

**TITLE: CHANGES IN PLASMA AND PLATELET BDNF LEVELS
INDUCED BY S-CITALOPRAM IN MAJOR DEPRESSION.**

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ABSTRACT

BACKGROUND: Neuroplastic processes are thought to be involved in the pathophysiology of major depression. It has been reported that serum brain-derived neurotrophic factor (BDNF) is decreased in depressed patients.

OBJECTIVES: Compare BDNF levels in depressed patients and healthy controls in platelet poor plasma and in washed platelets. Observe the effects of 8 and 24 week treatment with S-citalopram on these levels.

METHODS: We assessed the levels of BDNF in platelet poor plasma and in washed platelets from 18 major depression patients, and compared them with 14 healthy controls. Blood samples were obtained from patients before and during treatment (8 and 24 weeks) with a selective serotonin reuptake inhibitor, S-citalopram.

RESULTS: A significant decrease in severity of depressive symptoms was observed from the first month of treatment with S-citalopram, and symptoms continued decreasing until the 6th month. Plasma BDNF levels in untreated patients appeared significantly increased ($p<0.01$), but reached values similar to those of controls at the 24th week. In contrast, levels of platelet BDNF appeared significantly decreased ($p<0.05$), but treatment also normalized levels so that values obtained were equivalent to those of controls.

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CONCLUSIONS: Untreated depressed patients showed increased plasma BDNF levels and decreased platelet BDNF levels, as compared with control subjects, and tend to normalize during treatment with S-citalopram for 24 weeks, with BDNF reaching levels similar to those in healthy controls at the 24th week in both samples. We observed that improvement in depressive symptoms was accompanied by normalization of plasma and platelet BDNF levels.

Keywords: major depression; brain-derived neurotrophic factor (BDNF); serum; plasma; platelets; S-citalopram.

Abbreviation: Brain-derived neurotrophic factor (BDNF); Hamilton Depression Rating Scale (HDRS); Enzyme-linked immunosorbent assays (ELISA); Plasma poor in platelet (PPP-BDNF); washed platelets-BDNF (Plt-BDNF).

INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, present in both the central and the peripheral nervous system (Lindsay et al. 1994; Lommatzsch et al. 1999), has been implicated in the pathophysiology of depression (Duman and Monteggia 2006; Duman et al. 1997). Low levels of serum BDNF have been described in patients with major depressive disorder who were antidepressant-free (Karege et al. 2002a and 2002 b) or naïve (Shimizu et al. 2003). Moreover, BDNF levels increased with chronic antidepressant treatment (Shimizu et al. 2003; Aydemir et al. 2005; 2006; Gervasoni et al. 2005; Gonul et al. 2005). Some studies (Shimizu et al. 2003) detected a negative correlation between the severity of depression and serum BDNF levels and that the response to the antidepressant treatment correlated with the increase in BDNF levels.

BDNF is found in both human serum and plasma (Fujimura et al. 2002; Radka et al. 1996; Rosenfeld et al. 1995). Interestingly, serum levels of BDNF have been found to be 100-200-fold higher than plasma levels (Radka et al. 1996; Rosenfeld et al. 1995), and that difference results from BDNF released from platelets during the clotting process (Fujimura et al. 2002) as human platelets contain a large amount of the BDNF in blood (Fujimura et al. 2002; Pliego-Rivero et al. 1997; Yamamoto and Gurney 1990). However, regulation and function of BDNF in peripheral blood is still poorly understood.

Given the difficulty of studying BDNF levels in the brain directly, there has been great interest in the accurate assessment of BDNF activity in the periphery. Serum, plasma, and whole blood BDNF content are commonly measured using enzyme-linked immunosorbent assays (ELISA) with relatively high specificity and sensitivity. Although a few recent studies suggest plasma BDNF content is also decreased in depressed subjects (Grassi-Oliveira et al. 2008; Karege et al. 2005; Lee et al. 2007; Piccinni et al. 2008), most of the studies have been performed with serum, and only two studies (Lee & Kim 2009; Pandey et al. 2010) have investigated differences in BDNF levels in platelets between major depression patients and control subjects. To the best of our knowledge, no studies have analyzed changes due to treatment in platelet BDNF levels.

