two measurements for systolic and diastolic BP were considered for the present analysis.

3.2.3 Laboratory exams and novel circulating biomarkers

Participants had blood collected after fasting for 10 to 14 hours. Samples were processed and part (42 aliquots of 0.5 mL per participant) was stored at -80°C for future analysis. Glucose, lipids and other parameters were determined immediately. They were submitted to a two-hour oral glucose tolerance test (individuals without known DM) or standardized meal test (individuals known to be diabetic). Plasma glucose was measured by the hexokinase method (ADVIA Chemistry; Siemens, Deerfield, Illinois, USA). Glycated hemoglobin was determined by high pressure liquid chromatography (Variant; Bio Rad, Hercules, California, USA), and insulin by immunoenzymatic assay (Centaur; Siemens, Deerfield, Illinois, USA).

An extended lipid panel (complete lipid profile and lipoprotein subfractions) was determined by the VAP-II (Atherotech, Birmingham, USA), a single vertical density gradient ultracentrifugation method, which simultaneously measures cholesterol concentrations of lipoprotein classes and subfractions after their separation using small amount of plasma[116,215,216]. Five lipoprotein classes: HDL, LDL-r [real LDL], VLDL, IDL, and lipoprotein (a) [Lp(a)], and subfractions, such as HDL_2 (larger buoyant) and HDL_3 (smaller dense), LDL_1 through LDL_4, and $VLDL_1$ through $VLDL_3$ (smaller denser) were identified. VAP-II also permits characterization of TRL-c, consisting on lipoprotein remnants ($VLDL_3$ + IDL) beyond real and direct evaluation of low-density lipoprotein cholesterol [LDLr-c, which corresponds to total LDL-c minus lipoprotein (a) and IDL-c]. To characterize the buoyancy of LDL, subfractions were classified into large and buoyant (LDL_1 and LDL_2) or smaller and denser particles (LDL_3 and LDL_4). VAP-II allows the identification of total cholesterol from the sum of cholesterol from all particles and non-HDL cholesterol (corresponding to the sum of LDLr-c, VLDL-c, IDL-c and Lp (a)-c or $LDL_{1+2+3+4}$)[116,215-217]. Variability coefficients for lipoprotein subfraction measurements were previously reported[116,215,216].

HOMA-IR was calculated by the equation: insulin (mU/ml) * glucose (mmol/L) / 22.5 and used as a marker of insulin resistance[218]. Branched-chain amino acids, as leucine, isoleucine and valine were determined using magnetic resonance spectroscopy. Total BCAA corresponds to the sum of the three amino acids.

3.2.4 Coronary artery calcium score

A subsample of 4,077 individuals from the São Paulo research center was submitted to multi-detector computed tomography to assess the presence of coronary calcium. This non-invasive examination estimates the presence and quantifies calcium in the coronary arteries, constituting a reliable method for the evaluation of subclinical atherosclerosis. CAC determination is based on a noncontrasted acquisition of a series of axial sections with 3 mm thickness covering the entire length of the heart. The images were acquired in a synchronized way to the electrocardiogram signal[174].

Tests were performed on a Brilliance Multi-slice tomography with 64 detectors (Philips Brilliance 64; Philips Healthcare, Best, Netherlands). The field of view was delimited to include the entire heart from the bifurcation of the pulmonary arteries to the cardiac *ictus* during an exhale pause. Test configurations included radiation dose of 120 Kv and mA adjusted for BMI and prospective acquisition of single-phase images in the middle of the cardiac cycle diastole (70%) and 2.5 mm collimation, 400 ms gantry rotation and reconstruction with standard filter. The images were analyzed using the Software Brilliance Workspace[200].

Calcification was defined as a hyperattenuating lesion with signal intensity above 130 Hounsfield units (HU) and area $\geq$ 3 adjacent pixels (at least 1 mm^2). The CAC calculation was obtained by an experienced cardiologist using semi-automatic software (Calcium Scoring, Philips Workstation*)* from the weighted sum of densities above 130 HU[174,219]. The absolute results of the Agatston score were evaluated in a categorized manner, according to the absence or presence of any amount of calcium detected in the coronary arteries (CAC = zero or CAC > zero, respectively), additionally we categorized as CAC above or below 100.

3.3 Criteria

3.3.1 Menopause

Menopause was diagnosed in women without menses for at least 12 months. Peri-menopause was defined as cessation of menstrual cycles from six to 11.9 months. Duration of menopause was calculated subtracting age at menopause from age at

baseline in years. Only natural causes of menopause were evaluated. Menopause ≤ 40 years of age was not included in this analysis.

3.3.2 Demographics and lifestyle

High schooling was defined by at least 11 years of education. Physically active individuals at leisure were considered when ≥ 150 minutes per week of moderate intensity or ≥ 75 minutes per week of high intensity physical activity were reported.

3.3.3 Diabetes and pre-diabetes

American Diabetes Association criteria were used for the diagnosis of glucose tolerance categories[220]. Impaired fasting plasma glucose (IFG) was diagnosed when values were ≥ 100 and < 126 mg/dL, with 2-hour post-glucose load < 140 mg/dL during an oral glucose tolerance test; impaired glucose tolerance (IGT) was defined by fasting plasma glucose (FPG) < 126 mg/dL and 2-hour post-glucose load ≥ 140 mg/dL and < 200 mg/dL. Prediabetes included IFG and IGT categories as well as glycated hemoglobin (HbA1c) between 5.7 and 6.4%. Diabetes was defined by fasting plasma glucose ≥ 126 mg/dL, 2-hour post-glucose load ≥ 200 mg/dL, or HbA1c ≥ 6.5%. Self-reported diabetes under treatment with antidiabetic drugs were also considered in this latter category[221].

3.3.4 Hypertension

Hypertension was diagnosed when mean levels of systolic BP measurements ≥ 140 mmHg, diastolic BP ≥ 90 mmHg, or use of antihypertensive drugs[222].

3.3.5 Anthropometric status[223]

The individual was classified as overweight when she had a BMI ≥ 25 until 29.9 kg/m^2, and obese when BMI ≥ 30 kg/m^2.

3.3.6 Metabolic syndrome

Metabolic syndrome was defined according to the consensus of international scientific societies[224,225]. Any three of the following criteria assure the diagnosis:

- Waist circumference ≥ 88 cm for women;
- Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg (or use of antihypertensive drugs);
- FPG ≥ 100 mg/dL (or previous diagnosis of diabetes);
- Triglycerides ≥ 150 mg/dL (or use of lipid-lowering drugs);
- HDL-c < 50 mg/dL for women (or use of lipid-lowering drugs).

3.3.7 Cardiovascular disease

CVD was defined as a positive history of any of the following cardiovascular events: acute myocardial infarction, angina pectoris, transient ischemic attack, stroke, and peripheral arterial disease.

3.4 Statistical analysis

Continuous variables were expressed by measures of central tendency (means and medians) and dispersion (standard deviation and interquartile interval) according to the distribution of the data. The categorical ones were described in absolute number and relative frequency. Normality of continuous variables was tested by Shapiro Wilk test. Due to the non-normal distribution provided for certain variables, it was necessary to employ transformations in order to achieve normality distribution.

Student t test and Mann-Whitney analysis were applied for comparisons of continuous variables between two groups; ANOVA with Bonferroni correction or Kruskal-Wallis test were performed when three or more subgroups of individuals were compared. Frequencies were compared by the Chi-square test. Univariate and multiple regression analysis (linear and logistic) were used when indicated. Subgroup and sensitivity analyses were performed when indicated. Detailed statistical approach for each objective are presented in each paper and in the poster.

The analyses were carried out using Stata version 12 (Stata-Corp LLC, College Station, Texas, USA). A p-value < 0.05 was considered significant.

4 RESULTS

This chapter of the thesis has been replaced by three papers published in different journals and one meeting presentation in a national scientific congress and published in its annal as abstract format. Therefore, content is arranged according to the requirements of each journal.

4.1 PAPER 1

Impact of menopause and diabetes on atherogenic lipid profile: Is it worth to analyse lipoprotein subfractions to assess cardiovascular risk in women?

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Abstract

Cardiovascular disease (CVD) is the leading cause of death in women at advanced age, who are affected a decade later compared to men. Cardiovascular risk factors in women are not properly investigated nor treated and events are frequently lethal. Both menopause and type 2 diabetes substantially increase cardiovascular risk in the female sex, promoting modifications on lipid metabolism and circulating lipoproteins. Lipoprotein subfractions suffer a shift after menopause towards a more atherogenic lipid profile, consisted of hypertriglyceridemia, lower levels of both total high density lipoprotein (HDL) and its subfraction HDL_2, but also higher levels of HDL_3 and small low-density lipoprotein (LDL) particles. This review discusses the impact of diabetes and menopause to the lipid profile, challenges in lipoprotein subfractions determination and their potential contribution to the cardiovascular risk assessment in women. It is still unclear whether lipoprotein subfraction changes are a major driver of cardiometabolic risk and which modifications are predominant. Prospective trials with larger samples, methodological standardizations and pharmacological approaches are needed to clarify the role of lipoprotein subfractions determination on cardiovascular risk prediction and intervention planning in postmenopausal women, with or without DM.

Keywords: menopause, women, cardiovascular risk, lipoprotein subfractions, diabetes mellitus

Introduction

Cardiovascular disease (CVD), particularly coronary artery disease (CAD)[1], is a major cause of death in women, who develop it about ten years later then men[2]. Traditional risk factors are present at a high frequency in individuals with CAD but are lacking in a not negligible proportion[3]. Risk calculators usually underestimate the real CVD risk in women and their CAD episodes are frequently fatal[4-6].

Hypercholesterolemia is the major driven cause for CVD in both sexes[7,8] and its treatment has been associated with significant reductions in morbidity and mortality[9-11]. Postmenopausal women tend to deteriorate lipid profile that becomes more atherogenic than their premenopausal counterpart[12,13]. After menopause, total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) usually increase, and these changes are accompanied by a decrease in high-density lipoprotein cholesterol (HDL-c) and an increase in triglycerides (TG)[14,15]. In addition to these major lipid abnormalities, also modifications in size and density of these lipoprotein particles are expected to happen after the loss of ovarian hormonal production[16-19]. This partially explains the increased cardiovascular risk in postmenopausal women[2,20], particularly among those with an earlier onset of menopause[21].

Hyperglycemia contributes to the elevation of cardiovascular risk of populations. Increasing prevalence rates of type 2 diabetes mellitus (DM) have been attributed to aging, modern lifestyle and obesity epidemic, which predisposes to several metabolic disturbances linked by the insulin resistance[22-24]. In men and women with DM a typical dyslipidemia was described, characterized by hypertriglyceridemia, low levels of HDL-c and increased proportion of small-dense LDL particles, known to be more prone to oxidation[25-27]. Elevated glucose levels have also been associated with dysfunctional lipoprotein subfractions, contributing to a more atherogenic lipid profile in both sexes[28-29]. Despite sharing these lipid abnormalities with the male sex, the diabetic woman has a more aggressive form of CAD and is more susceptible to death from CVD, mainly coronary events[30-31], suggesting that her lipid profile should be even more deleterious. These observations indicate the need of additional quantitative and/or qualitative laboratory procedures – such as determinations of lipoproteins subfractions – to clarify some sex-related differences.

To date, there is paucity of data describing lipoprotein subfractions in postmenopausal diabetic women[1,32-33]. It is unclear whether accurate analysis of subfractions of the several lipoproteins could be associated with improved identification

of women at higher risk, before and after menopause, with or without DM. In addition, menopausal hormonal replacement therapy (HRT) may impose unique risk to women. We review and discuss the differences in cardiovascular risk and lipoprotein subfractions in pre- and postmenopausal women and in diabetic ones. Understanding sex-related differences in lipid metabolism, as well as the impact of menopause and DM in women, may contribute to improve cardiovascular risk assessment in women. The keywords *postmenopausal and menopause, lipoprotein, lipoprotein subclass and subfractions, type 2 diabetes, analysis, cardiovascular risk* were selected for search in PubMed database, from 1980 to 2017, in English and/or Portuguese language.

Cardiovascular risk in women

CAD and stroke have been the leading causes of death in both sexes accounting for 25.1% of the total mortality[34]. Even in the younger women, high mortality rates following myocardial infarction (MI) have been reported[35]. In recent years, improvements in hospital treatment[5] have contributed to a 30% decrease in the number of women dying from cardiovascular events in USA[36] although these still cause more deaths than all other causes combined. Estimates of cardiovascular risk and clinical trials are commonly based on unbalanced samples and selection bias has limited gender comparisons of outcomes. Female sex is notably under-represented in clinical trials which frequently have a predominance of the men[37]. Also, there is evidence that women are undertreated and have cardiovascular risk factors less controlled compared to men[38], specially the diabetic population[39].

Apart from methodological concerns, atherogenesis *per se* could affect men and women distinctly. It is known that atherosclerosis involves inflammatory and thrombotic processes. In premenopausal women, smaller lipid cores, less calcium, and fewer thin-capped atheromas were described, and estrogen-related anti-inflammatory effects on atherosclerotic plaques seem to contribute to their stabilization[40]. The plaque in women is shown to have less inflammatory components than in men which can implicate in slower development of vulnerable plaques. Young women with acute coronary syndromes often present plaque erosion, while men and older women frequently show the classical pattern of ruptured plaque followed by thrombosis[40]. In carotid arteries, lower atheroma burden and more stable plaques were described in women. Despite the ability of estrogen to stabilize the atheroma, prothrombotic effects of this hormone

were reported. The reasons for sex-related differences in the development and progression of atherosclerosis are not completely understood[40-43].

Several scores have been proposed for cardiovascular risk assessment and the Framingham risk score is one of the mostly used[44-47]. It has been recognized that the Framingham risk score underestimates risk in women since those with subclinical atherosclerosis are often classified as at low risk[48]. In an update of this score, it was proposed that women should be classified as ‘‘high risk’’, ‘‘at risk’’ and ‘‘at ideal cardiovascular health’’. High-risk was defined by clinical evidence of CAD, peripheral artery disease and abdominal aortic aneurysm, or the presence of coronary risk equivalents, such as chronic kidney disease and DM, together with a 10-year predicted cardiovascular risk of ≥ 10%. At-risk women are those with at least one major risk factor [cigarette smoking, hypertension, dyslipidemia, obesity, poor diet, physical inactivity, family history of premature CVD, metabolic syndrome, evidence of advanced subclinical atherosclerosis (coronary calcification, carotid plaque, or increased carotid intima-media thickness), poor exercise capacity on treadmill test and/or abnormal heart rate recovery after stopping exercise, systemic autoimmune collagen-vascular disease (lupus or rheumatoid arthritis), history of preeclampsia, gestational diabetes, or pregnancy-induced hypertension]. “Ideal cardiovascular health” was defined by adequate total cholesterol and blood pressure levels, fasting plasma glucose and body mass index, with heart-healthy behaviours including healthy diet, smoking abstinence and regular physical activity[48-49].

CVD incidence in premenopausal women is significantly lower than men at the same age (1 woman : 3-10 men), but increases to an extent that the rate becomes similar at the age 65 years and higher by the age 75 years[50]. Among the epidemiological studies that examined cardiovascular risk in women, the Nurses’ Health Study included one of the biggest sample[51]. This reported that 82% of coronary events could be attributed to the absence of a low-risk lifestyle. The INTERHEART study[7] revealed that nine risk factors accounted for 94% of the population attributable risk, including smoking, dyslipidemia, hypertension, DM, abdominal obesity, physical inactivity, low daily fruit and vegetable consumption, alcohol overconsumption, and a low psychosocial index (depression, locus of control, perceived stress, and life events). These are shown to be important risk factors for the development of CVD in both sexes.

In clinical settings, health care professionals commonly underestimate cardiovascular risk in women who are not as properly treated for CVD as men[48,52].

Comparing sexes after MI, in every age, women are more likely to have a history of hypertension; however, concerning other risk factors, sex differences exist only before the age of 55, when women were more likely to have medical insurance, history of DM, heart failure or stroke, and higher Killip class on hospital admission[5]. Clinical symptoms of CAD also differ between sexes; men express classical symptoms such as angina, with pressure or squeezing to the chest, which can extend to the arms. Meanwhile, women tend to feel sharp, burning chest pain that can extend to neck, jaw, throat, abdomen or back and more frequently have atypical symptoms[53].

Sex differences could be raised concerning the efficacy of lipid lowering treatment. Statins have long been associated with reductions in total cholesterol, LDL-c as well as some increase in HDL-c concentration. Several meta-analyses reported significant reductions in cardiovascular outcomes with statins use for each 1 mmol/L decrease in plasma LDL-c[9,10]. Accumulated evidence has consistently shown that statins are equally effective in both sexes in the control of dyslipidemia and reduction of cardiovascular morbidity and mortality[54-55].

The deleterious impact of DM in cardiovascular morbidity and mortality is greater in women compared to men. In 2011, DM was responsible for 281,000 deaths in men and 317,000 in women, the majority from cardiovascular causes[56]. Despite being a strong risk factor for both sexes, a greater impact in mortality from CAD is seen in women than in men[57]. Its presence almost eliminated the sex-related difference in cardiovascular morbidity and mortality, approximating the risk level of the diabetic woman to the non-diabetic men[58]. Therefore, the diabetic woman needs special attention and optimized treatment of comorbidities to control risk factors and to decrease excessive cardiovascular mortality.

CVD is a major issue for women's health most predominantly at older age, although the younger women have a higher chance of fatality following coronary events. Despite lower absolute incidence compared to men, high mortality rates indicate the need to improve risk prediction, early diagnosis and adequate treatment of risk factors and comorbidities to enhance women quality of life and survival. The increased mortality rates conferred by presence of DM are more prominent in the female sex. A careful analysis of these disparities between sexes is necessary.

Lipoprotein subfractions: determinations and potentialities

Routinely, lipoproteins have been determined according to their molecular density (VLDL, LDL, and HDL) to assess cardiovascular risk. They have been classified by their size, charge, function, lipid core and apolipoprotein composition, and the resulting subgroups are called lipoprotein subfractions[59-60].

A considerable proportion of individuals that suffer from cardiovascular events shows either few or none of the traditional risk factors[59,61]. The assessment of lipoprotein subfractions and apolipoproteins (apo) represents a way to improve the cardiovascular risk prediction; in addition, they may enhance the accuracy of atherosclerosis detection, assist in treatment selection, and be useful for counselling first-degree relatives of patients with atherosclerosis[62].

Numerous methods for lipoprotein subfractions determination have been described, mostly for research purposes[62], such as analytic ultracentrifugation, vertical auto profile-II (VAP-II), density gradient ultracentrifugation, gradient gel electrophoresis, nuclear magnetic resonance (NMR) spectroscopy, immunoaffinity chromatography, 2-dimensional gel electrophoresis and ion-mobility analysis (Table 1). Heterogeneous techniques and nomenclature of lipoprotein subfractions limit data interpretation and study comparisons[60].

Table 1: Summary of main advantages and disadvantages of methods for lipoprotein subfractions determination.

Method	Advantages	Disadvantages
Analytic ultracentrifugation	Precision and reproducibility	Unfeasible for clinical practice, due to low availability high cost and time consuming
Vertical auto profile-II	Simple procedures and high sensitivity	Low correlation to NMR and electrophoresis
Gradient gel electrophoresis	Determination of LDL and HDL size distribution directly from blood samples	Accuracy depends on correct standards and quality control Provides only the size of predominant species or average size
Linear polyacrylamide gel	Useful for clinical labs since it is simple and fast	High cost

Nuclear magnetic resonance spectroscopy	No need of physically separation of the subfractions and fast procedure	Dependent of mathematical assumptions
Immunoaffinity chromatography / Ion mobility	Ability to isolate 2 HDL subfractions	Low availability and scarce data regarding efficiency
References: 59 - 78		

Analytic ultracentrifugation has been considered the gold standard of lipoprotein subclass analyses due to its precision and reproducibility, and used for validation of other techniques, but it is unfeasible for clinical practice[62]. This method is based on the lipoprotein ability to float when exposed to high gravitational forces. According to flotation rates, four LDL subfractions are grouped whose densities range from 1.025 to 1.060 g/mL[63].

The VAP-II uses a non-segmented continuous flow analyser for the enzymatic analysis of cholesterol in lipoprotein classes, allowing a profile analysis with only 40 µl of plasma[64-65]. Five subclasses for HDL, four for Lp(a), four for LDL, two for IDL and three for VLDL can be identified. The absorbance curve provides the density distribution of lipoprotein classes and subclasses in the centrifuge tube[66]. The procedures are simple and sensitivity for the lipoprotein density classification is high. However, some studies have shown low correlation of VAP with NMR and electrophoresis[67].

