Endothelial Progenitor Cell Mobilization and Platelet Microparticle Release Are Influenced by Clopidogrel Plasma Levels in Stable Coronary Artery Disease

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Background: Increased numbers of endothelial (EMP) and platelet (PMP) microparticles have been related to cardiovascular risk factors and coronary artery disease. Little is known about the early effects of statins and clopidogrel on these new biomarkers of vascular homeostasis. The aim of the present study was to evaluate pharmacokinetic interactions between atorvastatin and clopidogrel and their effects, alone or combined, on EMP, PMP, and endothelial progenitor cells (EPC).

Methods and Results: A prospective open-label study enrolled subjects with stable coronary disease (n=26). Drugs were given daily for 3 weeks (atorvastatin 80 mg, visits 1–3; clopidogrel 75 mg, visits 2–4). Counts of EPC (CD34+/CD133+/KDR+), EMP (CD51+) and PMP (CD42+/CD31+), and pharmacokinetic parameters over 24 h were assessed at each visit. Atorvastatin plasma concentrations were increased by concomitant therapy with clopidogrel (maximum serum concentration [Cmax], P=0.002; area under the clopidogrel or atorvastatin plasma concentration vs. time curve from 0 to the last detectable concentration [AUClast], P=0.03). After atorvastatin withdrawal there was an increase in clopidogrel plasma concentrations (Cmax, P=0.009; AUClast, P=0.039). PMP were inversely correlated with clopidogrel Cmax on visit 3 (rho=-0.57, P=0.006) and on visit 4 (rho=-0.54, P=0.01), and with clopidogrel AUClast on visit 3 (rho=-0.44, P=0.04), and on visit 4 (rho=-0.57, P=0.005). In addition, clopidogrel Cmax was correlated with EPC (CD133+/KDR+) on visit 4 (rho=0.48, P=0.025). No correlations of atorvastatin and MP or EPC were found.

Conclusions: The balance between platelet MP release and EPC mobilization seems influenced by clopidogrel plasma levels, suggesting a protective mechanism on coronary artery disease. (Circ J 2012; 76: 729–736)

Key Words: Clopidogrel; Endothelial progenitor cells; Platelets; Pharmacokinetics; Statin

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Methods

Patients
This is a prospective study with participants consecutively selected from the outpatient clinic of the cardiology division of Federal University of São Paulo. The trial protocol was conducted in accordance with the ethics standards of the institution on human experimentation, and approval was obtained from the local ethics committee. Patients were included after having signed written informed consent.

We included patients of both genders (n=26), aged 49–77 years, with diagnosed stable coronary artery disease, under stable statin therapy for at least 3 months. Patients with planned revascularization were excluded. Subjects with liver (alanine aminotransferase [ALT] >2.5-fold upper limit of normal) or renal disease (creatinine >2.0 mg/dl), those with uncontrolled comorbidities, such as diabetes (glycated hemoglobin [HbA1c] >7.5%), thyroid dysfunction (thyroid-stimulating hormone >8 μU/ml), body mass index >35 kg/m2, triglycerides >400 mg/dl, genetic dyslipidemias, heart failure class III/IV (according to the New York Heart Association), HIV, and those with any known intolerance to the study drugs, were also excluded from the protocol.

Study Design
Patients were selected to attend a clinical visit, at which time demographic data and laboratory samples were obtained for biochemistry. Eligible patients had prior statin use discontinued for 1 week, and they were then scheduled to 4 visits, to receive therapy with atorvastatin, clopidogrel alone and combined (Figure 1).

The study drugs were atorvastatin (Lipitor®; Pfizer Pharmaceutical, São Paulo, Brazil), and clopidogrel (Plavix®; Sanofi Winthrop Industrie, Ambarés & la-Grave, Bordeaux, France). In all visits, patients received study medication at the same time in the morning after a 12-h fasting period. Returned tablets were counted at each visit.

Blood Sample Collection and Assays
Biochemistry was analyzed in samples obtained after a 12-h fasting period in a central laboratory at Federal University of São Paulo using automated techniques.

Lipids and Biochemistry
Serum cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured using automated methods (Advia 2400; Siemens Healthcare Diagnostics, Tokyo, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation. ALT, creatinine and creatine kinase, were assayed using automated techniques (Advia 2400; Siemens Healthcare Diagnostics).

