Life Sciences 92 (2013) 845–851

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Effects of ezetimibe on markers of synthesis and absorption of cholesterol in high-risk patients with elevated C-reactive protein

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ARTICLE INFO

Article history:
Received 23 January 2013
Accepted 27 February 2013

Keywords:
Campesterol
β-Sitosterol
Desmosterol
Inflammation
C-Reactive protein
Statins
Ezetimibe

ABSTRACT

Aims: High-risk subjects with elevated C-reactive protein (CRP) are at high risk for cardiovascular events and frequently require potent statins or combined lipid-lowering therapy to achieve lipid targets and decrease inflammation. Our study aimed at evaluating the effects of three lipid-modifying therapies on LDL-cholesterol, CRP levels and markers of cholesterol absorption and synthesis.
Main methods: A prospective intervention study was performed in high cardiovascular risk individuals receiving atorvastatin 10 mg daily for four weeks. Those with CRP ≥ 2.0 mg/L were randomized to another four-week treatment period with atorvastatin 40 mg, ezetimibe 10 mg or the combination of atorvastatin 40 mg / ezetimibe 10 mg. Lipids, markers of cholesterol absorption (campesterol and β-sitosterol), and synthesis (desmosterol), as well as CRP were quantified at baseline and end of study.
Key findings: One hundred and twenty two individuals were included. Atorvastatin alone or combined with ezetimibe reduced both LDL-cholesterol and CRP ($P < 0.002$ vs. baseline; Wilcoxon); ezetimibe did not modify CRP. Ezetimibe-based therapies reduced absorption markers and their ratios to cholesterol ($P < 0.0001$ vs. baseline, for all; Wilcoxon), whereas atorvastatin alone increased campesterol/cholesterol and β-sitosterol/cholesterol ratios ($P < 0.05$ vs. baseline; Wilcoxon). In addition, ezetimibe also increased desmosterol and desmosterol/cholesterol ratio ($P < 0.0001$ vs. baseline; Wilcoxon).
Significance: These results contribute to understanding the link between cellular cholesterol homeostasis, inflammation and lipid-modifying therapies. Our findings highlight the broader benefit of combined therapy with a potent statin and ezetimibe decreasing inflammation, and preventing increase in cholesterol biosynthesis, an effect not observed with ezetimibe alone.

Introduction

High-risk patients require intensive lipid-modifying therapy to attain LDL-cholesterol goals (Cholesterol Treatment Trialists' CTT Collaboration et al., 2010). Statins are first-line lipid-lowering option, but combination with ezetimibe is often required to provide greater LDL-cholesterol reductions (Davis and Veltri, 2007; Catapano et al., 2006). Meta-analyses of statin trials have clearly demonstrated the beneficial effects of these drugs in reducing cardiovascular outcomes with a net benefit proportional to the magnitude of LDL-cholesterol reduction (Cholesterol Treatment Trialists' CTT Collaboration et al., 2010). Addition of ezetimibe, a cholesterol absorption inhibitor, to a statin, further reduces LDL-cholesterol in about 18–20% (Davis and Veltri, 2007; Catapano et al., 2006), but can be associated with an increment in endogenous cholesterol synthesis (Assmann et al., 2008).

In primary and secondary prevention studies, a significant residual risk can be demonstrated in spite of desirable LDL-cholesterol levels (Ridker et al., 1998, 2005, 2009; Chan et al., 2004; Sattar et al., 2007). Persistent inflammation, at least in part, seems to be related to the residual risk. In the PROVE IT-TIMI 22 trial (Ridker et al., 2005), subjects receiving high-dose atorvastatin remained at increased risk for recurrence of cardiovascular events when C-reactive protein levels persisted elevated. However, weak correlation was found between C-reactive protein and LDL-cholesterol, thus suggesting that other mechanisms beyond cholesterol reduction might be involved.

By blocking the endogenous cholesterol synthesis, statins increase intestinal absorption of sterols, both cholesterol and plant sterols (Miettinen and Gylling, 2003; Miettinen et al., 2003; Miettinen and Gylling, 2009), whereas ezetimibe is capable of preventing the increase in intestinal sterols absorption, but increases the endogenous cholesterol synthesis (Assmann et al., 2008).

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0024-3205
http://dx.doi.org/10.1016/j.lfs.2013.02.018

Table 1
Baseline clinical characteristics of the study population, by group.

Characteristic	Atorvastatin 40 mg N = 45	Ezetimibe 10 mg N = 40	Atorva/ezetimibe 40 mg/10 mg N = 37	*P*-value
Age, years, median (IQR)	60 (5–65)	61 (56–65)	65 (57–71)	**0.020**[a]
Male gender, n (%)	21 (47)	24 (57)	15 (41)	0.638
CHD, n (%)	30 (67)	27 (66)	31 (84)	0.142
High-risk condition[b], n (%)				
CHD or CHD risk equivalent	41 (91)	34 (83)	37 (100)	**0.031**[c]
FRS >20%	4 (9)	6 (17)	0 (0)	
Smokers, n (%)				
Past	6 (13)	10 (25)	5 (13)	0.554
Current	7 (16)	7 (17)	5 (13)	
Non-smokers	31 (71)	23 (58)	27 (74)	
Hypertension, n (%)	43 (95)	38 (95)	34 (91)	0.764
Diabetes, n (%)	20 (44)	17 (41)	16 (43)	0.721
Body weight, kg, mean (SD)	78 (13)	79 (16)	73 (11)	0.129
Body mass index, kg/m^2, median (SD)	29.8 (4.3)	29.7 (5.2)	28.8 (4.2)	0.556
Waist circumference, cm, mean (SD)	99.9 (8.7)	99.6 (11.0)	96.7 (9.1)	0.258
Hip circumference, cm, mean (SD)	106 (9)	105 (8)	104 (9)	0.663
Systolic blood pressure, mmHg, mean (IQR)[d,e]	135 (114–143)	130 (120–144)	133 (120–148)	0.464
Diastolic blood pressure, mmHg, median (IQR)[d,e]	72 (66–82)	76 (66–84)	71 (67–81)	0.916
Heart rate, bpm, mean (SD)[a]	65 (9)	65 (12)	67 (11)	0.762