The gradient gel electrophoresis determines LDL and HDL size distribution directly from blood samples. According to major peaks size and percent distribution, seven LDL subclasses, from larger buoyant LDL_1, LDL_{2a} and LDL_{2b} to the smaller and less dense LDL_{3a}, LDL_{3b}, LDL_{4a} and LDL_{4b} can be detected[62]. Also, five HDL subclasses, ranging from small dense HDL_{3c}, HDL_{3b}, and HDL_{3a} to larger HDL_{2a} and HDL_{2b}, can be determined. This method does not provide concentrations but the size of predominant species or average size[68]. The two-dimensional gel electrophoresis improved the ability of the gradient gel electrophoresis in recognizing new HDL subfractions: α1, α2, and α3, with sizes of 11.2, 9.51, and 7.12 nm, respectively[69]. Its use has been limited to specialized labs[62].

Lipoproteins subfractions determination can also be based on size and charge using linear polyacrylamide gel.The technique is simple and fast but expensive[70-71].

NMR spectroscopy allows quantification of lipoprotein subfractions given that each lipoprotein particle in plasma has its own characteristic lipid methyl signal. NMR uses a library of lipoprotein spectra reference in a linear least-square fitting computer program[72]. From the shape of the composite plasma methyl signal, the program computes the subclass signal amplitudes. Particle sizes derive from the sum of the diameter of each subclass multiplied by its relative mass percentage[60,62]. There is no need to physically separate the subfractions, which is a major advantage of the method. Lipoprotein subfractions identified are[72]:

- for VLDL: large VLDL/chylomicrons, medium VLDL, small VLDL
- for LDL, IDL, large LDL, medium small LDL, very small LDL
- for HDL, large HDL, medium HDL, small HDL

Immunoaffinity chromatography and the ion-mobility have been used for research purposes. The former is able to isolate 2 HDL subfractions through their content of apolipoprotein A-I and apolipoprotein A-II[62], while the latter determines concentrations of lipoprotein subfractions based on gas-phase differential electric mobility[60,73].

The availability of several techniques and different parameters to express lipoprotein subfractions (concentrations, percent distribution of the HDL subclasses relative to the total or by average particle diameter) should explain part of the contrasting results on their association with CVD. The most consistent finding is the association of gradient gel electrophoresis-determined HDL subfractions[74]. The amount of large HDL identified by NMR has been correlated with the gradient gel electrophoresis HDL_{2b} results, but other NMR HDL components have shown weaker correlations[74].

Regarding LDL phenotype, substantial agreements among gradient gel electrophoresis, VAP, NMR, and ion-mobility have been described[75]. Using any of these four methods, association of small, dense LDL with coronary atherosclerosis progression was demonstrated[76]. Furthermore, gradient gel electrophoresis, NMR and ion-mobility confirmed that the associations were independent of standard lipid measurements. A recent study on the comparison of ultracentrifugation, a novel electrophoretic method and two independent methods of NMR indicated ultracentrifugation as the most precise method for LDL particle determination with the lowest coefficient of variation. The electrophoresis showed a close precision, whereas NMR showed the highest coefficient of variation[77].

Meanwhile, lipoproteins are heterogeneous even within each subclass and differ not only in size, charge and density, but also in their lipid and protein composition. Lipidomics and proteomics use mass spectrometry to identify and quantify lipid and protein content in a cell, tissue or organ, respectively[78-80]. These methods involve the use of complex technology in several research settings and may even help determine typical and abnormal lipoprotein composition[81-82]. Changes in key components of lipoproteins under unusual circumstances, such as chronic inflammation and subclinical atherosclerosis, cause their remodelling, affect their functionality and contribute to the atherosclerotic process[83-85].

Evidence that certain lipoprotein subfractions enhance atherogenesis and increase cardiovascular risk emphasizes the importance of their determinations to improve the identification of those at higher risk[86-87]. Determination methods differ by their basic principles, technology, complexity and accuracy. Such diversity limits to compare results and to assure the real contribution for the improvement in cardiovascular risk prediction.

Also, apolipoprotein determination has shown to improve cardiovascular risk assessment. Apo B100 concentration reflects the atherogenic lipoproteins (VLDL, IDL and LDL), while apo A-I has been considered a HDL surrogate. Apo B-to-apo A-I ratio provides a balance between the atherogenic and anti-atherogenic cholesterol particles and its usefulness as a predictor of cardiovascular events was demonstrated[88-90]. Lower apo B-to-apo A-I ratio was reported in premenopausal compared to postmenopausal women and men[91]. Lipoprotein (a) has a similar structure to LDL, containing one apo-B molecule combined with an apo (a), known to diminish plasminogen activation and fibrin degradation, favouring thrombosis. It has been considered an independent cardiovascular risk factor[92-93]. There is no gender-related differences in lipoprotein (a) concentration, and a predictive value was observed only in men[94].

Standardization and cost reduction will be necessary for lipoprotein subfractions and apolipoprotein determinations reaching the clinical practice.

Lipid changes following menopause and hormonal replacement therapy

Women experience modifications on lipid profile and metabolism from child to adult life, during pregnancies and following menopause. Aging itself is associated with an increase in LDL-c, in part due to a reduction in its catabolism by the liver. However,

the higher levels of total cholesterol, LDL-c and apo-B found after menopause compared to premenopausal ones are not completely explained by aging[95]. A cross-sectional analysis of the Framingham Offspring Study[15], including 1,597 women and 1,533 men, showed higher LDL-c concentration in male sex, as expected. Additionally, in the postmenopausal compared to premenopausal women, increased LDL-c concentration was maintained after adjustments for age and several confounders.

Smaller denser Apo-B rich LDL particles are more frequent in postmenopausal women, while larger and buoyant LDL are decreased[17]. It is estimated that 14-30% of postmenopausal women have predominance of small dense LDL particles in contrast to only 5-7% in premenopausal counterpart[17,96]. Lower HDL-c/total cholesterol and apo-AI/apo-B ratios[17,96], as well as direct association of small LDL-c particles with TG levels, and inverse associations of HDL-c and Apo-AI with Apo-B were reported following menopause[96]. Increased TG rich lipoproteins are associated with higher proportions of small dense LDL. In postmenopausal period, affinity to the hepatic LDL receptor is reduced in small dense LDL-c that is more susceptible to oxidation, transendothelial transport and deposition in artery wall. This LDL subfraction has long been considered by the scientific community as an independent risk factor for CVD, although this is still controversial as some studies have failed to determine this association after several adjustments for confounding factors[97-106]. Small dense LDL is also considered an independent risk factor for the development of type 2 DM[107], particularly in women[108]. Meanwhile, large HDL particles – also named HDL_2 – play an essential role on reverse cholesterol transport and are considered cardioprotective[67,86,109]. In postmenopausal women, the latter seemed to be diminished, with a predominance of cholesterol-depleted smaller HDL particles[19,11-114]. These are not able to adequately transport cholesterol esters back to the liver, contributing to increased cholesterol concentrations in the blood.

In men, low levels of HDL_2 particles (larger buoyant particles) have been associated with CAD indicating worse and diffuse lesions[115]. A cross-sectional analysis of more than 1,000 women in UK showed that postmenopausal ones tended to decrease their total HDL-c concentrations together with a decrease in HDL_2, without any difference in the HDL_3 concentrations when compared to the premenopausal women[19]. Similar reductions in HDL_2 were reported in high-risk postmenopausal women with untreated breast cancer[116]. Other studies have confirmed lower levels of

large HDL_2 particles following menopause suggesting that HDL_2 concentrations might be influenced by the drop in female hormonal levels.

The role of sex hormones on lipid metabolism is supported by the demonstration that estrogenic therapy prevents decrease in LDL-c and increases in TG and VLDL-c concentration after menopause. Mechanisms by which female hormones interfere on lipid metabolism have been largely investigated. Estrogen is shown to increase both LDL receptor population in the liver, together with hepatic production of TG rich lipoproteins. Some authors have proposed that the lack of estrogen after menopause contributes to hypertriglyceridemia, low HDL-c and a predominance of small dense LDL particles, like the abnormalities seen in the metabolic syndrome[117]. This lipid profile is found in 15-25% of postmenopausal women and might in part be responsible for their increased cardiovascular risk[117]. The Very Large Lipid Database (VLDL 10B) study[118], in which more than a million-people had their lipoprotein subfractions measured by density gradient ultracentrifugation, supported that, after middle age, women presented a shift towards a more atherogenic lipid profile.

These findings have raised questions about the utility of hormonal replacement therapy (HRT) to prevent lipid metabolism abnormalities following menopause which could help in the prevention of CVD. Several clinical trials were conducted to investigate the effects of different schemes of HRT on the lipid profile after menopause[119-122], but those using accurate methods for the determination of subfractions of lipoproteins are less numerous[123-124]. In one study, 38 postmenopausal Brazilian women with formal indication for HRT were treated with continuous doses of 0.625 mg of conjugated equine estrogen (CEE) with (if they had uterus) or without 2.5 mg of medroxiprogesterone for 12 weeks. Lipoprotein subfractions were measured using an NMR spectroscopy at baseline and after treatment. Significant increases in larger VLDL and HDL particles, together with a decrease in the smaller HDL and VLDL particles were observed, but treatment did not induce significant differences in LDL subfractions[125].

Another trial evaluated the effect of estrogen alone or combined with medroxyprogesterone (1 mg of 17β-estradiol and/or 0.625 mg of CEE) for 3 months in 43 postmenopausal women[126]. Combined therapy resulted in a significant increase in the proportion of bigger HDL particles in circulation, also diminishing the absolute amount of smaller HDL particles. Other trials with estrogen alone in surgically induced menopause have shown a tendency for an increase in HDL and HDL_2, but a variety of

results were found for LDL particles[120-123]. Different HRT regimens, such as natural *vs* synthetic, transdermal *vs* oral, cyclic *vs* continuous, different progestogens or estrogens and doses have also been tested, but modifications in both lipid and lipoprotein subclasses are inconsistent across trials.

An interesting analysis of 243 postmenopausal women from the Healthy Women Study confirmed higher levels of large HDL particles measured by NMR spectroscopy between HRT users as compared to nonusers[127]. Despite lower levels of LDL-c, there were no differences in LDL subclasses or in coronary artery calcification (CAC) between the groups. As expected, having detectable CAC was associated with worse traditional lipid profile and increased atherogenic subfractions. Although an HRT-dependent shift on the proportions of lipoprotein subfractions could be expected in postmenopausal women, trials have not shown any benefit in cardiovascular morbidity or mortality[128-130]. Only in a subset of younger women who initiated on HRT immediately after menopause some beneficial effects were detected[131]. Scientific societies have not recommended estrogen replacement aiming at treating dyslipidemia or reducing cardiovascular risk in postmenopausal women[132-134].

Since aging and menopause provoke lipid changes (decreased HDL, especially HDL_2, increased small dense LDL and TG) that elevate cardiovascular risk in women partially controlled by HRT, several open questions need to be addressed to improve the prognosis of the atherosclerotic disease.

Disturbances in lipid profile and lipoprotein subfractions in diabetes and in postmenopausal diabetic women

Type 2 DM commonly coexists with obesity and both are characterized by states of low-grade inflammation and insulin resistance. Type 1 macrophages accumulated in the hypertrophic adipose tissue potentiate the pro-inflammatory cytokines secretion. Efflux of free fatty acids into circulation and the hepatic insulin resistance are responsible for the dyslipidemia in this condition[135-136]. Molecular mechanisms of the lipid metabolism disturbances in DM involve microRNAs, that are non-coding RNA molecules which regulate gene expression post-transcriptionally[137]. When microRNAs bind to their complementary sites at the 3′-untranslated regions of the target messenger RNAs (mRNAs) results in mRNA translational and repression or transcript degradation[138-139]. They have been proven to play important role on insulin resistance and on the regulation of liver metabolism affecting circulating lipids (miR-122, miR-33a,

miR-33b) and lipoprotein receptor. The relationship between insulin resistance and hypertriglyceridemia has been recognized, whereas through microRNA miR-34a, hypertriglyceridemia seems to favor the onset of DM[140-141].

Obesity and impairment in glucose tolerance are frequent pathophysiological conditions that generate lipid-related cardiovascular risk in women following menopause. As chronic inflammatory states, these conditions contribute to lipoprotein remodelling, compromising its function. Meanwhile, reduced estrogen levels contribute to a decrease in insulin sensitivity and aggravate metabolic disturbances[142]. Therefore, postmenopausal obese type 2 diabetic individuals are prone to a combination of disorders that markedly increases the risk of dying from cardiovascular events[143-144]. Obesity-induced efflux of free fatty acids provokes insulin-mediated skeletal uptake of free fatty acids and increased liver exposure, which results in a rise in hepatic secretion of VLDL, together with a retarded clearance of VLDL and chylomicrons, contributing to hypertriglyceridemia. This pattern of large VLDL, named $VLDL_1$, results in increased precursors of small dense LDL-c[145].

The typical pattern of dyslipidemia in DM – characterized by hypertriglyceridemia, low HDL-c and high small dense LDL-c levels – does not differ between sexes[146]. The HDL-c catabolism that occurs by the hepatic lipase and TG enrichment is elevated in conditions of insulin resistance[147]. Consequently, there is a reduction in HDL-c – that is predominantly from the HDL_{2b} subclass – as well as a relative or absolute increase in the smaller denser HDL_{3b} and HDL_{3c}[145]. Elevated non-HDL-c and predominance of small dense LDL particles to large buoyant LDL, known as phenotype B[145,148], raise atherogenicity even in near-normal limits of LDL-c. As these particles are prone to oxidative modification, oxidized LDL is more frequently found in diabetic individuals, contributing to accelerate atherogenesis.

Small dense LDL particles have reduced affinity to LDL receptors and a prolonged plasma residence time, which could result in an increment in LDL_{3a} and LDL_{3b} and a decrement in LDL_1 and LDL_{2a}[145]. Of note, the opposite and desirable profile, with higher concentration of large buoyant LDL, has been called phenotype A[145,148]. TG enrichment of these particles (VLDL and LDL) is due to the action of cholesteryl ester transfer protein (CETP), and hepatic lipase hydrolysis of TG and phospholipids[145,149].

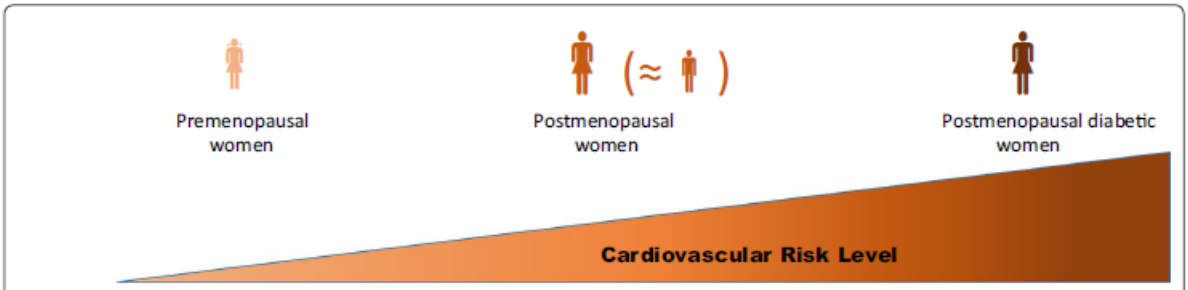
In addition, abnormalities on scavenger receptor class BI (SR-BI), that promotes selective uptake of HDL cholesteryl esters (HDL-CEs) into cells, have been described

in the type 2 DM. An overexpression of SR-BI in the liver accompanied by a reduction of HDL-c levels were reported[150]. In contrast, genetic deletion of SR-BI resulted in increased HDL-c and atherosclerosis. These HDL-c molecules seemed to have an altered composition, including a shift toward large, buoyant HDL particles, and a significant increase in plasma apo A-I, but not apo A-II in HDL particle[151].

Consequences of insulin resistance can be present in individuals with the metabolic syndrome even before the clinical diagnosis of DM[145,147]. Hyperglycaemia and hypoadiponectinemia are involved in the pathophysiology of the diabetic dyslipidaemia, but several questions remain unanswered[147].

Incidence of type 2 DM elevates after menopause[152] and that postmenopausal diabetic women are at increased cardiovascular risk compared to nondiabetic women at the same age and hormonal status[31]. Such risk is strongly related to modifications in the lipid metabolism which are dependent of both, menopause *per se* as well as the diabetic condition. For our best knowledge, the deleterious impact on lipid metabolism due to the presence of DM is similar in men and postmenopausal women.

The increased risk for atherosclerosis in postmenopausal diabetic women depends on low HDL-c levels, hypertriglyceridemia and predominance of small dense LDL particles[153]. Additionally, type 2 DM clusters with other disturbances from the spectrum of the metabolic syndrome, contributing to an elevated cardiovascular mortality[154]. Interestingly, the deleterious impact of DM in the LDL particle size seems to be greater in the diabetic women than in men[155-156] and postmenopausal diabetic women exhibited decreased large HDL particles (HDL_2) levels together with increased small HDL particles compared to normoglycemic women after menopause[32]. Figure 1 summarizes the main characteristics of structural and functional abnormalities of lipid metabolism during atherogenic process and aging and the impact of diabetes mellitus.



Plaque	Smaller lipid cores, less calcium, less inflammatory components and fewer thin-capped ateromas, with slower development of vulnerable plaques	Classical pattern of rupture followed by thrombosis	Classical pattern of rupture followed by thrombosis
Cardiovascular incidence	1 woman: 3-10 men Higher chance of fatality following coronary events	Similar at age 65 years and higher by age 75 years	Almost eliminated the sex-related difference in cardiovascular morbidity and mortality
Traditional lipid profile analyses	Maybe normal but progressing to →	↑ Total cholesterol ↑ LDL-c ↑ Triglyceride ↑ Apolipoprotein B ↓ HDL-c ↓ HDL-c/total cholesterol ↓ Apo-AI/Apo-B ratio	↑ Total cholesterol ↑ LDL-c ↑ Triglyceride ↑ Apolipoprotein B ↓ HDL-c ↓ HDL-c/total cholesterol ↓ Apo-AI/Apo-B ratio ↑ Hepatic VLDL secretion ↓ VLDL and chylomicrons clearance
Lipoprotein subfractions	Maybe normal but progressing to →	↑ Small dense LDL ↓ Larger and buoyant LDL ↓ Hepatic LDL receptor ↓ Large HDL particles	↑ Small dense LDL ↓ Large and buoyand LDL ↑ Small HDL particles ↓ Large HDL particles

Fig. 1 Main characteristics of structural and functional abnormalities of lipid metabolism during atherogenic process and aging and the impact of diabetes mellitus

Meanwhile, the hypothesis that estrogen therapy could alter lipids and improve cardiovascular risk profile and outcomes has been tested in both, diabetic and non-diabetic women[125,157-170]. Despite many studies that investigated the HRT effects on cardiovascular risk factors in postmenopausal diabetic women, just a few evaluated lipoprotein subfractions with conflicting results. Some authors described a significant increase in total HDL, predominantly on the HDL_2 subfraction, after intervention with combined HRT[170], while others failed to demonstrate any impact on HDL or LDL subfractions[33,168]. Due to the limited sample size and different HRT schemes used, studies available only generated hypothesis.

The effect of HRT on glucose homeostasis remains questionable[171]. A systematic review which included 16 trials with 17,971 postmenopausal women with type 2 DM demonstrated that estrogen replacement diminishes DM incidence and improves glycemic control[172], but there is no consensus yet.

To summarize, limited data on lipoprotein subfractions distribution in postmenopausal diabetic women, with or without dyslipidemia, are available. Different pharmacological approaches to ovarian failure still deserve comparisons, as well as different analytical methods to measure lipoprotein subfractions. Glycemic control level

may add a confounding factor among comparisons contributing partially for inconsistent results.

Worth of measurement of lipoprotein subfractions to the cardiovascular risk assessment in women

To date, there is insufficient evidence to recommend lipoprotein subfractions determination in clinical practice in both sexes at lower or higher cardiovascular risk[172]. Evidence that this measurement would impact on lipid-lowering treatment strategies is lacking either[173].

A small prospective nested case-control study in normal middle-aged women has previously demonstrated that baseline particle concentration was more predictive of future cardiovascular events than LDL particle size[174]. On the other hand, an analysis of 286 postmenopausal women from the Healthy Women Study confirmed an independent association of small dense LDL with higher CAC scores, suggesting a benefit from the addition of lipoprotein subfraction measurement for CVD prediction in this subset of individuals[175].

The largest prospective trial available included 27,673 healthy women followed for 11 years[176]. Traditional lipid profile and NMR-determined lipoprotein subclass number and size were measured at baseline. No extra benefit on cardiovascular risk prediction with lipoprotein subfractions measurement after adjustment for non-lipid risk factors was obtained[176]. Finally, a recent systematic review of 24 studies, in which the impact of LDL particles for cardiovascular outcomes was examined in both sexes, reported similar findings[177].

In summary, controversies in this matter persist[178] and it is questionable whether determination of lipoprotein subfractions could be useful in clinical settings. Several techniques for measurement are available, costs of the assays are high and the incremental benefit beyond traditional lipid measures may be minimal. Prospective studies demonstrating that advantages of lipoprotein subfractions to traditional lipid profile in the context of primary and secondary prevention of cardiovascular outcomes are needed.