Selective serotonin reuptake inhibitors (SSRIs) are one of the most common drugs used in major depression treatment. Interestingly, platelets possess different receptors for serotonin (5-HT_{2A}, 5-HT₃) and also the transporter (SERT) in their membranes (Hoyer et al. 2002; Stratz et al. 2008; Escobar et al. 2005; Blakely et al. 1991; Hoffman et al. 1991; Lesch et al. 1993; Ramamoorthy et al. 1993). Several studies have shown evidence of a link between increased risk of cardiovascular complications in clinically depressed patients (Carney & Freedland 2003) and a possible reduction of this risk after treatment with selective serotonin reuptake inhibitors (SSRIs) (Schlienger & Meier 2003; Maurer-Spurej et al. 2004; Sauer et al. 2003). Moreover, our group has recently published an in vitro study in which the effect of serotonin and the SSRI citalopram was evaluated on platelet function (Galan et al. 2009). Our studies demonstrated that serotonin accentuates platelet activation and potentiates procoagulant responses so increasing thrombogenesis on damaged vascular surfaces. Surprisingly, inhibition of

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3 serotonin reuptake by the SSRI confirmed the implication of serotonergic mechanisms
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5 in platelet activation and demonstrated a remarkable antithrombotic action of this drug
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8 in our experimental studies, as previously suggested by Atar and cols (Atar et al. 2006;
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15 The aims of this study were: firstly, to compare BDNF levels in depressed patients and
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17 healthy controls in platelet poor plasma and in washed platelets; and secondly, to
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19 observe the effects of 8 and 24 week treatment with S-citalopram on BDNF levels with
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21 simultaneous control for age.
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METHODS AND MATERIALS

Demographic and clinical data

Two groups were established for this study. The first group included major depression patients. Eighteen outpatients recruited at the Department of Psychiatry at Hospital Clínic, Barcelona, were consecutively enrolled in a longitudinal controlled study for 6 months. All patients were suffering from a current major depressive episode, single episode or recurrent, diagnosed according to Diagnostic and Statistical Manual for Mental Disorders Criteria (APA, 2003), using the structured clinical interview for DSM IV (SCID) (First et al. 1999). All patients had a 17-item Hamilton Depression Rating Scale (HDRS) (Hamilton 1960) total score of 18 or higher. Patients' ages ranged between 18 and 65 years. Exclusion criteria consisted of the presence of other major axis I disorders, including schizophrenia, bipolar disorders, anxiety disorders, substance-related disorders and eating disorders, as well as the presence of any acute physical disorders, exposure to any psychotropic drugs in the last month and to drugs known to affect either platelets or the coagulation system taken within the previous ten days.

The second group was the study control group. Fourteen healthy subjects with no history of chronic physical illness, substance abuse or mental diseases and not taking regular medications in the last month (including drugs known to affect either platelets or the coagulation system within the previous 10 days) were recruited. All subjects were free of chronic and acute physical illness during the 2 weeks before the study.

Experimental Design

Severity of depression was assessed using the 17-item HDRS, which is valid and reliable for the Spanish population. Clinical assessments were conducted at baseline, 2nd, 4th, 8th, 12th, 16th, 20th and 24th weeks of treatment. Clinical response was defined as HDRS < 50% and remission was defined as HDRS < 8 points. Blood extraction was conducted at baseline, and at 8 and 24 weeks of treatment.

After the baseline assessments and baseline blood samples were obtained, all patients were orally given S-citalopram as antidepressant therapy. Initially, this was 5 mg/day for 4 days, and increased to 10 mg/day on the fifth day. In the following visits, the psychiatrist increased the doses as required to a maximum of 40 mg/day. We used elevated doses based on the severity of clinical depression and on personal experience. In the first weeks, lorazepam was permitted in cases of anxiety or insomnia.

Written informed consent was obtained from each participating patient. The study was approved by the Ethics Committee at Hospital Clinic, Barcelona. We included all patients that took at least one dose of the drug.

