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Atorvastatin and hormone therapy effects on *APOE* mRNA expression in hypercholesterolemic postmenopausal women

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ABSTRACT

Menopause is associated with changes in lipid levels resulting in increased risk of atherosclerosis and cardiovascular events. Hormone therapy (HT) and atorvastatin have been used to improve lipid profile in postmenopausal women.

Effects of HT, atorvastatin and *APOE* polymorphisms on serum lipids and *APOE* and *LXRA* expression were evaluated in 87 hypercholesterolemic postmenopausal women, randomly selected for treatment with atorvastatin (AT, $n = 17$), estrogen or estrogen plus progestagen (HT, $n = 34$) and estrogen or estrogen plus progestagen associated with atorvastatin (HT + AT, $n = 36$). RNA was extracted from peripheral blood mononuclear cells (PBMC) and mRNA expression was measured by TaqMan® PCR. *APOE* $\varepsilon 2/\varepsilon 3/\varepsilon 4$ genotyping was performed using PCR-RFLP.

Total cholesterol (TC), LDL-c and apoB were reduced after each treatment ($p < 0.001$). Triglycerides, VLDL-c and apoAI were reduced only after atorvastatin ($p < 0.05$), whereas triglycerides and VLDL-c were increased after HT ($p = 0.01$). HT women had lower reduction on TC, LDL-c and apoB than AT and HT + AT groups ($p < 0.05$). *APOE* mRNA expression was reduced after atorvastatin treatment ($p = 0.03$). Although *LXRA* gene expression was not modified by atorvastatin, it was correlated with *APOE* mRNA before and after treatments. Basal *APOE* mRNA expression was not influenced by gene polymorphisms, however the reduction on *APOE* expression was more pronounced in $\varepsilon 3\varepsilon 3$ than in $\varepsilon 3\varepsilon 4$ carriers.

Atorvastatin down-regulates *APOE* mRNA expression and it is modified by *APOE* genotypes in PBMC from postmenopausal women.

1. Introduction

Several lines of evidence demonstrate that dyslipidemia is associated with high risk of cardiovascular heart disease (CHD) in women [1]. Menopause status has been associated with changes in lipid profile, specifically increase in plasma low density lipoprotein (LDL) cholesterol and triglycerides and reduction in high density lipoprotein (HDL) cholesterol, that result in increased risk of atherosclerosis and cardiovascular events [2].

Apolipoprotein (apo) E is a multifunctional protein that plays a key role in metabolism of cholesterol and triglycerides by binding to receptors in liver contributing with the clearance of chylomicrons, very low density lipoprotein (VLDL) and HDL from plasma [3]. Functionality of apoE has demonstrated to be determinant in maintenance of cholesterol homeostasis. apoE deficiency in mice leads to development of atherosclerosis and re-expression reduces the extend of the disease [4].

Hormone therapy (HT) has been used in primary and secondary prevention of CHD in postmenopausal women, however its long term efficiency at this respect remains controversial [5]. The influence of HT on serum lipids, decreasing total and LDL cholesterol and raising HDL cholesterol, support its beneficial cardiovascular effects [6].

The use of statins, inhibitors of endogenous cholesterol synthesis by competitive inhibition of hidroxi-metil-glutaril CoA reductase (HMGCR), has been largely described by numerous clinical trials to reduce cardiovascular events by lowering the cholesterolemia [7]. Nerveless, poorly investigated effects of statins in some segments of the population as older women make statin

Abbreviations: AT, women treated with atorvastatin; HT, hormone therapy or women treated with estrogen or estrogen plus progestagen; HT + AT, women treated with estrogen or estrogen plus progestagen associated with atorvastatin; PBMC, peripheral blood mononuclear cells; *APOE*, apolipoprotein E gene; *LXRA*, liver X receptor alpha gene; CHD, cardiovascular heart disease; CAD, coronary artery disease.

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effect on hypercholesterolemic postmenopausal women remain unclear.

