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Research Article

Does Major Depression and its Treatment Affect Platelet Activity?

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Abstract

Objectives: This study was undertaken to test the prevailing hypothesis that depressive illness is associated with platelet hypercoagulability and/or activation. A second hypothesis was that escitalopram treatment would attenuate these measures by 12 weeks, though probably not by 8 weeks of treatment.

Methods: Thirty patients diagnosed by structured interview to meet criteria for major depressive disorder without evidence of cardiovascular disease were enrolled for comparison to twenty-seven healthy subjects. A battery of rating scales and lab tests preceded the baseline assessments of platelet-rich plasma (PRP) aggregation and whole blood flow cytometry. The same measures were obtained again at weeks 8 and 12 of treatment. Twenty patients were deemed study completers. Mood rating scales were explored in associations with platelet reactivity.

Results: Platelets from untreated patients were not different from controls by flow cytometry (i.e., P-selectin surface labeling) but tended towards higher agonist-free PRP aggregation. Escitalopram treatment lowered agonist-free aggregation by 8 weeks. Agonist induction by ADP was non-discriminative in platelet group responses at pre-treatment, but by 12 weeks on escitalopram the ADP-induced surface P-selectin was down regulated with an up-regulation of ADP-induced PRP aggregation. During all phases of the study there were negative associations between mood rating scales and platelet reactivity.

Limitations: Small study population.

Conclusions: The first hypothesis was not confirmed that untreated depression would be associated with higher platelet reactivity. However, escitalopram lowered certain aspects of platelet reactivity. Treatment with a SSRI therefore appears to have complex effects on platelets depending on which measure is studied.

Introduction

Serotonergic and adrenergic signals play important roles in major depressive disorder (MDD) and in platelet activation and aggregation. Recent studies have reported the resting level of platelet activation is high in depressed patients [1-3]. However, not all prior studies agree [4] and it may depend on the choice of measurement [2]. Platelet activation is pivotal in the atherosclerotic process and MDD has been established as a relative risk (RR) factor for cardiovascular disease (CVD) [5-8]. Complicating the picture is the fact that depression is a risk factor independent of, but often concomitant with, a cluster of other behavioral CVD risk factors: type A personality, life style choices, social isolation, poor medication adherence, smoking, and lack of exercise [9].

Platelets may be considered a peripheral model of serotonergic neurons [10]. The acute effects of a serotonin selective reuptake inhibitor (SSRI) on platelets have been studied extensively [11,12]. SSRIs rapidly deplete platelets of serotonin. This effect probably explains some rare case reports of prolonged bleeding times and increased risk of gastrointestinal bleeding when taking a SSRI [4,13]. SAD-HART, a landmark prospective study of treatment for depression in CVD, examined the effects of the SSRI, sertraline, on heart outcomes [14]. In that study, post-myocardial infarction (MI) depressed patients were given sertraline or placebo as add-ons to standard post-MI care. Sertraline lowered subsequent cardiovascular events and prolonged life span when administered to these post-MI subjects [15]. An observed reduction in platelet activation after 16 weeks of treatment of sertraline treatment suggested that the favorable survival rate was mediated by the action of the antidepressant on platelets [16]. However, whether the reduced platelet activation after the SSRI was more closely related to restoration of euthymia and reduction in perceived stress (i.e., the CNS response to the SSRI) remained debatable and complex to untangle. Likewise, at least two epidemiological studies have questioned whether there is any long-term protective cardiovascular value to SSRI treatments of heart-healthy depressed patients [17,18]. In order to examine whether there is an anti-platelet effect of SSRI treatment in heart-healthy depressed patients, we have examined a number of standard platelet biomarkers linked to the atherosclerotic process and determined if the antidepressant favorably modifies them.