Obtaining blood samples

Blood samples were drawn from the antecubital vein of major depression patients and of healthy controls; and collected into citrate/phosphate/dextrose (CPD) at a final citrate concentration of 19 mM. Blood extractions were performed between 9 and 10 a.m. to

minimize the effects of possible circadian rhythm alterations to BDNF concentrations. Patients and controls had not been exposed to drugs known to affect either platelets or the coagulation system within 10 days prior to blood extraction.

Preparing plasma and platelet BDNF samples

Plasma poor in platelet (PPP-BDNF) samples were obtained by centrifugation of citrated blood at 2200 rpm (15 min, 22°C). Platelets were separated by centrifugation (1100 rpm, 22° C, 15 min,) to obtain platelet rich plasma. Platelets were then washed, as previously described (Diaz-Ricart et al. 2002), by washing three times with equal volumes of citrate/citric acid/dextrose (93 mM sodium citrate, 7 mM citric acid and 140 mM dextrose), pH 6.5, and containing 5 mM adenosine and 3 mM theophylline. The final pellet was resuspended at a concentration of 0.8×10^6 platelets/ μ l in a Hanks' balanced salt solution supplemented with dextrose (2.7 mM) and NaHCO₃ (4.1 mM), and maintained for 30 min at 37°C. Platelets were counted in a Beckman/Coulter MD II System Hematology Analyzer (Beckman Coulter, CA, USA).

To obtain the sample with platelet-BDNF (Plt-BDNF), washed platelets were diluted 4 times with lysis buffer (100 mM Tris-HCl, pH 7.4, 2% Triton X-100), containing 10 mM ethylene glycol bis (β -aminoethylether)-N,N,N',N'-tetraacetic acid, 4 mM ethylenediaminetetraacetic acid, 2 mM phenylmethyl sulfonyl fluoride, 2 mM benzamidine, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, and 2 mM sodium orthovanadate, as protease and phosphatase inhibitors, leaving a final platelet concentration of 0.2×10^6 platelets/ μ l. Platelet suspensions were treated with lysis buffer for 30 min (at 4°C).

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3 Triton-insoluble residues, corresponding to the polymerized cytoskeletal fraction, were
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5 kept together with the supernatant containing free BDNF. PPP-BDNF and Plt-BDNF
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7 samples were immediately frozen in dry-ice and stored at -20 °C.
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10 11 12 13 14 15 **Measurement of PPP- and Plt-BDNF** 16

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19 PPP- and Plt-BDNF was measured with the BDNF Emax® ImmunoAssay Kit
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21 (Promega, Wallisellen, Switzerland), which is designed for the sensitive and specific
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23 detection of BDNF based on the antibody sandwich principle. Before the assay, a
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25 further step of acid treatment was performed, according to the manufacturer's
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27 instructions, to increase the amount of BDNF by release of the BDNF in the
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29 membranes. Insoluble residues were pelleted by centrifugation in a microfuge to avoid
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31 being pipetted with the samples.
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39 The assay was performed according to the manufacturer's instructions with washing
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41 between steps to remove excess reagents. All assays were performed in duplicate.
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43 Briefly, 96-well plates were coated with anti-BDNF monoclonal antibody. After
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45 blocking unspecific binding sites, undiluted samples and BDNF standards were
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47 incubated in duplicates. The BDNF in the samples was detected in two steps, first with
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49 an antihuman BDNF polyclonal antibody, which in turn was detected with an anti-IgY
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51 antibody conjugated to horseradish as a tertiary reactant. Subsequently, a chromogenic
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53 substrate was added and colour changed in proportion to the amount of BDNF. The
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55 reaction was stopped with 1 M HCl and the absorbance measured at 450 nm in a
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57 MultiSkan Ascent® (Thermo Electron Corporation, Finland). The concentrations of the
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3 samples in each plate were calculated according to a standard curve. Results were
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5 expressed as pg/ml. In the case of Plt-BDNF, results were always expressed as content
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7 of BDNF considering an average physiologic platelet count equivalent to 0.2×10^6
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17 **Statistical analysis**
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22 Normality of the variables PPP-BDNF and Plt-BDNF levels have been tested
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24 respectively with the Kolmogorov-Smirnov test ($Z=0.851$, $p=0.464$; $Z=0.642$,
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26 $p=0.804$). Data are expressed as Mean $\pm$ standard error of the mean (SEM). The
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28 differences among the various conditions were tested by a general linear model for
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30 repeated measurements. One-way ANOVA test for independent experiments was
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32 applied when multiple comparisons were required, and student's t-test for paired data
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34 was used for comparisons between two different conditions. The level of statistical
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36 significance was established at least with $p<0.05$.
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RESULTS