Polymorphisms in the apoE gene (*APOE*), mainly those that encode $\varepsilon2/\varepsilon3/\varepsilon4$ protein isoforms, have been related to basal serum lipids and CHD risk. Compared with $\varepsilon3$ allele, the $\varepsilon2$ allele is associated with lower levels whereas $\varepsilon4$ is associated with higher levels of LDL cholesterol [8]. Moreover, $\varepsilon2/\varepsilon3/\varepsilon4$ *APOE* genotypes also were reported to modified lipid-lowering response to statins [9] and HT [10,11].

Transcriptional regulation of *APOE* in human tissues is mediated by several factors. Increasing concentration of intracellular free cholesterol has been shown to stimulate *APOE* transcription in macrophages and adipocytes as well as nuclear factors liver X receptor alpha (*LXRA*) and beta (*LXRB*) that are key regulators of *APOE* expression in these tissues [12]. Moreover, cholesterol-lowering drugs could control *APOE* expression by regulation of intracellular cholesterol pool.

The present study aims to evaluate *APOE* and *LXRA* mRNA expression in peripheral mononuclear cells of hypercholesterolemic postmenopausal women and their relationship with *APOE* genotypes and HT and atorvastatin treatment.

2. Materials and methods

2.1. Study design and protocol

This randomized controlled study aims to evaluate the effects of atorvastatin and HT on *APOE* mRNA expression. Eighty-seven natural postmenopausal, hypercholesterolemic and Caucasian-descent Brazilian women (aged 50–65 years) were selected at the Dyslipidemia Section of the Dante Pazzanese Institute of Cardiology (Sao Paulo City, Brazil) from 2003 to 2005. Subjects with thyroid, liver or renal disease, diabetes, hypertriglyceridemia [triglycerides > 400 mg/dl (4.52 mmol/l)] or under treatment with lipid-lowering drugs were not included in the study. Moreover, all women were not smoking and had no family history of coronary artery disease (CAD). The sample size to estimate *APOE* mRNA values was calculated using a pilot sample study (*APOE* mRNA mean value: 0.04685, SD: 0.02949) considering $\alpha = 0.1$ and a relative error of the mean estimation of 0.15. The minimum sample size needed for the study was 47 individuals.

All participants had LDL cholesterol higher than 130 mg/dl (3.36 mmol/l), even after a wash-out period of four weeks on a low-fat diet, accompanied by nutritionists. All women were treated with placebo (1 tablet/day) for 4 weeks and this time was established as baseline period. Following they were randomly distributed in five groups using the parallel group method for randomization. Briefly, patients meeting inclusion criteria were selected by analyzing the medical chart and their names were registered in a waiting list. Afterwards, the patients were recruited and, after accordance with their participation, they were randomly allocated into one of the five groups of treatments. Each group received 12 weeks of the active treatments: atorvastatin (10 mg/day, $n = 17$); estradiol monotherapy (2 mg/day, $n = 19$); estradiol associated with norethisterone acetate (NETA, 1 mg/day, $n = 15$); estradiol (2 mg/day) plus atorvastatin (10 mg/day, $n = 18$); and finally, estradiol (2 mg/day) plus NETA (1 mg/day) combined with atorvastatin (10 mg/day, $n = 18$). Further analysis was performed using three groups, considering patients under hormone therapy (HT, $n = 34$), under monotherapy with atorvastatin (AT, $n = 17$) and patients using association of HT plus atorvastatin (HT + AT, $n = 36$). Every woman assigned to each group completed the 12-week period of treatment.

Before and after the treatments, the patients were evaluated for serum concentrations of lipids, apoAI and apoB, and *APOE* and *LXRA* mRNA expression. The response to atorvastatin was monitored by reduction of LDL cholesterol and other serum lipids. There were no changes in trial outcomes during the study. ALT and CK were determined in order to detect possible liver and muscle adverse drug reactions, but such effects and others were not reported by the patients therefore changes in methods were not necessary. The study protocol was approved by the local Ethical Committees (protocol number # 164) and informed consent was obtained from each participant.