Categorical variables expressed as n (%); numerical variables presented as mean (SD) or median (IQR), as appropriate. Comparisons between groups used Chi-square or ANOVA–Tukey.
$P < 0.05$
CHD, coronary heart disease; FRS, Framingham risk score; IQR, interquartile range; SD, standard deviation.
[a] Atorvastatin 40 mg/Ezetimibe 10 mg > Atorvastatin 40 mg.
[b] High-risk condition defined by NCEP/ATPIII[16].
[c] Atorvastatin 40 mg/Ezetimibe 10 mg > Ezetimibe 10 mg.
[d] Systolic and diastolic blood pressure, and heart rate are the average of 3 measurements with 2 min apart.
[e] Log-transformed variables for comparisons.

Markers of cholesterol synthesis in the Framingham Offspring Study were associated with reduction in cardiovascular disease risk and, in contrast, absorption markers were associated with a two-fold increased risk (Matthan et al., 2009).

Despite the wide use of ezetimibe, an inhibitor of intestinal cholesterol absorption, in clinical practice, the beneficial effect of this lipid-modifying drug on cardiovascular events remains controversial (Rossebø et al., 2008; Kastelein et al., 2008).

Therefore, to better understand the mechanisms involved in the persistent high cardiovascular risk of subjects with elevated C-reactive protein levels, but low LDL-cholesterol levels following lipid lowering therapy, we examined the effects of different lipid-modifying regimens on C-reactive protein levels, markers of cholesterol absorption and synthesis.

Materials and methods

Design and study population

A prospective, randomized, open label study, with three parallel arms and blinded endpoints was performed. Patients were recruited from the outpatient unit of dyslipidemias of the Federal University of Sao Paulo from January 2011 to April 2012.The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee (*Comitê de Ética em Pesquisa da Universidade Federal de São Paulo, São Paulo, SP, Brazil,* #1841/2011). The protocol design and procedures were explained to all patients prior to inclusion, and they signed a voluntary written informed consent for the participation in this study. This procedure is approved by our ethics committee.

Eligible patients were men and women, 30 to 75 years, at high risk for coronary heart disease, characterized by at least one of the following conditions: established coronary heart disease or equivalent (NCEP III, 2001); diagnosis of type 2 diabetes; or those whose Framingham risk score was > 20%. Subjects should be at statin therapy to attain LDL-cholesterol levels below 100 mg/dL and hs-CRP ≥ 2.0 mg/L prior to inclusion (NCEP III, 2001). Patients with unstable coronary or non-coronary conditions during the three months preceding recruitment, those with any planned revascularization procedure, or any known inflammatory non-cardiovascular condition (infections, chronic inflammatory diseases, or neoplasms) that might increase C-reactive protein were not included (Glynn et al., 2009). Other exclusion criteria were liver, renal or gastrointestinal disease, uncontrolled metabolic disorders, or other condition that might affect the tolerability or safety of the treatments. Exclusion criterion during the study was low adherence (less than 85%) to the lipid-lowering regimen. Three-hundred and thirty-two subjects were screened. After the 4-week run-in period with atorvastatin 10 mg, one-hundred and twenty-two patients were eligible, were randomized and completed the study protocol. Screen failures were mainly due to low C-reactive protein levels (<2.0 mg/L) in the run-in phase. The major characteristics of this study population are presented in Table 1.

After an initial clinical evaluation, eligible patients had prior lipid-lowering therapy discontinued, received nutrition counseling, in accordance with the NCEP/ATP III guidelines (NCEP III, 2001), and simultaneously initiated a 4-week run-in period with atorvastatin 10 mg, to conform to guidelines for high-risk patients and to homogenize the sample at baseline. Baseline blood samples were obtained at the end of the run-in period for laboratory analyses. After confirming the eligibility criteria, patients were randomized to receive atorvastatin 40 mg, ezetimibe 10 mg or combination of atorvastatin 40 mg plus ezetimibe 10 mg daily for another 4-week period. Lifestyle changes were reinforced and adherence to the study drugs was evaluated at each visit. Study design and procedures are shown in Fig. 1.

Study drugs

Atorvastatin (Lipitor ®, IPR Pharmaceuticals, Porto Rico) and ezetimibe (Zetia®, Schering-Plough Products, Las Piedras, Porto Rico) were provided by Pfizer and Merck Co, respectively.