A total of 18 patients and 14 controls were included in the study. By the 8th week of treatment, 2 patients had dropped out as they failed to attend follow-up visits, and by the 24th week of treatment, 2 further patients had dropped out as they did not complete the course of pharmacological treatment. Thus, 14 patients completed the study. As shown in Table 1, both patient and control groups were mainly comprised of women. There was a statistical difference between patients' and controls' HDRS scores ($p < 0.001$), which shows that controls did not have symptoms that could interfere in the analysis of results.

Patients had experienced, on average, 1.9 previous episodes of depression, while this was the first episode for eight subjects. Six patients had had two episodes, three patients had experienced a third episode and one had had more than three episodes.

Effect of treatment with S-citalopram on the HDRS score

Average doses of S-citalopram were 28 mg/day. One patient was prescribed antipsychotic (aripiprazole) due to psychotic symptoms. A significant improvement in the mean scores of HDRS was detected from the 4th week of treatment with S-citalopram, as summarized in Figure 1. At 8th week of treatment all patients were responsive, and from the 12th week, all were in remission. However, there was a significant difference between patients' HDRS scores of at the end of study and controls' HDRS scores (3.93 ± 3.95 vs 0.62 ± 1.19 , respectively; $p < 0.01$).

There was a significant difference in HDRS between first episode patients and recurrent patients ($p<0.05$) at the end of study. First episode patients at the end of study did not show significant differences to control subjects regarding HDRS score. In all HDRS measures, recurrent patients showed increased scores (more symptoms) with respect to the first episode of depression. At baseline measure there were no significant differences between naïve and recurrent patients. There was no difference in HDRS between male and female participants in any measure.

Assessment of BDNF levels in major depression and healthy subjects

Levels of PPP-BDNF from untreated patients appeared significantly increased versus healthy subjects (496.8 ± 132.3 pg/ml vs. 311.5 ± 90.6 pg/ml; $p<0.01$). Levels of PPP-BDNF decreased during the treatment period. This tendency was less evident, but significant, at the 8th week (454.39 ± 167.65 pg/ml vs. 311.5 ± 90.6 pg/ml; $p<0.05$) but reached levels similar to control subjects after 24 weeks of treatment (369.9 ± 151.5 pg/ml).

In contrast, Plt-BDNF levels of untreated patients appeared significantly lower than healthy subjects (458.5 ± 163.9 pg/ml vs. 793.8 ± 232.7 pg/ml; $p<0.05$). As with the PPP-BDNF, antidepressive treatment SSRI normalized Plt-BDNF levels. All these results are summarized in the Figure 2.

There were no differences in PPP- and Plt-BDNF levels between first episode and recurrent depressive patients. No significant differences in PPP- and Plt-BDNF were found with respect to age.

We analyzed BDNF levels in male and female subjects separately. Plt-BDNF levels were significantly lower in both male and female untreated depressed patients compared with healthy subjects of both genders (Men: 213 ± 56 pg/ml vs. 640 ± 13.2 pg/ml; $p < 0.05$ and women: 540.3 ± 12 pg/ml vs. 788.1 ± 261.7 pg/ml; $p < 0.05$), although PPP-BDNF levels were significantly increased at baseline in female but not in male depressed patients compared with corresponding controls (women: 486.6 ± 145.2 pg/ml; $p < 0.01$). There were no differences in any measure at the 24th week of treatment.