2.2. Biochemical measurements

After placebo (baseline period) and after each treatment, blood samples were collected from all women after an overnight (12 h) fast. Serum total cholesterol, HDL cholesterol and triglycerides (TG) were measured by routine enzymatic colorimetric methods. Plasma apoAI and apoB were measured by nephelometry. LDL and very low-density lipoprotein (VLDL) cholesterol were estimated by Friedewald formula [13]. Serum ALT and CK concentrations were determined by kinetic methods.

2.3. DNA extraction and APOE genotyping

Genomic DNA was extracted from EDTA-anticoagulated whole blood samples using a salting-out method [14]. *APOE* polymorphisms rs7412 and rs429358 that determinate the *APOE* alleles $\varepsilon2$, $\varepsilon3$ and $\varepsilon4$ were analyzed by polymerase chain reaction followed by restriction fragment analysis (PCR-RFLP) as previously described [15]. The accuracy of the genotyping was assessed by re-analyzing all RFLP profiles by an independent investigator without any change, and 15% of the samples were retested in order to avoid mistyping errors.

2.4. Blood samples and isolation of mononuclear cells

EDTA-anticoagulated blood samples for mRNA expression were obtained after baseline and each treatment. Peripheral blood mononuclear cells (PBMC) were isolated and immediately used for RNA extraction. Blood was diluted in phosphate buffered saline (1:1) and this suspension was layered in Hystopaque-1077 (Sigma–Aldrich, MO, USA) and centrifuged for 30 min at $400 \times g$ at room temperature. PBMC were collected from the interphase and immediately used for RNA extraction [16].

2.5. RNA isolation, cDNA synthesis and mRNA quantification

Total RNA was extracted from PMBC using TRIzol® Reagent (Invitrogen-Life Technologies, CA, USA) following the manufacturer's suggested protocol. RNA was dissolved in DEPC-treated water and the concentration was measured by spectrophotometry using the NanoDrop® (NanoDrop Technologies Inc., DE, USA).

cDNA was produced from 1 μg of total RNA by Superscript™ II Reverse Transcriptase (Invitrogen-Life Technologies, Carlsbad, CA, USA) and *APOE* and *LXRA* mRNA was measured by TaqMan® quantitative PCR (qPCR) assay. Among six reference genes tested [ubiquitin C (*UBC*), glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), beta-2-microglobulin (*B2M*), hypoxanthine phosphoribosyl-transferase I (*HPRTI*), succinate dehydrogenase complex, subunit A (*SDHA*) and hydroxymethyl-bilane synthase (*HMBS*)], *HPRT1* was chosen as the most stable according to the analysis by GeNorm software [http://medgen.ugent.be/genorm]. The assays ID Hs00171168_m1 and Hs00173195 were used to access the *APOE* and *LXRA* mRNA detection, respectively. The sequence of primers and probes used for *HRPT1* are described as follow: forward, 5′-TGACACTGGCAAAACAATGCA-3′; reverse,