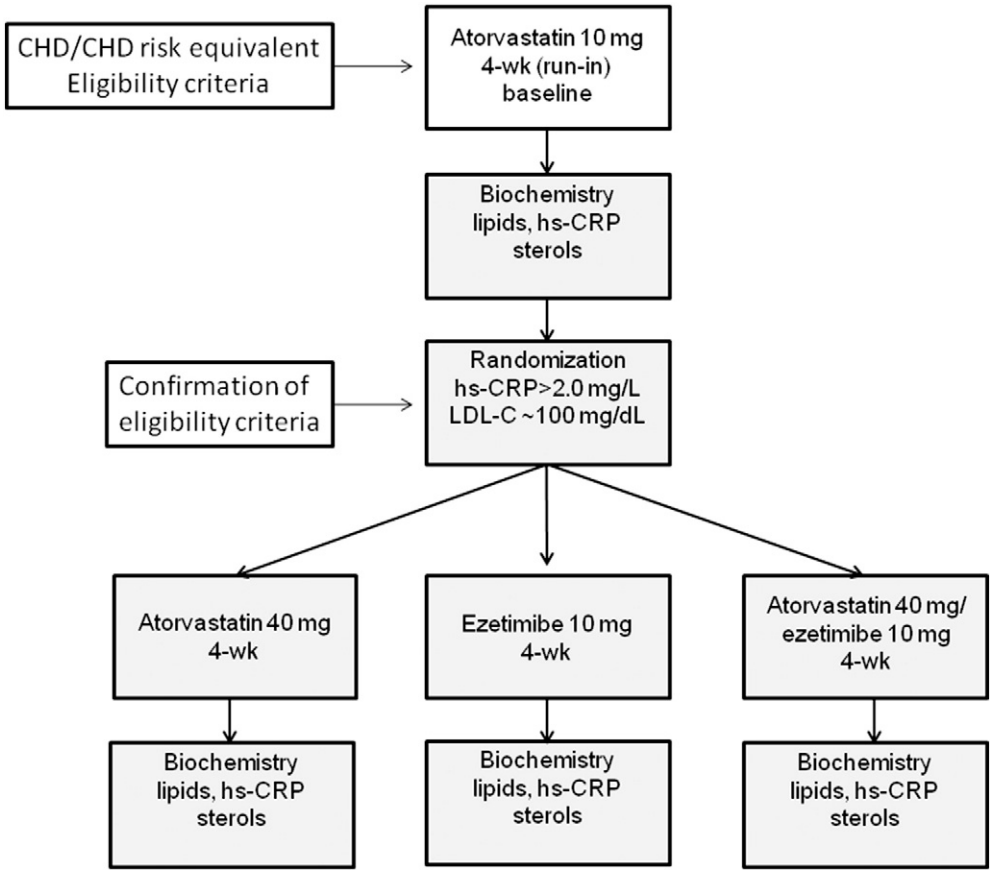

Fig. 1. Study design and procedures. Patients with CHD or CHD risk equivalent on previous statin treatment and with LDL-cholesterol <100 mg/dL and hs-CRP ≥ 2.0 mg/L were selected. Those who met eligibility criteria initiated a 4-week run-in period with atorvastatin 10 mg. Baseline blood samples were obtained at the end of the run-in period for biochemistry, lipids, hs-CRP, and sterols. If hs-CRP > 2.0 mg/L they were randomized to receive atorvastatin 40 mg, ezetimibe 10 mg or combination of atorvastatin 40 mg plus ezetimibe 10 mg daily for another 4-week period. Biochemistry, lipids, hs-CRP, and sterols were collected at the end of study. CHD, coronary heart disease; hs-CRP, high-sensitivity C-reactive protein.

Blood sample collection and assays

Lipids and biochemistry

Biochemical analyses were performed in samples obtained after a 12-hour fasting period according to the study protocol, and they were assayed in a central laboratory of our university. Serum total cholesterol, HDL-cholesterol, and triglycerides were determined by automated methods (Advia 2400, Siemens Healthcare Diagnostics, Tokyo, Japan). LDL-cholesterol was calculated using the Friedewald formula when triglycerides were below 400 mg/dL (Friedewald et al., 1972). Glycated hemoglobin was assayed by high-performance liquid chromatography (Tosho G2, Tosho Inc., Tokyo, Japan); high-sensitivity C-reactive protein (hs-CRP) was determined by nephelometry (Array 360 CE/AL, Beckmann Coulter, Inc. Brea, CA, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamaglutamil transpeptidase (GGT), creatinine and creatinekinase (CK), were assayed by automated techniques (Advia 2400, Siemens Healthcare Diagnostics, Tokyo, Japan). Thyroid-stimulating hormone (ultrassensitivity radioimmunoassay technique, TSH) was determined by automated technique.

Campesterol, β-sitosterol and desmosterol

For the quantification of plasma levels of β-sitosterol and campesterol (markers of sterol absorption), as well as for desmosterol (precursor of endogenous cholesterol synthesis), ultra-performance liquid chromatography (UPLC) and mass spectrometry (MS) were employed, as previously reported (Kasmas et al., 2012; Ramos et al., 2011). Briefly, the method consisted of liquid-liquid extraction followed by separation in the UPLC system and detection with an atmospheric pressure chemical ionization (APCI) ion source mass spectrometer operating on "single ion monitoring" for each sterol (β-sitosterol, campesterol, and desmosterol). The MS system (Quattro Premier-XE, Waters Co., UK) was adjusted to monitor single ions formed by an APCI ion source. The sterols were detected as their free forms, i.e., non-esterified, with the ions being monitored with a mass to charge ratio (m/z) of 367.30 for desmosterol, 397.25 for β-sitosterol, and 383.60 for campesterol. The levels of compounds were determined by comparison of peak response against a calibration curve, ranging from 0.5 μg/mL to 10.0 μg/mL. Samples presenting levels higher than 10.0 μg/mL were diluted to compare with calibration levels. Absolute values of plasma sterols were reported as μg/mL. As these sterols are transported by lipoproteins (Miettinen et al., 1990), absolute values of sterols were corrected for total plasma cholesterol and reported as their ratios to cholesterol, and expressed as μg/mg of cholesterol.

