

Antiplatelet Properties of Escitalopram in Patients with the Metabolic Syndrome: A Dose-Ranging *In Vitro* Study

Dan Atar¹, Alex Malinin², Alex Pokov², Louis van Zyl³, Nancy Frasure-Smith^{4,5}, Francois Lesperance⁵ and Victor L Serebruany^{*,2}

¹Division of Cardiology, Aker University Hospital and Faculty of Medicine, University of Oslo, Oslo, Norway; ²HeartDrug Research LLC, Johns Hopkins University, Baltimore, MD, USA; ³Department of Psychiatry, Queen's University, Kingston, ON, Canada; ⁴Department of Psychiatry, McGill University, Montreal, QC, Canada; ⁵Department of Psychiatry, University of Montreal, Montreal, QC, Canada

There is an increasing body of evidence suggesting that selective serotonin reuptake inhibitors exhibit clinical benefit beyond treating depression, by simultaneously inhibiting platelet activity. We recently demonstrated that escitalopram (ESC), but not its major metabolites, inhibits multiple platelet biomarkers in healthy volunteers. Considering that the metabolic syndrome represents one of the major risk factors for vascular disease, we here determined how ESC affects platelet activity in such patients. We assessed the *in vitro* effects of preincubation with escalating (50–200 nM/l) concentrations of ESC on platelet aggregation, expression of major surface receptors by flow cytometry, and quantitatively by platelet function analyzers. Blood samples were obtained from 20 aspirin-naïve patients with documented metabolic syndrome. Pretreatment of blood samples with medium (150 nM/l), or high (200 nM/l) doses of ESC resulted in a significant inhibition of platelet aggregation induced by ADP ($p = 0.007$) and by collagen ($p = 0.004$). Surface platelet expression of GPIIb (CD42, $p = 0.03$), LAMP-3 (CD63, $p = 0.04$), and GP37 (CD165, $p = 0.03$) was decreased in the ESC-pretreated samples. Closure time by the PFA-100 analyzer was prolonged after the 200 nM/l dose ($p = 0.02$), indicating platelet inhibition under high shear conditions. On the other hand, the lowest tested concentration of ESC (50 nM/l) did not affect platelet activity in these patients. The *in vitro* antiplatelet characteristics of ESC in patients with the metabolic syndrome are similar to those in healthy volunteers. However, higher ESC doses are required to induce equally potent platelet inhibition. These data justify prospective *ex vivo* studies with the highest therapeutic dose to determine the potential clinical advantage of ESC in high-risk patients with vascular disease. *Neuropsychopharmacology* (2007) **32**, 2369–2374; doi:10.1038/sj.npp.1301355; published online 14 March 2007

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INTRODUCTION

Mood disorders, in general, and clinical depression, in particular, are associated with an increase in risk for acute vascular events (Lett *et al*, 2004). Platelet inhibition has been implicated in the reduction in secondary vascular events (Antithrombotic Trialists' Collaboration, 2002). Because of the evidence that selective serotonin reuptake inhibitors (SSRI) may influence platelet activation, it has been suggested that SSRI treatment may reduce the increased risk for vascular events, which is associated with depression. In the Sertraline Antidepressant Heart Attack Randomized Trial (SADHART), there were numerically fewer severe cardiovascular adverse events in the SSRI

treatment arm than in the placebo arm (14.1 vs 22.4%; HR = 0.77, 95% CI (0.51–1.16), but the sample was not large enough to draw any definite conclusions (Glassman *et al*, 2002). However, the platelet substudy performed within the framework of this clinical trial demonstrated that treatment with SSRI was associated with diminished platelet/endothelial activation despite widespread coadministration of antiplatelet regimens including aspirin and clopidogrel (Serebruany *et al*, 2003). In addition to sertraline (Serebruany *et al*, 2001), paroxetine (Javors *et al*, 2000), citalopram (Plenge *et al*, 1991), and fluoxetine (Tassini *et al*, 2002) have also been reported to exhibit antiplatelet effects, suggesting a class effect. Moreover, the ENRICH trial also provide convincing evidence that use of SSRIs is indeed associated with vascular protection by reducing risks for myocardial infarction (adjusted HR, 0.57; 95% CI, 0.38–0.85), as well as the risk of death (adjusted HR, 0.58; 95% CI, 0.36–0.94) (The ENRICH Trial Investigators, 2003).