Final remarks

Despite the lower incidence of CVD in adult women compared to men, their sex-related protective effect vanishes after menopause. This phase of women life *per se*

imposes deterioration of their lipid profile and weight gain is a frequent manifestation that could aggravate their predisposition to metabolic disturbances. The cardiovascular risk scores and health care professionals commonly underestimate their risk, and higher mortality and morbidity after coronary events have been reported in women. Consequently, women are less properly treated for CVD than men.

The deleterious impact of type 2 DM in cardiovascular risk may be superior in women compared to men, emphasizing the importance of improving the risk assessment, especially in postmenopausal diabetic women.

Since plasma lipoproteins constitute a major cardiovascular risk factor, a deeper analysis of their subfractions might contribute to understanding why lipid-dependent cardiovascular risk in women is increased. A more atherogenic lipid profile – hypertriglyceridemia, lower levels of both HDL-c and HDL_2, higher levels of both HDL_3 and small dense LDL – are usual after menopause, and modifications in lipoprotein subfractions are also expected in the presence of hyperglycemia. Therefore, postmenopausal diabetic women should be aggressively treated against dyslipidemia as well as against other risk factors.

Nowadays, no evidence supports that replacement of ovarian hormones has benefits in reducing cardiovascular events and mortality in different subgroups of women.

Finally, prospective trials including large samples of postmenopausal women, with or without DM, at different treatments and metabolic control, should be conducted to clarify whether lipoprotein subclass analysis would improve identification of higher-risk individuals. Considering that these determinations are expensive, cost-effectiveness studies are also necessary to address the worth of the addition of lipoprotein subfraction analysis in clinical practice.

List of abbreviations:

CVD: cardiovascular disease

CAD: coronary artery disease

TC: total cholesterol

HDL-c: high density lipoprotein cholesterol

LDL-c: low density lipoprotein cholesterol

TG: triglycerides

DM: diabetes mellitus

HRT: hormone replacement therapy

MI: myocardial infarction

VAP: vertical auto profile method

NMR: nuclear magnetic resonance

Apo: apolipoprotein

CEE: conjugated equine estrogen

CETP: cholesteryl ester transfer protein

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Consent for publication

Not applicable.

Availability of data and material

Not applicable.

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4.2 PAPER 2

Changes in lipoprotein subfractions following menopause in the Longitudinal Study of Adult Health (ELSA-Brasil)

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Highlights:

- There is little evidence on the association between duration of menopause and lipoprotein subfractions.
- A more deleterious lipid panel was observed in the first two years after menopause.
- No associations were found between time since menopause and lipoprotein cholesterol subfractions.
- Menopause is accompanied by higher triglyceride-rich lipoprotein subfractions.

Abstract

Introduction: It is unclear how aging and menopause-induced lipid changes contribute to the elevated cardiovascular risk in menopausal women. We examined the association between status and duration of menopause with lipid profile in the ELSA-Brasil.

Methods: This is a cross-sectional analysis of baseline data of women from the ELSA-Brasil, stratified by duration of menopause into 5 groups: pre-menopause, <2 yrs, 2-5.9 yrs, 6-9.9 yrs and ≥10 yrs of menopause, excluding menopause <40 yrs or non-natural cause, use of lipid-lowering drugs or hormone replacement. Comparisons were performed using ANOVA with Bonferroni correction. Associations of menopause categories and time since menopause with lipid variables obtained by vertical auto-profile were tested using multiple linear regression.

Results: From 1,916 women, postmenopausal groups had unadjusted higher total cholesterol, LDL-c, real LDL-c, IDL-c, VLDL-c, triglycerides, non-HDL-c, $VLDL_3$-c, triglyceride-rich lipoprotein remnants (TRL-c) and buoyant LDL-c concentrations than pre-menopausal, with no difference among menopausal groups. In multiple linear regression, duration of menopause <2 years was significantly associated with TRL-c [7.21 mg/dL (95% CI 3.59-10.84)] and VLDL3-c [2.43 mg/dL (95%CI 1.02-3.83)]. No associations of menopausal categories with HDL-c or LDL-c subfractions were found, also no associations of time since menopause with lipid subfractions.

Conclusions: In a large sample of Brazilian women, deterioration of the lipid profile following menopause was confirmed, which could contribute to increased cardiovascular risk. Our findings suggest a postmenopausal elevation in triglyceride-rich lipoprotein remnants. How lipoprotein subfractions change after its onset warrants investigation in studies with appropriate design.

Key words: menopause; cardiovascular risk; lipoprotein subfractions; triglyceride-rich lipoprotein remnants; low-density lipoprotein; very low-density lipoprotein.

1. Introduction

Atherosclerotic cardiovascular disease (ASCVD) is a major public health problem worldwide, and the leading cause of death in both sexes[1]. Premenopausal women have lower ASCVD incidence compared to men at similar age, but this sex-related difference is narrowed following menopause, when women exhibit increased morbidity and mortality risk[2]. There is still room for investigation of non-traditional factors that could contribute to cardiovascular risk in postmenopausal women.

After menopause, a more atherogenic lipid profile has been described, characterized by increases in low-density lipoprotein cholesterol (LDL-c) and triglyceride levels, together with decreases in high-density lipoprotein cholesterol (HDL-c)[3-5]. Increase in the highly atherogenic particle, the small dense LDL-c, has been consistently reported in diabetes, obesity and metabolic syndrome, and in association with cardiovascular outcomes in both sexes[6]. However, the HDL-c decrease after menopause has been challenged. Despite increases in HDL-c during the peri- and post-menopausal periods[7-9], progression of atherosclerosis is still observed[10-12]. HDL is a highly heterogeneous lipoprotein that carries more than 80 types of proteins and more than 100 different lipid species; its proteome contributes to a wide array of functions, such as in inflammation and oxidative stress and can be affected by systemic inflammatory and metabolic conditions[13]. Although there is evidence supporting the association of some HDL-c subfractions with CVD in both sexes, their role is an issue of ongoing debate[14,15].

Lipoprotein subfractions differ according to their size, charge, composition, density and function. Several methods for subfractionation of major lipoproteins are available but they are expensive and time-consuming, limiting their use in clinical practice. Some of the heightened risk for ASCVD in postmenopausal women has been attributed to an increase in small dense LDL-c and reduction in HDL_2-c[16,17], but recent data dispute this[12].

Remnant lipoprotein cholesterol (TRL-c) is an emerging ASCVD risk factor. TRL-c represents the sum of the small very low-density lipoprotein cholesterol ($VLDL_3$-c) plus the intermediate-density lipoprotein cholesterol (IDL-c), which are triglyceride-enriched precursors to LDL in the fasting state. TRL-c levels were associated with

endothelial dysfunction, unstable carotid plaques, carotid intimal-media thickness, and with coronary artery disease[18-20].

Studies involving the subfractionation of major lipoproteins conducted in large samples of postmenopausal women are scarce[21]. The marked reduction in oestrogen production following menopause contributes to endothelial dysfunction and disturbances in the lipid profile. Reproductive life span, age at menopause onset, and menopause duration influence the risk for cardiovascular events in women[22]. Aging itself contributes to changes to the lipid profile and it is still unclear how each factor impacts on lipoproteins and their subfractions.

Despite knowledge that the progressive decline in oestrogen levels during menopause is accompanied by lipid abnormalities, few studies have investigated the association of menopause duration with lipid variables[8] or lipoprotein subfractions[12] and none using ultracentrifugation (vertical auto-profile technique). We examined whether the status and duration of menopause were associated with lipoprotein subfractions at time of baseline in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil).

2. Methods

2.1 Study design and population

This is a cross-sectional analysis of baseline data of women included in the Sao Paulo site of the ELSA-Brasil, which is a multicentre prospective cohort designed to evaluate cardiovascular disease and diabetes and their biological, behavioural, environmental, occupational, psychological and social risk factors[23]. The main study enrolled 15,105 civil servants aged 35 to 74 years from six Brazilian cities. Details on objectives and methodological aspects were previously reported[23-27]. Participants had an initial interview using validated questionnaires at the job site and were scheduled for clinical examinations and laboratory tests in the research centre. Baseline evaluations were carried out from August 2008 through December 2010. Participants living in São Paulo underwent additional examinations such as the quantification of plasma lipoprotein subfractions and were included in this analysis. Local ethics committees approved the study and participants signed informed consent.

The current sample included pre-, peri- and postmenopausal women aged 35-74 years, stratified into five groups according to the status and duration of menopause

(Group 1: pre-menopause; Group 2: peri-/post-menopause with duration of menopause <2 years; Group 3: post-menopause with duration of menopause from 2 to 5.9 years; Group 4: post-menopause with duration of menopause from 6 to 9.9 years and Group 5: post-menopause with duration of menopause ≥10 years). Menopause was defined as cessation of menstruation ≥12 months. Peri-menopause was defined as cessation of menstrual cycles from 6 to 11.9 months. Exclusion criteria were menopause before 40 years of age, non-natural causes of menopause, use of lipid-lowering drugs and hormone replacement therapy (HRT). From a total of 5,061 participants from the Sao Paulo centre, 2,726 were women. After excluding 810 women who met exclusion criteria, 1,916 women were included in our analysis.

2.2 Clinical assessment

Sociodemographic data, lifestyle factors and medical history were self-reported. Duration of menopause was calculated subtracting age at natural menopause from age at baseline in years. Body weight and height were measured using calibrated electronic scales and a fixed rigid stadiometer. Body mass index (BMI) was calculated as weight (kilograms) divided by squared height (meters). Waist circumference was measured with an inextensible tape. Blood pressure (BP) was taken three times after a 5-minute rest in the sitting position. The mean of the two last measurements weas used. Leisure-time physical activity was evaluated by the International Physical Activity Questionnaire[24].

2.3 Laboratory tests

After overnight fasting, blood samples were taken, immediately centrifuged and analysed or stored in -80°C for future analyses. Plasma glucose was measured by the hexokinase method (ADVIA Chemistry; Siemens, Deerfield, Illinois, USA).

An extended lipid panel (complete lipid profile and lipoprotein subfractions) was determined by the VAP-II (Atherotech, Birmingham, USA), a single vertical density gradient ultracentrifugation method, which simultaneously measures cholesterol concentrations of lipoprotein classes and subfractions after their separation[25]. Five lipoprotein classes: HDL, LDL-r, VLDL, IDL, and lipoprotein (a) [Lp(a)], and subfractions, such as HDL_2 and HDL_3, LDL_1 through LDL_4, and $VLDL_1$ through $VLDL_3$ are identified[25]. VAP-II also permits characterization of TRL-c, beyond real and direct evaluation of low-density lipoprotein cholesterol [LDLr-c, which corresponds to total

LDL-c minus lipoprotein (a) and IDL-c]. To characterize the buoyancy of LDL, subfractions were classified into large and buoyant (LDL_1 and LDL_2) or smaller and denser particles (LDL_3 and LDL_4). Variability coefficients for lipoprotein subfraction measurements were previously reported[25].

2.4 CV risk factor definitions

Central obesity was diagnosed when waist circumference was ≥88 cm. Hypertension was defined by systolic BP ≥140 mmHg or diastolic BP ≥90 mmHg or use of antihypertensive drugs. Diabetes was defined as a report of a previous diagnosis of diabetes, use of medication for diabetes or meeting a diagnostic value for diabetes according to the American Diabetes Association criteria [fasting plasma glucose ≥126 mg/dL, glycated hemoglobin A1c (HbA1c) ≥6.5% or 2-h plasma glucose during 75g-oral glucose tolerance test ≥200 mg/dL[23]]. Hypercholesterolemia was defined by LDL-c levels ≥130 mg/dL[26-27]. Hypertriglyceridemia was defined by triglycerides ≥150 mg/dL and low HDL-c by values <50 mg/dL. Traditional colorimetric assays for cholesterol quantification were used for the diagnosis of the conditions above. Women were considered physically active if they performed at least 150 minutes of moderate-intensity or 75 minutes of high-intensity leisure-time aerobic physical activity or combined equivalent of both per week. Excessive alcohol intake was defined as ingestion ≥140g of any kind of alcohol per week.

2.5 Statistical analysis

Data are expressed as medians and interquartile ranges due to non-normal distribution of variables. Women were categorized into one of five groups according to menopausal status and duration, as previously mentioned. ANOVA with Bonferroni correction for log-transformed continuous variables was used for comparisons among groups and the chi squared test for categorical variables. Multiple linear regression was employed in which lipid variables were considered dependent variables, while independent variables were five menopausal categories. Lipid variables were log-transformed for these analyses showing similar results to the non-transformed ones; therefore, in the table, linear regression results are presented as non-transformed values. Premenopausal women were considered the reference group. Variables selected for adjustments were based on the p-value from the crude analysis or scientifically-based. Model 1 was adjusted for age, education level and race (self-

reported skin colour). In model 2, adjustments for central obesity, hypertension, diabetes, physical activity, smoking and alcohol consumption were added. Additional multiple linear regression models for the association of selected VAP-II lipid variables with menopausal status (yes or no) and time since menopause (as a continuous variable only in postmenopausal women), using the same adjustments, were performed.

Statistical analyses were performed using Stata version 12 (StataCorp LLC,College Station, Texas, USA). A p-value <0.05 was considered significant.

3. Results

From 2,726 female participants in Sao Paulo site, after excluding 810 women who met exclusion criteria, 1,916 women were included in our analysis (1,121 premenopausal and 795 postmenopausal women). The mean age of the participants was 49.6 years (SD 8.5). The sample was characterized by high education level (89% had completed at least high school) with a predominance of self-reported white skin colour.

Baseline characteristics of the five groups of participants, stratified according to menopause categories are shown in Table 1. Notably, there was a predominance of premenopausal women; significant differences between groups were observed for most of the variables. Postmenopausal women had higher waist circumference, systolic and diastolic BP, and plasma glucose when compared to premenopausal women. Comparisons among groups showed higher levels of total cholesterol, LDL-c, LDLr-c, IDL-c, VLDL-c, triglycerides, non-HDL-c, $VLDL_3$-c, TRL-c and buoyant LDL-c in all postmenopausal groups when compared to the premenopausal one, but no differences across menopausal categories (groups 2 to 5) were observed. A worse crude lipid profile of women with menopause <2 years compared to pre-menopause was observed, with a subsequent near-plateau of median values of lipoproteins and subfractions (Figure 1 Supplement). Compared to the premenopausal group, higher median HDL-c and HDL_3-c values starting six years after menopause were found, with stabilization afterwards. No difference in HDL_2-c levels between groups was detected. Median values of dense LDL-c were significantly lower in the premenopausal group only when compared to the women whose menopause occurred ≥10 years.

Frequencies of central obesity, hypertension, diabetes and dyslipidemia were significantly lower before menopause (Table 1). Frequency of current smokers differed among groups, but not of alcohol consumers and physically active individuals.

Association of menopause status (yes or no) with lipid variables was initially tested in a multiple linear regression model (data not shown in table). Menopause was associated with $VLDL_3$-c [β 1.72 mg/dL (95%CI 0.69-2.76)], TRL-c [β 5.58 mg/dL (95%CI 2.91-8.25)] and buoyant LDL-c [β 4.84 mg/dL (95%CI 0.07-9.62)], independently of age, education level, race, central obesity, hypertension, diabetes, physical activity, smoking and alcohol consumption.

Table 2 exhibits crude and adjusted results of the linear regression analyses for the association of menopause categories with selected lipid variables. Duration of menopause <2 years was significantly associated with higher TRL-c [7.21 mg/dL (95% CI 3.59-10.84)] and $VLDL_3$-c [2.43 mg/dL (95%CI 1.02-3.83)], adjusted for age, education level, race, central obesity, diabetes, hypertension, smoking, alcohol consumption and physical exercise (model 2). Duration between 2 and 5.9 years was also associated with higher TRL-c [4.56 mg/dL (95% CI 1.40-7.73)], while between 6 and 9.9 years only with $VLDL_3$-c [1.73 mg/dL (95%CI 0.15-3.31)]. No association of menopause categories with HDL-c and LDL-c subfractions was observed. No significant differences were observed when additional adjustment for total cholesterol or presence of hypercholesterolemia was performed (data not shown). When time since menopause was taken as continuous variable, no association was found with any lipoprotein subfractions, after adjustments for the same confounding variables (data not shown).

4. Discussion

Our findings reinforce that menopause is associated with a more atherogenic lipid profile in women from the ELSA-Brasil study. Shortly after menstrual cessation, crude elevated levels of almost all cholesterol-carrying particles (total cholesterol, LDL-c, LDLr-c, IDL-c, VLDL-c, triglycerides, non-HDL-c, $VLDL_3$-c, TRL-c and buoyant LDL-c) were observed, with no further increments after this period. We interpreted such alterations as partially dependent on the effects of oestrogenic decline on lipoprotein metabolism. In the long-term, aging *per se* might be inducing lipoprotein disturbances[7], since no differences were found when comparing the postmenopausal groups.

Interestingly, in multiple linear regression, no association of menopause status or duration with HDL-c subfractions or dense LDL-c was found, but there were associations between menopause status and higher $VLDL_3$-c, TRL-c and buoyant LDL-c. When evaluating different categories of menopause, duration <2 years was independently associated with higher TRL-c and VLDL3-c suggesting a role for oestrogen deficiency in these metabolic alterations. Also, age-adjustment was an attempt to individualize the effect of menopause on lipid profile, although in physiological terms aging and menopause occur concomitantly.

Endogenous oestrogen may protect against atherosclerosis through facilitation of nitric oxide-mediated vasodilation, reduction of inflammatory activation and cell-adhesion, improvement in endothelial function, as well as decreasing LDL-c and increasing HDL-c. Therefore, its gradual decline during the peri-menopause may negatively impact risk for cardiovascular events. Previous studies have even suggested an increased risk for ASCVD in women with shortened oestrogenic exposure during the life course, such as those with late-onset of menarche, early menopause, or shorter duration of reproductive life span[22]. A systematic review and meta-analysis indicated higher risk of ASCVD and mortality in women who experienced premature or early-onset of menopause[28], which could be at least partly due to oestrogen decline. Our findings regarding the lipid profile in those participants who had more recent menopause (<2 years) suggest that changes in some particles, mainly TRL-c and VLDL3-c, could in part be due to decreasing exposure to endogenous oestrogen. Despite our analysis having added an important observation in this issue, prospective studies on the role of short- and long-term hypoestrogenism on cardiovascular risk in postmenopausal women are still needed. Whether right after menopause onset there is an elevation in triglyceride-rich lipoprotein remnants followed by a plateau should be investigated in studies with appropriate design. Although serum oestrogen levels were not available in our study, we speculate that the decline could be much smaller after the first years from menopause.

Initial years post-menopause of our participants were accompanied by higher lipoprotein cholesterol levels, but no further difference was observed in the long-term. Previous studies have already investigated the complex relationship between menopause, aging and lipoprotein abnormalities[17-18]. In line with our results, total cholesterol, LDL-c and apolipoprotein B levels of participants in the *Study of Women's Health across the Nation* increased 12 months before and after menopause,

maintaining a plateau afterwards[7]. Their HDL-c and apolipoprotein A1 levels increased slowly and progressively over time, indicative of a chronological aging effect. Also, an analysis of the *Multi-Ethnic Study of Atherosclerosis* revealed an association of higher levels of large HDL particles, measured by nuclear magnetic ressonance (NMR), with increased carotid intimal-media thickness close to menopause and an inverse association later in life[12]. The authors suggested an altered function of large HDL-c early after menopause. We also did not find associations of HDL-c subfractions with menopause status, but it is important to mention that the technique for lipoprotein subfraction determination was different. The method of lipoprotein subfractions determination, whether NMR or VAP-II or other techniques, may explain at least in part discrepancies between our results and those of previously published studies.

Our finding of a slight increase in HDL could be an unexpected result after menopause, although some controversial findings were reported[7,9,11]. Our study has included a selected sample of highly educated women, with unusual high-normal HDL-c levels which could be contributing to an absence of association between menopause duration and HDL-c subfractions. Apparent high frequencies of hypercholesterolemia could be attributed to the less strict definition used (LDL-c ≥ 130 mg/dL).

Considering the proatherogenic, proinflammatory and prothrombotic properties of remnant lipoproteins, they have been associated with subclinical atherosclerosis and CAD[20] and in women, are also considered an independent ASCVD risk factor[30]. Our findings are in agreement with the aforementioned, as $VLDL_3$-c and TRL-c were independently associated with menopause. Recently, data regarding 228 postmenopausal women from a cross-sectional analysis of the ATENA Project found a direct association between VLDL-c and c-IMT and independent associations between high concentrations of IDL-c and TRL-c with the presence of carotid plaques[21]. Another study confirmed the association of TRL-c with CAD in postmenopausal women. Findings from the *Fenofibrate Intervention and Event Lowering in Diabetes* (FIELD) study support a deleterious impact of triglyceride-enriched particles on cardiovascular risk, as fenofibrate reduced cardiovascular events in hypertriglyceridemic individuals of both sexes. Fenofibrate also improved the lipoprotein profile more significantly in women than in men. Therefore, the contribution of the remnant lipoprotein cholesterol for residual risk warrants additional investigation.