Hypotheses of Our Study

As a baseline hypothesis we aligned with most previous studies, namely, that in heart-healthy depressed patients, platelet reactivity should be higher than in healthy control subjects. Our secondary hypothesis was that by treating patients with the SSRI, escitalopram (ESC), a down regulation of platelet reactivity should occur by 12 weeks, but not by 8 weeks of treatment in keeping with most earlier studies [2,12,19,20]. Another hypothesis was that even if a direct down-regulatory effect of the SSRI should occur, the improvement in mood elicited by the SSRI should simultaneously contribute to the down-regulation of platelet reactivity and manifest as a positive correlation between final platelet reactivity and final mood rating scores. Even though we analyzed many biomarkers in our blood samples (listed below), for the sake of statistical power the decision a priori was to focus on the literature most often quoted in depression studies, namely: (a) flow cytometry of anti-P-selectin-stained whole blood, and (b) platelet aggregometry of plasma-rich plasma (PRP).

Material and Methods

Study population

This study was approved by the Institutional Review Board of Loyola University Medical Center and was conducted according to the principles in the Declaration of Helsinki. Subjects between 20-65 years of age who met DSM-IV criteria for primary major depressive disorder (MDD), first episode or recurrent type, who were otherwise physically healthy and mentally capable to give informed consent, were candidates. Their index episode had to be ≥ 1 month duration and they could not have had psychopharmacological treatment the preceding four weeks. A minimum score of 18 on the 17-item Hamilton Depression Scale (HAM-D17) was required for study admission. Additional exclusion criteria stipulated that subjects be free of the following conditions: any inflammatory condition including gum disease, hypertension, dyslipidemia, diabetes mellitus, history of smoking or substance abuse in the preceding 6 months, and history of heart disease. Female subjects could not be pregnant, lactating, or taking oral contraceptives. Sexually active females were expected to practice reliable contraception during their participation in the study.. Screening blood samples (collected post-fasting) were used to ensure normalcy in complete blood count, complete metabolic panel, lipid profile, thyroid function and urinalysis (including pregnancy test). The presence of any clinically significant abnormalities led to non-acceptance in the study and an alternative course of care. The presence of active suicidality and/or other Axis I diagnoses were other exclusion criteria.

Thirty (n=30) MDD patients were enrolled in the study. Their demographic distribution is shown in Table 1. Immediately after baseline blood collection, ESC was prescribed starting at a low dose with titration at the discretion of the psychiatrist. Over the course of treatment, despite best clinical practices, 10 patients were lost to attrition (1 became actively suicidal, 4 dropped due to side effects and 5 dropped for reasons unrelated to the study). The twenty patients who reached 8 weeks were considered "completers". One of them was withdrawn from the study after the 8th week due to nonresponse (that individual's 8 week data was carried forward).

Healthy control subjects

Eligible healthy control (HC) subjects were screened in much the same way as the MDD subjects, after providing written informed consent approved by the Institutional Review Board. They were enrolled only if screening tests were within normal range. Once eligible, their baseline visit was scheduled and identical procedures were used as for the MDD group. The main exclusion criteria for HC subjects was any kind of medical or mental illness (including gum disease, substance use, mental illness or substance use amongst first degree relatives), or if they were pregnant or lactating females. The HC HAM-D and Beck Depression Inventory scores had to be less than 5 to be considered a HC. A total of twenty-seven HC subjects were enrolled.

Study design

Two clinic screenings (Screening-1 and -2) preceded all baseline measurements, a design which allowed for some acclimation to the clinic before the experiments began. Screening visit-1 involved collection of blood and urine samples to obtain complete blood counts with differential, complete metabolic panel with electrolytes, thyroid function, lipid profile, hCG pregnancy test and a toxicology screen. Screening-2 involved a physical exam, gum exam, followed by a structured diagnostic interview and battery of mood rating instruments: Mini International Neuropsychiatric Interview (MINI), Family History Questionnaire, Gynecologic History Questionnaire, Hamilton Rating Scale for Depression (HAM-D) and Anxiety (HAM-A), Beck Depression Inventory (BDI), Beck Scale for Suicide (BSS) and Clinical Global Impression (CGI). In most cases the baseline visit occurred within 2-3 days after the second screening visit, unless the mandatory four-week antidepressant washout period had to occur first. If the subject was taking only maintenance anti-anxiety and/or hypnotic medications at the time of the baseline assessment, he/she was allowed to continue because these types of agents are not known to affect platelets and therefore their continued use was extended in those few cases.