The metabolic syndrome represents a constellation of risk factors including insulin resistance, dyslipidemia, hypertension, and obesity, resulting in an elevated risk for vascular

*Correspondence: Dr VL Serebruany, HeartDrug Research Laboratories, Osler Medical Center, 7600 Osler Drive, Suite 307, Towson, MD 21204, USA, Tel: +1 410 847 9490, Fax: +1 443 583 0205, E-mail: Heartdrug@aol.com
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occlusion. There are numerous reports suggesting that individuals with metabolic syndrome are at increased risk of cardiovascular (Shaw *et al*, 2006) and cerebrovascular (Ovbiagele *et al*, 2006) events.

Escitalopram oxalate (LEXAPRO™, CIPRALEX™) is the S-enantiomer of the racemic bicyclic phthalane derivative of citalopram. The mechanism of antidepressant action of escitalopram (ESC) is presumed to be linked to enhanced serotonergic activity in the central nervous system resulting from its inhibition of neuronal reuptake of serotonin (5-HT). *In vitro* and *in vivo* studies in animals indicate that ESC is a highly SSRI with minimal effects on norepinephrine and dopamine neuronal reuptake. ESC is at least 100-fold more potent than the R-enantiomer with respect to inhibition of 5-HT reuptake and inhibition of 5-HT neuronal firing rate (Thase, 2006).

We recently showed that preincubation of whole blood with escalating doses of ESC resulted in significant decreases in platelet aggregation and major surface receptor expression in human volunteers (Atar *et al*, 2006). In order to examine the platelet-related properties of ESC in a sample of individuals at high cardiovascular risk but not yet treated with any antiplatelet agents including aspirin, we carried out a similarly designed study in volunteers with metabolic syndrome.

METHODS

Patients

The study was designed in an open-label, prospective, dose ranging *in vitro* manner, and was approved by the local Institutional Review Board. Written informed consent was obtained from all patients, who were informed of the strict compliance rules and compensated for outpatient visits and blood draws. Patients aged ≥ 21 years were eligible. The study population consisted of patients with documented metabolic syndrome (NCEP, 2002). Based on the present National Cholesterol Education Program's Adult Treatment Panel III recommendation, the metabolic syndrome was diagnosed when three of the following five characteristics were present: abdominal obesity (waist circumference > 102 cm for men, and > 88 cm for women), triglycerides (> 150 mg/dl), HDL cholesterol (< 40 mg/dl for men, and < 50 mg/dl for women), blood pressure ($> 130/85$ mmHg), and/or fasting glucose (> 110 mg/dl). Explicit demonstration of insulin resistance was not required for diagnosis; however, most patients meeting the above-mentioned criteria were insulin resistant based on the discordance of high plasma insulin and glucose levels. Because the presence of type II diabetes does not exclude the diagnosis of metabolic syndrome (Grundy *et al*, 2004), these patients were eligible for study inclusion. Patients were excluded for a history of bleeding diathesis, drug or alcohol abuse, prothrombin time greater than 1.5 times control, platelet count $< 100\,000/\text{mm}^3$, hematocrit $< 25\%$, or creatinine > 4.0 mg/dl, surgery or angioplasty performed within 3 months or planned for the future, history of gastrointestinal or other bleeding, history of drug-induced disorders, trauma, cancer, rheumatic diseases, coronary artery disease, or stroke. Patients participating in other investigational drug trials within 1 month of completion were also

excluded. No patients had received intravenous platelet glycoprotein IIb/IIIa inhibitors, thienopyridines, NSAIDs, dipyridamole, or aspirin for the last 6 months. The treating physician was blinded to the platelet biomarker data, and laboratory personnel were not aware of the ESC dose.

Samples

Blood samples were obtained in order to perform platelet activity tests including conventional plasma aggregometry, whole-blood flow cytometry, and functional studies utilizing cartridge-based platelet analyzers. All patients underwent blood sampling after 2 h of fasting and at least 30 min of rest (supine, nonstressed). Four Vacutainer tubes (4.5 ml containing 3.8% sodium citrate; total of 18 ml whole blood-citrate mixture) were collected from each patient between 0800 and 1000 hours to avoid diurnal variations. One tube incubated with buffer solution was kept as an internal control. Three tubes were incubated with escalating doses of ESC for 60 min at 37°C to achieve final concentrations of 50, 100, and 200 nM/l. Plasma concentrations of ESC ranged from a subtherapeutic to the highest current recommendation, mimicking the clinical scenario in patients receiving SSRI therapy. The average steady-state concentration of 50 nM/l (range 20–125 nM/l) is achieved at a daily peroral dose of 10 mg ESC (data on file, H Lundbeck A/S, Copenhagen Valby, Denmark). Fresh solutions of ESC were prepared the same morning that each platelet study was performed. To avoid possible observer bias, blood samples were coded and blinded. Sampling procedures and platelet studies were performed by individuals unaware of the protocol.