Our study has limitations related to the cross-sectional design that precludes inferences of causation and to the small numbers of participants in menopausal

groups. The unavailability of hormone determinations limits our ability to establish the status and duration of menopause in a more precise, quantitative way. However, the follow-up of the ELSA-Brasil cohort should improve understanding of lipoprotein abnormalities developing during ovarian aging and their association with outcomes. Additionally, characteristics of the population studied limit generalization of our results since it is composed of a highly educated sample, with high-normal median HDL-c levels in all groups of women.

In summary, in a large sample of Brazilian women, deterioration of the lipid profile following menopause was confirmed, which could contribute to increased ASCVD risk. Our findings suggest a postmenopausal elevation in triglyceride-rich lipoprotein remnants. How lipoprotein subfractions change after its onset warrants investigation in studies with appropriate design to explore their role in potentiating ASCVD risk in postmenopausal women.

Data statement:

Data will be made available upon request.

Ethical statement:

Local ethics committees approved the study and participants signed informed consent. The work has been carried out in accordance with ethical guidelines.

Author's contribution:

Marília I H Fonseca: conceptualization, methodology, analysis, visualization, writing/revision. Bianca de Almeida-Pititto: conceptualization, methodology, analysis, visualization, writing/revision. Isabela M Bensenor: conceptualization, writing/revision. Paulo A Lotufo: conceptualization, writing/revision. Peter P Toth, Steven R Jones and Michael J Blaha: writing/revision. Krishnaji R Kulkarni: determination of lipoprotein subfractions, revision. Sandra R G Ferreira: conceptualization, visualization, writing/revision, supervision. All authors contributed to the revision of this article. All authors read and approved the final manuscript.

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Conflicts of interest:

KRK is currently an employee of VAP Diagnostics Laboratory and previously employed by Atherotech Diagnostics Laboratory. Other authors declare no conflicts of interest.

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Supplementary data:

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.maturitas.2019.09.005.

Table 1 - Number (percentages) of participants or medians (interquartile interval) of their clinical variables according to menopause status and duration.

	Group 1 Pre-menopause	**Group 2 Menopause <2 years**	**Group 3 Menopause 2-5.9 years**	**Group 4 Menopause 6-9.9 years**	**Group 5 Menopause ≥10 years**	**P-value**
	N = 1,121	N = 133	N = 235	N = 172	N = 255	-
Age, years	44 (41-47)	51 (49-53)[a]	54 (51-57)[ab]	57 (56-59)[abc]	64 (60-67)[abcd]	<0.001
Race						0.054
- White	649 (58.2)	87 (65.9)	136 (58.4)	98 (57.7)	169 (67.3)	
- Mulatto	244 (21.9)	24 (18.2)	48 (20.6)	30 (17.7)	33 (13.2)	
- Black	164 (14.7)	12 (9.1)	37 (15.9)	25 (14.7)	30 (12.0)	
- Asian	52 (4.7)	7 (5.3)	10 (4.3)	16 (9.4)	17 (6.8)	
- Indigenous	6 (0.5)	2 (1.5)	2 (0.9)	1 (0.6)	2 (0.8)	
Education level						<0.001
- ≤ Elementary	64 (5.7)	17 (12.8)	36 (15.3)	34 (19.8)	59 (23.1)	
- High school	518 (46.2)	54 (40.6)	93 (39.6)	58 (33.7)	71 (27.8)	
- College/university	539 (48.1)	62 (46.6)	106 (45.1)	80 (46.5)	125 (49.0)	
Waist circumference, cm	83 (76-92)	86 (78-96)	86 (79-95)[a]	86 (78-94)	89 (80-100)[a]	<0.001
Body mass index, kg/m^2	26.1 (23.3-29.9)	26.9 (23.6-30.4)	26.7 (23.8-30.1)	26.5 (23.2-30.0)	27.7 (24.1-31.5)[a]	0.023
Systolic BP, mmHg	110 (102-119)	113 (105-124)	117 (106-128)[a]	116 (106-126)[a]	122 (111-133)[abcd]	<0.001

Diastolic BP, mmHg	71 (65-78)	73.0 (67-81)	73 (67-80)[a]	73 (67-81)	74 (67-81)[a]	<0.001
Glucose, mg/dL	99 (94-106)	103 (96-110)	103 (98-113)[a]	107 (101-114)[ab]	109 (102-117)[abc]	<0.001
Total cholesterol, mg/dL	205 (183-231)	232 (196-259)[a]	231 (209-261)[a]	233 (208-259)[a]	233 (207-263)[a]	<0.001
LDL-c, mg/dL	125 (105-146)	143 (117-168)[a]	145 (123-167)[a]	143 (122-167)[a]	144 (123-168)[a]	<0.001
LDLr-c, mg/dL	103 (85-122)	117 (95-138)[a]	118 (99-137)[a]	116 (100-135)[a]	115 (99-138)[a]	<0.001
IDL-c, mg/dL	14 (10-19)	19 (14-25)[a]	19 (15-25)[a]	18 (14-25)[a]	19 (14-24)[a]	<0.001
VLDL-c, mg/dL	21 (17-27)	26 (19-32)[a]	24 (19-31)[a]	25 (19-31)[a]	24 (19-32)[a]	<0.001
HDL-c, mg/dL	56 (49-66)	58 (51-68)	59 (51-70)[a]	62 (53-72)[a]	59 (50-70)[a]	<0.001
Triglycerides, mg/dL	89 (68-123)	108 (79-150)[a]	104 (79-139)[a]	110 (83-149)[a]	109 (84-149)[a]	<0.001
Non-HDL-c, mg/dL	147 (126-172)	170 (145-200)[a]	170 (145-196)[a]	169 (147-197)[a]	170 (146-195)[a]	<0.001
$VLDL_3$-c, mg/dL	12 (10-15)	15 (12-17)[a]	14 (12-17)[a]	14 (12-17)[a]	14 (11-18)[a]	<0.001
HDL_2-c, mg/dL	16 (12-20)	16 (13-20)	16 (13-21)	17 (14-22)	17 (12-21)	0.017
HDL_3-c, mg/dL	41 (36-46)	42 (38-47)	43 (38-49)[a]	44 (39-50)[a]	43 (38-49)[a]	<0.001
TRL-c, mg/dL	26 (21-34)	33 (27-43)[a]	33 (27-40)[a]	32 (26-42)[a]	33 (26-43)[a]	<0.001
Buoyant LDL-c, mg/dL	40 (30-53)	49 (37-68)[a]	53 (40-66)[a]	49 (38-61)[a]	50 (37-64)[a]	<0.001
Dense LDL-c, mg/dL	45 (33-61)	47 (34-64)	48 (32-65)	49 (36-64)	50 (36-70)[a]	0.002
Lipoprotein(a)-c, mg/dL	6 (4-9)	6 (5-10)	7 (5-10) [a]	7 (5-9)	7 (4-10)	0.004
Current smoker	162 (14.5)	23 (17.2)	49 (20.9)	40 (23.3)	32 (12.6)	0.004
Alcohol consumers	28 (7.9)	4 (8.3)	5 (5.7)	6 (10.2)	7 (9.9)	0.859
Physically active	199 (18.4)	28 (22.0)	36 (16.0)	28 (16.9)	45 (18.3)	0.694
Central obesity	386 (34.4)	59 (44.4)	97 (41.3)	68 (39.5)	136 (53.3)	<0.001
Hypertension	164 (14.6)	29 (21.8)	66 (28.1)	47 (27.3)	114 (44.7)	<0.001
Diabetes	100 (8.9)	17 (12.8)	42 (17.9)	32 (18.6)	65 (25.5)	<0.001
Hypercholesterolemia	428 (38.2)	79 (59.4)	144 (61.3)	104 (60.5)	148 (58.0)	<0.001
Hypertriglyceridemia	193 (17.2)	36 (27.1)	55 (23.4)	51 (29.7)	77 (31.1)	<0.001
Low HDL-c	312 (27.8)	24 (18.1)	41 (17.5)	28 (16.3)	44 (17.7)	<0.001

BP: blood pressure; LDL-c: total low-density lipoprotein cholesterol; LDLr-c: real low-density lipoprotein cholesterol; IDL-c: intermediate-density lipoprotein cholesterol; VLDL-c: very low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; TRL-c: triglyceride-rich lipoprotein remnants; Dense LDL-c: sum of LDL_{3+4}-c; Buoyant LDL-c: sum of LDL_{1+2}-c.

Groups compared using ANOVA (log-transformed continuous variables) with Bonferroni correction or chi-square test.

P value < 0.05 [a]versus Group 1; [b]versus Group 2; [c]versus Group 3; [d]versus Group 4.

Table 2 - Coefficients (95% confidence interval) in mg/dL obtained using multiple linear regression for the association of selected lipid variables and menopause categories.

	Pre-menopause	**Menopause <2 yrs**	**Menopause 2-5.9 yrs**	**Menopause 6-9.9 yrs**	**Menopause ≥10 yrs**
HDL_2-c					
Crude	Reference	0.17 (-1.01-1.34)	**1.01 (0.09-1.94)**	**1.24 (0.18-2.29)**	**0.92 (0.03-1.82)**
Model 1	Reference	-0.35 (-1.58-0.89)	0.23 (-0.89-1.35)	0.05 (-1.30-1.40)	-0.83 (-2.39-0.73)
Model 2	Reference	-0.15 (-2.28-1.97)	-0.19 (-1.66-2.05)	-0.95 (-3.34-1.44)	-1.33 (-4.19-1.54)
HDL_3-c					
Crude	Reference	**1.87 (0.46-3.28)**	**2.72 (1.61-3.83)**	**3.55 (2.28-4.82)**	**2.50 (1.43-3.58)**
Model 1	Reference	1.03 (-0.45-2.51)	**1.44 (0.10-2.77)**	**1.72 (0.10-3.34)**	-0.28 (-2.14-1.58)
Model 2	Reference	0.84 (-1.84-3.52)	0.78 (-1.56-3.12)	0.18 (-2.83-3.19)	-1.01 (-4.63-2.60)

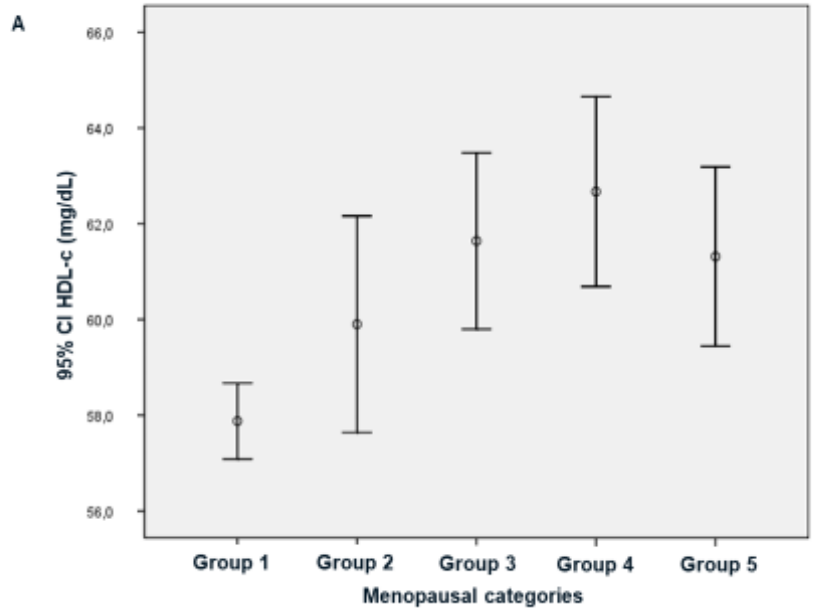
$VLDL_3$-c					
Crude	Reference	**2.42 (1.53-3.30)**	**1.51 (0.81-2.21)**	**2.27 (1.47-3.06)**	**1.90 (1.22-2.57)**
Model 1	Reference	**2.01 (1.07-2.96)**	**0.93 (0.08-1.78)**	**1.48 (0.45-2.51)**	0.82 (-0.37-2.00)
Model 2	Reference	**2.43 (1.02-3.83)**	1.12 (-0.11-2.35)	**1.73 (0.15-3.31)**	0.97 (-0.92-2.87)
TRL-c					
Crude	Reference	**7.75 (5.60-9.89)**	**6.40 (4.70-8.09)**	**6.83 (4.90-8.76)**	**6.25 (4.61-7.89)**
Model 1	Reference	**6.29 (3.99-8.57)**	**4.26 (2.19-6.32)**	**3.83 (1.33-6.33)**	1.97 (-0.90-4.85)
Model 2	Reference	**7.21 (3.59-10.84)**	**4.56 (1.40-7.73)**	3.49 (-0.12-8.03)	2.60 (-2.29-7.49)
Dense LDL-c					
Crude	Reference	2.27 (-1.84-6.37)	2.46 (-0.78-5.70)	3.58 (-0.11-7.26)	**7.17 (4.04-10.30)**
Model 1	Reference	0.87 (-3.51-5.26)	0.11 (-3.84-4.07)	0.61 (-4.18-5.41)	3.05 (-2.46-8.56)
Model 2	Reference	-0.23 (-7.52-7.05)	-1.26 (-7.62-5.09)	2.42 (-5.78-10.61)	-1.42 (-11.255-8.41)
Buoyant LDL-c					
Crude	Reference	**10.97 (7.38-14.57)**	**11.41 (8.58-14.24)**	**8.75 (5.52-11.97)**	**9.49 (6.75-12.23)**
Model 1	Reference	**8.57 (4.75-12.40)**	**7.88 (4.43-11.33)**	3.49 (-0.69-7.67)	1.66 (-3.15-6.46)
Model 2	Reference	6.34 (-0.15-12.83)	4.69 (-0.97-10.35)	0.38 (-6.92-7.68)	0.37 (-8.39-9.13)

TRL-c: triglyceride-rich lipoprotein remnants; Dense LDL-c: sum of LDL_{3+4}-c; Buoyant LDL-c: sum of LDL_{1+2}-c.

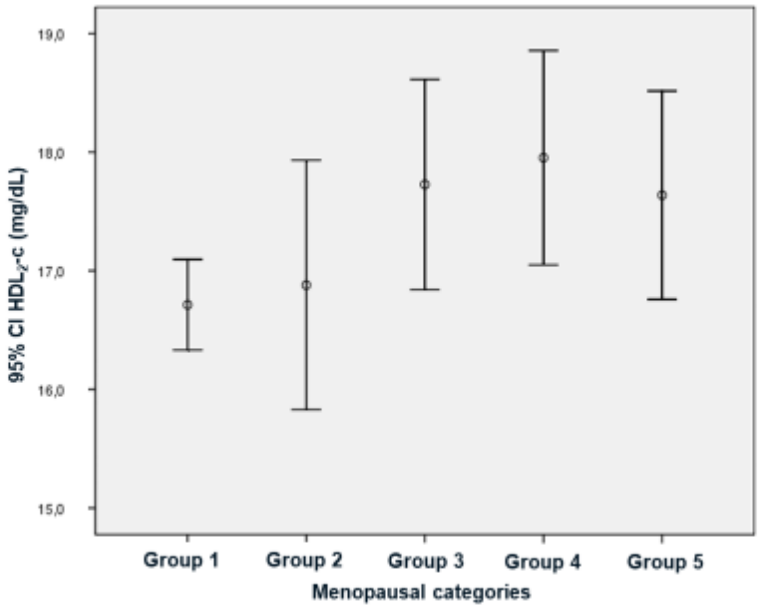
Model 1: adjusted for age, education level and race. Model 2: adjusted for age, educational level, race, central obesity, hypertension, diabetes, physical activity, smoking and alcohol consumption.

Supplementary Figure 1:

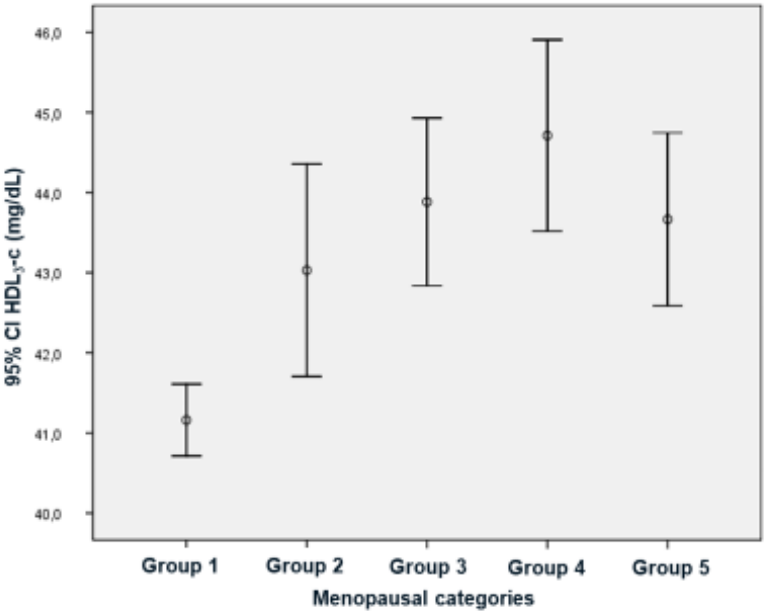
Figure 1: Median values (95% CI) in mg/dL of selected lipoproteins according to menopausal categories.



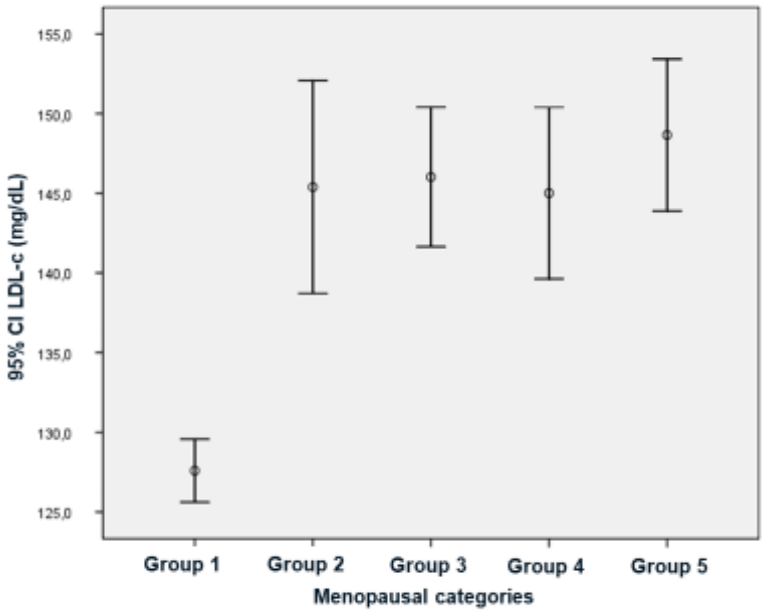
B


19,0
18,0
17,0
16,0
15,0
95% CI HDL2-c (mg/dL)
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

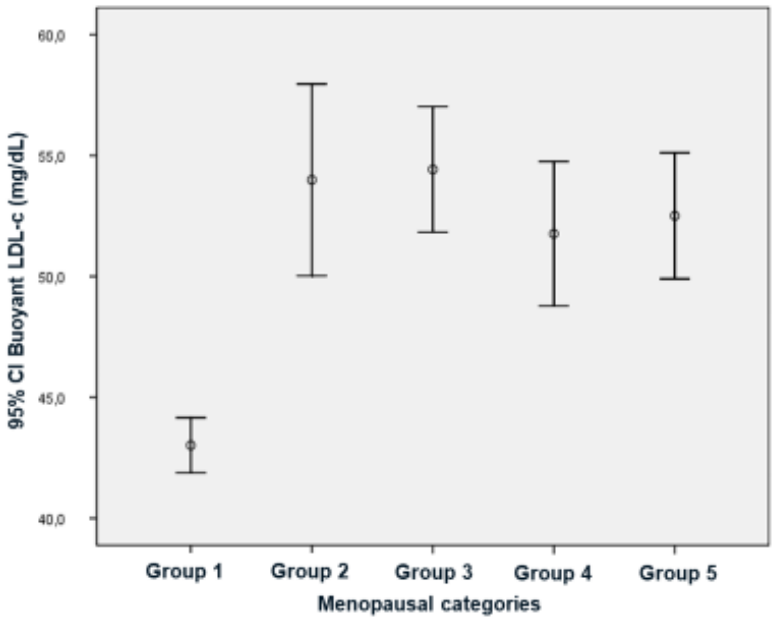
C


46,0
45,0
44,0
43,0
42,0
41,0
40,0
95% CI HDL3-c (mg/dL)
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