Depression and anxiety ratings

Two rating scales were used to quantify depression throughout the study: the 17-item HAM-D [21] and the 21-item Beck Depression Inventory (BDI) [22]. Because anxiety often accompanies depression, and anxiety is known to acutely cause platelet activation [23], we also quantified anxiety using the HAM-A [24] and the Beck Anxiety Inventory [25].

Course of treatment

This was an open label outpatient study. Patients received ESC (LexaproR courtesy of Forest Laboratories, New York, NY) over a dose range of 10-40 mg per day taken once daily or in divided doses. Average dose of ESC was 26.67 ± 9.81 mg/d. Patients returned for follow-up visits at weeks 1, 2, 4, 8 and 12 at which times the following rating scales were repeated: HAM-D, HAM-A, BDI, BSS and CGI. An Adverse Events Inventory was also administered at each visit. The most commonly reported side effects, which accounted for many of the dropouts, were excessive sedation, gastrointestinal and sexual side effects. At each visit the ESC dose could be adjusted at the discretion of the physician based on clinical response and tolerability (but always in the 10-40 mg/d range). No further dose adjustment occurred after the week-8 visit. No other types of therapeutic interventions (e.g., psychotherapy) were used. Samples of blood drawn at baseline (week 0), week-8 and week-12 were used to monitor plasma ESC levels. In the presence of suitable blood levels of ESC, a 50% reduction in the baseline HAM-D score was considered to be a "treatment response". An end-of-study score of HAM-D ≤ 7 constituted "remission". Patients who failed to meet either of these outcome measures were deemed either "partial responders" or "non-responders" depending on their final HAM-D scores. The one patient, mentioned above, who was withdrawn from the study after eight weeks of uninterrupted ESC treatment, completed all the end-of-study assessments for data to be carried forward into the final data analysis. In the final analysis of treatment outcomes: 16 patients were treatment responders (80% of total; of which 13 patients were remitters), 3 patients (15%) had a partial response, and 1 patient (5%) failed to respond.

Operational restrictions

By standard operating procedures (SOP), certain rules were imposed on the subjects prior to arrival at the clinic to minimize the likelihood of outside influences on platelet activation. Specifically, subjects were instructed not to take aspirin (previous 240 hours), antihistamines (previous 72 hours), acetaminophen (previous 72 hours), vitamins C or E (previous 72 hours), sleeping pills (previous 72 hours), caffeinated beverages (8 hours), physical exertion (8 hours) or tobacco products (though none were smokers).

Sampling conditions

Upon arrival, the subjects reclined in a quiet room for 20 minutes. A needle was inserted into the antecubital vein and the first 3 ml of blood was discarded to avoid tissue factors. Additional blood, 39 ml, was collected from the same venipuncture using four 12 cc plastic syringes. After gentle mixing, 1 ml of citrated blood was used for flow cytometry. Without delay the remainder of the citrated blood was centrifuged (180 g for 10 min at RT) to yield platelet rich plasma (PRP); and subsequently the loose pellet was centrifuged (2,000 g for 10 min at RT) to yield platelet poor plasma (PPP). The platelet aggregation assays were performed no later than 4 hrs after the venipunc-

ture. Meanwhile, the heparinized tube on ice (4.5 ml blood) was centrifuged (2,000g for 10 min at 3˚C) and the plasma was frozen at -80˚C for later determination of the antidepressant blood level.

Flow Cytometry Analysis

Step-1 involved pre-equilibration of the whole blood to 37ºC. Platelet activation was stimulated without mechanical mixing by the additions of either: saline, 5 µM ADP (BioData, Horsham, PA), 500 µg/ml arachidonic acid (BioData), 10 µg/ml collagen (ChronoLog, Havertown, PA) or 6.25 µM TRAP (Thrombin Receptor Activating Peptide; Sigma, St. Louis, MO). Three minutes thereafter, the blood was fixed by addition up to 1% paraformaldehyde. Following this fixation step, excess paraformaldehyde was removed and the cells were labeled with CD61-FITC antibody-conjugate (to identify platelets) and an antibody targeting P-selectin (human platelet CD62P-PE antibody-conjugate to identify activated platelets). CD61-FITC and CD62-PE were obtained from Becton-Dickinson Immunocytometry Systems (San Jose, CA). Once labeled, the blood samples were analyzed on an EPICS XL flow cytometer (Beckman-Coulter, Miami, FL). The technique was determined to yield coefficients of variation (CVs) ± SEM for repeated blood drawings as follows: 14.9 % ± 4.9 SEM for intensity of unstimulated platelets and 10.2% ± 2.8 SEM for ADP-stimulated platelets (a separate group of n=5 healthy females and n=5 healthy males were recruited, drawn and analyzed on different days for making these CV determinations).