DISCUSSION

One finding of the present study is that we have detected lower levels of Plt-BDNF in acute major depressive patients when compared to healthy subjects. This suggests an involvement of neurotrophic factors in major depression, in line with earlier reports (Shimizu et al. 2003; Aydemir et al. 2005), that proposed decreased neuroplasticity in depression. Two studies (Lee and Kim 2009; Pandey et al. 2010) found the same results before treatment and indicated that reduced platelet BDNF contents circulating as stored BDNF could be associated with lower serum BDNF level in patients with major depression. However, they did not study the effects of treatment in platelet BDNF levels. Our results also show that Plt-BDNF levels in remitted patients were similar to values obtained in healthy controls, which could indicate a relationship between normalization at a biochemical level and clinical improvement due to biological treatment.

Another finding of the present study, although unexpected, is that PPP-BDNF levels appear significantly higher in depressive patients with respect to healthy controls. Furthermore, we have shown that PPP-BDNF levels in remitted patients were equivalent to those measured in healthy controls. These results make our study the first one to demonstrate that antidepressant treatment normalizes both PPP- and Plt-BDNF levels in patients with major depression. However, there are studies that describe lower PPP-BDNF levels in acute depressive patients than in controls (Grassi-Oliveira et al. 2008; Karege et al. 2005; Lee et al. 2007; Piccinni et al. 2008), or even report no differences between control and acute depressive patients (Lee and Kim 2009; Bocchio-Chiavetto et al. 2010). A recent meta-analysis showed a reduction of BDNF plasma

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3 levels in depression (Bocchio-Chiavetto et al. 2010). It is well known that
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5 measurements of BDNF levels are highly dependent on the methodology used (Brunoni
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7 et al, 2008). The accuracy and the reproducibility of BDNF determination in serum has
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9 been validated (Trajkovska et al. 2007) although standardized protocols for plasma
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11 collection and BDNF dosage have to be developed. One possible explanation could be
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13 related to the methodological differences between this work and previous ones. It is a
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15 fact that plasma levels of BDNF present a high variance. Lommatzsch et al. (2005)
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17 found that PPP-BDNF levels decreased significantly with increasing age, weight or
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19 cholesterol, whereas platelet levels did not. Furthermore, there is evidence for the
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21 stability of BDNF levels in platelets or serum (Trajkovska et al., 2007), whereas in
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23 plasma it circulates for less than one hour (Kishino et al. 2001; Poduslo et al. 1996).
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32 It has been elegantly demonstrated that the amount of BDNF in serum is nearly
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34 identical to the amount of BDNF found in washed lysate platelets (Fujimura et al.
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36 2002). Thus, the difference between serum and plasma BDNF levels seems to reflect the
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38 amount of BDNF stored in circulating platelets. Indeed, previous clinical studies
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40 calculated the amount of Plt-BDNF by subtracting the BDNF levels in serum and
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42 plasma (Lommatzsch et al. 2005; 2007; Trajkovska et al. 2007). Our study would
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44 support this equivalence showing the same tendency on Plt-BDNF as other studies had
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46 on serum levels of BDNF (Karege et al. 2002a; 2002 b; Shimizu et al. 2003; Sen et al.
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48 2008). Platelet BDNF could represent a long-term marker of varying BDNF levels over
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50 a period of several days. The precursors of platelet or megakaryocyte cells cannot
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52 produce BDNF, however they can actively take up BDNF from external sources
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54 (Fujimura et al. 2002), probably derived from both the circulating plasma pool and from
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resident cells in the brain (Karege et al. 2002b) or other organs (Lommatzsch et al. 1999; Fujimura et al. 2002; Pan et al. 1998; Nakahashi et al. 2000).

Some studies (Fujimura et al. 2002; Karege et al. 2005) reported an alteration of serum BDNF levels in depression patients that could be related to altered BDNF secretion from platelets. However, our study shows that serum-BDNF could be independent of a defect in platelet release. Increased platelet reactivity in patients with major depression has been described (Carney et al. 2003; 2004; Ataoglu & Canan 2009). This state might result in a partial release of the platelets contents, including BDNF and other platelet activation markers that could consequently be found in the plasma phase. In fact, our results show increased levels of BDNF in plasma and decreased levels inside platelets in untreated depressed patients compared with healthy subjects. These data would be in agreement with a pre-activation state of platelets in major depression. Treatment with an SSRI would improve this preactivated state and explain the increase of BDNF inside platelets and the parallel decrease in plasma BDNF, even reaching values similar to those of healthy subjects.