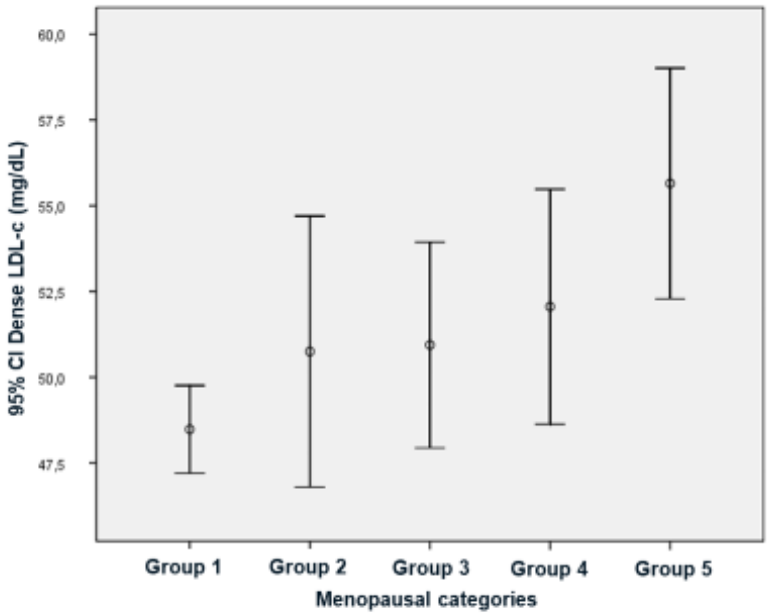
D


155,0
150,0
145,0
140,0
135,0
130,0
125,0
95% CI LDL-c (mg/dL)
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

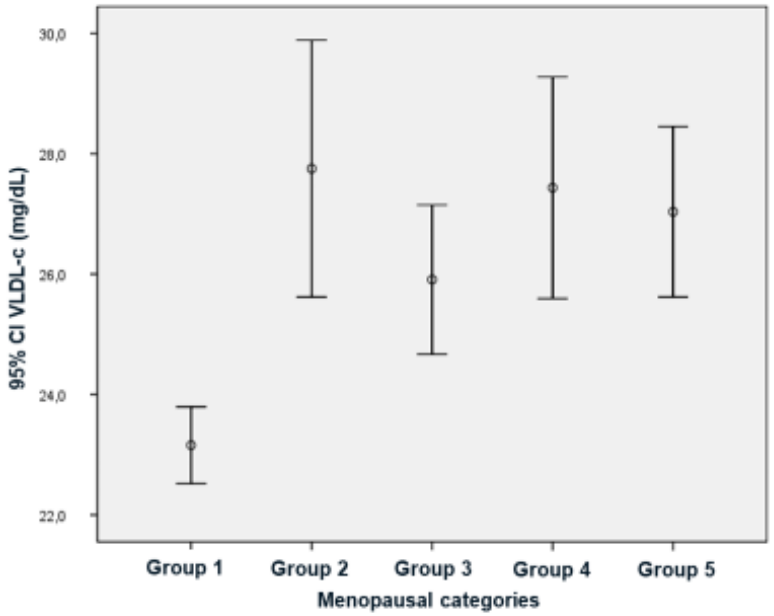
E


60,0
55,0
50,0
45,0
40,0
95% CI Buoyant LDL-c (mg/dL)
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

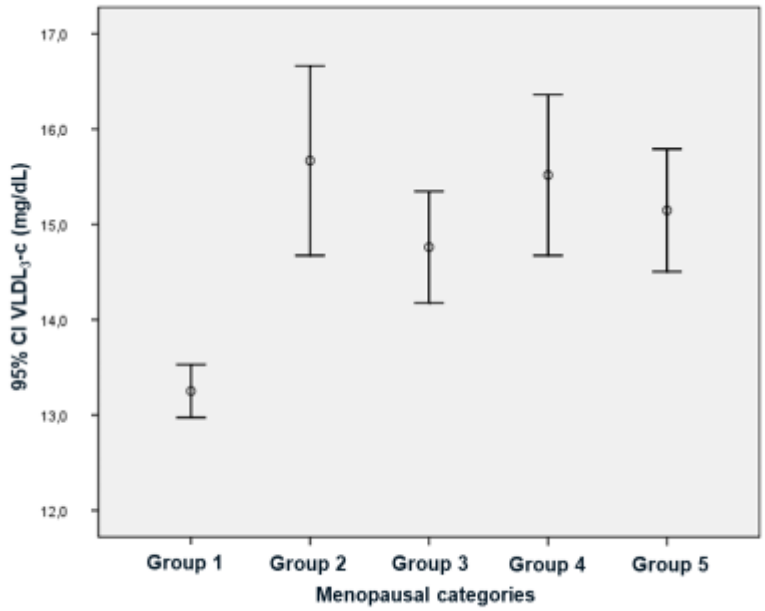
F


95% CI Dense LDL-c (mg/dL)
60,0
57,5
55,0
52,5
50,0
47,5
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

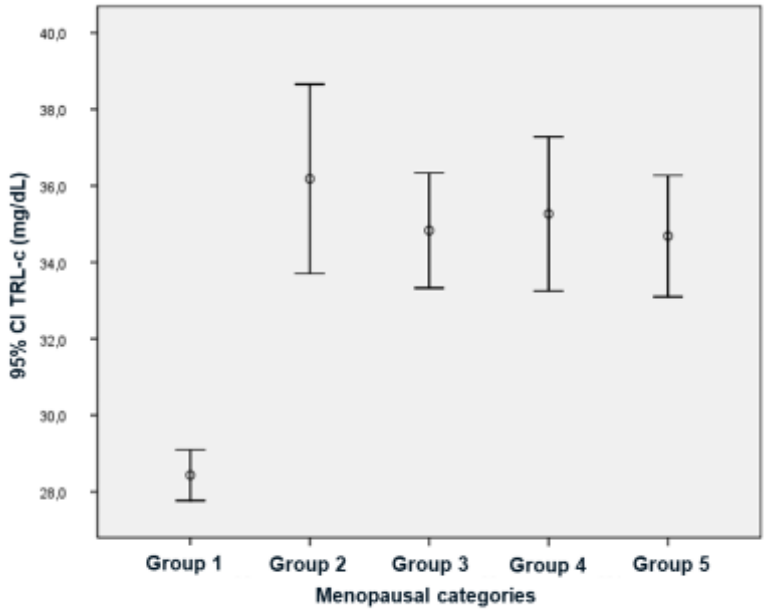
G


95% CI VLDL-c (mg/dL)
30,0
28,0
26,0
24,0
22,0
Group 1
Group 2
Group 3
Group 4
Group 5
Menopausal categories

H



I



A: HDL-c; B: HDL_2-c; C: HDL_3-c; D: LDL-c; E: Buoyant LDL-c; F: Dense LDL-c; G: VLDL-c; H: $VLDL_3$-c; I: TRL-c.

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4.3 PAPER 3

Menopause *per se* is associated with coronary artery calcium score: results from the ELSA-Brasil

Short title: Menopause and CAC in the ELSA-Brasil

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Abstract

Background: Menopause and aging deteriorate the metabolic profile, but little is known how they independently contribute to structural changes in coronary arteries. We compared a broad cardiometabolic risk profile of women according to their menopausal status and investigated if menopause *per se* is associated with presence of calcium in coronary arteries (CAC) in the ELSA-Brasil.

Methods: All participants except perimenopausal women, those who had menopause <40 years or from non-natural causes or reported use of hormone therapy were included. Sample was stratified according to menopause and age categories (pre-menopause ≤45 years, pre-menopause >45 years and post-menopause); their clinical profile and CT-determined CAC compared using Kruskal-Wallis and chi-squared test for frequencies. Associations of CAC (binary variable) with menopause categories adjusted for traditional and non-traditional covariables were tested using logistic regression.

Results: From 2,047 participants aged 51±9 years, 1,175 were premenopausal (702 ≤45 years) and 872 postmenopausal women. Mean values of anthropometric variables, blood pressure, lipid and glucose parameters, branched-chain amino acids (BCAA) and insulin resistance index (HOMA-IR), as well as frequencies of morbidities, were more favorable in premenopausal, particularly in younger ones. In crude analyses, CAC >0 was associated with triglyceride-rich lipoprotein remnants, dense low-density lipoprotein, BCAA and other variables, but not with HOMA-IR. Menopause was independently associated with CAC >0 (OR 2.37 [95%CI 1.17-4.81]) when compared to the younger premenopausal group.

Conclusion: Associations of menopause with CAC, independently of traditional and non-traditional cardiovascular risk factors, suggest that hormonal decline *per se* may contribute to calcium deposition in coronary arteries.

Introduction

Cardiovascular disease (CVD) is the major cause of death worldwide[1-3] but large studies conducted in developing world, particularly in the female sex, are scarce. Coronary artery disease stands out as determinant of morbidity and mortality especially after menopause[4] when cardiovascular risk in women resembles that of men at the same age[5]. Traditional cardiovascular risk factors do not account for the excessive risk in postmenopausal women, and the decline in oestrogen levels could be a contributing factor. To date, it is unknown if menopause, independently of age and other risk factors, increases cardiovascular risk, which could imply in public health interventions to this specific population stratum.

Worsening of insulin sensitivity occurs after menopause competing to increase the prevalence of dyslipidemia, hypertension and diabetes[6]. Among the lipid metabolism abnormalities, increase in triglyceride levels, decrease in high-density lipoprotein cholesterol (HDL-c) and a predominance of small dense low-density lipoprotein (LDL) have been reported with some controversial results[7-16]. Recently, our group found higher levels of triglyceride-rich lipoprotein remnants (TRL-c) in postmenopausal women from the ELSA-Brasil[17], which could be contributing to their risk excess[18,19]. Considering the need of improving prediction of cardiovascular events in women, the role of branched-chain amino acids (BCAA) has been investigated. Increased BCAA in circulation have been associated with cardiometabolic disorders, particularly insulin resistance and diabetes, and may represent an early marker of CVD[20-25].

Loss of cardiovascular protection in postmenopausal women is partially dependent of hypoestrogenism since this is associated with changes in the lipid profile, endothelial function, inflammation and coagulability[26,27]. However, cardiovascular benefits from hormone therapy (HT), which ameliorates dyslipidemia, are controversial[28-37]. Deepening the knowledge on underlying mechanisms of the arterial system damage as well as on the impact of interventions

in lipoprotein subfractions and other biomarkers could contribute to improve management of cardiovascular risk in the female population.

Compared to men at the same age, women have been under investigated, underdiagnosed and undertreated for CVD[1-3]. Early detection of atherosclerosis by computed tomography-detected calcium in the coronary arteries (CAC) was shown to improve prediction of cardiovascular events in both sexes[38,39]. Any amount of calcium in arterial wall has been considered indicative of atherogenesis and has prognostic importance[38]. It has been suggested that CAC is even more strongly related with mortality in women than in men[40]. Whether oestrogen is directly related to CAC and when calcium deposition begins during the physiological hormonal decline are still under debate. Associations of oestrogen with CAC have been poorly investigated and results are inconsistent[30,41-43].

The large sample of the Longitudinal Study of Adult Health (ELSA-Brasil)[44] enables the evaluation of new biomarkers and the use of accurate methods for detection of arterial structural abnormalities. We hypothesized that deepening analyses of circulating lipids and other biomarkers like BCAA could help differentiating women at higher risk for atherosclerosis. As a sub-study of ELSA-Brasil, we compared a broad cardiometabolic risk profile of women stratified by their menopausal status and independent associations of menopause with presence of calcium in coronary arteries.

Methods

Study population

Details of objectives and methodology of the ELSA-Brasil were reported elsewhere[44]. Briefly, this is a multicenter cohort study designed to evaluate chronic diseases, which included at baseline 15,105 civil servants from six universities in Brazil and was approved by local ethics committees. The present cross-sectional analysis was based on baseline data of pre- and

postmenopausal women followed at the University of São Paulo center. Exclusion criteria were non-natural cause of menopause (n = 420), occurrence before 40 years of age (n = 40), current use of HT (n = 97) and perimenopausal women (last menstrual cycle ≥6 and <12 months; n = 122). The final sample included 2,047 women.

Trained interviewers applied questionnaires to participants who were invited for a visit in the site for clinical examinations, collection of biological material and imaging exams. A sub-sample underwent computed tomography (CT) to quantify CAC and blood determinations of lipoprotein subfractions and BCAA.

Variables of interest

Menopause was defined as at least 12 months of menstrual cessation. Data were self-reported by the participants, who were stratified in: premenopausal aged ≤45 years, premenopausal aged >45 years and postmenopausal. Menopause was the independent variable of major interest. Women were grouped according to absence (CAC =0) or any amount of CT-detected calcium in coronary arteries (CAC >0).

Other variables of interest included sociodemographic data (skin color and education level), life habits (smoking and leisure-time physical activity), anthropometry, blood pressure, medications (lipid-lowering agents), laboratory data (glucose, lipid profile, insulin and BCAA) and presence of morbidities (obesity, hypertension, diabetes, dyslipidemia and CVD). Weight was measured using a digital scale with 0.1 kg of precision and height by a fixed stadiometer with 0.1 cm precision; these measurements were used to calculate body mass index (BMI). Waist circumference was obtained using an inelastic tape at the midpoint between the last rib and the iliac crest. Systolic and diastolic blood pressure were obtained in triplicate after resting seated; mean values of the two last measurements were considered in analyses.

Obesity was defined by BMI ≥30 kg/m^2 and hypertension by systolic blood pressure ≥140 mmHg, diastolic ≥90 mmHg or use of antihypertensive drugs. Diabetes mellitus was self-

reported or diagnosed by fasting plasma glucose ≥126 mg/dL or 2-hour plasma glucose after 75-g glucose load ≥200 mg/dL or glycated hemoglobin ≥6.5%. Hypercholesterolemia was diagnosed by LDL-c ≥130 mg/dL, low HDL-c by levels <50 mg/dL and hypertriglyceridemia by levels ≥150 mg/dL. CVD was defined by self-reported history of myocardial infarction, angina *pectoris*, stroke, transient ischemic attack or peripheral arterial disease. High schooling was defined by at least 11 years of education, physically active at leisure by ≥150 minutes per week of moderate intensity physical activity or ≥75 minutes per week of high intensity physical activity. Current smoking was self-reported.

Laboratory

Blood samples were collected after overnight fasting and aliquots were immediately analyzed or stored at -80°C for future determinations. Plasma glucose was determined by the glucose oxidase method and insulin by enzyme-linked immunosorbent assay (Monobind, Lake Forest, CA, USA). Homeostasis model assessment (HOMA-IR) was used to assess insulin resistance[45]. BCAA (leucine, isoleucine and valine) concentrations were obtained using magnetic resonance spectroscopy (LabCorp, Raleigh, NC, USA). For a participants' sample, lipid profile was assessed by vertical ultracentrifugation (VAP-II, Atherotech, Birmingham, USA) that allows separation and quantification of cholesterol from lipoprotein subfractions. VAP-II provided concentrations of total cholesterol (CT), lipoprotein cholesterol (a) [Lp(a)-c], very low-density lipoprotein cholesterol (VLDL-c), $VLDL_3$-c (dense VLDL rich in cholesterol), LDLr (real LDL, which consists of total LDL-c= $LDL_{1+2+3+4}$), total LDL-c (LDLr-c + Lp(a)-c + intermediate density lipoprotein cholesterol [IDL-c]), dense LDL (LDL_3 and LDL_4), buoyant LDL (LDL_1 and $LDL_{2)}$, IDL-c, total HDL-c, HDL_2 (large and buoyant), HDL_3 (small dense HDL particle), TRL-c (which consists of $VLDL_3$-c + IDL-c) and non-HDL cholesterol[46-48]. Variability coefficients for the VAP-II parameters were previously reported[46].

Calcium in the coronary arteries

Participants were submitted to a 64 detectors chest CT to assess calcium in coronary arteries (Brilliance multi-slice, Philips Brilliance 64; Philips Healthcare, Best, Netherland). This procedure was based on non-contrasted acquisition of a series of axial images covering the entire length of the heart. Images were acquired with electrocardiogram gating[49] and included the entire heart from the bifurcation of the pulmonary arteries to the apex during expiratory pause. Default settings included 120 Kv, mA adjusted for BMI, prospective acquisition of single-phase images at mid-diastole, collimation of 2.5 mm, gantry rotation of 400 ms and reconstruction with standard filter.

Calcification was defined as a lesion with signal intensity above 130 Hounsfield units (HU) and area ≥3 adjacent pixels (at least 1 mm^2). An experienced cardiologist used a semi-automatic software to calculate Agatston score from the weighted sum of densities above 130HU *(Calcium Scoring, Philips Workstation)*[49]. Results were evaluated as absolute values of the Agatston score as a continuous variable and subsequently categorized into the absence or presence of any amount of calcium in coronary arteries (CAC = 0 or >0). A categoric variable evaluating CAC > or <100 was also generated, and the frequency of individuals with this amount of calcium was compared across groups of women.

Statistical analysis

Variables were summarized by measures of central tendency and dispersion for continuous variables and frequencies for categorical variables. Comparisons among groups were carried out by Kruskal-Wallis with post-hoc Dunn test for continuous variables or chi-squared test for frequencies. CAC was the outcome (dependent variable) and menopausal status the independent variable of main interest. Multiple logistic regression was employed to assess associations of variables with CAC >0. Variables that differed among menopausal status groups in the univariate analysis at a p-value <0.20 were entered in logistic regression models. Odds ratios (OR) and 95% confidence intervals (95% CI) were provided using the younger

premenopausal group as reference. Additional analysis taking the older premenopausal group as reference and another subgroup analysis excluding participants with CVD were performed. A p-value <0.05 was considered significant. Stata package, version 12, was used for analysis (StataCorp LLC, College Station, Texas, USA).

Results

From 2,047 participants, 1,175 were pre-menopause and 872 postmenopausal women. Mean age was 51±9 years; 47% had high schooling, 60% reported white skin color. Frequencies of current smokers (13.7; 16.5 and 17.4%, p = 0.120) and physically actives (18.5; 18.4 and 18.0%; p = 0.960), respectively in premenopausal ≤45 years, premenopausal >45 years and postmenopausal groups, did not differ. Lipid-lowering therapy was more frequent in postmenopausal women than in premenopausal women aged ≤45 and >45 years (20.6; 2.4 and 7.8%, respectively, p <0.001). For the postmenopausal group, median age at menopause was 50 years (interquartile range [IQR]: 47-52), time since menopause was 7 years (IQR: 4-13) and 264 women reported previous use of HT.

Main characteristics of the sample according to age and menopausal status are shown in Table 1. Postmenopausal women were older and had higher systolic blood pressure, fasting plasma glucose, HOMA-IR and worse lipid profile when compared to premenopausal ones of both age groups. Differences in BMI and BCAA concentrations were observed only in relation to the premenopausal group aged ≤45 years. Higher frequencies of morbidities (hypertension, diabetes, dyslipidemias, CVD) and CAC >0 were detected in the postmenopausal group. Frequencies of individuals with CAC >100 were low in premenopausal women aged ≤45 and >45 years (0.2 and 2.0%, respectively), but significantly higher in the postmenopausal ones (11.0%, p < 0.001 between groups). Due to low frequencies of CAC >100, association of this variable was not tested in regression models.

OR for the associations of CAC with variables of interest are shown in Table 2. CAC >0 was directly associated with menopause, age, smoking, systolic blood pressure, fasting plasma glucose, TRL-c, dense LDL-c, buoyant LDL-c, BCAA and frequencies of obesity, hypertension, diabetes, dyslipidemias, use of lipid-lowering therapy and CVD. No association between CAC and high schooling, HOMA-IR, HDL_2-c, HDL_3-c and low HDL-c was found.

Logistic regression models are depicted in Table 3. In the crude model, taking the premenopausal women aged ≤45 years as reference, chance for having CAC >0 was higher in the older premenopausal and also in the postmenopausal women (OR 3.70 [95% CI 2.16-6.35] and OR of 17.79 [95% CI 11.12-28.47], respectively). In model 1, adjusted for age, smoking, traditional cardiovascular risk factors, BCAA and HOMA-IR, menopause was associated with CAC>0, in relation to the younger premenopausal group. In model 2, including metabolic variables as continuous instead of categorical, results were similar to the previously described ones. No association of CAC >0 was observed in adjusted models when comparing postmenopausal to the totality of premenopausal women. Associations of CAC >0 with age (OR 1.12 [95% CI 1.09-1.15]), smoking (OR 2.16 [95% CI 1.47-3.16]), physical activity (OR 1.47 [95% CI 1.02-2.14]), CVD (OR 1.86 [95% CI 1.02-3.39]), hypertriglyceridemia (OR 1.48 [95% CI 1.06-2.08]), hypercholesterolemia (OR 1.46 [95% CI 1.08-1.98]) were found in model 1, but not with skin color, obesity, hypertension, diabetes, lipid-lowering therapy, HOMA-IR and BCAA. A sensitivity analysis excluding participants with CVD showed similar results (adjusted OR 1.59 [95% CI 0.83-3.06] and 2.14 [95% CI 1.03-4.46] for premenopausal >45 and postmenopausal women, respectively). When taking older premenopausal women as reference, postmenopausal ones had similar risk for CAC >0 (OR 1.44 [95% CI 0.92-2.26]).

Discussion

Our findings support that menopause *per se* – regardless of age and related metabolic consequences – may be associated with coronary artery disease assessed by an accurate method of atherosclerosis detection. Changes in traditional risk factors in postmenopausal women are considered underlying mechanisms of the increased cardiovascular risk observed in this phase of life. Despite these factors having diminished the strength of the association, menopause and CAC remained associated even including extended adjustments when compared to younger premenopausal women. We reinforced a deleterious relationship between dense LDL-c particles and TRL-c with calcium deposition in coronary arteries but found no independent association of CAC with insulin resistance or BCAA.