Platelet Aggregometry

Platelet aggregometry was performed on a four-channel aggregometer (BioData PAP-4, BioData Corp., Horsham, PA). The technique records the increase in light transmission through a stirred suspension of PRP maintained at 37° C. Aggregation was induced in separate aliquots by the following 50 µl additions: saline, ADP (2.5 x 10-5M), arachidonic acid (AA: 5.0 mg/ml), collagen (1.9 mg/ml), or epinephrine (EPI: 100 µg/ml). Values are expressed as percentages of aggregation, representing the percentage of light transmission standardized to untreated PRP and PPP samples yielding 0% and 100%, respectively.

Escitalopram blood levels

Plasma concentrations of ESC were measured in the laboratory of Dr. Lindsay Devane according to a previously described HPLC method [26]. Since the determinations were performed on all samples after conclusion of the study, the values only allowed ascertainment of patient compliance with the protocol but not dose adjustments during the course of the study.

Statistical Analyses

The differences in group means (i.e., pre- and post-treatment) were regressed on sex and age. Repeated measures ANOVA was used to test changes in each biomarker over time (also using Student's t-test post-hoc to test some predicted pre- versus post- comparisons). The relationships were adjusted for factors that might have affected the results such as the presence or absence of classical CVD risk factors. The Pearson correlation coefficient was determined to correlate absolute values from the platelets with HAM-D and HAM-A scores. In addition, the changes in depression rating scores (i.e., HAM-D from pre- to post-treatment) were correlated with changes in each biomarker.

Results

General statement

There were no obvious demographic differences to distinguish the MDD patients from the healthy controls (Table 1). Beyond this key comparison, and for space considerations, only those findings which achieved statistical merit between patients and controls will be mentioned. That is, no group differences were observed (i.e., MDD versus controls) when the blood samples were treated and compared with collagen, epinephrine, or TRAP as agonists – be the endpoint aggregometry or flow cytometry – and, therefore, these parameters are not listed as they fell within normal limits. We have likewise restricted the analysis herein to +saline/untreated, +ADP and +AA treated conditions, where findings were obtained.

Table 1. Demographics of Patients with Major Depressive Disorder and Healthy Control Subjects

	MDD Subjects	Healthy Control Subjects	p Value
Enrollment			
At Baseline	30	27	
At Week 8	20		
At Week 12	19		
Demographics			
Age (±SD)	37.1 (11.7)	38.4 (12.2)	0.84[a]
BMI (±SD)	29.4 (5.4)	26.5 (6.1)	0.09[a]
Weight in kg (±SD)	78.0 (14.2)	74.6 (15.3)	0.31[a]
Female (Pre vs Menopausal)	77% (74%)	74% (79%)	0.82[b](0.70)[b]
Caucasian	47% (N=14)	59.3% (N=16)	0.34[b]
Non-Caucasian	53% (N=16)	40.7 (N=11)	
Hx of Tobacco Use (not current use)	Yes= 2 No= 28	Yes=3 N=24	0.55[b]

[a]Two-sample t test

[b]Chi-square test

Pre-escitalopram group comparisons

Concerning flow cytometry (Table 2), the citrated blood from untreated MDD patients showed no significant group differences compared to the HC subjects in any dimension of platelet activation. The patients showed pre-ESC surface P-selectin levels in unstimulated blood which were statistically the same as the healthy controls, and although the in vitro addition of agonists led predictably to higher values of surface P-selectin in each group, the increases due to +ADP or +AA were comparable in MDD and HC alike.