Platelet Aggregation

Platelet-rich plasma. The whole blood-citrate mixture was centrifuged at 1200 g for 5 min in order to obtain platelet-rich plasma, which was kept at room temperature for use within 1 h. Platelet counts were determined for each sample with a Coulter Counter ZM (Coulter Co., Hialeah, FL). Platelet numbers were adjusted to $3.50 \times 10^8/\text{ml}$ with homologous platelet-poor plasma. Platelet aggregation was induced by 5 μmol ADP and 5 $\mu\text{g/ml}$ collagen obtained from Chronolog Corporation (Havertown, PA). Aggregation studies were performed using a four-channel Chronolog Lumi-Aggregometer (model 560 - Ca). Aggregation was expressed as the maximum percentage of light transmittance change (% max) from baseline at the end of the recording time, using platelet-poor plasma as a reference. Aggregation curves were recorded for 6 min and analyzed according to internationally established standards (Ruggeri, 1994).

Platelet Function Analyzers

PFA-100. Using the Dade Behring (Miami, FL) platelet function analyzer (PF-100™) instrument, the blood-citrate mixture is aspirated under a constant negative pressure and contacts an ADP- and collagen-coated membrane. The blood then passes through an aperture that induces high shear and simulates primary hemostasis after injury to a small blood vessel under flow conditions. The time to

aperture occlusion (the closure time) is recorded in seconds and is inversely related to the degree of shear-induced platelet reactivity (Mammen *et al*, 1998).

Ultegra[®]. A rapid platelet-function assay with an ADP cartridge (RPFA, Ultegra[®], Accumetrics Inc., San Diego, CA) was used. The Ultegra[®] analyzer is a turbidometric optical detection system, which measures platelet-induced aggregation as an increase in light transmittance. The test cartridge contains a lyophilized preparation of human fibrinogen-coated beads, platelet agonist, buffer, and preservative. Fibrinogen-coated microparticles are used in the Ultegra[®] RPFA cartridge to bind to available platelet receptors. When the activated platelets are exposed to the fibrinogen-coated microparticles, agglutination occurs in proportion to the number of available platelet receptors. To ensure consistent and uniform activation of the platelets, both iso-TRAP (thrombin receptor-activated peptide) and ADP are incorporated into the assay cartridge. The Ultegra[®] analyzer is designed to measure this agglutination as an increase in light transmittance. The whole blood-citrate mixture is added to the cartridge, and agglutination between platelets and coated beads is recorded. Ultegra[®] RPFA assay results are reported as platelet aggregation units (PAU) (Smith *et al*, 1999).

Whole-Blood Flow Cytometry

The surface expression of platelet receptors was determined by flow cytometry using the following monoclonal antibodies: CD 41 antigen (GP IIb/IIIa), CD 42b (GP Ib), CD 62p (P-selectin), PAC-1 (GP IIb/IIIa activity), CD 31 (platelet/endothelial cell adhesion molecule (PECAM)-1), CD 51/CD 61 (vitronectin receptor), CD 63 (LIMP or LAMP-3), CD 107a (LAMP-1), CD 151 (PETA-3), CD154 (CD40-ligand), CD 165 (GP37) (PharMingen, San Diego, CA); CD36 (thrombospondin, GPIV), WEDE15, and SPAN12 (Beckman Coulter, Brea, CA). Formation of platelet-leukocyte aggregates was assessed by dual labeling with pan-platelet marker (CD151), and then with CD14, the macrophage receptor for endotoxin lipopolysaccharides. The blood-citrate mixture (50 µl) was diluted with 450 µl Tris-buffered saline (TBS) (10 mmol/l Tris, 0.15 mol/l sodium chloride) and mixed by inverting an Eppendorf tube gently two times. The appropriate primary antibody was then added (5 µl) and incubated at 37°C for 30 min, and then a secondary antibody was applied if needed. After incubation, 400 µl of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on a Becton Dickinson FACScan flow cytometer (San Diego, CA) measuring fluorescent light scatter as described previously (Ault, 1993). All parameters were collected using four-decade logarithmic amplification. The data were collected in list mode files and then analyzed. P-selectin was expressed as percent positive cells, whereas all other antigens were expressed as log mean fluorescence intensity.