We employed CT-determined CAC that is considered a distinguished method for evaluating subclinical atherosclerosis, particularly in asymptomatic individuals being predictive of cardiovascular outcomes[38,39,51]. Recently, a large study suggested that CAC in women is a death predictor even stronger than in men[40]. Thus, such an examination might enable cardiovascular risk reclassification and suggest more appropriate therapies in women.

Risk stratification based on CAC has more commonly used four categories indicating gradual increments in the risk of a coronary event[38,52-54]. A CAC =0 indicates a low probability of cardiovascular outcomes while having any amount of CAC detectable confirms coronary atherosclerosis[38]. Considering the sensitivity of this exam to detect small deposition of calcium, and the lower frequency of higher amounts of calcium detectable in some populations, investigators have shown that any amount of CAC detectable in the CT is associated with a significantly higher risk of major cardiovascular events (especially coronary, with a RR 4.30 [95% CI 3.50-5.20]) when compared to individuals with CAC =0[55]. In our study, participants were not recruited from hospital settings, which could have contributed to low rates of high CAC scores. Our stratification into two categories was previously employed with satisfactory discrimination of cardiovascular risk[55,56].

The role of aging for cardiovascular risk is wide recognized and the fact that menopause occurs in parallel limits to dissociate independent deleterious effects[57-59]. Although unquestionable ovarian hormones decline concurs with increased atherosclerotic risk after menopause, mechanisms are not completed understood. Our methodological approach was an attempt to innovate and seems to contribute to this field. In our study, postmenopausal women were about twice as likely to have CAC than younger premenopausal ones, after adjusting for several variables including age. This finding should be valued and reinforces an alert to the cardiovascular health of women which has been frequently neglected[3]. Our study arouses the need to deepen investigation on how direct vascular action of oestrogen and/or indirect effects in metabolism contribute to increased women's risk. It is known that oestrogen participates in regulating lipid metabolism, inflammatory activity and coagulation[27,60,61] and that it promotes nitric oxide-mediated vasodilation and inhibits platelet and inflammatory activity on healthy endothelium. However, when endothelial dysfunction and atherosclerosis are present, oestrogen seems to have antagonistic effects, reducing vasodilation and generating inflammation and plaque instability[27]. In the present study, determination of circulating hormones was not available but low oestrogen levels were surely presumable in our sample of postmenopausal women. We also supposed that this physiological event of woman life would have a specific relation with structural arterial abnormalities independently of the aging impact. As a matter of fact, a previous autopsy study including 56 women found an inverse association of oestrogen with the presence and amount of calcium in the coronary arteries[62], suggesting that oestrogen could modulate the deposition of calcium in arteries, favoring to the hypothesis raised herein. More recently, a contrasting result was reported in 1,947 postmenopausal women from the *Multi-Ethnic Study of Atherosclerosis* (MESA), in which no association between oestrogen and CAC was detected[41]. Of notice, estradiol levels diminish and become very low in postmenopausal women. As the postmenopausal ovary continues to produce testosterone, a

relatively higher circulating level of androgens may play a role in calcium deposition and/or plaques in the coronaries of peri- and postmenopausal women[42,43]. Inconsistencies in the literature[30,41-43] reinforce the importance of reporting our findings obtained in this large sample of the ELSA-Brasil, that has markedly contributed to the field of cardiometabolic diseases' determinants.

Considering previously described effects of oestrogen on insulin sensitivity, glucose and lipid metabolism[60,61], we performed a broad evaluation of the cardiometabolic risk profile of the participants according to their menopausal status. Insulin resistance as estimated by the HOMA-IR index differed between pre- and postmenopausal women, as did BCAA (although BCAA levels were only slightly different between groups, $p<0.001$). It is known that insulin sensitivity deteriorates with aging[63]. Also, there is evidence that hypoestrogenism contributes to insulin resistance and a Brazilian study previously published found an independent association between CAC and insulin resistance index, suggesting a role for insulin resistance in mediating calcium deposition in the coronary arteries[64], which could be partly modulated by oestrogen. Meanwhile, no studies that we are aware of have investigated the association of CAC with BCAA, although studies have observed associations of BCAA with cardiovascular events[25,65] and several metabolic disturbances linked by the insulin resistance[24]; however, none of these risk markers were independently associated with CAC in our study. We deepened the evaluation of lipid profile atherogenicity by determining lipoprotein subfractions in a large female sample whose gender has been understudied. A worse lipoprotein subfraction profile was confirmed in the postmenopausal group, and both TRL-c and dense LDL were associated with CAC, which is in agreement with several studies[31,66-68].

Only 8.2% of all postmenopausal participants of the ELSA-Brasil were under current HT and duration was quite variable (data not shown). Studies on the effect of HT on CAC are uncommon, with variable results[31-34], largely attributed to methodological differences (type,

dose and route of administration of hormones). Apparently, HT, especially oestrogen alone, is associated with lower CAC when initiated early after menopause, reinforcing the "timing hypothesis" in cardiovascular prevention[32,33]. We decided to exclude current HT users due to its potential confounding factor in our results.

Our study has several limitations and strengths. The arbitrary cut off of 45 years for premenopausal women was based on the median age-distribution in our sample of premenopausal women, which guaranteed appropriate numbers for each group. ELSA-Brasil included participants from 35 years of age and 90% of premenopausal women were younger than 50 years. We acknowledge the difficulty to disentangle the age-effects from menopausal status, although the overlap among age-groups was very small. When the totality of premenopausal women was taken as reference, no association of menopause with CAC >0 was observed. Another limitation is related to the lack of accurate information about cessation of menstrual cycles, since timing of menopause itself could be an indicator of increased cardiovascular risk, and this was not evaluated in our analysis. Strengths of our study were the substantial sample size, that allowed analysis of novel cardiometabolic risk markers, stratifications and adjustments for several confounding factors. Because deterioration of metabolic profile of menopausal women was expected, such adjustments were essential in an attempt to identify an association of CAC and menopause *per se*. As a matter of fact, associations of exposures with CAC were attenuated in adjusted analyses. Our cross-sectional design impedes to infer causality but contributes to the knowledge in this field indicating the need for further investigations. Since CT-detected CAC is an expensive procedure, at least for developing countries, in terms of public health care, the cost-benefit of this approach to improve cardiovascular risk detection in women has to be evaluated. Finally, the absence of hormone determinations (gonadotropins and oestrogen) have limited establishing more accurately the reproductive phase of women at the time our analyses were performed.

Conclusion

In summary, by comparing a broad cardiometabolic risk profile, we reinforced that menopause is accompanied by insulin resistance, atherogenic lipid profile and increments in BCAA. These conditions did not fully explain the association between CAC and menopause in our study sample. We suggest that there might be an independent association of menopause with CAC, consistently with the hypothesis that female hormones decline plays a role in atherosclerosis. This finding should motivate deeper investigations on this issue with appropriate design aiming to mitigate cardiovascular risk in women.

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Table 1: Median values (interquartile ranges) of clinical variables or numbers (percentages) of the participants with disorders stratified by menopausal status and age.

	Pre-menopause		**Post-menopause**	**p-value**
	≤ 45 years	**> 45 years**	**N = 872**	
	N = 702	**N = 473**		
• ***Clinical variables***				
Age, years	42 (39-44)	48 (47-50)[a]	58 (54-63)[a,b]	< 0.001
Body mass index, kg/m^2	26 (23-29)	27 (24-30)[a]	27 (24-31)[a]	< 0.001
Waist circumference, cm	82 (75-91)	85 (78-95)[a]	87 (80-96)[a,b]	< 0.001
Systolic blood pressure, mmHg	108 (102-117)	112 (104-124)[a]	119 (107-129)[a,b]	< 0.001
Diastolic blood pressure, mmHg	70 (65-78)	72 (66-79)[a]	73 (67-80)[a]	< 0.001
Glucose, mg/dL	98 (93-105)	102 (97-109)[a]	107 (100-115)[a,b]	< 0.001
HOMA-IR	1.51 (0.92-2.59)	1.59 (0.89-2.62)	1.77 (1.03-2,99)[a,b]	<0.001
Total cholesterol, mg/dL	202 (180-225)	212 (189-238)[a]	227 (202-256)[a,b]	< 0.001
LDL-c, mg/dL	122 (103-143)	130 (106-151)[a]	139 (117-165)[a,b]	< 0.001
LDLr-c, mg/dL	101 (85-119)	108 (88-125)[a]	113 (93-135)[a,b]	< 0.001
IDL-c, mg/dL	13 (10-18)	15 (11-20)[a]	18 (14-24)[a,b]	< 0.001
VLDL-c, mg/dL	21 (16-27)	22 (17-27)	25 (19-32)[a,b]	< 0.001
HDL-c, mg/dL	55 (48-65)	58 (49-67)[a]	59 (51-70)[a,b]	<0.001
Triglycerides, mg/dL	88 (66-124)	93 (70-124)	112 (83-149)[a,b]	<0.001
$VLDL_3$-c, mg/dL	12 (10-15)	13 (11-15)	14 (12-17)[a,b]	<0.001
HDL_2-c, mg/dL	16 (12-20)	16 (12-21)	16 (13-21)[a]	0.023
HDL_3-c, mg/dL	40 (36-45)	41 (37-47)[a]	43 (38-49)[a,b]	<0.001
TRL-c, mg/dL	26 (20-33)	28 (22-36)[a]	32 (26-41)[a,b]	<0.001
Buoyant LDL-c, mg/dL	38 (29-50)	44 (32-57)[a]	48 (36-62)[a,b]	<0.001
Dense LDL-c, mg/dL	45 (33-61)	45 (34-63)	49 (35-66)[a,b]	0.001
Lipoprotein (a), mg/dL	6 (4-9)	6 (5-9)	7 (5-10)[a,b]	<0.001
BCAA, µmol/L	374 (335-414)	383 (343-432)[a]	388 (349-441)[a]	<0.001
Valine, µmol/L	207 (183-228)	212 (190-238)[a]	218 (192-244)[a]	<0.001
Leucine, µmol/L	127 (111-144)	131 (115-148)[a]	132 (113-150)[a]	0.027
Isoleucine, µmol/L	40 (31-47)	41 (31-50)	41 (32-52)[a]	0.020
• ***Frequencies of disorders***				
Obesity	161 (22.9)	130 (27.5)	253 (29.0)	0.022

Hypertension	80 (11.4)	115 (24.3)	336 (38.5)	<0.001
Diabetes	45 (6.4)	74 (15.6)	214 (24.5)	<0.001
Hypercholesterolemia	239 (34.1)	210 (44.4)	466 (53.4)	<0.001
Low HDL-c	214 (30.5)	114 (24.1)	159 (18.2)	<0.001
Hypertriglyceridemia	126 (18.0)	85 (18.0)	260 (29.8)	<0.001
CAC > zero	20 (3.0)	46 (10.4)	264 (35.8)	<0.001
Cardiovascular disease	17 (2.4)	21 (4.4)	50 (5.7)	0.006

HOMA-IR: insulin resistance index; LDL-c: total low-density lipoprotein cholesterol; LDLr-c: real low-density lipoprotein cholesterol; IDL-c: intermediate-density lipoprotein cholesterol; VLDL-c: very low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; TRL-c: triglyceride-rich lipoprotein remnants; Dense LDL-c: sum of LDL_{3+4}-c; Buoyant LDL-c: sum of LDL_{1+2}-c; BCAA: branched-chain amino acids; CAC: coronary artery calcium. Groups compared by Kruskal-Wallis with post-hoc Dunn test or chi-square test. $P < 0.05$: [a]versus pre-menopause ≤45 years; [b]versus pre-menopause >45 years.

Table 2: Crude odds ratios (95% confidence intervals) for the association of CAC > 0.

	Odds ratio	**95% confidence interval**
Menopause	8.74	6.54-11.69
Lipid-lowering therapy	3.38	2.48-4.62
Hypertension	2.96	2.31-3.80
Cardiovascular disease	2.55	1.59-4.10
Diabetes	2.48	1.87-3.29
Hypertriglyceridemia	2.13	1.65-2.76
Hypercholesterolemia	1.59	1.25-2.01
Smoking	1.56	1.15-2.11
Obesity	1.38	1.07-1.79
Physically active	1.25	0.92-1.69
White skin color	1.22	0.95-1.57
Age	1.15	1.13-1.17
Body mass index	1.03	1.01-1.06
TRL-c	1.03	1.02-1.04
Systolic blood pressure	1.03	1.02-1.04
Plasma glucose	1.02	1.01-1.02
Dense LDL-c	1.01	1.01-1.02
HOMA-IR	1.01	1.00-1.02
Buoyant LDL-c	1.00	1.00-1.01
BCAA	1.00	1.00-1.01

Univariate logistic regression used. CAC: coronary artery calcium; TRL-c: triglyceride-rich lipoprotein remnants; LDL-c: low-density lipoprotein cholesterol; Dense LDL-c: sum of LDL_{3+4}-c; Buoyant LDL-c: sum of LDL_{1+2}-c; HOMA-IR: insulin resistance index; BCAA: branched-chain amino acids; HDL-c: high-density lipoprotein cholesterol.

Table 3: Crude and adjusted *odds ratios* and 95% confidence intervals for the associations of CAC > 0 and groups of women.

	Crude	**Model 1**	**Model 2**
Pre-menopause	Reference	Reference	Reference
Post-menopause	8.74 (6.54-11.69)	1.50 (0.95-2.37)	1.47 (0.92-2.34)
Pre-menopause ≤ 45 years	Reference	Reference	Reference
Pre-menopause > 45 years	3.70 (2.16-6.35)	1.72 (0.92-3.22)	1.63 (0.89-2.98)
Post-menopause	17.79 (11.12-28.47)	2.37 (1.17-4.81)	2.20 (1.16-4.18)

Values obtained by multiple logistic regression. Model 1: adjusted for age, skin color, smoking, physical activity, hypertension, diabetes, obesity, hypertriglyceridemia, hypercholesterolemia, cardiovascular disease, lipid-lowering therapy, insulin resistance index and branched-chain amino acids. Model 2: adjusted for age, skin color, smoking, physical activity, blood pressure, fasting glucose, body mass index, TRL-c, dense LDL-c, insulin resistance index, branched-chain amino acids, cardiovascular disease and lipid-lowering therapy.

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4.4 MEETING PRESENTATION

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Could serum branched-chain amino acids (BCAA) contribute to the increased cardiovascular risk in postmenopausal women?

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Introduction: Postmenopausal woman has increased cardiovascular risk, not all explained by traditional risk factors. Recently, BCAA have been associated with several cardiometabolic abnormalities in both sexes. Independent association with menopause was scarcely investigated, which could contribute to the increased risk of postmenopausal women. We tested association of serum BCAA with menopausal status (pre- or post-menopause).
Methods: This is a cross-sectional analysis of baseline data of women from the Sao Paulo centre of ELSA-Brasil stratified according to menopausal status. Exclusion criteria included menopause < 40 years and non-natural cause. Traditional risk factors and NMR spectroscopy-determined BCAA (valine, leucine and isoleucine) were compared using Mann–Whitney or chi-squared test. Multiple linear regression (BCAA as dependent variable) was performed to test independent association with menopausal category, adjusted for several factors. Additional analysis included comparisons of BCAA across 4 groups by combining presence/absence of metabolic syndrome (MS) and menopausal status: pre-menopause MS−, pre-menopause MS+, post-menopause MS− and post-menopause MS+.
Results: A total of 2,258 women (50.7 ± 8.8) were included. Postmenopausal women were older and had higher blood pressure, glucose, lipids, BCAA levels and higher prevalence rates of cardiometabolic disturbances than pre-menopausal ones. In multiple linear regression, central obesity, diabetes, hypertriglyceridemia, low HDL-c (components of the MS) were significantly associated with higher serum BCAA when the entire sample or menopausal categories were considered, but menopause was not independently associated with BCAA. Comparisons of BCAA within MS− and MS+ groups showed no difference between pre- and postmenopausal women. However, when comparing premenopausal or postmenopausal women, each with and without MS, higher BCAA levels were consistently found in MS+ groups. Conclusion: Our finding did not support that menopause is independently associated with circulating levels of BCAA in ELSA-Brasil, but the classical components of the MS.

Keywords: Menopause; Women; Branched-chain amino acids; BCAA; Metabolic syndrome.

5 FINAL CONSIDERATIONS AND CONCLUSIONS

Considering our set of results, we propose that menopause *per se* is a condition associated with changes in lipid profile and lipoprotein subfractions, calcium deposition in coronary arteries and possibly with overall cardiovascular risk in women, independently of age and other traditional risk factors.

As a background for this thesis, in our first paper[226], we reviewed the impact of menopause and diabetes on lipids and lipoprotein subfractions. With regards to the methods for lipoprotein subfractionation, we concluded that these techniques should be reserved for research purposes, as they do not improve cardiovascular risk assessment in women. We believe that there is still need to deepen the investigation of menopausal effects on lipoprotein subfractions.

In our second paper[227], our findings reinforced that menopause was associated with a more atherogenic lipid profile, including higher $VLDL_3$-c and TRL-c, but not with HDL-c subfractions or dense LDL-c. Of notice, more prominent lipid disturbances were observed in the early years post-menopause, suggesting a role for oestrogen deficiency in these metabolic alterations. Prospective studies on the role of short- and long-term hypoestrogenism on cardiovascular risk in postmenopausal women are needed.

In paper 3, CAC > 0 showed to be associated with TRL-c and dense LDL-c, but not with BCAA nor HOMA-IR. We also found that postmenopausal women were about twice as likely to have CAC > 0 than younger premenopausal ones, after several adjustments. This is relevant and should alert to the cardiovascular risk in female sex.

When tested[228] if menopause was independently associated with BCAA; this was not confirmed in our ELSA-Brasil sample. Major cardiovascular risk factors seem to explain most of the BCAA levels in women, although further studies are needed.

We conclude that menopause is associated with changes in lipoprotein fractions and subfractions and with calcium deposition in the coronary arteries. Despite the difficulty to disentangle the age-effects from menopausal ones, it seems that natural menopause, independently of age and other risk factors, deteriorates cardiovascular health and might be considered an independent risk factor. More investigation on the oestrogen effects on lipoprotein fractions and subfractions,

coronary calcium deposition and cardiovascular events is needed[229,230]. Long-term prospective studies regarding the effects of interventions on these parameters may help answering some clues raised in this thesis, contributing to change cardiovascular outcomes in women.

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7 APPENDIX

ATTACHMENT 1 - Informed consent form of the ELSA-Brasil

ID NUMERO:							

Código Formulário: TCL
Versão: 10/06/2009

ELSA BRASIL

Termo de Consentimento Livre e Esclarecido (TCLE)

a. Declarou que compreendeu as informações apresentadas no TCLE e deu consentimento para participação no estudo

[ ] Não

[ ] Sim

b. Declarou concordar que amostras de sangue sejam armazenadas para análises futuras sobre as doenças crônicas em estudo.

