

# Effects of simvastatin/ezetimibe on microparticles, endothelial progenitor cells and platelet aggregation in subjects with coronary heart disease under antiplatelet therapy

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## Abstract

It is not known whether the addition of ezetimibe to statins adds cardiovascular protection beyond the expected changes in lipid levels. Subjects with coronary heart disease were treated with four consecutive 1-week courses of therapy (T) and evaluations. The courses were: T1, 100 mg aspirin alone; T2, 100 mg aspirin and 40 mg simvastatin/10 mg ezetimibe; T3, 40 mg simvastatin/10 mg ezetimibe, and 75 mg clopidogrel (300 mg initial loading dose); T4, 75 mg clopidogrel alone. Platelet aggregation was examined in whole blood. Endothelial microparticles (CD51), platelet microparticles (CD42/CD31), and endothelial progenitor cells (CD34/CD133; CDKDR/CD133, or CD34/KDR) were quantified by flow cytometry. Endothelial function was examined by flow-mediated dilation. Comparisons between therapies revealed differences in lipids (T2 and T3 < T1 and T4 for total cholesterol, LDL-C, and triglycerides;  $P < 0.002$  for all), as well as for endothelial function (T2 > T1 and T4,  $P = 0.001$ ). Decreased platelet aggregation was observed after aspirin (arachidonic acid, T1 < T3 and T4,  $P = 0.034$ ) and clopidogrel (adenosine, T3 and T4 < T1 and T2,  $P < 0.0001$ ) therapy. Simvastatin/ezetimibe diphosphate did not change platelet aggregation, the amount of circulating endothelial and platelet microparticles, or endothelial progenitor cells. Cardiovascular protection following therapy with simvastatin/ezetimibe seems restricted to lipid changes and improvement of endothelial function not affecting the release of microparticles, mobilization of endothelial progenitor cells or decreased platelet aggregation.

Key words: Endothelial microparticles; Platelet microparticles; Platelet aggregation; Endothelial progenitor cells; Simvastatin/ezetimibe

## Introduction

Endothelial dysfunction has been associated with decreased circulating endothelial progenitor cells (1,2) and increased circulating endothelial microparticles (3,4). Both conditions are influenced by classic risk factors (4-6) and inflammatory status (7,8).

The pleiotropic effects of statin therapy include increased nitric oxide bioavailability, antithrombotic, and anti-inflammatory effects (9,10). However, the benefits of vascular protection may vary according to the potency of statin or pharmacokinetic interactions.

An increase in the number of circulating platelet microparticles was reported in subjects with coronary heart disease 1 week after rosuvastatin withdrawal, despite continuing use of clopidogrel, suggesting that statins play a role in antiplatelet activation (11). Furthermore, pharmacokinetic interaction between clopidogrel and atorvastatin has been described and related to the amount of circulating platelet microparticles (12). Interactions between clopidogrel and simvastatin may also be expected because they are metabolized by the same hepatic microsomes (13,14).

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However, a decrease in the number of platelet and endothelial microparticles has been seen after therapy with simvastatin in diabetic and hypertensive subjects (15), while *in vitro* studies have revealed increased release of microparticles related to a simvastatin-mediated decrease of protein prenylation (16). In addition, because ezetimibe increases endogenous cholesterol synthesis (17), its combination with simvastatin may perturb the effects of the statin on microparticles when given alone.

Therefore, taking these interesting and controversial aspects of vascular protection into account, this study was designed to evaluate the effects of simvastatin/ezetimibe therapy on microparticles, endothelial progenitor cells and platelet aggregation in patients with stable coronary heart disease.

## Material and Methods

Participants were recruited from the cardiology outpatient unit at Universidade Federal de São Paulo. The trial protocol was conducted in accordance with the ethics standards of the institution and approval was obtained from the local Ethics Committee. Patients were included after having signed written informed consent. Eligible subjects were those of both genders, 35-75 years of age, and with stable coronary heart disease documented by clinical symptoms and a previous coronary angiogram. We excluded patients with planned revascularization and those with comorbidities such as uncontrolled diabetes (glycated hemoglobin >8.0%), liver (alanine aminotransferase >2.5 times the upper limit of normal) or renal (creatinine >2.0 mg/dL) diseases, thyroid dysfunction (thyroid-stimulating hormone >8  $\mu$ U/mL), genetic hyperlipidemias, New York Heart Association class III/IV heart failure, and those with any known intolerance or contra-indication to the study drugs. Major characteristics of the study population are shown on Table 1.