Determination of EPC, EMP and PMP
Measurements of EPC were performed as previously reported. Briefly, blood mononuclear cells were separated on Ficoll density gradient centrifugation. The cells were incubated for 30 min with the following mouse antibodies: CD34 FITC (BD Biosciences, Franklin Lakes, NJ, USA); CD133 APC (Miltenyi Biotec, Auburn, CA, USA) and KDR PE (R&D Systems, Minneapolis, MN, USA). As controls, we used mouse antibody isotypes: a minimum of 500,000 events was acquired for flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). EPC counts are expressed as percentage (%) of total progenitor cells in the lymphocyte gate. For EMP and PMP quantification, blood sample was collected in citrated tubes and centrifuged to obtain the platelet-rich plasma (PRP).
The PRP was then centrifuged to obtain the platelet-poor plasma (PPP). The PPP was then incubated for 20 min with the mouse antibodies CD42 FITC and CD31 PE (BD Biosciences), for PMP identification and with CD51-FITC (BD Biosciences), to detect EMP. Isotypes were used as controls. We used disposable containers (TruCOUNT Tubes; BD Biosciences) to determine microparticle number per microliter of PPP.

Pharmacokinetics

Drug Analysis  
Blood samples from a suitable antecubital vein were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10; 12; 16; and 24h after dosing of the compound of reference. Plasma levels of atorvastatin were measured on validated liquid chromatography with mass spectrometry (LC-MS/MS) using an API 4000 system (Applied Biosystems, Canada). For clopidogrel, we used a fast, sensitive and specific LC-MS/MS bioanalytical method validated for determination of unchanged clopidogrel in human plasma.

Platelet Function Tests
Platelet adhesion and aggregation were tested in anti-coagulated whole blood collected using citrate buffer tubes. We used the IMPACT-R test (Matis Medical, Beersel, Belgium), which is a device for testing platelet function under close to physiological arterial flow conditions (1,800/s for 2 min). Briefly, laminar flow over the polystyrene surface of the well is achieved following the cone and plate principle. Upon application of a blood sample (130 μl) into the polystyrene well, plasma proteins immediately adhere to the well surface and become attractive for platelets, resulting in platelet adhesion and aggregation on the well surface under flow conditions. Following washing of excess blood and staining of the covered platelet aggregates (representing platelet adhesion and aggregation), and the average size of the aggregates (in μm²; representing platelet aggregation). To test for clopidogrel efficacy, platelet activation was stimulated by adenosine diphosphate (ADP). Clopidogrel irreversibly blocks the ADP receptor P2Y12 on platelet cell membranes. This receptor plays an important role in platelet aggregation, specifically in the cross-linking of platelets by fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIIa pathway.

Statistical Analysis
Categorical variables are presented as number (%); numerical data are expressed as mean±SD or median (interquartile range).

<table>
<thead>
<tr>
<th>Table 2. Lipid Level, Microparticle and EPC Changes</th>
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<tbody>
<tr>
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<tr>
<td><strong>Lipids</strong></td>
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<tr>
<td>Total cholesterol</td>
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<tr>
<td>LDL-C</td>
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<td>HDL-C</td>
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<tr>
<td>Triglycerides</td>
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<tr>
<td><strong>Endothelial progenitor cells</strong></td>
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<tr>
<td>CD34+KDR+</td>
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<tr>
<td>CD34+CD133+</td>
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<tr>
<td>KDR+/CD133+</td>
</tr>
<tr>
<td><strong>Microparticles</strong></td>
</tr>
<tr>
<td>Endothelial</td>
</tr>
<tr>
<td>Platelet</td>
</tr>
</tbody>
</table>

Data given as mean±SD.

EPC, endothelial progenitor cell; ATV, atorvastatin (80 mg daily); CLO, clopidogrel (75 mg daily); LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Statins were discontinued 1 week before baseline visit. Total cholesterol on visit 1, visit 2 and visit 3 <visit 4; LDL-C on visit 1 and visit 2 <visit 4; HDL-C on visit 2 <visit 1 and visit 4; triglyceride levels on visit 2 and visit 3 <visit 4. ANOVA-Tukey. Friedman test.
Continuous variables were tested for distribution of normality using the Kolmogorov-Smirnov test. Variables were compared between time points using ANOVA-repeated measures or Friedman test. Correlations were examined using the Spearman test. The first-order terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase in a log-linear plot. Half-life (t1/2) was derived from this rate constant (t1/2=ln(2)/ke). The maximum serum concentration of clopidogrel (Cmax) and the time taken to achieve this concentration (Tmax) were obtained directly from the curves. The areas under the clopidogrel and atorvastatin plasma concentration vs. time curves from 0 to the last detectable concentration (AUClast) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity (AUC0-inf) was done by adding the Clast/ke to the calculated AUClast (where Clast=the last detectable concentration). Tests were 2-tailed and statistical significance was set at P<0.05. All analyses were done SPSS 18.0 for Windows (SPSS, Chicago, IL, USA).

Results

Patients
All patients completed the study protocol with full adherence to atorvastatin and clopidogrel. Participants were predominantly middle-aged overweight men, and all subjects had stable coronary disease. All patients had diagnosis of hypertension, and one-third of participants had diabetes mellitus. Major baseline subject characteristics (visit 1) are given in Table 1.