Table 2. Whole Blood Flow Cytometry: Percent Platelets Labeled with P-Selectin

Group	Week-0	Week-8	Week-12
MDD Subjects			
+ Saline	4.2 ± 0.9 (n=27)	4.5 ± 1.0 (n=20)	4.4 ± 1.1 (n=18)
+ADP	27.4 ± 2.4 (n=27)	21.3 ± 2.7 (n=19)	16.7 ± 2.8 (n=18) *
+AA	23.1 ± 2.5 (n=27)	21.2 ± 2.7 (n=19)	16.1 ± 2.9 (n=18) £
Healthy Controls			
+ Saline	4.3 ± 0.9 (n=26)	NA	NA
+ADP	23.6 ± 2.6 (n=24)	NA	NA
+AA	25.4 ± 2.6 (n=24)	NA	NA

* P= 0.06 compared to healthy controls; trending lower compared to pre-treatment (p =0.16).
£ P=0.03 compared to healthy controls; trending lower compared to pre-treatment (p =0.16).
No significant differences were noted between week-8 and week-12 values.
All values represent mean ± SEM.

The story was similar, but not identical, in regard to PRP aggregation (Table 3). First, the pre-ESC baseline aggregatory response to +saline (with stirring) showed the hypothesized higher average in untreated MDD patients. The mean value was ≈2X higher than in the HC subjects (Table 3). Unfortunately, variability between subjects in the baseline PRP values precluded statistical significance (p = 0.20). The PRP variability at baseline (+saline condition) was primarily due to an outlier patient who registered 71% maximal platelet aggregation (ultimately a responder to treatment), plus 6 patients in the range 10-22% at baseline, no obvious clustering amongst patient dropouts or non-responders, and the highest value in the HC +saline group was 12% of maximum. The in vitro addition of agonists at baseline yielded comparable levels of aggregation in untreated MDD patients compared to HC subjects (+ADP and +AA shown in Table 3). Thus, except for the non-significant trend higher with +saline PRP aggregation, the platelet activation and aggregation values were relatively normal in MDD patients prior to treatment (Tables 2 and 3 first columns).

Table 3. Platelet-Rich Plasma Aggregometry: Percent Maximal Response

Groups	Week-0	Week-8 Treatment	Week-12 Treatment
Major Depressives			
+ Saline	20.7 ± 5.1 (n=27) *	5.4 ± 0.7 (n=17) **	4.4 ± 0.5 (n=16) £
+ADP	58.0 ± 4.3 (n=27)	63.3 ± 5.5 (n=20)	64.6 ± 5.5 (n=16) β
+AA	71.7 ± 5.2 (n=27)	67.1 ± 7.3 (n=19)	66.1 ± 8.4 (n=16)
Healthy Controls			
+ Saline	9.8 ± 6.5 (n=17)	NA	NA
+ADP	64.5 ± 4.4 (n=25)	NA	NA
+AA	77.0 ± 5.3 (n=26)	NA	NA

* Not significantly different than healthy controls (P=0.20).
** Not significantly different than healthy controls (P=0.35), but significantly low (P= 0.04) compared to week-0 (paired t-test).
£ Not significantly different than healthy controls (p=0.28) or Week-0 (p=0.21).
β Significantly higher (p=0.01) compared to week-0 (paired t –test).
No significant differences were noted between week-8 and week-12 values.
All values represent mean ± SEM.

Escitalopram comparisons

Unmetabolized ESC is the major compound found in plasma. The principal metabolite is S-demethylcitalopram (S-DCT); it is a weak inhibitor of serotonin reuptake and does not contribute appreciably to the therapeutic efficacy of the parent compound. The metabolite is known to be present at approximately one third the ESC level [27,28]. In our study, the group of MDD completers had mean blood parent compound ESC levels of 54.6 ng/ml and 48.6 ng/ml of parent compound at week-8 and week-12, compared to 17.1 ng/ml and 16.8 ng/ml of the metabolite at week-8 and week-12. There were no significant differences between these values at week-8 or week-12, indicating that steady state had been achieved. There was no evidence of medication non-compliance amongst the completers. Post hoc analyses failed to reveal any correlations between platelet activation or aggregation markers and the ESC blood levels.