Subjects were instructed to discontinue statin therapy for a week and to attend four consecutive study visits according to the study protocol (Figure 1). Clinical parameters and laboratory samples for biochemistry and flow cytometry were obtained at each visit. Flow-mediated dilation was evaluated with the application of four specific therapies (T): T1, 100 mg aspirin alone; T2, 100 mg aspirin and 40 mg simvastatin/10 mg ezetimibe; T3, 40 mg simvastatin/10 mg ezetimibe and 75 mg clopidogrel (300 mg on the first day); and T4, 75 mg clopidogrel alone. The study drugs were Aspirina Prevent<sup>®</sup> (Bayer, Brazil), Vytorin<sup>®</sup> (Merck, Brazil), and Plagrel<sup>®</sup> (Sandoz, Brazil).

## Laboratory assays

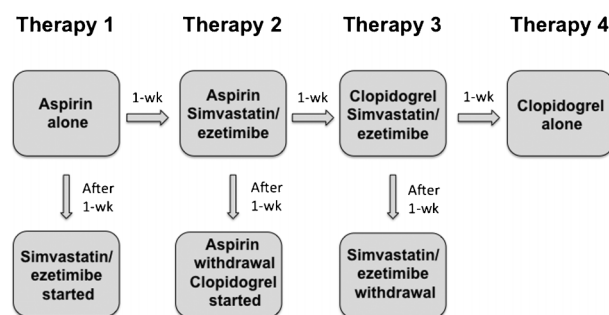
Blood samples were obtained after a 12-h fasting period. Lipids were determined using automated assays (Advia 2400, Siemens Healthcare Diagnostics, Japan) and LDL cholesterol was estimated by the Friedewald equation (18).

**Table 1.** Baseline characteristics of the study population.

Characteristic	Values
Age (years)	62 (57-70)
Gender	
Male	13 (65%)
Female	7 (35%)
Medical history	
Myocardial infarction	13 (65%)
Stable angina	11 (55%)
Percutaneous intervention	11 (55%)
Surgical revascularization	2 (10%)
Ischemic stroke	1 (5%)
Hypertension	18 (90%)
Diabetes	9 (45%)
Medications	
Beta blocker	14 (70%)
Angiotensin-converting enzyme inhibitors	11 (55%)
Angiotensin receptor blockers	4 (20%)
Calcium channel blockers	6 (30%)
Diuretics	11 (55%)
Aspirin	20 (100%)
Nitrates	1 (5%)
Oral antidiabetic agents	9 (45%)
Insulin	1 (5%)

Data are reported as number with percent in parentheses or median and interquartile range.

Measurements of endothelial progenitor cells (EPC) were performed as previously reported (3,11,12). Briefly, the cells were incubated for 30 min with fluorescently labeled mouse anti-human antibodies: CD34 FITC (BD Biosciences, USA), CD13 APC (Miltenyi Biotec, USA), and



**Figure 1.** Study design. Therapy 1: 100 mg aspirin alone; Therapy 2: 40 mg simvastatin/10 mg ezetimibe plus 100 mg aspirin; Therapy 3: 300 mg clopidogrel (initial dose) followed by 75 mg plus 40 mg simvastatin/10 mg ezetimibe; Therapy 4: 75 mg clopidogrel alone. Laboratory tests, including platelet aggregation analyses, flow-mediated dilation, and quantification of endothelial progenitor cells, platelet microparticles, and endothelial microparticles were performed at the end of each 1-week (wk) therapy. All patients followed the consecutive therapies without drug washout.

KDR PE (R&D Systems, USA). Mouse antibody isotypes were used as controls. A minimum of 500,000 events was acquired for flow cytometry (FACSCalibur; BD Biosciences). EPC counts are reported as the percentage of total progenitor cells in the lymphocyte gate. For quantitation of endothelial microparticles (EMPs) and platelet microparticles (PMPs), blood samples were collected in citrated tubes and centrifuged (160 g, 10 min) to obtain the platelet-rich plasma (PRP). The PRP was then centrifuged (1500 g, 6 min) to obtain the platelet-poor plasma (PPP). The PPP was incubated for 20 min with fluorescently labeled mouse anti-human CD42 FITC and CD31 PE (BD Biosciences) antibodies for PMP identification, and with CD51 FITC (BD Biosciences) to detect EMP. Mouse antibody isotypes were used as controls. We used disposable containers (TruCOUNT Tubes; BD Biosciences) to determine the number of microparticles per microliter of PPP.