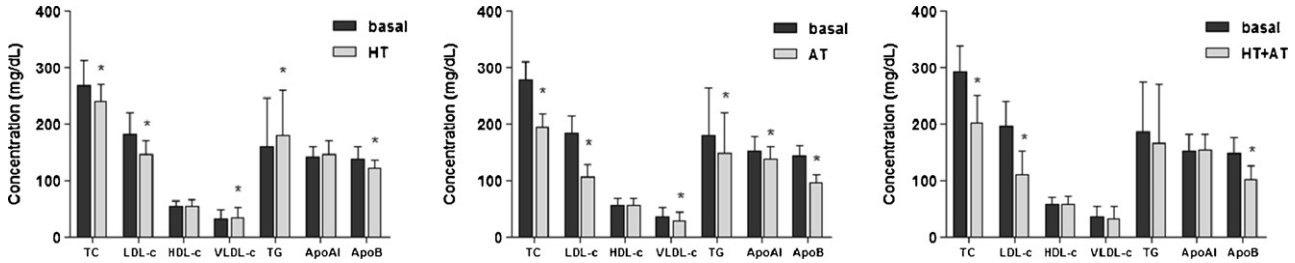

Fig. 1. Serum lipid profile of postmenopausal women before and after hormone therapy, atorvastatin or combined treatments. Values are presented as mean ± SD and compared by paired *t*-test or Wilcoxon test for paired samples. HT, hormone treatment; AT, atorvastatin treatment; HT + AT, treatment with atorvastatin associated with hormone therapy; TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; VLDL-c, very low density lipoprotein cholesterol; TG, triglycerides; ApoAI, apolipoprotein AI; ApoB, apolipoprotein B.

5′-GGTCCTTTTCACCAGCAAGCT-3′; and probe, VIC-CCTTGGTCAGGCAGTAT-MGB/NFQ. The qPCR assays were carried out in 96 well plates using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA).

2.6. Statistical analysis

Statistical analyses were performed using the software STATA/SE 8.0 for windows (StataCorp, TX, USA). Genotype and allele frequencies were estimated by gene counting. Categorical variables were compared by chi-square test. Continuous variables were previously tested for distribution using K–S test and skewed variables were logarithmically transformed and compared appropriately by independent or paired *t*-test (two variables) or one-way ANOVA (three variables). Variables without normal distribution after log transformation were compared by Wilcoxon test (two variables) for independent or pared samples or Kruskal–Wallis test (three variables). Tukey test was used for multiple comparisons when three variables had significant difference. Significance was considered at $p < 0.05$.

3. Results

Clinical characteristics, basal serum lipids and *APOE* allele frequencies of postmenopausal women are presented in Table 1. Serum lipids at baseline and after treatments for HT, AT and HT + AT groups are shown in Fig. 1. No differences were observed in basal serum lipids among HT, AT and HT + AT groups. Total cholesterol, LDL cholesterol and apoB concentrations were reduced after all treatments ($p < 0.001$). Triglycerides, VLDL cholesterol and apoAI were reduced after atorvastatin treatment ($p < 0.05$), whereas triglycerides and VLDL cholesterol were increased in HT group ($p = 0.01$).

Relative frequencies for *APOE* ε2/ε3/ε4 alleles are described in Table 1. Due to the absence of ε2ε2 carriers and the low frequency of ε2ε3 and ε4ε4 genotype carriers, these individuals were not included in inferential analysis. Therefore, data from carriers of only ε3ε3 and ε3ε4 were compared in this sample, where it was not possible to associate *APOE* genotypes with basal concentrations of total, LDL, HDL and VLDL cholesterol and triglycerides, apoAI and apoB at baseline and after treatments ($p < 0.05$; data not shown). Similarly, no association was detected among *APOE* genotypes and serum lipids after treatments when analyzed each group separately ($p < 0.05$; data not shown).

APOE mRNA expression in PBMC was similar among the three treatment groups at baseline (data not shown). *APOE* expression in PBMC was reduced after atorvastatin treatment (10 mg/day) in AT group ($p = 0.03$), but it was not modified by HT or HT + AT treatments (Fig. 2). Although *LXRA* expression was not affected by atorvastatin or HT treatments (data not shown), it was positively correlated with *APOE* mRNA expression before ($r = 0.45$, $p < 0.001$) and after treatments ($r = 0.44$, $p < 0.001$) as shown in Fig. 3.

PBMC *APOE* mRNA expression was not influenced by *APOE* genotypes in postmenopausal women at baseline (Fig. 4A) and treatments (data not shown). However, reduction of *APOE* mRNA levels in response to atorvastatin was more pronounced in women carrying ε3ε3 genotypes (57% of mean reduction) than in ε3ε4 genotype carriers (33% of mean reduction) (Fig. 4B).

Table 1
Clinical, biochemical and *APOE* polymorphism data of postmenopausal women.