Statistical analysis

Categorical variables are presented as n (%), and compared by Pearson's Chi square test. Numerical variables are expressed as mean (standard deviation, SD) or median (interquartile range, IQR). Continuous variables were tested for distribution of normality by Kolmogorov–Smirnov test. Variables with non-Gaussian distribution were log-transformed for comparisons. For between-groups comparisons ANOVA–Tukey test was used; for within-group comparisons, the Student's paired t-test was employed. To compare non-cholesterol sterols, non-parametric tests were used (Kruskal–Wallis for between-groups comparisons and Wilcoxon, for within-group comparisons).

Percent changes in selected parameters were tested by Kruskal–Wallis test. Comparisons of selected variables according to diabetes

status were performed using unpaired Student's t-test. Spearman's correlation tests were performed. Statistical significance was set at a P-value < 0.05. All analyses were made using the SPSS 17.0 for Windows (SPSS Inc, Chicago, IL).

Results

Study population

Clinical characteristics of the study population show that patients were comparable regarding most of the baseline characteristics, excluding age. Subjects allocated to atorvastatin 40 mg/ezetimibe 10 mg were slightly older than those assigned to atorvastatin 40 mg. Forty percent of the subjects presented diabetes mellitus. Other characteristics are presented in Table 1.

Laboratory parameters

Baseline samples obtained after a run-in period with atorvastatin have shown that groups were comparable regarding laboratory parameters. Lipids were similar at baseline, and median LDL-cholesterol levels were <100 mg/dL. In addition, after a 4-week run-in period receiving atorvastatin 10 mg, median levels of hs-CRP were above 4.0 mg/L in all treatment arms. Other laboratory parameters are presented in Table 2.

Four-week treatment with atorvastatin 40 mg/ezetimibe 10 mg resulted in greater decrease in total and LDL-cholesterol than in other groups. Other laboratory variables were comparable at this time point (Table 3).

Atorvastatin 40 mg and the combination of atorvastatin 40 mg plus ezetimibe 10 mg reduced total- and LDL-cholesterol and hs-CRP. Treatment with ezetimibe 10 mg increased LDL-cholesterol levels, when compared with atorvastatin 10 mg, and did not modify hs-CRP (Table 3).

Campesterol, β-sitosterol and desmosterol were comparable at baseline. Ratios of these sterols to cholesterol were also similar among groups after a run-in period with atorvastatin 10 mg (Table 4).

At the end of study, markers of sterols absorption (plasma campesterol and β-sitosterol) and their ratios to cholesterol were lower in ezetimibe 10 mg when compared with atorvastatin 40 mg or atorvastatin 40 mg/ezetimibe 10 mg; these markers were lower in atorvastatin 40 mg / ezetimibe 10 mg than in atorvastatin 40 mg alone. In addition, desmosterol plasma levels and desmosterol/cholesterol ratio were higher in ezetimibe group, when compared with other groups (Table 4).

Ezetimibe-based therapies reduced absorption markers and their ratios to cholesterol ($P < 0.0001$ vs. baseline, for all; Wilcoxon), whereas atorvastatin alone increased campesterol/cholesterol and β-sitosterol/cholesterol ratios ($P < 0.05$ vs. baseline; Wilcoxon). In addition, ezetimibe also increased desmosterol and desmosterol/cholesterol ratio ($P < 0.0001$ vs. baseline; Wilcoxon) (Table 4).

Percent changes in lipid parameters, hs-CRP, and in markers of cholesterol absorption and synthesis are presented in Supplementary Table 1, and Figs. 1 and 2. Percent change in campesterol and β-sitosterol differed in ezetimibe and atorvastatin/ezetimibe vs. atorvastatin group, whereas changes in desmosterol plasma levels differed in ezetimibe vs. atorvastatin and atorvastatin/ezetimibe.

No correlation between values of LDL-cholesterol and markers of cholesterol absorption and synthesis were observed both at baseline and end of study (Fig. 3). High-sensitivity C-reactive protein levels did not correlate with campesterol or β-sitosterol both at baseline and end of study. Desmosterol levels were inversely correlated with C-reactive protein at baseline and end of study (rho $= -0.15$, $P = 0.046$, and rho $= -0.36$, $P = 0.036$, Spearman's correlation test).

As forty percent of subjects had diabetes mellitus, percent changes in selected parameters were tested. No differences in changes in lipid parameters were observed between diabetic and non-diabetic subjects, in all groups (data not shown). In subjects receiving atorvastatin 40 mg, percent changes in campesterol ($P = 0.046$ vs. non-diabetic) and β-sitosterol ($P = 0.015$ vs. non-diabetic) were greater in those without diabetes compared with diabetic individuals (Supplementary Table 2).