Platelet aggregation was examined by multiple electrode platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma, Germany) performed as previously reported (11). Aggregation tests induced by collagen, thrombin receptor-activating peptide 6, adenosine diphosphate, and arachidonic acid were performed in duplicate.

Flow-mediated dilation (FMD) of the brachial artery was assessed at each visit by ultrasound (Hewlett-Packard SONOS 5500, USA) using a high-frequency transducer, as previously reported (7,11). Briefly, patients were required to fast and abstain from nitrates, alcohol, and vasoactive medications for the previous 24 h. A pneumatic cuff was placed on the extended left arm after a 20-min rest, when baseline images were acquired. The cuff was inflated (>200 mmHg) to occlude blood flow for 5 min and images were acquired during the next 2 min after cuff release. The greatest vasodilation was recorded.

Endothelium-independent responses following nitrates were not performed because of the validity of FMD measurements alone (19).

The effects of the four therapies on biochemistry, platelet aggregation, flow-mediated dilation, and circulating microparticles were tested using ANOVA-repeated measures followed by the Tukey test when appropriate. The size of the study sample was based on a previous study of our group with a similar design (11). Comparisons of the percentages of circulating EPCs obtained with the therapies were analyzed by the Wilcoxon test. Triglycerides were log transformed. Tests were two-tailed and significance was set at  $P < 0.05$ .

## Results

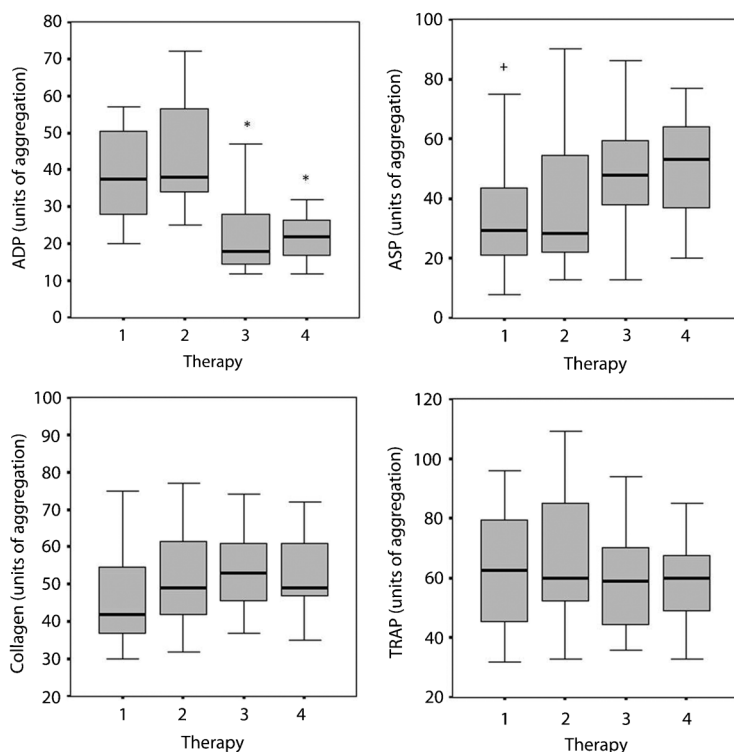
There were no adverse events during the study, drugs were well tolerated, and complete adherence to the study medications was observed in all visits for all patients. Laboratory assay results were not affected by the study treatments, except for lipids and platelet aggregation. Total cholesterol and LDL cholesterol were reduced by 32 and 45%, respectively, after 1 week of simvastatin/ezetimibe therapy; LDL cholesterol was <100 mg/dL in 95% of subjects and LDL cholesterol was <70 mg/dL in 65% of patients. A substantial rise in cholesterol was also observed 1 week after withdrawal of lipid-lowering therapy, corresponding to a 41% increase in total cholesterol and an 83% increase in LDL cholesterol. Significant changes of triglycerides were also observed between visits (Table 2).