Variable	Data
Number of individuals	87
Age, years	57.6 ± 3.9
Body mass index, kg/m^2	29.3 ± 4.9
Serum lipids, mg/dl	
Total cholesterol	280 ± 44
Triglycerides	176 ± 87
HDL cholesterol	57 ± 12
LDL cholesterol	189 ± 40
VLDL cholesterol	35 ± 17
Apolipoprotein AI	149 ± 25
Apolipoprotein B	145 ± 24
APOE polymorphism	
ε2 allele	0.04
ε3 allele	0.79
ε4 allele	0.17

Continuous variables are presented as mean ± SD. Genotyping data is presented as relative frequencies for ε2, ε3 or ε4 allele. HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; *APOE*, apolipoprotein E gene. Conversion factor to convert to International System of units (SI) are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for lipoproteins (g/l).

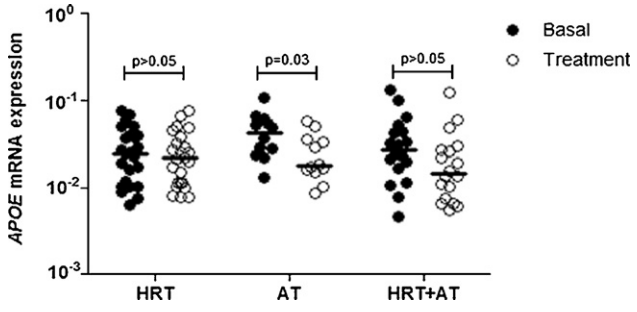

Fig. 2. *APOE* mRNA expression in mononuclear cells before and after hormone therapy, atorvastatin or combined treatments. Values are shown as dispersion plot with bars indicating median values and compared by Wilcoxon test for paired samples. *APOE*, apolipoprotein E gene; HT, hormone treatment; AT, atorvastatin treatment; HT + AT, treatment with atorvastatin associated with hormone therapy. Basal and treatment represent expression values before and after treatments.

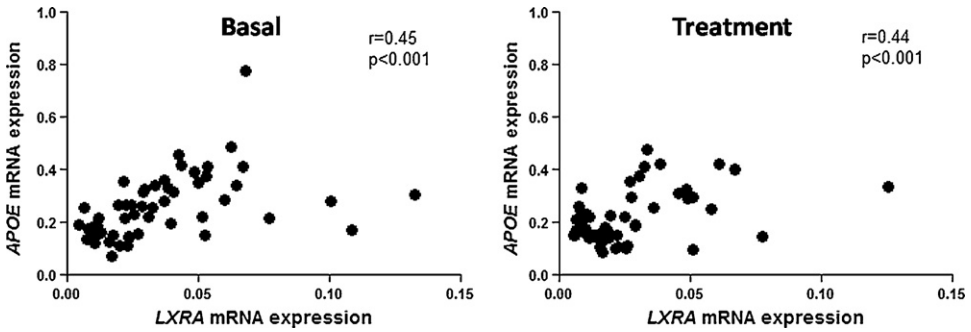

Fig. 3. Correlation analysis between *APOE* and *LXRA* mRNA expression in mononuclear cells at baseline and after treatments. *APOE*, apolipoprotein E gene; *LXRA*, liver X receptor α gene. Data are presented in a scatter plot and correlations between variables were evaluated using spearman coefficient.

4. Discussion

Lipid-lowering effects of both HT and statins have been previously described in postmenopausal hypercholesterolemic women [17,18]. Moreover, association of both drugs has not demonstrated additional benefits over statin monotherapy in improving lipid profile and consequently in prevention of cardiovascular events [17,19]. However, this study did not aim to evaluate the lipid lowering effect of these drugs due to the small sample size. Therefore this work focused mainly in the analysis of molecular mechanisms regulating *APOE* expression and their relation with response to treatments.

Relative frequencies of *APOE* alleles observed in this work were similar to early studies, which European descendant populations were analyzed [20,21], even those reported *APOE* allele frequencies in Brazilian European descendant samples that studied total population [22,23] and only women [10,15] (ε2 allele: 0.04–0.08; ε3 allele: 0.70–0.83; and ε4 allele: 0.11–0.23).