Discussion

Our study demonstrated that in high-risk patients with acceptable LDL-cholesterol levels, but presenting high levels of hs-CRP, the lipid lowering strategy may produce different effects on inflammation decrease and in sterols metabolism. Thus, the choice of the drugs might take into account other parameters beyond LDL-cholesterol targets. These aspects seem important since no correlation between LDL-cholesterol reduction and hs-CRP decrease was obtained in other study (Ridker et al., 2009).

We have chosen atorvastatin, an effective statin with relatively long half- life, in order to counter balance the stimulus for endogenous

Table 2
Baseline laboratory variables, obtained after a run-in period with atorvastatin 10 mg, by group.

Variable	Atorvastatin 40 mg N = 45	Ezetimibe 10 mg N = 40	Atorva/Ezetimibe 40/10 mg N = 37	*P*-value
Total cholesterol, mg/dL, median (IQR)[a]	175 (147–187)	160 (139–195)	170 (147–209)	0.740
HDL-cholesterol, mg/dL, median (IQR)[a]	44 (37–52)	44 (38–53)	47 (41–53)	0.669
LDL-cholesterol, mg/dL, median (IQR)[a]	89 (71–106)	82 (72–114)	94 (75–117)	0.422
Triglycerides, mg/dL, median (IQR)[a]	158 (101–217)	137 (83–175)	126 (112–174)	0.303
Glucose, mg/dL, median (IQR)[a]	108 (97–115)	102 (94–113)	105 (95–123)	0.328
HbA1c, mg/dL, median (IQR)[a]	6.20 (5.90–6.55)	6.30 (5.75–7.00)	6.30 (5.93–6.75)	0.867
AST, IU/L, median (IQR)[a]	22 (19–27)	22 (21–27)	23 (19–27)	0.982
ALT, IU/L, mean (IQR)[a]	23 (18–33)	23 (19–34)	23 (17–30)	0.738
GGT, IU/L, mean (IQR)[a]	41 (25–57)	32 (25–49)	37 (23–64)	0.744
CK, IU/L, median (IQR)[a]	128 (80–178)	122 (81–197)	104 (75–147)	0.390
hs-CRP, mg/L, median (IQR)[a]	4.50 (2.80–6.73)	4.20 (2.60–6.50)	5.05 (2.42–8.98)	0.366
Creatinine, mg/dL, median (IQR)[a]	0.83 (0.74–0.99)	0.92 (0.75–1.12)	0.92 (0.78–1.06)	0.645
TSH, μUI/mL, median (IQR)[a]	2.09 (1.34–3.10)	1.54 (1.11–2.94)	1.57 (1.24–3.00)	0.180
Red blood cells, 10^6/mm^3, mean (SD)	4.81 (0.40)	4.95 (0.55)	4.81 (0.40)	0.270
Leukocytes, 10^3/mm^3, mean (SD)	7380 (1914)	7540 (1670)	7710 (2308)	0.754
Platelets, 10^3/mm^3, mean (SD)	269110 (64758)	254000 (55341)	259500 (60273)	0.507

Numerical variables presented as mean (SD) or median (IQR), as appropriate. Comparisons between groups used ANOVA–Tukey.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; Atorva, atorvastatin; CK, creatinekinase; GGT, gamaglutamil transpeptidase; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; TSH, thyroid-stimulating hormone; SD, standard deviation.
[a] Log-transformed variables for comparisons.

Table 3
Laboratory variables, obtained after a 4-week treatment period, by group.

Variable	Atorvastatin 40 mg N = 45	Ezetimibe 10 mg N = 40	Atorva/ezetimibe N = 37	*P*-value between groups	*P*-value vs. baseline Atorva group	*P*-value vs. baseline Eze group	*P*-value vs. baseline Atorva/Eze group
Total cholesterol, mg/dL, median (IQR)[a]	147 (130–174)	185 (161–205)	133 (109–161)	<**0.0001**[b]	<**0.0001**[c]	<**0.0001**[d]	<**0.0001**[e]
HDL-cholesterol, mg/dL, median (IQR)[a]	43 (39–52)	46 (40–52)	48 (39–54)	0.781	0.525	0.108	0.474
LDL-cholesterol, mg/dL, median (IQR)[a]	74 (59–95)	105 (94–123)	62 (48–75)	<**0.0001**[b]	**0.001**[c]	<**0.0001**[d]	<**0.0001**[c]
Triglycerides, mg/dL, median (IQR)[a]	128 (90–166)	129 (87–182)	100 (77–143)	**0.037**[e]	<**0.0001**[c]	0.075	<**0.0001**[c]
glucose, mg/dL, median (IQR)[a]	105 (99–125)	103 (93–116)	102 (95–121)	0.573	0.403	0.797	0.210
HbA1c, mg/dL, median (IQR)[a]	6.20 (5.90–6.70)	6.30 (5.80–6.80)	6.35 (5.93–6.88)	0.730	0.203	0.767	0.368
AST, IU/L, median (IQR)[a]	23.5 (20.3–29.8)	23.0 (21.0–25.0)	27.0 (20.5–32.0)	0.412	0.230	0.620	**0.008**[d]
ALT, IU/L, mean (IQR)[a]	27.0 (22.3–33.7)	26.0 (21.0–33.0)	24.0 (19.5–33.0)	0.454	0.082	0.187	**0.042**[d]
GGT, IU/L, median (IQR)[a]	41.0 (24.3–53.7)	35.0 (22.0–57.0)	40.0 (21.0–59.5)	0.887	0.793	0.447	0.176
CK, IU/L, median (IQR)[a]	135 (81–210)	123 (83–190)	109 (79–160)	0.420	0.164	0.648	0.116
hs-CRP, mg/L, median (IQR)[a]	3.00 (2.03–6.18)	4.10 (2.70–5.60)	2.10 (1.10–6.15)	0.082	**0.001**[c]	0.436	**0.002**[c]
Creatinine, mg/dL, median (IQR)[a]	0.85 (0.74–1.02)	0.87 (0.73–1.02)	0.89 (0.76–1.02)	0.387	0.578	0.259	0.645
TSH, μUI/mL, median (IQR)[a]	1.84 (1.44–2.97)	1.92 (1.26–2.64)	1.83 (1.30–3.23)	0.778	0.080	**0.047**[d]	0.552
Red blood cells, $10^6/mm^3$, mean (SD)	4.82 (0.38)	4.91 (0.49)	4.81 (0.41)	0.555	0.877	0.624	0.826
Leukocytes, $10^3/mm^3$, mean (SD)	6910 (1549)	7240 (1875)	8050 (2458)	**0.036**[f]	**0.032**[c]	0.380	0.344
Platelets, $10^3/mm^3$, mean (SD)	263230 (63676)	265030 (66432)	258280 (65298)	0.899	0.295	**0.024**[d]	0.929