Antiplatelet tests showed decreases in aggregation in response to arachidonic acid ( $P = 0.034$ ) and adenosine diphosphate ( $P < 0.0001$ ) for patients treated with aspirin and clopidogrel, respectively. When simvastatin/ezetimibe

**Table 2.** Effects of therapy on clinical parameters and biochemistry.

	T1	T2	T3	T4
SBP (mmHg)	135 ± 3	130 ± 2	126 ± 3	126 ± 3
DBP (mmHg)	80 ± 2	78 ± 1	77 ± 2	78 ± 1
HR (bpm)	66 ± 2	68 ± 2	68 ± 2	66 ± 2
CK (U/L)	137 ± 16	138 ± 21	151 ± 26	140 ± 22
Creatinine (mg/dL)	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Glucose (mg/dL)	108 ± 8	116 ± 8	116 ± 7	107 ± 4
AST (U/L)	20 ± 2	22 ± 2	21 ± 2	19 ± 1
ALT (U/L)	22 ± 2	25 ± 3	27 ± 3	23 ± 3
TC (mg/dL)	185 ± 10	127 ± 7*	120 ± 7*	170 ± 9
HDL-C (mg/dL)	43 ± 2	44 ± 3	45 ± 3	45 ± 3
LDL-C (mg/dL)	110 ± 8	61 ± 6*	53 ± 6*	97 ± 7
Triglycerides (mg/dL)	156 ± 15	109 ± 7*	98 ± 7*	140 ± 14

Data are reported as means ± SE. Therapy: T1: 100 mg aspirin; T2: 40 mg simvastatin/10 mg ezetimibe plus 100 mg aspirin; T3: 300 mg clopidogrel (initial dose) followed by 75 mg plus 40 mg simvastatin/10 mg ezetimibe; T4: 75 mg clopidogrel alone. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; CK: creatine kinase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; HDL-C and LDL-C: high- and low-density lipoprotein cholesterol, respectively. \* $P < 0.0001$  TC and LDL-C on T2 and T3 < T1 and T4; \* $P < 0.002$  triglycerides on T2 and T3 < T1 and T4 (ANOVA-Tukey).



**Figure 2.** Platelet aggregation in response to therapies (T): T1: 100 mg aspirin; T2: 40 mg simvastatin/10 mg ezetimibe plus 100 mg aspirin; T3: 300 mg clopidogrel (initial dose) followed by 75 mg plus 40 mg simvastatin/10 mg ezetimibe; T4: 75 mg clopidogrel alone. Decreased platelet aggregation was observed after therapy with aspirin [arachidonic acid (ASP) \*T1<T3 and T4, P=0.034, ANOVA-Tukey] and clopidogrel [adenosine diphosphate (ADP) \*T3 and T4<T1 and T2, P<0.0001, ANOVA-Tukey].

therapy was given to patients taking aspirin or clopidogrel, it did not modify the antiplatelet effects obtained when these antiplatelet agents were given alone (Figure 2).

Systolic and diastolic blood pressure, as well as heart rate, were unchanged throughout the study. FMD was improved by simvastatin/ezetimibe therapy (P=0.001); an increase of 249% in FMD was observed after 1 week of therapy. Conversely, 1 week after simvastatin/ezetimibe withdrawal, FMD returned to baseline values (Table 3).

Endothelial and platelet microparticles were not affected by any of the treatments, and the percentage of circulating endothelial progenitor cells remained unchanged throughout the study (Table 3).

## Discussion

Patients with coronary heart disease should receive an antiplatelet drug and are usually given lipid-lowering therapy to achieve an LDL cholesterol of less than 70 mg/dL

**Table 3.** Effects of therapy on flow-mediated dilation, endothelial progenitor cells, endothelial and platelet microparticles.