One of the major strengths of our study involves the use of a single antidepressant drug. In the Brunoni (2008) meta-analysis antidepressant type is associated with the magnitude of induced neuroplastic changes and therefore contributes to the heterogeneity of observed studies. To the best of our knowledge, only two studies have investigated the changes in BDNF levels related to escitalopram treatment. The recent Matrisciano (2009) study did not find significant changes in BDNF values for escitalopram-treated patients after 5 weeks and 6 months of treatment. This result is in contrast with the one reported by Aydemir (2006), who found an increase in BDNF

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3 serum levels in 20 women affected with major depression after 6 weeks of treatment
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5 with escitalopram, and there was a concurrent significant decrease in their depressive
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7 symptoms. BDNF tendencies found in serum in the latter study are in agreement with
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9 our results in platelets.
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15 In addition, the longest follow-up is the Piccinni (2008) study where patients were
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17 followed up for 1 year, but they used more than one antidepressant. This work showed
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19 that the clinical improvement paralleled the normalization of PPP-BDNF after one
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21 month of treatment; while, at each moment of evaluation, patients' serum BDNF levels
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23 were lower than those of control subjects. We performed a 24-week treatment follow-up
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25 at three time-points (baseline, and 8th and 24th weeks). Our results show that Plt-BDNF
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27 levels in remitted patients reach values similar to those of control subjects and that PPP-
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29 BDNF levels in patients tended to normalize along with the treatment, whereas other
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31 studies only performed a 6 or 8 week follow-up and therefore might underestimate the
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33 increase of BDNF levels (Aydemir et al., 2005; 2006; Yoshimura et al. 2007; Basterzi et
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35 al. 2009; Huang et al. 2008).
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43 A few limitations should be kept in mind when evaluating our results. First, the sample
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45 size was relatively small, which may have limited our ability to determine meaningful
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47 differences and we would consider these results preliminary until replication.
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49 Furthermore, the symptom assessments were limited to HDRS and may have missed
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51 some components of the depressive syndrome. Further studies are needed to analyze the
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53 influence of cyclic changes in hormonal status on BDNF levels, which were not
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55 considered in this study. Another consideration is that the best sample for the study of
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57 BDNF (plasma, serum or platelets) is not well established. It would be advisable to
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reach a consensus on standardizing the methodology used to conduct research on BDNF in blood samples in order to avoid variability.

A potentially interesting application of Plt-BDNF measures could be as a biomarker of antidepressant efficacy. This suggests that the measure may be used to screen for novel antidepressant agents or possibly even predict an individual's response to an antidepressant treatment at an early time point after treatment initiation. This could be used to monitor the treatment. Our results indicate that Plt-BDNF could be more reliable than PPP-BDNF.

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TABLE 1: Sex, age and depression symptoms of depressed patients and control subjects at baseline Controls subjects and depressed subjects no showed differences between age and sex, but they showed statistical differences between symptoms.

	Depressed patients (n= 18)	Control Subjects (n= 14)	p value
Sex (Female/Male)	15/3	11/3	0.84
Age (years)	38,50 ± 16,08	39,07 ± 16,12	0.92
HDRS	24 ± 4,35	0,62 ± 1,19	<0.001

HDRS, Hamilton Depression Rating Scale.

Age and HDRS scores are shown in mean±SD.