Statistical Analysis

Analysis of covariance (ANOVA) was used to test for significance of differences in platelet aggregation, results of Ultegra[®], Dade-PFA 100[™], and receptor expression

between baseline and post-ESC incubation. Normally, distributed data were expressed as mean \pm standard error ($m \pm SE$), and skewed data as median (range). All *p*-values were two sided. The whole analysis was performed using the SPSS v9.0 program (SPSS Inc., Chicago, IL).

RESULTS

Demographics and Clinical Characteristics

Twenty patients with documented metabolic syndrome were screened for study eligibility, met the inclusion criteria, and underwent platelet-function assessment. Demographic and clinical characteristics are shown in Table 1.

Study participants were mostly middle aged, and predominantly Caucasian. The male/female ratio (11/9) was fairly even. The biochemical characteristics were strongly indicative of the metabolic syndrome, with the minority of patients being tobacco users, hypertensives, or diabetics. Some of the patients were treated with anti-

Table 1 Patient Characteristics

Variables		
	Patients with MS (n = 20)	Healthy volunteers (n = 20)*
Demographics		
Age (years \pm SD)	59.4 \pm 48.0	32.0 \pm 8.2
Gender, n (% male)	11 (55)	9 (45)
Caucasian	14 (70)	17 (85)
African-American	6 (30)	3 (15)
Asian	—	—
Risk factors		
Total cholesterol (mg/dl \pm SD)	257.3 \pm 41.9	NA
Triglycerides (mg/dl \pm SD)	174.3 \pm 26.9	NA
HDL-C (mg/dl \pm SD)	34.9 \pm 6.0	NA
LDL-C (mg/dl \pm SD)	187.6 \pm 45.4	NA
Tobacco use, n (%)	7 (35)	8 (40)
Waist circumference		
Male	104.3 \pm 8.2	NA
Female	101.3 \pm 7.4	NA
Hypertension, n (%)	8 (40)	—
Diabetes, n (%)	7 (35)	—
Medications, n (%)		
Beta-blockers	8 (40)	—
ACE-inhibitors	7 (35)	—
Ca channel blockers	5 (25)	—
Angiotensin receptor blockers	4 (20)	—
Glitazones	5 (25)	—
Metformin	11 (55)	—

NA, not available.

*Data have been reported previously (Atar *et al*, 2006).

Table 2 Platelet Activity Biomarkers

Biomarker	Baseline	50 nM/l	100 nM/l	200 nM/l
<i>Conventional platelet aggregation, %</i>				
ADP 5 μ M	74.2 \pm 9.1	71.3 \pm 8.3	56.2 \pm 5.5*	48.6 \pm 7.0*
Collagen 5 μ g/ml	79.0 \pm 7.9	77.5 \pm 8.3	79.2 \pm 8.6	56.8 \pm 6.2*
<i>Platelet function analysers</i>				
PFA-100, s	201.1 \pm 24.6	199.3 \pm 24.7	205.3 \pm 26.1	233.6 \pm 31.5*
Ultegra, PAU	133.9 \pm 17.8	129.1 \pm 15.7	138.0 \pm 22.1	131.9 \pm 17.5
<i>Flow cytometry</i>				
CD31	59.4 \pm 12.8	61.1 \pm 9.3	60.0 \pm 10.3	59.2 \pm 10.4
CD41b	384.8 \pm 49.2	379.8 \pm 51.0	383.5 \pm 51.8	378.6 \pm 48.4
PAC-1	9.9 \pm 1.9	9.9 \pm 2.1	9.5 \pm 2.1	9.1 \pm 1.9
CD42b	183.3 \pm 17.5	181.2 \pm 16.1	162.8 \pm 9.1*	166.6 \pm 10.6*
CD51/61	8.8 \pm 1.9	8.5 \pm 1.9	8.4 \pm 1.7	8.5 \pm 1.5
CD62p	9.6 \pm 1.7	10.0 \pm 1.6	9.3 \pm 1.7	9.7 \pm 1.6
CD63	10.7 \pm 2.1	10.2 \pm 2.1	8.0 \pm 1.1*	7.9 \pm 1.1*
CD107a	5.7 \pm 1.5	5.8 \pm 1.5	5.9 \pm 1.5	5.8 \pm 1.5
CD151+14	123.8 \pm 15.1	121.5 \pm 19.4	122.9 \pm 16.6	117.1 \pm 16.4
CD154	5.6 \pm 1.8	6.2 \pm 1.2	5.6 \pm 1.5	5.5 \pm 1.6
CD165	37.6 \pm 2.2	36.8 \pm 1.9	32.5 \pm 3.8*	32.5 \pm 4.7*
WEDE-15	43.7 \pm 3.9	44.6 \pm 4.0	44.0 \pm 4.3	45.7 \pm 3.5
SPAN-12	28.7 \pm 4.2	30.2 \pm 5.6	30.1 \pm 6.0	27.5 \pm 4.9
Thrombospondin	14.4 \pm 2.8	14.8 \pm 2.5	14.1 \pm 2.6	14.5 \pm 2.7