[ ] Não

[ ] Sim

ATTACHMENT 2 - Baseline questionnaire applied to female participants of the ELSA-Brasil

ID NUMERO:							

Código Formulário: MUL
Versão: 09/07/2009

ELSA BRASIL

Informações Administrativas:

0a. Data da entrevista: ☐☐/☐☐/☐☐☐☐ 0b. Nº Entrevistador(a): ☐☐☐

MULHERES (MUL)

Entrevistador(a): ASSINALE SE O SEXO DO(A) PARTICIPANTE [ ] Mulher [ ] Homem **(PULE PARA A QUESTÃO 01 DO BLOCO DIE)**	
Entrevistador(a): Diga à participante: "*As próximas perguntas são importantes para conhecer aspectos específicos da saúde das mulheres. Vamos falar primeiro sobre a sua menstruação*".	
01. Que idade a Sra. tinha quando menstruou pela primeira vez?	
|___|___| anos	
[ ] NÃO SABE/NÃO QUER RESPONDER	
[ ] Nunca menstruei **(PULE PARA A QUESTÃO 16; ANTES LEIA O CABEÇALHO)**	
02. A Sra. ainda menstrua?	
[ ] Sim **(PULE PARA A QUESTÃO 06)**	
[ ] NÃO SABE/NÃO QUER RESPONDER	
[ ] Não --------→	03. Há quanto tempo a Sra. parou de menstruar? LEIA AS ALTERNATIVAS
	[ ] Há menos de 6 meses
	[ ] Entre 6 meses e 1 ano
	[ ] Há mais de 1 ano
	[ ] NÃO SABE/NÃO QUER RESPONDER

	04. Que idade a Sra. tinha quando sua menstruação parou definitivamente?
	|___|___| anos
	[ ] NÃO SE APLICA
	[ ] NÃO SABE/NÃO QUER RESPONDER
	05. Porque não menstrua mais? LEIA AS ALTERNATIVAS. Se for o caso, pode ser escolhida mais de uma opção de resposta.
	[ ] Menopausa natural
	[ ] Cirurgia para retirada de útero (histerectomia)
	[ ] Cirurgia para retirada de dois ovários
	[ ] Outros tratamentos (hormônios, quimioterapia ou radiação)
	[ ] Outra razão. Especifique:
	[ ] NÃO SABE/NÃO QUER RESPONDER
06. De quanto em quanto tempo a Sra. costuma/costumava ficar menstruada? LEIA AS ALTERNATIVAS	
[ ] Menos de 25 dias	
[ ] Entre 25 e 34 dias	
[ ] Entre 35 e 59 dias	
[ ] Entre 60 dias e 6 meses incompletos	
[ ] Entre 6 meses e um ano	
[ ] Mais de um ano	
[ ] Tinha ciclos irregulares	
[ ] NÃO SABE/NÃO QUER RESPONDER	

Entrevistador(a): Diga à participante: *"Vamos falar agora sobre gravidez"*	
07. A Sra. já esteve grávida? Considere todas as gestações, incluindo aquelas que resultaram em filho nascido vivo ou morto, em aborto espontâneo/perda, aborto provocado e gravidez ectópica/nas trompas.	
[ ] Sim	
[ ] Não [ ] NÃO SABE/NÃO QUER RESPONDER	**(PULE PARA A QUESTÃO 14)**
08. Que idade a Sra. tinha quando engravidou pela primeira vez?	
|___|___| anos	
[ ] NÃO SABE/NÃO QUER RESPONDER	
09. Considerando todas as suas gestações, quantas terminaram com: LEIA AS ALTERNATIVAS	
|___|___| Nascidos-vivos	
|___|___| Nascidos-mortos	
|___|___| Abortos	
|___|___| Outras (gravidez tubária (nas trompas), mola e etc)	
[ ] NÃO SABE/NÃO QUER RESPONDER	
10. Apenas confirmando o número total de vezes que a Sra. engravidou foi:	
|___|___| GESTAÇÕES	
[ ] NÃO SABE/NÃO QUER RESPONDER	

11. Em alguma gravidez, parto ou pós-parto, a Sra. teve pré-eclampsia/eclampsia (pressão alta e inchação nas pernas que podem levar à perda do bebê, especialmente na primeira gravidez)?	
[ ] Sim	
[ ] Não	
[ ] NÃO SABE/NÃO QUER RESPONDER	
12. Em alguma gravidez, a Sra. teve ganho de peso maior do que 30 kg?	
[ ] NÃO SABE/NÃO QUER RESPONDER	
[ ] Não	
[ ] Sim --------→	13. Em quantas gestações?
	|___|___| gestações
	[ ] NÃO SABE/NÃO QUER RESPONDER
14. Mantendo relações sexuais com freqüência, alguma vez a Sra. já tentou engravidar durante um ano completo ou mais e não conseguiu?	
[ ] Sim	
[ ] Não	
[ ] NÃO SABE/NÃO QUER RESPONDER	
15. Algum médico já lhe deu o diagnóstico de síndrome de ovários policísticos? LEIA AS ALTERNATIVAS.	
[ ] Sim, com base em exame clínico	
[ ] Sim, confirmado por ultra-som	
[ ] Sim, mas desconhece com que base foi feito o diagnóstico	
[ ] Não	
[ ] NÃO SABE/NÃO QUER RESPONDER	

Entrevistador(a): Se a participante tiver dito que não menstrua mais (ver Questão 02) **PULE PARA A QUESTÃO 21; ANTES DA PERGUNTA LEIA O CABEÇALHO**. Diga à participante: *"As próximas perguntas se referem aos métodos anticoncepcionais"*.	
16. **ATUALMENTE**, a Sra. (ou seu marido/parceiro) usa algum método para evitar a gravidez?	
[ ] Sim	
[ ] Não [ ] NÃO SABE/NÃO QUER RESPONDER	**(PULE PARA A QUESTÃO 21; ANTES DA PERGUNTA LEIA O CABEÇALHO)**
17. Qual método anticoncepcional a Sra. (ou seu marido/parceiro) usa atualmente? Se for o caso, escolha mais de uma opção de resposta. **Entrevistador(a):** ENTREGUE O CARTÃO MUL01	
[ ] Pílula (comprimido oral)	
[ ] Injeções contraceptivas	
[ ] Implante hormonal	
[ ] Anel (contraceptivo hormonal intravaginal)	
[ ] DIU com hormônio (Mirena)	
[ ] DIU sem hormônio	SE SOMENTE ALGUM DESSES ITENS TIVER SIDO ASSINALADO, **PULE PARA A QUESTÃO 21; ANTES DA PERGUNTA LEIA O CABEÇALHO.**
[ ] DIU não especificado	
[ ] Camisinha masculina (condom)	
[ ] Ligadura/laqueadura de trompas (esterilização feminina)	
[ ] Parceiro fez vasectomia (esterilização masculina)	
[ ] Outro (especifique):	
[ ] NÃO SABE/NÃO QUER RESPONDER	
Entrevistador(a): RECOLHA O CARTÃO MUL01	

18. Que idade a Sra. tinha quando começou a usar o método hormonal atual (pílula, injeções contraceptivas, implante hormonal, anel (contraceptivo hormonal intravaginal) ou DIU com hormônio)?
|___|___| ANOS
[ ] NÃO SABE/NÃO QUER RESPONDER
19. Por quanto tempo usa esse método hormonal atual (somar o tempo de uso apenas do método hormonal atual, **EXCLUINDO** os períodos que interrompeu)?
|___|___| ANOS (SE MENOS QUE 1 ANO MARQUE 00)
[ ] NÃO SABE/NÃO QUER RESPONDER
20. Qual é o nome comercial/genérico do método hormonal atual (pílula, implante ou injeções contraceptivas) que usa? **Entrevistador(a):** ENTREGUE O CARTÃO MUL02
|___|___| CÓDIGO DO MEDICAMENTO
[ ] Outro --➔ 20a. Qual?
[ ] NÃO SABE/NÃO QUER RESPONDER
Entrevistador(a): RECOLHA O CARTÃO MUL02
Diga à participante: "***Agora, gostaríamos de saber sobre os métodos anticoncepcionais que a Sra. usou no passado***".
21. A Sra. já usou anticoncepcionais hormonais para evitar filhos ou qualquer outro motivo (por exemplo, para tratar acne/espinhas, para regular ou suprimir a menstruação) **QUE NÃO ESTEJA USANDO ATUALMENTE**?
[ ] Sim
[ ] Não **(PULE PARA A QUESTÃO 26; ANTES DA PERGUNTA LEIA O CABEÇALHO)** [ ] NÃO SABE/NÃO QUER RESPONDER

22. Qual método anticoncepcional hormonal a Sra. já usou? Se for o caso, escolha mais de uma opção de resposta. LEIA AS ALTERNATIVAS
[ ] Pílula (comprimido oral)
[ ] Injeções contraceptivas
[ ] Implante hormonal
[ ] Anel (contraceptivo hormonal intravaginal)
[ ] DIU com hormônio (Mirena)
[ ] NÃO SABE/NÃO QUER RESPONDER
ENTREVISTADOR(A): Leia a frase ***NÃO CONSIDERE O MÉTODO ANTICONCEPCIONAL ATUAL***, SOMENTE para as mulheres que estejam usando método anticoncepcional HORMONAL atualmente (ver **Questão 17**).
23. Que idade a Sra. tinha quando começou a usar esse (ou o primeiro desses) método(s) anticoncepcional(is) hormonal(is)? **NÃO CONSIDERE O MÉTODO ANTICONCEPCIONAL ATUAL**
|___|___| ANOS
[ ] NÃO SABE/NÃO QUER RESPONDER
24. Ao todo, **NO PASSADO**, durante quanto tempo a Sra. usou esse(s) método(s) anticoncepcional (is) hormonal (is)? Se for o caso, exclua os períodos em que interrompeu o uso. **NÃO CONSIDERE O MÉTODO ANTICONCEPCIONAL ATUAL**
|___|___| ANOS (SE MENOS QUE 1 ANO MARQUE 00)
[ ] NÃO SABE/NÃO QUER RESPONDER

25. Há quanto tempo a Sra. parou de usar esse (ou o último desses) método(s) anticoncepcional(is) hormonal(is) que a Sra usou **NO PASSADO**? **NÃO CONSIDERE O MÉTODO ANTICONCEPCIONAL ATUAL**
|___|___| ANOS (SE MENOS QUE 1 ANO MARQUE 00)
[ ] NÃO SABE/NÃO QUER RESPONDER
Leia à participante: ***"Por fim, vamos lhe fazer algumas perguntas sobre terapia hormonal, ou seja, sobre hormônios que são tomados antes ou depois da menstruação parar. Isso não inclui hormônios usados para prevenir gravidez"***
26. A Sra. usa ou já usou medicamento com hormônios femininos (como estrógeno ou progesterona) para aliviar sintomas da menopausa, para prevenir doença como osteoporose ou qualquer outro motivo?
[ ] Sim
[ ] Não **(PULE PARA A QUESTÃO 01 DO BLOCO DIE)** [ ] NÃO SABE/NÃO QUER RESPONDER
27. Que idade a Sra. tinha quando usou pela primeira vez?
|___|___| ANOS [ ] NÃO SABE/NÃO QUER RESPONDER
28. **ATUALMENTE,** a Sra. ainda usa medicamento com hormônios femininos (como estrógeno ou progesterona) para aliviar sintomas da menopausa, para prevenir doença como osteoporose ou qualquer outro motivo?
[ ] Sim
[ ] Não --➔ 29. Há quanto tempo parou?
|___|___| ANOS (SE MENOS QUE 1 ANO MARQUE 00) **(PULE PARA A QUESTÃO 31)**
[ ] NÃO SABE/NÃO QUER RESPONDER
[ ] NÃO SABE/NÃO QUER RESPONDER

30. Qual o nome comercial/genérico do medicamento com hormônio feminino que a Sra. usa atualmente? **Entrevistador(a):** ENTREGUE O CARTÃO MUL03	
|___|___| CÓDIGO DO MEDICAMENTO	
[ ] Outro --→	30a. Qual?
[ ] NÃO SABE/NÃO QUER RESPONDER	
Entrevistador(a): RECOLHA O CARTÃO MUL03	
31. Por quanto tempo a Sra. usa/usou medicamento com hormônio feminino (somar todo o tempo, excluindo os períodos que interrompeu)?	
|___|___| ANOS (SE MENOS QUE 1 ANO MARQUE 00)	
[ ] NÃO SABE/NÃO QUER RESPONDER	

ATTACHMENT 3 - Paper 1 published, available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5384156/

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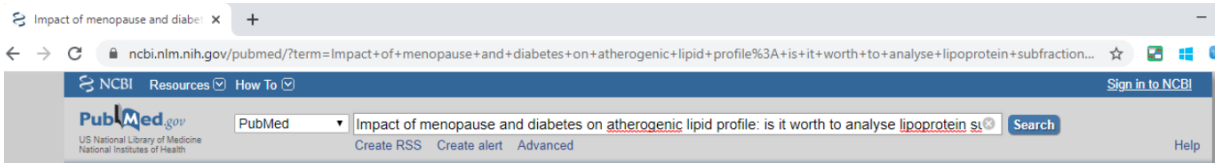
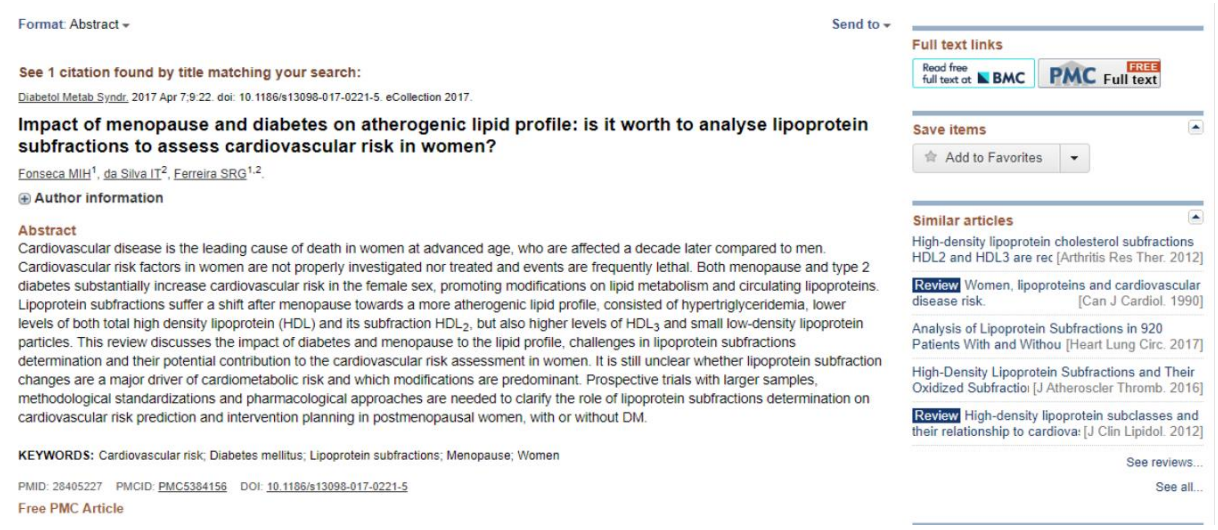
Diabetology & Metabolic Syndrome

REVIEW Open Access

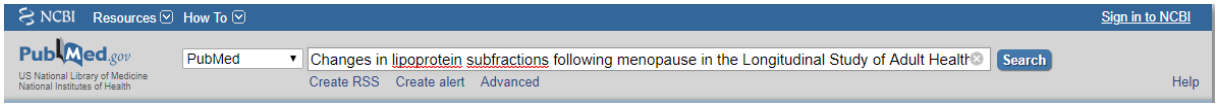
Impact of menopause and diabetes on atherogenic lipid profile: is it worth to analyse lipoprotein subfractions to assess cardiovascular risk in women?

Marília Izar Helfenstein Fonseca[1], Isis Tande da Silva[2] and Sandra Roberta G. Ferreira[1,2*]





ATTACHMENT 4 - Paper 2 published, available at: https://linkinghub.elsevier.com/retrieve/pii/S0378-5122(19)30420-7



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Maturitas. 2019 Dec;130:32-37. doi: 10.1016/j.maturitas.2019.09.005. Epub 2019 Sep 12.

Changes in lipoprotein subfractions following menopause in the Longitudinal Study of Adult Health (ELSA-Brasil).

Fonseca MIH[1], de Almeida-Pititto B[2], Bensenor IM[3], Toth PP[4], Jones SR[5], Blaha MJ[6], Lotufo PA[7], Kulkarni KR[8], Ferreira SRG[9], ELSA-Brasil Research Group.

Author information

Abstract

INTRODUCTION: It is unclear how aging and menopause-induced lipid changes contribute to the elevated cardiovascular risk in menopausal women. We examined the association between lipid profiles and menopausal status and duration of menopause in the Longitudinal Study of Adult Health (ELSA-Brasil).

METHODS: This is a cross-sectional analysis of baseline data from women in the ELSA-Brasil, stratified by duration of menopause into 5 groups: pre-menopause, <2 years, 2-5.9 years, 6-9.9 years and ≥10 years of menopause, excluding menopause <40 years or of non-natural cause; also excluded were women using lipid-lowering drugs or hormone replacement. Comparisons were performed using ANOVA with Bonferroni correction. Associations of menopause categories and time since menopause with lipid variables obtained by vertical auto-profile were tested using multiple linear regression.

RESULTS: From 1916 women, postmenopausal groups had unadjusted higher total cholesterol, LDL-c, real LDL-c, IDL-c, VLDL-c, triglycerides, non-HDL-c, $VLDL_3$-c, triglyceride-rich lipoprotein remnants (TRL-c) and buoyant LDL-c concentrations than pre-menopausal women, with no difference among postmenopausal groups. In multiple linear regression, duration of menopause <2 years was significantly associated with TRL-c [7.21 mg/dL (95% CI 3.59-10.84)] and VLDL3-c [2.43 mg/dL (95%CI 1.02-3.83)]. No associations of menopausal categories with HDL-c or LDL-c subfractions were found, and nor were associations of time since menopause with lipid subfractions.

CONCLUSIONS: In a large sample of Brazilian women, deterioration of the lipid profile following menopause was confirmed, which could contribute to the increased cardiovascular risk. Our findings suggest a postmenopausal elevation in triglyceride-rich lipoprotein remnants. How lipoprotein subfractions change after the onset of menopause warrants investigation in studies with appropriate designs.

KEYWORDS: Cardiovascular risk; Lipoprotein subfractions; Low-density lipoprotein; Menopause; Triglyceride-rich lipoprotein remnants; Very low-density lipoprotein

PMID: 31706433 DOI: 10.1016/j.maturitas.2019.09.005

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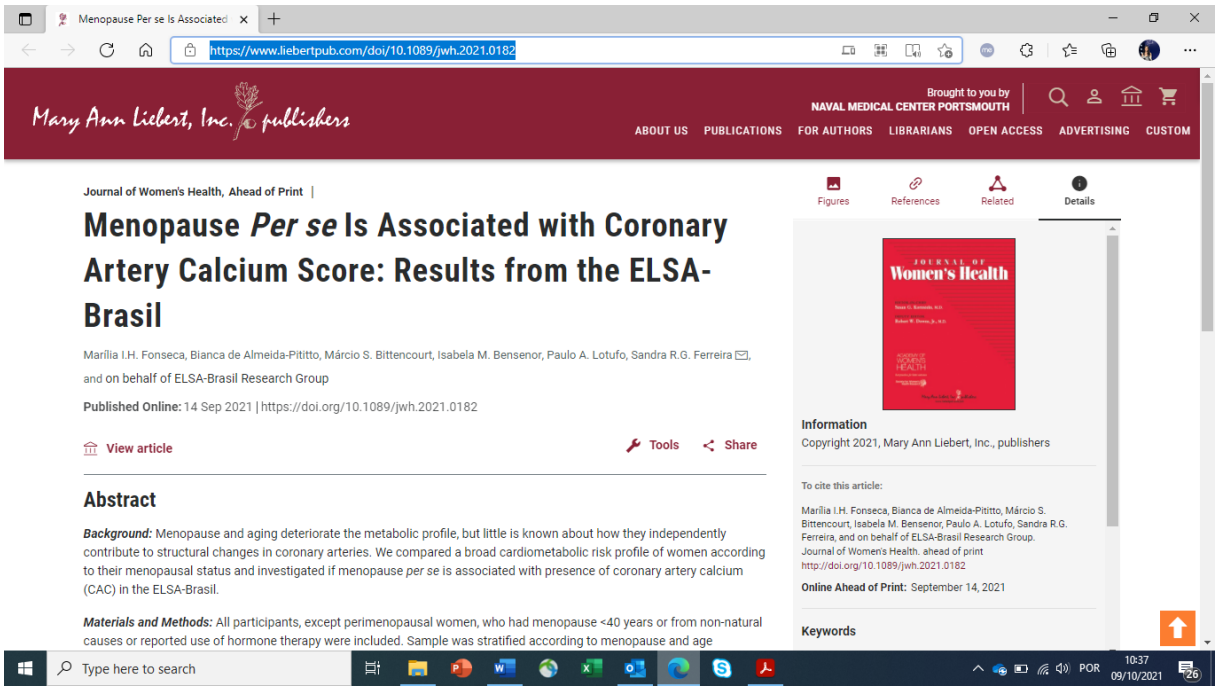
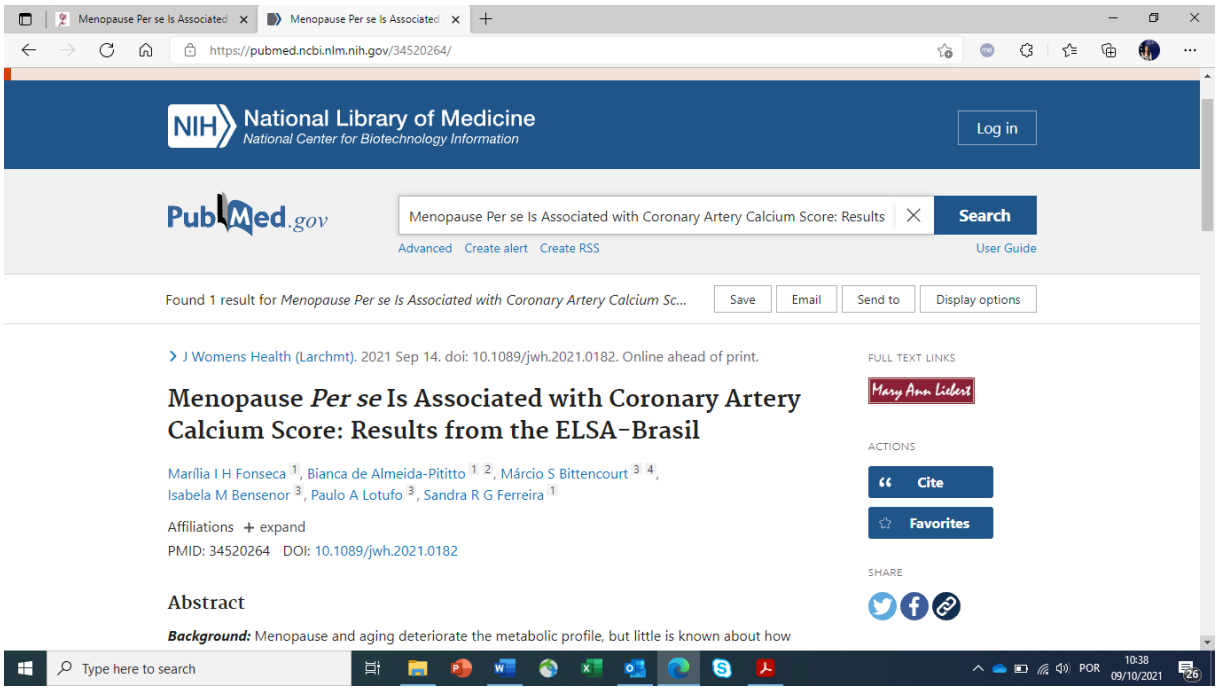
ATTACHMENT 5 - Paper 3 published, available at: [Menopause Per se Is Associated with Coronary Artery Calcium Score: Results from the ELSA-Brasil | Journal of Women's Health (liebertpub.com)]





ATTACHMENT 6 – Meeting presentation as a poster number P60 presented at 22nd Brazilian Diabetes Society Congress 2019 and published, available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6794747/pdf/13098_2019_Article_473.pdf

Diabetol Metab Syndr 2019, **11**(Suppl 1):82
https://doi.org/10.1186/s13098-019-0473-3

Diabetology & Metabolic Syndrome

MEETING ABSTRACTS **Open Access**

22nd Brazilian Diabetes Society Congress

Natal, Brazil. 16–18 October 2019
Published: 16 October 2019

P60

Could serum branched-chain amino acids (BCAA) contribute to the increased cardiovascular in postmenopausal women?