Treatment effects on platelets

No changes from baseline were observed after ESC treatment as evaluated by flow cytometry of surface P-selectin in the saline-treated blood. Control (+saline only) values remained minimal. There was lower in vitro ADP-induced platelet surface P-selectin while on SSRI therapy: from 27.4% at baseline to 21.3% at week-8 (-22.3%), and to 16.7% at week-12 (-39.1%, p=0.06 by week-12) (Table 2). A similar effect was seen with in vitro addition of AA that reached statistical significance at week-12 (p=0.03; Table 2). Adding to this were post-ESC findings – though not of identical nature - with PRP aggregation (Table 3). The control +saline aggregation response of PRP was attenuated after 8 weeks of the SSRI treatment with no further reduction by 12 weeks of treatment (p=0.04 paired t test, Table 3). The average control (+saline) response by PRP after 12 weeks of treatment went lower than in untreated HC subjects, although this did not reach statistical significance post-ESC (Table 3). Opposite effects were obtained comparing the two output assays in terms of post-ESC values of ADP-stimulated platelets. That is, although post-ESC platelets with the in vitro addition of ADP or AA were less potent in terms of inducing surface P-selectin (assayed by flow cytometry compared to pre-ESC ;Table 2), the post-ESC +ADP responses were consistently more potent in terms of aggregation of PRP compared to pre-ESC (p=0.01 paired t test; Table 3).

HAM-D correlational findings

Despite the observation that untreated MDD patients showed control levels of surface P-selectin or PRP aggregation values (Tables 2 and 3), a series of negative correlations emerged when the PRP values were plotted in association with mood symptoms (Table 4). For instance, the distribution of HAM-D

scores pre-ESC was negatively correlated with the distribution of ADP-induced aggregation of PRP pre-ESC (r = -0.461, p =0.016: Table 4A). [By comparison, a similar significant correlation was not found amongst the flow cytometry responses to +ADP (r= -0.057, p = 0.630, data not shown)]. Significant negative correlations between HAM-D scores and PRP aggregation were found across every phase of the study and by using multiple agonists (Table 4). The HAM-D scores at week 8 and 12 were negatively correlated with the extents of aggregation of PRP induced by ADP (e.g., week 12 values, r= -0.740, p= 0.006: Table 4A). Likewise, the PRP aggregation responses to AA were negatively correlated with HAM-D scores at baseline Pre-ESC (r= -0.462, p=0.015: Table 4A). No hint of platelet-mood correlation at any phase of the study was found if flow cytometry values were used instead of the PRP aggregation values.

Table 4. Platelet-Rich Plasma Aggregometry Negative Associations With Mood Severity

PRP Aggregation Measures	Mood Measure	Phase of Escitalopram Treatment	Correlation (r value)	P Value
A. Agonists With Main Group Effects				
ADP-induced % max.	HAM-D	Pre-treatment (n = 27)	-0.461	0.016
AA-induced % max.	HAM-D	Pre-treatment (n = 27)	-0.462	0.015
ADP-induced % max.	HAM-D	Week-8 (n=19)	-0.433	0.057
ADP-induced % max.	HAM-D	Week-12 (n=16)	-0.740	0.006
ADP-induced % max.	HAM-A	Week-12 (n=16)	-0.642	0.024
B. Agonists Without Main Group Effects				
EPI-induced % max.	HAM-A	Pre-treatment (n = 27)	-0.395	0.046
EPI-induced % max.	HAM-D	Pre-treatment (n = 27)	-0.361	0.070
EPI-induced % max.	HAM-A	Week-8 (n= 19)	-0.548	0.053
EPI-induced % max.	HAM-D	Week-8 (n= 19)	-0.731	0.005
COL-induced % max.	HAM-A	Week-12 (n= 16)	-0.765	0.004
COL-induced % max.	HAM-D	Week-12 (n= 16)	-0.855	0.0001

We next explored whether PRP aggregation responses to the addition of collagen or epinephrine – the two agents which had shown no hint of group effects - might nonetheless be correlated with HAM-D scores as was the unexpected case with +ADP and +AA (Table 4A). Again, to our surprise, negative correlations were found with collagen-induced and epinephrine-induced PRP aggregations at all stages of the study when correlated with HAM-D scores (Table 3B). Having many reproducible and strong negative correlations with PRP across all four agonists and during different phases of treatment, argues for more than chance association with depressive symptoms. HAM-A scores were also negatively associated with PRP aggregatory responses (not shown for space reasons).