Numerical variables presented as mean (SD) or median (IQR), as appropriate. Comparisons between groups used ANOVA–Tukey. Comparisons within group (vs. baseline) used paired T-test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; Atorva, atorvastatin; CK, creatinekinase; Eze, ezetimibe; GGT, gamaglutamil transpeptidase; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; TSH, thyroid-stimulating hormone; SD, standard deviation.
[a] Log-transformed variables for comparisons.
[b] Atorvastatin 40 mg and atorvastatin 40 mg/ezetimibe 10 mg < ezetimibe 10 mg and atorvastatin 40 mg/ezetimibe 10 mg < atorvastatin 40 mg.
[c] Baseline > end of study.
[d] Baseline < end of study.
[e] Ezetimibe > Atorvastatin 40 mg/Ezetimibe 10 mg.
[f] Atorvastatin 40 mg < atorvastatin 40 mg/ezetimibe 10 mg.

cholesterol synthesis following the use of ezetimibe. Combined therapy was considered an appropriate strategy to reduce both LDL-cholesterol levels and hs-CRP.

The study shows that the ineffectiveness of ezetimibe alone to achieve lipid targets or decrease hs-CRP levels was probably related to the increase in endogenous cholesterol synthesis observed with this drug.

Atorvastatin can increase sterols absorption in the intestine by two mechanisms: the blockade of cholesterol synthesis (Smahelová et al., 2005), causing up regulation of the NPC1L1 expression, and the interference with the ABCG5/G8 transporters, decreasing the extrusion of cholesterol and phytosterols to the intestine lumen (Kajinami et al., 2004). Therefore, the combined use of ezetimibe in subjects treated with atorvastatin appears to be a very interesting lipid-lowering approach. In fact, previous studies of our group have demonstrated that statins, especially those with short half-life, increase cholesterol absorption and do not reduce cholesterol synthesis effectively (Kasmas et al., 2012).

Table 4
Values of campesterol, β-sitosterol and desmosterol, and their ratios to cholesterol, at baseline and end of study, by group.

Variable	Atorvastatin 40 mg N = 45	Ezetimibe 10 mg N = 40	Atorvastatin/ezetimibe N = 37	*P*-value (between group)	*P*-value (atorvastatin)	*P*-value (ezetimibe)	*P*-value (atorvastatin/eze)
Campesterol, μg/mL (IQR)							
Baseline	0.42 (0.30–0.73)	0.45 (0.31–0.61)	0.54 (0.34–0.93)	0.635	**0.164**	<**0.0001**[a]	<**0.0001**[a]
End of study	0.46 (0.36–0.71)	0.17 (0.12–0.29)	0.33 (0.18–0.43)	<**0.0001**[b]			
ratio to cholesterol[a]							
Baseline	0.25 (0.18–0.44)	0.28 (0.18–0.35)	0.29 (0.20–0.54)	0.697	**0.014**[c]	<**0.0001**[a]	<**0.0001**[a]
End of study	0.34 (0.24–0.46)	0.10 (0.08–0.15)	0.25 (0.19–0.37)	<**0.0001**[b]			
β-sitosterol, μg/mL (IQR)							
Baseline	0.34 (0.25–0.48)	0.37 (0.29–0.53)	0.57 (0.27–0.73)	0151	**0.082**	<**0.0001**[a]	<**0.0001**[a]
End of study	0.38 (0.29–0.61)	0.18 (0.14–0.30)	0.29 (0.17–0.47)	<**0.0001**[d]			
Ratio to cholesterol[a]							
Baseline	0.19 (0.16–0.36)	0.22 (0.16–0.29)	0.30 (0.21–0.46)	0.159	**0.007**[c]	<**0.0001**[a]	**0.005**[a]
End of study	0.25 (0.18–0.40)	0.10 (0.07–0.14)	0.21 (0.15–0.36)	<**0.0001**[d]			
Desmosterol, μg/mL (IQR)							
Baseline	0.11 (0.02–0.14)	0.13 (0.00–0.16)	0.12 (0.11–0.16)	0.177	0.583	<**0.0001**[c]	0.077
End of study	0.11 (0.00–0.14)	0.21 (0.14–0.27)	0.11 (0.00–0.14)	<**0.0001**[f]			
Ratio to cholesterol[e]							
Baseline	0.06 (0.00–0.08)	0.06 (0.00–0.09)	0.07 (0.00–0.08)	0.878	0.852	<**0.0001**[c]	0.675
End of study	0.07 (0.00–0.09)	0.08 (0.07–0.13)	0.06 (0.00–0.10)	**0.008**[f]			