Several studies have evaluated the impact of apoE isoforms on basal serum lipids and, despite some controversial data that reported no differences on LDL cholesterol levels among *APOE* genotypes in hypercholesterolemic individuals [22,24], ε2 allele is classically associated with lower total and LDL cholesterol and apoB whereas ε4 allele has demonstrated to have opposite effects in comparison with the common allele ε3 [9]. These differences could be explained by structural and biophysical properties of apoE isoforms [25].

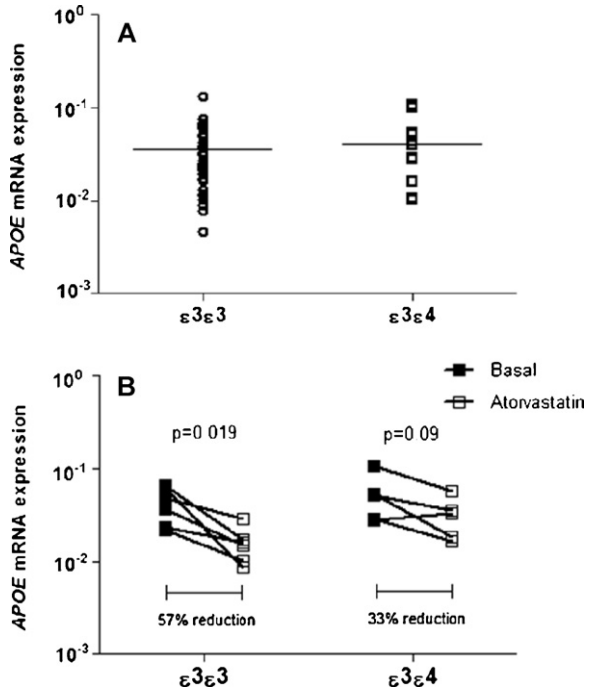

Fig. 4. Relationship between *APOE* genotypes and mRNA expression in mononuclear cells at baseline and in response to atorvastatin treatment. Values are presented as dispersion plot and compared by Wilcoxon test for independent samples (ε3ε3 vs ε3ε4 in total population in panel A) or Wilcoxon test for paired samples (basal vs treatment in panel B). *APOE*, apolipoprotein E gene.

Influence of *APOE* genotypes on basal serum lipids was also evaluated in postmenopausal women. HT nonuser women carrying ε4 alelle had higher LDL cholesterol than women with ε3 or ε2 alelles [10]. In our study, no differences on basal lipids or lipid-change after treatments according to *APOE* genotypes, however association of genotypes with plasma lipids and response was not the primary objective of this study, because the limited sample analyzed that was meanly focused in expression analysis. The small size of the sample is an important limitation of our study, which could restrict the power of statistical inference tests and then to hide possible associations between genotypes and basal plasma lipids or response to pharmaceutical interventions.

Conclusions from studies that investigated interaction between *APOE* genotypes and response to HT and statins in postmenopausal women remain controversial. Concerning HT response, whereas Tsuda et al. described that in Japanese women ε2 and ε3 allele carriers had better response to HT in reduction of total and LDL cholesterol than those women with ε4 allele [11], in Brazilian women the presence of ε4 allele may benefit more from HT than women with other *APOE* genotypes [10]. Moreover, three month HT induced more accentuated increase of triglycerides in Spanish women carrying ε2 allele, but no differences were observed on total or LDL cholesterol variation [26]. On the contrary, no difference was observed according to *APOE* genotypes on change of serum lipid profile when long-term HT was analyzed after five years follow-up [27]. Regarding statin response, despite some results are contradictory and so far inconclusive, in general *APOE* ε3 homozygotes get a larger benefit from statin than *APOE* ε4 carriers in terms of LDL decrease, whereas those with the ε2 allele have an ever greater reduction in LDL cholesterol during statin medication [9]. Nevertheless, it merits to be mentioned that several studies demonstrated no affect for the ε2/ε3/ε4 polymorphism on lipid profile in response to statin treatment [28,29]. However, studies from mRNA expression analysis in postmenopausal women under HT or statin therapy are scarce and they are important to provide additional information helping to elucidate the contribution of *APOE* to lipid-lowering response in this population.