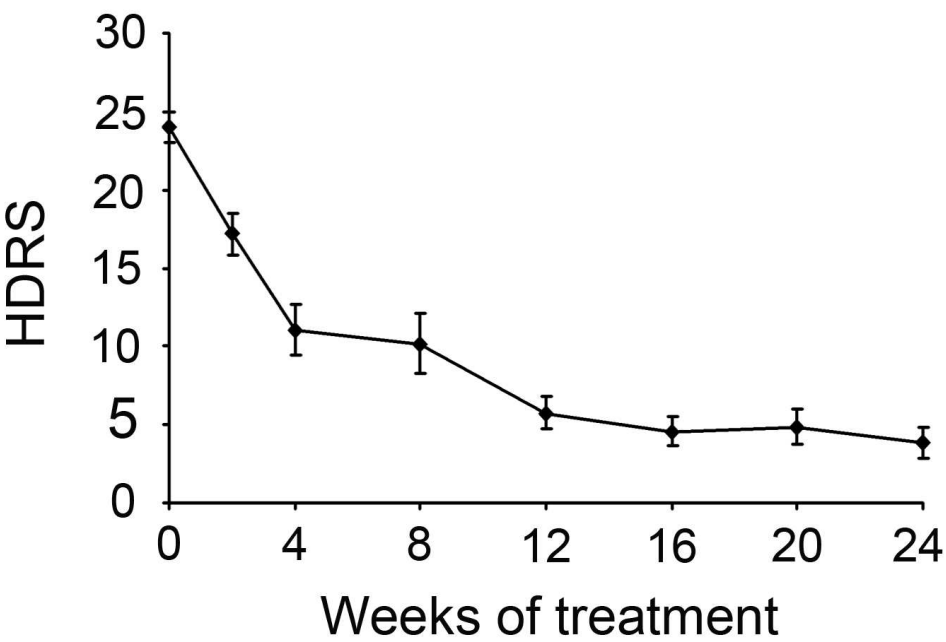


FIGURE 1: HDRS total score of the patients at baseline and during treatment with S-citalopram for 24 weeks. This figure shows the mean values of HDRS. Clinical response was defined as HDRS < 50% and remission was defined as HDRS < 8 points. Hamilton Depression Rating Scale (HDRS).
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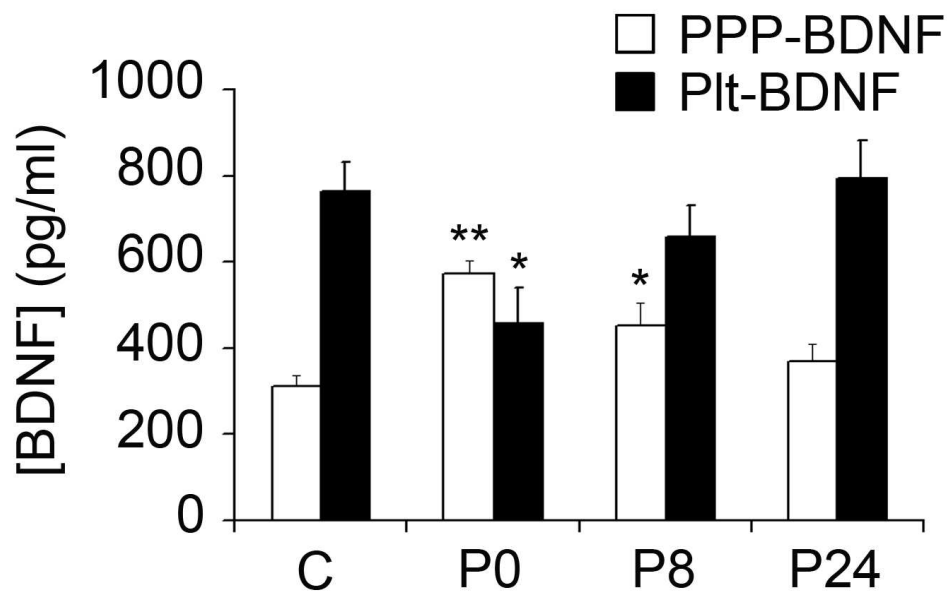


FIGURE 2: Plasma BDNF levels and levels of platelet BDNF in depressive patients and controls, at baseline and during treatment. This figure shows the mean values of BDNF in pg/ml.

PPP-BDNF: Plasma poor in platelet BDNF.

Plt- BDNF : Washed platelets-BDNF.

C: Controls; P0: patients at baseline; P8: patients after 8 weeks of treatment; P24: patients after 24 weeks of treatment.

** p<0.01; *p<0.05

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