Values expressed as median (IQR).
IQR, interquartile range.
[a] Baseline > end of study.
[b] Ezetimibe 10 mg < atorvastatin 40 mg and atorvastatin 40 mg/ezetimibe 10 mg; atorvastatin 40 mg/ezetimibe 10 mg < atorvastatin.
[c] Baseline < end of study.
[d] Atorvastatin 40 mg > ezetimibe 10 mg.
[e] Ratios are expressed as μg/mg cholesterol.
[f] Ezetimibe 10 mg > atorvastatin 40 mg and atorvastatin 40 mg/ezetimibe 10 mg.

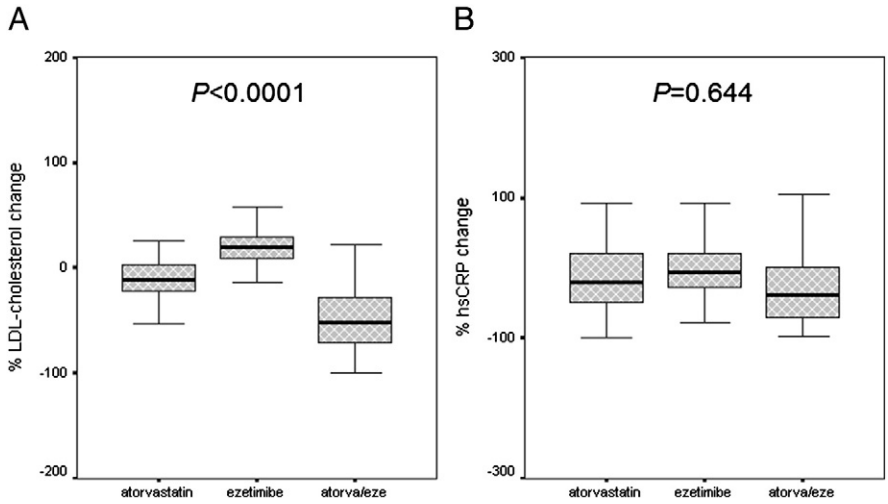

Fig. 2. Box-plots representing percent changes in LDL-cholesterol levels (A) and hs-C-reactive protein (B) in groups, according to treatment. Boxes represent median, 25th and 75th percentiles and whiskers are extreme values. (A) Percent change in LDL-cholesterol differed in ezetimibe vs. atorvastatin and atorvastatin/ezetimibe; percent change in LDL-cholesterol differed in atorvastatin vs. atorvastatin/ezetimibe ($P < 0.0001$, Kruskal–Wallis); (B) No differences in percent changes in hs-C-reactive protein between groups were observed ($P = 0.644$, Kruskal–Wallis).

High-risk patients often need combined therapy to achieve LDL-cholesterol goals, and persistent inflammation is a condition that is not completely reversed when statins are given (Ridker et al., 2008, 2009). Although there is no formal indication for statins aiming at lowering hs-CRP, studies have demonstrated a beneficial effect of these drugs (Ridker et al., 2008, 2009). Part of these effects are related to decrease in cholesterol synthesis that leads to lower expression of small G-proteins that participate in the signaling cascade for transcription of pro- and anti-inflammatory genes, and increases nitric oxide biosynthesis, with a net benefit on atherosclerosis (Landmesser et al., 2005; Zhou and Liao, 2010). In this study, a reduction of ~50% from the baseline hs-CRP levels was achieved with atorvastatin or atorvastatin/ezetimibe, but not with ezetimibe alone, what has been highlighted in other studies (Liu et al., 2009; Ye et al., 2012; Rudofsky et al., 2012).

Previous studies have indicated an increased absolute event risk in patients in primary or secondary prevention of cardiovascular disease if hs-CRP levels were elevated regardless the LDL-cholesterol levels (Ridker et al., 1998, 2005; Sattar et al., 2007). Ezetimibe acts through inhibiting the Niemann–Pick C1 Like 1 (NPC1L1) transporter at the enterocyte with an important synergism with statins in reducing LDL-cholesterol levels (Davis and Veltri, 2007). However, ezetimibe also increases endogenous cholesterol synthesis, with a potential harmful effect on vascular function and inflammation attributed to intermediate compounds generated in the cholesterol biosynthesis pathway (Rho, Rac, and the downstream target Rho-kinase), which are down regulated by statins (Liu et al., 2009; Ye et al., 2012; Rudofsky et al., 2012; 28).