The main contribution of our work is the measurement of *APOE* mRNA levels according to *APOE* genotypes and the exploration of gene expression in response to HT and atorvastatin treatments.

Hepatocytes and macrophages are adequate samples to evaluate cholesterol transport and of lipid-lowering drugs effects, however collecting these specimens is not very convenient in human subjects. We and others [30] have analyzed mRNA expression using

PBMC that would became macrophages in peripheral tissues. However, modulation of *APOE* expression by atorvastatin may not be similar in all tissues and these characteristics could be a limitation in the interpretation of our results.

Although there are only few studies, influence of statin treatment on *APOE* expression has been previously explored using in vitro an in vivo models. Using an in vitro approach, Llaverias and co-workers [31] reported that 24 h of treatment with 5 μM of atorvastatin reduces *APOE* mRNA and protein expression in THP-1 derived macrophages. On the contrary, the same treatment did not alter *APOE* expression using lipid loaded macrophages [32].

In humans, lower *APOE* mRNA expression was detected in PBMC from diabetic patients with hyperlipidemia when compared to healthy controls, but there was no differences between hyperlipidemic diabetic patients who had not received lipid-lowering treatment and those that were treated with 5–10 mg/day of simvastatin [30]. These results differ from the down-regulation of *APOE* expression by atorvastatin reported in the present work, however some differences in the model of study could explain this divergence. First, the statin effects may vary according to the dose and type of statin used and it is known that atorvastatin has more potent effect than simvastatin, when used at similar dose. On the other hand, diabetic patients evaluated by Guan et al. diverge pathophysiologically from our sample of postmenopausal women with hypercholesterolemia.

A possible mechanism that may explain the reduction of *APOE* mRNA is related to the inhibitory effect of statins on the synthesis of oxysterols, which are LXR ligands and would lead to a decreased expression of LXR target genes. LXR regulates *APOE* expression in macrophages and adipocytes though direct interaction with two duplicated enhancer elements placed downstream of the gene and responsive to LXRs [12]. Besides the up regulation of gene expression, it was demonstrated that LXR activation by incubating with a LXR agonist increases apoE secretion in HepG2 cells [33].

In our sample, although no reduction of *LXRA* expression by atorvastatin was detected, positive correlation between *APOE* and *LXRA* expression was observed before and after treatments. However, we only measured mRNA levels of *LXRA* and the transcriptional activity of LXRα by interacting with the *APOE* promoter was not evaluated. Additionally, *APOE* mRNA reduction by atorvastatin was *APOE* genotype dependant in our sample that could give some additional explanation of influence of genotypes on variation in response to statins. However further studies using a larger sample size and if possible more adequate cellular models are necessary.

HT effects on *APOE* expression have been investigated mainly in brain tissues where HT seems to confer neuronal protection and regeneration [34]. Estradiol treatment in cultured neurons causes a rapid (4 h) elevation of apoE [35]. Additionally, when ovariectomized mice were continuously treated with estradiol [36], *APOE* up-regulation at acute treatment (five days) was observed in brain tissues, but this effect was lacked with longer estradiol exposure (14–49 days). On the other hand, estradiol administration increased hepatic apoE levels in mice without affecting *APOE* mRNA [37]. No differences on PBMC *APOE* mRNA were detected after HT treatment in our study, however according with above mentioned early findings long term treatment and posttranscriptional regulation could explain this fact. Moreover, an additional limitation of our work is the measurement of mRNA levels but not apoE protein, which would have enable us to elucidate posttranscriptional regulation of *APOE* in postmenopausal women.

5. Conclusion

APOE mRNA expression in PBMC was down-regulated by atorvastatin in a process probably mediated by LXRα though reduction of oxysterols and this was influenced by *APOE* genotypes, nevertheless HT and HT associated to atorvastatin did not influence *APOE* expression.

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