Discussion

It should be noted that in two previous attempts to address platelet activation in depression, we have reported [29,30] no changes in platelet surface P-selectin in heart-healthy depressed patients after 8 weeks on antidepressants (either of two non-SSRIs). But, because other groups have since shown down-regulation of P-selectin following antidepressants, a number of methodological issues required scrutiny [19,20,31]. Most recent reports indicate that SSRIs as well as non-SSRIs (i.e., norepinehrine reuptake inhibitors: NRI) and even psychotherapy, all down regulate platelet reactivity in heart-healthy depressed patients. One report [20] showed that 3 months' treatment with either ESC or nortriptyline elicits down-regulation of platelet aggregation, but the result was confined to mood responders as opposed to non-responders.

Our baseline hypothesis was that platelets from untreated MDD patients would show higher than normal activation as assessed by flow cytometry (i.e., more P-selectin surface labeling) and also by PRP aggregation. We had assumed this would occur with all types of agonist stimulations. In fact, this proved not the case in several ways (Tables 2 and 3). There was a trend for higher PRP aggregation with +saline (i.e., no agonist) but it seemed a function of wide variability and therefore our hypothesis that untreated depression is associated with higher platelet activity should be rejected. Indeed, when examining the spread of platelet aggregation values in correlation with HAM-D and HAM-A scores, we found the more seriously depressed patients (who were also by-and-large the more anxious ones) were correlated within their group with the lowest agonist-induced values of PRP aggregation (Table 4's surprising negative correlations).

Some insight into our findings may come from the established fact that acute life stressors elicit activation of circulating platelets [32]. A connection is made because some studies have used acute life stress paradigms to show platelet responses tend to be more amplified in depressed patients relative to healthy subjects [23]. Aschbacher and colleagues reported [23] that acute platelet activation in response to psychological stress positively correlates with rating scores of depression [23]. They later reported [33] that individual robust platelet activation to psychological stress is essentially a stable trait. In accordance with report, we had enforced certain SOPs during the blood drawings to minimize emotional stress. As described under the Methods section, our subjects acclimated to the phlebotomy setting prior to their baseline visit and they also habituated by reclining for 20 minutes in a quiet room before the baseline venipuncture. Such habituation was not mentioned in the protocols of earlier investigators with whom our present findings may disagree. We speculate, therefore, that the earlier reports may have inadvertently allowed the depressed patients to respond more intensely to the stress of the phlebotomy than healthy controls did. Hence, if their patients had had chance to acclimate, the findings might not have been so different from healthy controls.

Our next hypothesis was that ESC would attenuate platelet activity by week-12 of treatment. We collected week-8 samples to compare with two previous studies of non-SSRIs which had gone 8 weeks [29,30]. In those previous studies with non-SS-

RI's we found no down-regulation of platelet reactivity after 8 weeks [29,30]. Now, the 12-week time point aligns with most previous investigators [12,19,20,34]. Indeed, we now report that ESC significantly attenuated the ADP and AA-induced P-selectin expression after 12 weeks, but not after 8 weeks (Table 2). Hence, our second hypothesis was confirmed that ESC required 12 weeks to induce a clear decrease in platelet P-selectin activation. This effect, however, was not seen by PRP aggregometry (Table 3).

What methodological differences between flow cytometry and PRP aggregometry might explain the differences between these results shown in Tables 2 and 3? One is that flow cytometry is considered gentler and another is that flow cytometry uses whole blood rather than enriched plasma. PRP has undergone centrifugal force during preparation as well as mechanical stirring during aggregometry. Also, flow cytometry can be viewed as a "snap-shot" of P-selectin at one instant, while PRP aggregometry runs for 5 minutes and often has wave components. PRP aggregometry tracings have a primary wave followed by a secondary wave of aggregation. The secondary wave appears due to mechanical stirring-induced dense granule secretion and it is considered irreversible. It is during the secondary wave that serotonin is released from platelets. On this basis, the PRP secondary aggregometry could theoretically be more sensitive to the effect of the SSRI. Perhaps this explains why the use of PRP aggregometry was able to detect diminished platelet responses post-ESC with saline in the absence of agonist, but the use of flow cytometry was unable to do so. Conversely, the flow cytometry assay seemed more able to detect agonist-mediated differences between SSRI-treated and untreated patients who may involve difference in pathways independent of serotonin release (Table 2 versus Table 3).