The increase in endogenous cholesterol synthesis precursor, desmosterol, observed with this drug may be an explanation for the lack of efficacy of ezetimibe in reducing hs-CRP levels when given alone (Thongtang et al., 2012). In fact, the augmented cholesterol synthesis determines an increase in the expression of intermediate compounds, such as farnesyl pyrophosphate or geranyl geranyl pyrophosphate, related to the translocation of small signaling proteins to the membrane (Landmesser et al., 2005; Zhou and Liao, 2010). By specific stimuli, these small proteins can increase the transcription of genes related to inflammation and thrombosis. In addition, increased activity of Rho/Rho-kinase decreases the phosphorylation of

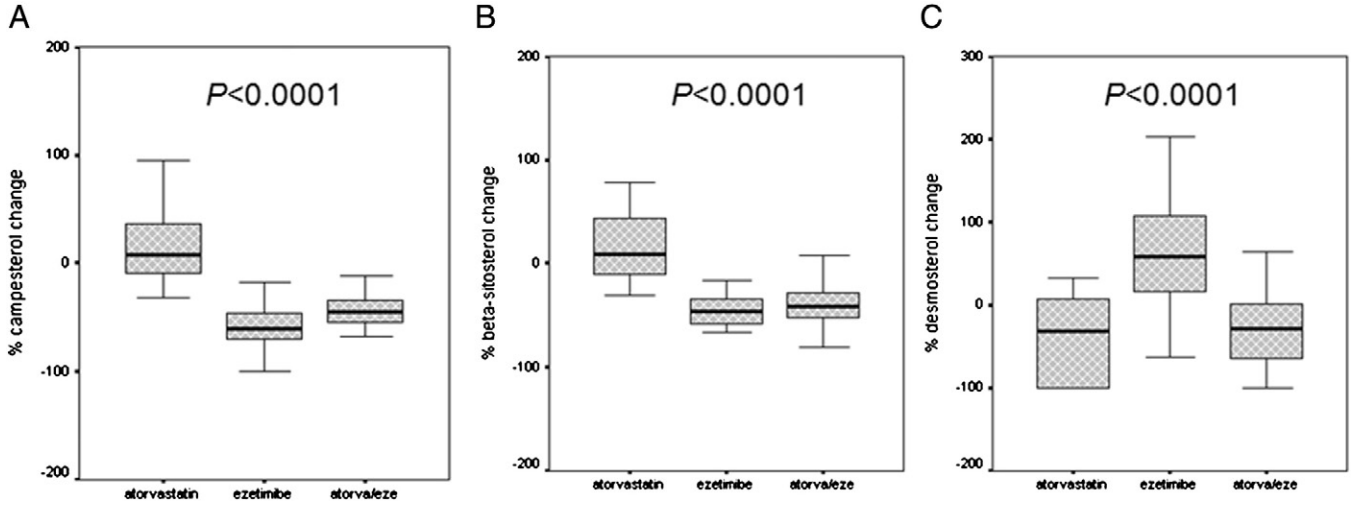

Fig. 3. Box-plots representing percent changes in campesterol (A), β-sitosterol (B) and desmosterol (C) plasma levels in groups according to treatment. (A) Percent change in campesterol and (B) β-sitosterol differed in ezetimibe and atorvastatin/ezetimibe vs. atorvastatin ($P < 0.0001$, Kruskal–Wallis); (C) Percent change in desmosterol differed in ezetimibe vs. atorvastatin and atorvastatin/ezetimibe ($P < 0.0001$, Kruskal–Wallis).

nitric oxide (NO) synthase, thus reducing NO release. Statins can increase NO release due to decrease in the Rho/Rho-kinase expression, which increases the PI 3-kinase/Akt pathway (Landmesser et al., 2005; Zhou and Liao, 2010).

A meta-analysis explored the effects of statins, ezetimibe and their combination on circulating non-cholesterol sterols (Descamps et al., 2011), reinforcing our findings that statins and ezetimibe have inverse effects on cholesterol absorption and synthesis markers, and that when added to a statin, ezetimibe counters the compensatory increase in cholesterol absorption induced by statins. Recently, Thongtang et al. (2012) demonstrated that patients on high-potency statins had the highest levels of cholesterol absorption markers and the lowest levels of cholesterol synthesis markers, with a greater benefit from addition of ezetimibe on these markers, if further reduction of LDL-cholesterol was needed. However, the association between markers of cholesterol absorption and cardiovascular disease has been addressed in a systematic review and meta-analysis by Genser et al. (2012), without clear evidence that moderate fluctuations on plasma concentrations of plant sterols can affect cardiovascular disease risk.

Conclusions

In summary, our results are timely and contribute to better understanding the link between cellular cholesterol homeostasis, inflammation and lipid-modifying therapies. Our findings highlight the broader benefit of combined therapy with a potent statin and ezetimibe decreasing LDL-cholesterol and inflammation, and preventing increase in cholesterol synthesis, an effect not observed with ezetimibe alone.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2013.02.018.

Author contribution

LNM, ACA, HTB and MCI conducted research; FAF and MCI designed research; LCL, CNF, SPB, and JBA, provided essential materials; FAF, CNF, FAF and MCI analyzed data and performed statistical analysis; MCI, LCL and FAF wrote paper and had primary responsibility for final content.

All authors read and approved the final manuscript.

Conflict of interest statement

None.

Acknowledgments

SPB and LCL equally contributed for this research. This study was funded by a research grant #2009/50052-1 from FAPESP (Foundation for Research of the State of Sao Paulo, Brazil). SPB has received research grant from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil).

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