Parameter	T1	T2	T3	T4
FMD (%)	6.11 ± 1.86	15.28 ± 2.49*	12.48 ± 1.56	5.93 ± 1.35
EPC (% cells)				
34/KDR	0.020 ± 0.005	0.010 ± 0.004	0.020 ± 0.004	0.010 ± 0.003
34/133	0.0511 ± 0.00718	0.0563 ± 0.01473	0.0432 ± 0.00662	0.0700 ± 0.01328
133/KDR	0.020 ± 0.011	0.010 ± 0.011	0.020 ± 0.008	0.030 ± 0.008
Microparticles (n/μL plasma)				
EMP	1087 ± 336	1365 ± 403	1627 ± 364	1835 ± 917
PMP	32304 ± 5123	31139 ± 6422	33999 ± 4784	38705 ± 6395

Data are reported as means ± SE. Therapy: T1: 100 mg aspirin; T2: 40 mg simvastatin/10 mg ezetimibe plus 100 mg aspirin; T3: 300 mg clopidogrel (initial dose) followed by 75 mg plus 40 mg simvastatin/10 mg ezetimibe; T4: 75 mg clopidogrel alone. FMD: flow-mediated dilation; EPC: endothelial progenitor cells (CD34+/KDR+, CD34+/CD133+, and CD133+/KDR+); EMP: endothelial microparticles (CD51+); PMP: platelet microparticles (CD42+/CD31+). \*P=0.001 FMD on T2>T1 and T4 (ANOVA-Tukey).

(20,21). This study showed that the LDL cholesterol target could be achieved with simvastatin/ezetimibe but that these agents did not contribute to additional antiplatelet effects beyond those already obtained by clopidogrel or aspirin. Despite an early and pronounced improvement on endothelial function with this lipid-lowering regimen, no changes in the amount of microparticles or circulating endothelial progenitor cells were seen. Taken together, the study showed that simvastatin/ezetimibe therapy can be administered in combination with clopidogrel to patients with coronary heart disease, but potential vascular benefits in addition to lipid changes may not be seen.

Two recent meta-analyses of statin treatment clearly showed the importance of a 2-3 mM reduction of LDL cholesterol to obtain increased benefit in prevention of major cardiovascular events (22,23). Although usually safe, the use of high doses of statins may be accompanied by an increase in adverse muscle effects (24,25). The effectiveness of simvastatin/ezetimibe to achieve LDL cholesterol targets is well recognized, but some pleiotropic effects of statins may not be fully expressed when they are used together with other lipid lowering strategies, (e.g., the combined use of ezetimibe and simvastatin) (26). In this regard, our study shows that the antiplatelet effect of aspirin or clopidogrel was unaffected by the concomitant simvastatin/ezetimibe therapy. In addition, this lipid lowering therapy changed neither the number of circulating microparticles nor the percentage of endothelial progenitor cells. However, due to safety and ethical reasons, the effect of simvastatin/ezetimibe alone on platelet aggregation and microparticles, without antiplatelet therapy, could not be assessed in this high-risk patient group.

Simvastatin is a prodrug with the active metabolites formed after biotransformation by the P450 cytochrome (CYP) 3A4 system (27). Clopidogrel is metabolized in the liver by several P450 cytochrome isoenzymes, including

CYP 3A4 to form its active thiol metabolite (28). A prior study reported pharmacokinetic interactions between statins and clopidogrel that decreased the antiplatelet activity of clopidogrel (29). However, no compelling evidence derived from clinical studies indicates the need to avoid concomitant use of any specific statin with clopidogrel (30). In fact, a recent study comparing simvastatin 80 mg with simvastatin 10 mg plus ezetimibe 10 mg showed that FMD responses after treatment were of similar magnitude and closely related to the changes in LDL cholesterol (31). In our study, additional improvement in FMD appeared to be associated with the low LDL cholesterol levels that were achieved.

Our study tested the early effects of combined lipid lowering therapy in subjects with stable coronary heart disease and currently using antiplatelet therapies because pharmacokinetic interactions between clopidogrel and statins detected in previous studies affected clopidogrel levels and the number of microparticles released shortly after the start of statin therapy or its withdrawal (11,12).

The large IMPROVE-IT trial (32) comparing ezetimibe/simvastatin versus simvastatin monotherapy in patients with acute coronary syndromes is ongoing and will provide definitive data regarding long-term safety and clinical benefits of combined lipid-lowering therapy in subjects receiving antiplatelet therapy.

To summarize, our findings suggest that simvastatin/ezetimibe use is an effective lipid-lowering therapy for patients with stable coronary heart disease, including concomitant use with clopidogrel. However, the potential benefit of this lipid-lowering therapy for vascular protection seems restricted to its lipid-lowering properties and improvement of endothelial function.

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