Concerning our statistical findings that HAM-D and HAM-A scores negatively associated with measures of PRP aggregation (Table 4), we determined that these mood scale correlations did not extend to their change scores (pre-treatment ratings minus post-treatment ratings). Nonetheless, the fact remains that although negative correlations were unexpected with absolute HAM-D and HAM-A values, the results were found across agonists and essentially independent of ESC treatment. To explain this observation, a study we reported previously [35] is worth reconsidering. In that study, also of platelets from depressed patients, the focus was on desensitization of PRP aggregation after exposure to epinephrine. We found no group differences in baseline aggregometry due to the acute in vitro addition of epinephrine, but there was significantly less desensitization at 4, 20, 30, or 60 minutes post-epinephrine in the MDD patients compared to the HC group [35]. We did not study ADP and other agonists. In fact, that blunted desensitization in the depressed patient platelets manifested as a delay in the onset of desensitization beginning between 0.5 – 2 minutes after epinephrine. After that delay, a mono-exponential desensitization time course was observed which did not differ comparing MDD to controls. That older study also demonstrated the extent of desensitization was proportional ($p = 0.02$) to the density (Bmax) of high affinity α2-adrenoceptors as detected prior to adding epinephrine by radioligand binding. We now can reinterpret those early results in line with our current study; namely, the possible existence of an elevation of endogenous epinephrine in MDD such that the platelets of depressed patients had likely already undergone compensatory mechanisms which desensitized the process. This may be applied to the present finding of negative correlations with mood (Table 4) which showed the worst MDD symptoms (presumably those had the highest endogenous epinephrine) in association with the lowest (most pre-desensitized) agonist-induced PRP aggregation. Thus, the prior time course of platelet exposure to stress hormones in the blood may be a critical factor.

In summary, our present findings add information concerning the complex status of platelets vis-a-vis a higher relative risk that depression imposes on future CVD. Notably, our untreated heart-healthy MDD patients at baseline showed neither elevated platelet P-selectin activation nor elevated aggregation (Tables 2 and 3). Indeed, the platelets from depressed patients were less (not more) reactive to agonist-induced aggregations than those of healthy controls as plotted in negative correlations with the severity of mood. We speculate this observation could align with epinephrine elevation in the blood leading to desensitized platelet responses [35]. With respect to ESC, our finding is consistent with most of the literature, lowering agonist-induced P-selectin activation of platelets in flow cytometry (Table 2). The fact that it took 12 weeks, not 8 weeks, for ESC treatment, confirms the observation of others that the SSRI takes time to produce an anti-platelet effect. Whether this is a direct pharmacological effect or related to the overall mood improvement and associated reduction in stress perception is still not clear. Finally, it should be kept in mind that many other physiological signals, especially related to HPA axis and immune system activation and inflammatory mediators, are likely confounding factors that could independently predispose to CVD.

Limitations of the study

We acknowledge that the study has certain limitations. The sample size of twenty participants is fairly small and theoretically the obtained results might be different with a larger sample size. Additionally, this was an open label study that did not include a placebo control group nor did it include a comparison group with a different psychiatric diagnosis such as generalized anxiety disorder. A few of our subjects were maintained on antianxiety/hypnotic medication which may have confounded the obtained results. Lastly, Axis I comorbidity was excluded using the MINI structured interview schedule rather than the SCID.

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Author contributions

JP and AH designed the study and wrote the protocol. AH and EM were responsible for all clinical aspects of the study and clinical data acquisition. JP, DH, WJ and JF were responsible for the assays and the interpretation of the findings. JS was responsible for statistical analyses. CLD conducted the escitalopram blood level analyses. JP wrote the first draft of the manuscript. All authors have contributed and have approved the final version of the manuscript.

Statement of Conflict of interest

None declared

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