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Diabetology & Metabolic Syndrome 2019, **11(Suppl 1)**:P60

Introduction: Postmenopausal woman has increased cardiovascular risk, not all explained by traditional risk factors. Recently, BCAA have been associated with several cardiometabolic abnormalities in both sexes. Independent association with menopause was scarcely investigated, which could contribute to the increased risk of postmenopausal women. We tested association of serum BCAA with menopausal status (pre- or post-menopause).
Methods: This is a cross-sectional analysis of baseline data of women from the Sao Paulo centre of ELSA-Brasil stratified according to menopausal status. Exclusion criteria included menopause < 40 years and non-natural cause. Traditional risk factors and NMR spectroscopy-determined BCAA (valine, leucine and isoleucine) were compared using Mann–Whitney or Chi squared test. Multiple linear regression (BCAA as dependent variable) was performed to test independent association with menopausal category, adjusted for several factors. Additional analysis included comparisons of BCAA across 4 groups by combining presence/absence of metabolic syndrome (MS) and menopausal status: pre-menopause MS−, pre-menopause MS+, post-menopause MS− and post-menopause MS+.
Results: A total of 2,258 women (50.7 ± 8.8) were included. Postmenopausal women were older and had higher blood pressure, glucose, lipids, BCAA levels and higher prevalence rates of cardiometabolic disturbances than pre-menopausal ones. In multiple linear regression, central obesity, diabetes, hypertriglyceridemia, low HDL-c (components of the MS) were significantly associated with higher serum BCAA when the entire sample or menopausal categories were considered, but menopause was not independently associated with BCAA. Comparisons of BCAA within MS− and MS+ groups showed no difference between pre- and postmenopausal women. However, when comparing premenopausal or postmenopausal women, each with and without MS, higher BCAAlevels were consistently found in MS+ groups.
Conclusion: Our finding did not support that menopause is independently associated with circulating levels of BCAA in ELSA-Brasil, but the classical components of the MS.
Keywords: Menopause; Women; Branched-chain amino acids; BCAA; Metabolic syndrome.

Abstract published at Diabetology & Metabolic Syndrome 2019, 11(Suppl 1):P60

Could serum branched-chain amino acids (BCAA) contribute to the increased cardiovascular risk in postmenopausal women?

Fonseca MIH[1], Almeida-Pititto B[2], Bensenor IM[3], Lotufo PA[3], Ferreira SRG[1], on behalf of ELSA-Brasil investigators.

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INTRODUCTION AND OBJECTIVES

- Postmenopausal women have increased cardiovascular risk, not all explained by traditional risk factors.[1]
- Recently, BCAA have been associated with several cardiometabolic abnormalities in both sexes.[2,3]
- Independent association with menopause was scarcely investigated, which could contribute to the increased risk of postmenopausal women.
- We tested association of serum BCAA with menopausal status (pre- or post-menopause).

METHODS

- Cross-sectional analysis of baseline data of women from ELSA-Brasil, SP center; stratified according to menopausal status.
- Exclusion criteria included menopause <40 yrs and non-natural cause of menopause.
- Traditional risk factors and NMR spectroscopy-determined BCAA (valine, leucine and isoleucine) were compared using Mann-Whitney or chi-squared test.
- Multiple linear regression (BCAA as dependent variable) was performed to test independent association with menopausal category, adjusted for several factors.
- Additional analysis included comparisons of BCAA across 4 groups by combining presence/absence of metabolic syndrome (MS) and menopausal status: pre-menopause MS-, pre-menopause MS+, post-menopause MS- and post-menopause MS+.

Table 1: Characteristics of participants according to menopausal status.

	Pre-menopause	Post-menopause	P-value
N	1,175	1,083	
Age, years	44 (41-47)	57 (53-62)	**<0.001**
White ethnicity	684 (58.6)	683 (64.0)	**0.001**
High educational level	566 (48.2)	532 (49.1)	**<0.001**
Waist circumference, cm	83 (76-92)	87 (79-95)	**<0.001**
Waist-to-hip ratio	0.82 (0.78-0.88)	0.86 (0.81-0.91)	**<0.001**
Body mass index, kg/m²	26 (23-30)	27 (24-30)	**<0.001**
Systolic blood pressure, mmHg	110 (102-120)	117 (107-128)	**<0.001**
Dyastolic blood pressure, mmHg	71 (65-78)	73 (67-80)	**<0.001**
Fasting plasma glucose, mg/dL	100 (95-106)	106 (100-114)	**<0.001**
HbA1c, %	5.2 (4.8-5.6)	5.5 (5.1-5.9)	**<0.001**
BCAA, µmol/L	377 (338-421)	387 (347-441)	**<0.001**
Valine, µmol/L	210 (186-233)	217 (192-243)	**<0.001**
Leucine, µmol/L	129 (113-146)	132 (113-149)	**0.027**
Isoleucine, µmol/L	40 (31-48)	41 (32-52)	**0.024**
Current smokers	174 (14.8)	177 (16.3)	0.315
Heavy alcohol consumers	29 (7.7)	30 (8.1)	0.841
Physically active	209 (18.5)	210 (20.0)	0.357
Central obesity	417 (35.5)	497 (45.9)	**<0.001**
Presence of CVD	38 (3.2)	58 (5.4)	**0.013**
Hypertension	195 (16.6)	391 (36.1)	**<0.001**
Diabetes	119 (10.1)	246 (22.7)	**<0.001**
Hypercholesterolemia	463 (39.4)	589 (54.4)	**<0.001**
Hypertriglyceridemia	231 (19.7)	323 (29.8)	**<0.001**
Low HDL-c	321 (27.3)	188 (17.4)	**<0.001**
Use of lipid-lowering drugs	54 (4.6)	213 (19.7)	**<0.001**

Data are presented as median (IR) for continuous variables and number (%) for categorical variables. P values between groups were obtained using Mann-Whitney test for continuous variables and chi-square test for categorical variables.

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1. Saeed A et al. Prevention of cardiovascular disease in women. Methodist Debakey Cardiovasc. 2017;13:185-92.
2. Hu W et al. Relationship between branched-chain amino acids, metabolic syndrome, and cardiovascular risk profile in a chinese population: a cross-sectional study. Int J Endocrinol. 2016;8173905.
3. Flores-Guerrero JL et al. Plasma branched-chain amino acids and risk of incident type 2 diabetes: results from the PREVEND prospective cohort study. J Clin Med. 2018;7:513.

RESULTS AND DISCUSSION

- A total of 2,258 women (50.7 ± 8.8 years) were included.
- Postmenopausal women were older and had higher blood pressure, glucose, lipids, BCAA levels and higher prevalence rates of cardiometabolic disturbances than pre-menopausal ones (Table 1).
- In multiple linear regression, central obesity, diabetes, hypertriglyceridemia, low HDL-c (components of the MS) were significantly associated with higher serum BCAA when the entire sample or menopausal categories were considered, but menopause was not independently associated with BCAA (Table 2), nor with its individual components (data not shown).
- Comparisons of BCAA within MS- and MS+ groups showed no difference between pre- and postmenopausal women (Figure 1).
- However, when comparing pre- or postmenopausal women, each with and without MS, higher BCAA levels were consistently found in MS+ groups (Table 3).

Table 2: Coefficients and 95% CI from multiple linear regression models for the contributing factors for BCAA in women.

BCAA	β (95% CI)
Age	-0.17 (-0.64-0.29)
High educational level	1.35 (-4.07-6.77)
White race	-5.41 (-10.82-0.02)
Central obesity	**27.41 (21.90-32.91)***
Presence of CVD	7.75 (-5.00-20.50)
Hypertension	6.03 (-0.54-12.60)
Diabetes	**37.70 (30.20-45.19)***
Hypercholesterolemia	3.25 (-2.03-8.53)
Hypertriglyceridemia	**30.53 (24.30-36.76)***
Low HDL-c	**14.89 (8.55-21.23)***
Menopausal status	1.86 (-6.08-9.80)
Use of lipid-lowering drugs	**12.69 (4.16-21.25)***
Physically active	1.08 (-5.51-7.67)

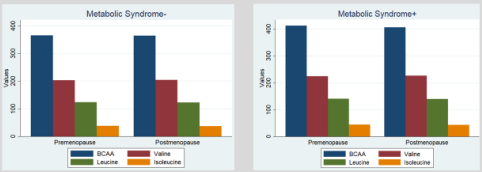
Values obtained by multiple linear regression. Adjusted R-squared 0.22. *p-value <0.05.
BCAA: branched-chain amino acid. CVD: cardiovascular disease HDL-c: high-density lipoprotein cholesterol.

Table 3: Comparison of BCAA according to MS presence and menopause.

	WITHOUT Metabolic Syndrome (Group 1 vs. 2)			WITH Metabolic Syndrome (Group 3 vs. 4)			Group 1 vs. 3	Group 2 vs. 4
	Pre- (1)	Post- (2)	P-value	Pre- (3)	Post- (4)	P-value	P-value	P-value
N	848	485		327	598			
BCAA, µmol/L	366 (327-405)	365 (325-409)	0.879	413 (366-460)	407 (366-459)	0.650	**<0.001**	**<0.001**
Valine, µmol/L	203 (181-225)	204 (183-228)	0.295	225 (202-254)	227 (203-254)	0.635	**<0.001**	**<0.001**
Leucine, µmol/L	124 (109-140)	123 (107-141)	0.338	141 (125-157)	140 (121-156)	0.178	**<0.001**	**<0.001**
Isoleucine, µmol/L	38 (30-46)	37 (29-47)	0.477	45 (35-54)	44 (35-54)	0.550	**<0.001**	**<0.001**

Data presented as median (IR). P-value obtained using Mann-Whitney test between groups. BCAA: branched-chain amino acid.

Figure 1: Median levels of BCAA according to MS presence and menopause.



CONCLUSION

Our finding did not support that menopause is independently associated with circulating levels of BCAA in ELSA-Brasil, but the classical components of the MS.

Certificamos que o trabalho **COULD SERUM BRANCHED-CHAIN AMINO ACIDS (BCAA) CONTRIBUTE TO THE INCREASED CARDIOVASCULAR IN POSTMENOPAUSAL WOMEN?** autoria de **FONSECA MIH, ALMEIDA-PITITTO B, BENSENOR IM, LOTUFO PA, FERREIRA SRG** foi apresentado sob a forma **PÔSTER**, durante o XXII Congresso da Sociedade Brasileira de Diabetes, realizado de 16 a 18 de outubro de 2019, no Centro de Convenções de Natal/RN.

Natal, 18 de outubro de 2019

Realização SBD DIABETES 2019 Natal

Tadeu Alencar
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Hermelinda Pedrosa
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ATTACHMENT 7 - University of Sao Paulo records of the PhD candidate (Janus system)

NUSP: 10102718 Nome: Marilia Izar Helfenstein Fonseca

Ficha do Aluno

	Curso	Área	Nº Sequencial	Situação	Visualizar
➔	Doutorado Direto	Epidemiologia (6141)	1	Matrícula Regular	

Preparar para imprimir

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Saúde Pública
FICHA DO ALUNO

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Curso:	Doutorado Direto
Programa:	Epidemiologia
Data de Matrícula:	08/07/2016
Início da Contagem de Prazo:	08/07/2016
Data Limite para o Depósito:	10/05/2021
Orientador:	Prof(a). Dr(a). Sandra Roberta Gouvea Ferreira Vivolo - 08/07/2016 até o presente. Email: sandrafv@usp.br
Co-orientador:	Prof(a). Dr(a). Bianca de Almeida Pititto - 14/10/2019 até o presente. Email: bapititto@unifesp.br
Proficiência em Línguas:	Inglês, Aprovado em 08/07/2016
Data de Aprovação no Exame de Qualificação:	Aprovado em 07/08/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 08/07/2016

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Matrícula Regular em 27/07/2020
Impresso em: 14/03/2021 09:45:39

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6141 - 10102718/1 - Marilia Izar Helfenstein Fonseca

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
PSP5121-1/3	Bioestatística	07/03/2017	23/05/2017	90	6	100	A	N	Concluída
Atividade do Programa	Publicação do trabalho "Impact of menopause and diabetes on atherogenic lipid profile: is it worth to analyse lipoprotein subfractions to assess cardiovascular risk in women?". Diabetology & Metabolic Syndrome. 9, Article number: 22 (2017), p. 13. DOI: 10.1186/s13098-017-0221-5	07/04/2017	07/04/2017	-	3	-	-	-	-
PSP5119-1/3	Princípios da Epidemiologia	06/03/2018	15/05/2018	90	6	94	A	N	Concluída
PSP5117-1/3	Análise de Dados Epidemiológicos	13/08/2018	08/10/2018	60	4	93	A	N	Concluída
PSP5118-1/4	Delineamento e Introdução a Análise Epidemiológica	14/05/2019	24/06/2019	60	4	82	A	N	Concluída
EPI5713-2/2	Introdução ao R para a Análise de Dados	03/06/2019	07/07/2019	30	2	100	A	N	Concluída
MPR5720-5/4	Estudos Epidemiológicos Transversais (Faculdade de Medicina - Universidade de São Paulo)	12/08/2019	15/09/2019	45	3	100	A	N	Concluída
Atividade do Programa	Publicação do capítulo "Classificação e diagnóstico do diabetes mellitus" no manual tecnológico Diretrizes Sociedade Brasileira de Diabetes 2019-2020, São Paulo, editora Clannad, 2019, v. 1, p. 19-26.	12/08/2019	03/12/2019	-	2	-	-	-	-
PSP5123-1/3	Análise Multinível em Estudos Epidemiológicos	04/10/2019	29/11/2019	60	4	100	A	N	Concluída
Atividade do Programa	Participou do XXII Congresso da Sociedade Brasileira de Diabetes, com a apresentação do trabalho "Could serum branched-chain amino acids (BCAA) contribute to the increased cardiovascular in postmenopausal women?", publicado nos anais do evento, Natal, RN, 2019, p. 27.	16/10/2019	18/10/2019	-	1	-	-	-	-
Atividade do Programa	Participou do XXII Congresso da Sociedade Brasileira de Diabetes, com a apresentação do trabalho "Menopause per se is associated with calcium deposition in coronary arteries: results from the Elsa-Brasil", publicado nos anais do evento, Natal, RN, 2019, p. 71.	16/10/2019	18/10/2019	-	1	-	-	-	-
EPI5714-1/1	Diabetes Mellitus e Síndrome Metabólica: Aspectos Fisiopatológicos e Epidemiológicos	02/03/2020	06/04/2020	30	2	100	A	N	Concluída
MPR5730-6/4	Epidemiologia Clínica (Faculdade de Medicina - Universidade de São Paulo)	09/04/2020	17/06/2020	90	0	-	-	N	Turma cancelada
PSP5128-1/1	Métodos e aplicação de análise de dados não paramétricos	14/05/2020	02/07/2020	60	0	-	-	N	Turma cancelada
PSP5128-1/2	Métodos e aplicação de análise de dados não paramétricos	01/10/2020	26/11/2020	60	4	100	B	N	Concluída
MCM5908-2/2	Revisão Sistemática e Meta-Análise: Princípios Teóricos e Práticos (Faculdade de Medicina - Universidade de São Paulo)	04/11/2020	01/12/2020	60	0	-	-	N	Pré-matrícula indeferida
HNT5762-2/3	Revisão Sistemática e Meta-Análise	30/11/2020	13/12/2020	30	0	-	-	N	Matrícula cancelada

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	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	40	42
Estágios:			
Total:	0	40	42

Créditos Atribuídos à Tese: 152

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Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula Regular em 27/07/2020
Impresso em: 14/03/2021 09:45:39

ATTACHMENT 8 – Curriculum lattes of the PhD candidate

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Endereço para acessar este CV: http://lattes.cnpq.br/4126410286246941

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Última atualização do currículo em 21/01/2021

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Identificação

Nome Marília Izar Helfenstein Fonseca

Nome em citações bibliográficas FONSECA, M. I. H.;Fonseca, Marília Izar Helfenstein;Fonseca, Marilia Izar Helfenstein;Fonseca, M I;Fonseca, M H;Fonseca, MIH;FONSECA, MARÍLIA;Marília Izar Helfenstein Fonseca;Fonseca M;M Fonseca;FONSECA, M.;FONSECA, MARÍLIA I.H.

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ATTACHMENT 9 – Curriculum lattes of the counselor Sandra R G Ferreira Vivolo

Sandra Roberta Gouvea Ferreira Vivolo

Bolsista de Produtividade em Pesquisa do CNPq - Nível 1B

Endereço para acessar este CV: http://lattes.cnpq.br/6633883139386818

ID Lattes: **6633883139386818**

Última atualização do currículo em 03/03/2021

Possui graduação em Medicina pela Pontifícia Universidade Católica de Campinas (1981), mestrado em Medicina (Endocrinologia Clínica) pela Universidade Federal de São Paulo (1986) e doutorado em Medicina (Endocrinologia Clínica) pela Universidade Federal de São Paulo (1988). É professora titular na Faculdade de Saúde Pública da Universidade de São Paulo, onde ocupou cargos de chefia de departamentos e presidente de Comissão de Pós-graduação. Tem bolsa de produtividade em pesquisa do CNPq, nível 1-B. Tem experiência na área de Medicina, com ênfase em Epidemiologia e Nutrição, atuando principalmente nos seguintes temas: diabetes mellitus, síndrome metabólica, hipertensão arterial, obesidade, nutrição e microbiota intestinal. **(Texto informado pelo autor)**

Identificação

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ATTACHMENT 10 – Curriculum lattes of the co-advisor Bianca de Almeida-Pititto

Bianca de Almeida Pititto

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ID Lattes: **8433932854107690**

Última atualização do currículo em 11/03/2021

Possui formação em Clínica Médica, mestrado em fatores de risco cardiovascular pela Endocrinologia da Universidade Federal de São Paulo - UNIFESP (2003) e doutorado em intervenção para prevenção de diabetes mellitus e impacto em fatores de risco cardiovascular pela Faculdade de Saúde Pública-USP (2009), tendo sido "visiting student" no MRC Epidemiology Unit of Cambridge University-UK (2007). Finalizou pós-doutorado pela Faculdade de Saúde Pública ? USP (2013), estudando fatores de risco cardiovascular não tradicionais em coorte de estudo epidemiológico, ELSA-SP. Curso de pós-graduação Latu Senso em Geriatria na UNIFESP e na USP. É médica concursada do Departamento de Medicina Preventiva-UNIFESP, atuando na graduação e residência médica e orientação de pós-graduação pelo Programa de Pós Graduação em Endocrinologia, na área de epidemiologia das doenças crônicas não transmissíveis e fatores de risco cardiometabólico.s Áreas de atuação: epidemiologia, diabetes mellitus, obesidade, fatores de risco cardiovascular, prevenção e epidemiologia do ciclo vital (eventos precoces da vida). **(Texto informado pelo autor)**

Identificação

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Nome em citações bibliográficas Almeida-Pititto B;Almeida-Pititto, Bianca de;Almeida B;Almeida-Pititto Bianca;B. Almeida-Pititto;ALMEIDA-PITITTO, B.;ALMEIDA-PITITTO, BIANCA;DE ALMEIDA-PITITTO, BIANCA;DE ALMEIDA-PITITTO, B.;ALMEIDA, BIANCA;PITITTO, BIANCA ALMEIDA

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