Lipids, EPC and microparticles
Treatment with atorvastatin was effective to maintain LDL-C levels <100 mg/dl. It was noted, however, that 1-week discontinuation of this lipid-lowering agent was followed by increase in all measured lipid parameters (Table 2). The numbers of EMP and PMP per μl of PPP, as well as the percentages of the 3 subpopulations of EPC were unchanged throughout the study (Table 2). An inverse correlation, how-
ever, was seen between $C_{\text{max}}$ and the number of PMP at visit 3 ($\rho=-0.57, P=0.006$, Spearman test) and at visit 4 ($\rho=-0.54, P=0.01$, Spearman test; Figure 2). In addition, an inverse correlation was also observed between the AUC$_{\text{last}}$ of clopidogrel and PMP on visit 3 ($\rho=-0.44, P=0.04$, Spearman test), and visit 4 ($\rho=-0.57, P=0.005$, Spearman test; Figure 2). Furthermore, the clopidogrel AUC$_{\text{last}}$ was positively correlated with the EPC (CD133+/KDR+) on visit 4 ($\rho=0.48, P=0.025$, Spearman test).

**Pharmacokinetics**

Major pharmacokinetic parameters are given in Table 3 for

**Table 3. Pharmacokinetic Parameters**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Atorvastatin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>3.46±6.19</td>
<td>1.40±0.69</td>
<td>1.88±2.19</td>
<td>NA</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>127±103</td>
<td>200±160</td>
<td>183±161</td>
<td>NA</td>
</tr>
<tr>
<td>$C_{\text{last}}$ (ng/ml)</td>
<td>11.6±31.8</td>
<td>6.7±14.2</td>
<td>1.4±0.9</td>
<td>NA</td>
</tr>
<tr>
<td>AUC$_{\text{last}}$ (ng·h·ml$^{-1}$)</td>
<td>523±352</td>
<td>659±444</td>
<td>524±421</td>
<td>NA</td>
</tr>
<tr>
<td>AUC$_{\text{inf}}$ (ng·h·ml$^{-1}$)</td>
<td>583±427</td>
<td>787±609</td>
<td>540±421</td>
<td>NA</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>6.2±3.1</td>
<td>5.7±4.2</td>
<td>7.1±3.7</td>
<td>NA</td>
</tr>
<tr>
<td>ke (1/h)</td>
<td>0.13±0.04</td>
<td>0.15±0.06</td>
<td>0.12±0.06</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Clopidogrel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>NA</td>
<td>2.33±2.62</td>
<td>3.38±1.66</td>
<td>1.50±0.89</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>NA</td>
<td>6.7±4.07</td>
<td>8.83±5.56</td>
<td>12.51±11.46</td>
</tr>
<tr>
<td>$C_{\text{last}}$ (ng/ml)</td>
<td>NA</td>
<td>1.04±1.99</td>
<td>2.87±4.53</td>
<td>1.12±2.71</td>
</tr>
<tr>
<td>AUC$_{\text{last}}$ (ng·h·ml$^{-1}$)</td>
<td>NA</td>
<td>33.40±27.99</td>
<td>47.89±35.82</td>
<td>45.28±32.63</td>
</tr>
<tr>
<td>AUC$_{\text{inf}}$ (ng·h·ml$^{-1}$)</td>
<td>NA</td>
<td>263±770</td>
<td>84.60±104.77</td>
<td>49.20±44.25</td>
</tr>
<tr>
<td>AUC$_{\text{extr}}$ (%)</td>
<td>NA</td>
<td>19.13±27.96</td>
<td>26.44±21.63</td>
<td>9.95±11.28</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>NA</td>
<td>32.99±93.53</td>
<td>9.83±6.12</td>
<td>7.37±8.32</td>
</tr>
<tr>
<td>ke (1/h)</td>
<td>NA</td>
<td>0.15±0.15</td>
<td>0.11±0.11</td>
<td>0.16±0.11</td>
</tr>
</tbody>
</table>

Data given as mean ± SD.

ATV, atorvastatin (80 mg daily); CLO, clopidogrel (75 mg daily); $T_{\text{max}}$, time to reach maximum concentration; NA, not applicable; $C_{\text{max}}$, maximum achieved concentration; $C_{\text{last}}$, last detectable concentration; AUC$_{\text{last}}$, area under the plasma concentration vs. time curve from 0 to the last detectable concentration; AUC$_{\text{inf}}$, extrapolation of area under the curve to infinity; AUC$_{\text{extr}}$, percent extrapolation of area under the concentration curve; $t_{1/2}$, half-life; ke, first-order terminal elimination rate constant.