*p-value <0.05 when compared with the baseline values.

hypertensive and glucose-lowering agents. None of the patients received statins or other lipid-profile modulators, SSRIs, or antiplatelet medications.

Platelet Biomarkers

The combined platelet data are summarized in Table 2.

Platelet Aggregation in Platelet-Rich Plasma

Preincubation with high therapeutic (100 nM/l), and very-high(supra-) therapeutic (200 nM/l) concentrations of ESC resulted in a significant but moderate inhibition of ADP-induced platelet aggregation, whereas no changes in ADP-stimulated platelet aggregation were observed after incubation with 50 nM/l. Contrary to ADP, the collagen-induced aggregation was reduced only after preincubation with the highest dose of ESC.

Platelet-Function Analyzers

A delay of the closure time (PFA-100 analyzer, collagen-epinephrine cartridge) was recorded, indicating platelet inhibition under high shear conditions utilizing high (200 nM/l) concentrations of ESC. Lower doses of ESC did not affect the PFA-100 readings significantly. There were no changes in the platelet activation units measured by the Ultegra analyzer independently of the ESC dose.

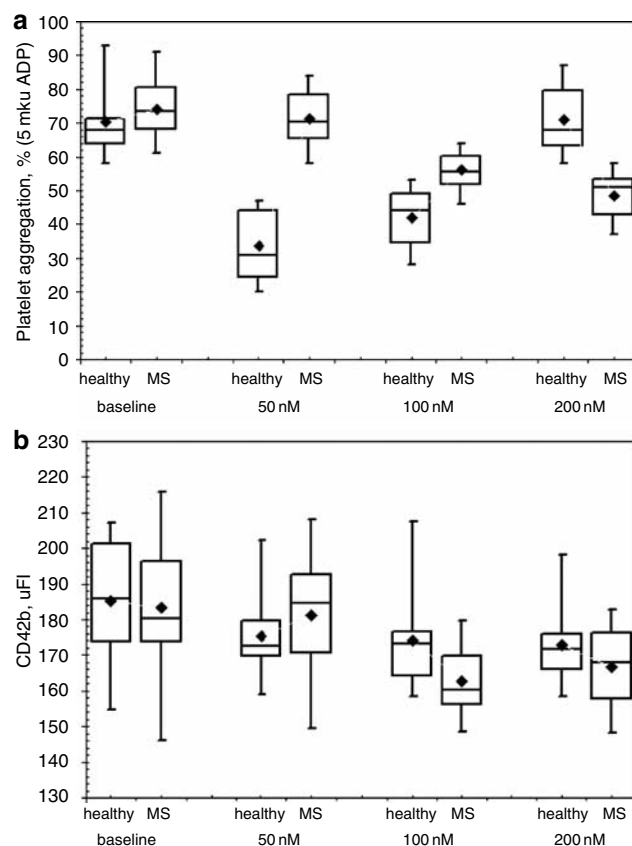


Figure 1 (a–b) Vertical bar and Whisker charts of platelet aggregation and receptor expression after preincubation with identical concentrations of ESC. The boxes show median, upper and lower quartiles of the data, whereas the whiskers indicate the minimum and maximum values. The black square inside each box corresponds to the mean value.

Whole-Blood Flow Cytometry

Incubation with ESC in the 100–200 nM/l range reduced the expression of GPIb (CD42), LAMP-3 (CD63), and GP37 (CD165) moderately, but significantly. Other platelet receptors tested were not affected.

DISCUSSION

The data from the present study expand our previous observation suggesting that ESC in doses within the therapeutic range directly inhibits certain biomarkers of platelet activity. This phenomenon is not only observed in normal human volunteers, but is reproducible in patients with documented metabolic syndrome as well. The antiplatelet property of ESC *in vitro* is mild, clearly dose-dependent, and requires higher blood levels of ESC to reach a similar extent of platelet inhibition in patients with metabolic syndrome when compared with healthy volunteers. The differences in the antiplatelet response after ESC between the patients with metabolic syndrome and volunteers (data retrieved from a previously reported study (Atar *et al*, 2006) are presented in Figure 1.

In the therapeutic range (50–100 nM), ESC causes dose-dependent inhibition of platelet activity, which was stronger

in healthy volunteers. In contrast, the threshold of 'platelet defense' was higher in the patients with metabolic syndrome suggesting that higher doses of ESC are required to reach the similar antiplatelet potency.

Although the precise role of ESC in patients with the metabolic syndrome is not established, the association between platelet activation and the metabolic syndrome is not entirely new. In fact, every risk factor and/or clinical symptom associated with the metabolic syndrome including insulin resistance (Anfossi *et al*, 2003), vascular inflammation (Esposito *et al*, 2004), dyslipidemia (Arruzazabala *et al*, 2002), hypertension (Serebruany *et al*, 2006), and obesity (Anfossi *et al*, 2004) may *per se* cause platelet activation. As a combination of the above-mentioned features, the metabolic syndrome obviously represents a high-risk thrombophilic state because of the activation of primary platelet hemostasis (Arteaga *et al*, 2006; Jesri *et al*, 2005), hypercoagulation (Nieuwdorp *et al*, 2005), and impaired fibrinolysis (Trost *et al*, 2006). The present data are indirectly in agreement with the concept of platelet hyperactivity in patients with the metabolic syndrome based on the fact that it takes higher doses of ESC to exhibit antiplatelet efficacy in these patients than in normal volunteers. The clinical utility of these findings is far from being conclusive, but the results justify further research into the use of ESC in depressed patients with metabolic abnormalities.

Interestingly, there are several reports showing that patients with the metabolic syndrome have a higher prevalence of clinical depression than observed in the general population. For example, in the SOPKARD registry (Gil *et al*, 2006), 31% of middle-aged women with elevated fasting plasma glucose levels were diagnosed with clinical depression. This statement seems especially true for women with elevated fasting glucose blood levels in whom depression, in combination with the metabolic syndrome, is diagnosed as often as in 31% of middle-aged women as suggested. However, in the Northern Finland Birth Cohort Study (Herva *et al*, 2006) depression and anxiety were not common in younger adults with the metabolic syndrome. On the other hand, increased visceral fat has been found to be an independent predictor of depression in young women with the metabolic syndrome, suggesting an association between mood disorders and metabolic abnormalities (Kahl *et al*, 2005). Unhealthy lifestyle and female gender have also been implicated with the higher depression rates in hypertensive patients with the metabolic syndrome (Bonnet *et al*, 2005). Moreover, The Third National Health and Nutrition Examination Survey carried out in the United States reported that women with a history of a major depressive episode were twice as likely to have the metabolic syndrome as those with no history of depression (Kinder *et al*, 2004). Even though such postulates do not necessarily hold true for males, the NIH twins-study unequivocally proves the impact of genetic predisposition for the development of the link between depression and the metabolic syndrome (McCaffery *et al*, 2003).

Lastly, stronger resistance to the antiplatelet potency of ESC in metabolic patients may be an outcome of the enhanced inflammation known to be prevalent in such patients (Shoelson *et al*, 2006; Soro-Paavonen *et al*, 2006). Circulating inflammatory biomarkers such as C-reactive

protein predominantly activate platelets, and may contribute substantially to the hyperactive hemostasis (Kahn *et al*, 2006).

In conclusion, the *in vitro* antiplatelet characteristics of ESC in patients with the metabolic syndrome are similar to those of healthy volunteers. However, higher ESC doses are required to induce equally potent platelet inhibition. These data justify prospective *ex vivo* studies to determine the potential clinical advantage of ESC in high-risk patients with vascular disease. If indeed ESC confers meaningful antiplatelet properties in the clinical setting, this will represent a valuable addition to the therapeutic armamentarium for improving mood disturbances, and may potentially lead to the expansion of the indications of SSRIs beyond mood and anxiety disorders.

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