**Figure 3.** Mean concentration of atorvastatin according to treatment. Visit 1, atorvastatin 80 mg 1st day; visit 2, atorvastatin 80 mg 7th day + clopidogrel 1st day; visit 3, atorvastatin 21st day + clopidogrel 14th day; visit 4, clopidogrel 21st day.
atorvastatin and clopidogrel. We found lower $C_{\text{max}}$ and $AUC_{\text{last}}$ of atorvastatin on visit 1 than on visits 2 and 3, when clopidogrel was added (Figure 3; $P=0.0022$, ANOVA for repeated measures). Conversely, atorvastatin had little influence on clopidogrel serum levels (Figure 4; $P=0.034$, ANOVA for repeated measures, without differences between treatments according to the Tukey-Kramer test).

**Platelet Function Tests**

Table 4 lists the results for platelet adhesion and aggregation under steady-state conditions, for atorvastatin plus clopidogrel and clopidogrel alone. We found that neither adhesion nor aggregation were significantly modified when atorvastatin was discontinued.

**Discussion**

Recently, Ryu and Kim reported that clopidogrel therapy was associated with decreased EMP in vitro.\(^{36,27}\) The present study found an inverse relationship between clopidogrel serum levels and the amount of PMP. We investigated the effects of clopidogrel in patients who were not receiving aspirin or any anti-thrombotic agent, alone or combined with a potent statin.

Regarding microparticles, EMP are the most studied and their increase has been associated with uncontrolled cardiovascular risk factors, triggering mechanisms of endothelial apoptosis.\(^{36,27}\) In some circumstances, their release appears to have a protective role on endothelium integrity.\(^{28}\) In contrast, the significance of PMP count is less understood, but pronounced increase seems related to thrombosis.\(^{29,30}\) These microparticles can also stimulate the development of collateral vessels, acting as messengers of ischemic conditions.\(^{31}\) Taking these data together, the lower presence of EMP and PMP in patients with coronary artery disease seems to indicate a greater vascular stability, suggesting low rates of apoptosis of endothelial cells, and decreased platelet consumption.

Contrary to our expectations, we were unable to demonstrate changes in the amount of microparticles with use of high-dose atorvastatin. The highest atorvastatin dose is the most used dose in randomized clinical trials. This is in agreement with a recent meta-analysis showing the highest benefit among patients who had the highest LDL-C reduction,\(^{32}\) which is usually correlated with the highest statin dose. We found pharmacokinetic interactions between atorvastatin and clopidogrel, with increases in the plasma concentration of atorvastatin. Because atorvastatin is not a prodrug, this interaction probably had little effect on its lipid-lowering effectiveness, as well as on the anti-platelet actions of clopidogrel, as suggested by previous studies.\(^{33-35}\) Controversial results in the literature, however, may reflect a lower dose of statins or lack of an appropriate study design, because drug introduction and withdrawal were not assessed.\(^{36,37}\)

Clopidogrel, but not atorvastatin, plasma levels were correlated with the CD133+/KDR+ subpopulation of EPC, which is considered an immature lineage of EPC, and a marker of disease severity.\(^{38}\) This effect of clopidogrel, not seen with atorvastatin, may have been related to chronic and effective use of statins in the present subjects. We selected patients with relatively low levels of cholesterol, which may have attenuated the effects of the homing of EPC, thus decreasing EPC

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**Table 4.** Platelet Function vs. Treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ATV+CLO</th>
<th>CLO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion (%)</td>
<td>2.34±2.73</td>
<td>1.06±1.00</td>
<td>0.058</td>
</tr>
<tr>
<td>Aggregation (mm$^2$)</td>
<td>29.65±9.49</td>
<td>31.58±8.07</td>
<td>0.178</td>
</tr>
</tbody>
</table>

Data given as mean±SD.

ATV, atorvastatin (80 mg daily); CLO, clopidogrel (75 mg daily).

Comparisons made using Friedman test.
mobilization.39 But, even with significant changes to lipid profile, either by use of high-dose or by abrupt withdrawal of atorvastatin, no changes in the amount of circulating EPC or microparticles were observed.

Study Limitations

The present study had a relatively small sample size, but we were able to demonstrate changes in surrogate endpoints. We tested patients under the highest atorvastatin dose that is the most used dose in randomized clinical trials, but without a loading dose of clopidogrel, therefore the present results cannot be extrapolated to other drug regimens.

In summary, the present study has demonstrated a pharmacokinetic interaction between atorvastatin and clopidogrel, an inverse correlation between clopidogrel plasma concentration and the release of PMP, and a positive correlation with mobilization of EPC, thus suggesting a new potential benefit for protection in coronary artery disease.

A definitive clinical statin-clopidogrel interaction has yet to be demonstrated in a large-scale randomized clinical trial.

Acknowledgments

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