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**The lack of genotype-phenotype relationship between platelet serotonin concentration and serotonin transporter gene promoter polymorphism in healthy subjects**

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## **Abstract**

A polymorphism in the serotonin transporter gene (5-HTTLPR) is frequently studied for association with antidepressant treatment response, different personality traits, and psychiatric disorders. Baseline platelet serotonin (5-HT) concentration has been proposed to indicate a good or a poor treatment response to antidepressant drugs and to be associated with particular symptoms in psychiatric disorders. The aim of the study was to elucidate the genotype-phenotype relationship between platelet 5-HT concentration and 5-HTTLPR in healthy subjects. The frequency of 5-HTTLPR genotypes and alleles, as well as platelet 5-HT concentration were evaluated in 434 male and 86 female unrelated healthy medication-free Caucasian subjects of Croatian origin. A two-way ANOVA revealed no significant difference in platelet 5-HT concentration subdivided according to the particular 5-HTTLPR genotype, no significant effect of sex, no significant effect of genotype, and no significant interaction between sex and genotype on platelet 5-HT concentration. In addition, one-way ANOVAs did not detect significant effects of homozygous *S/S* genotype, or homozygous *L/L* genotype on platelet 5-HT concentration. Our results showed a lack of significant association between platelet 5-HT concentration and 5-HTTLPR variants, suggesting that there is no functional relationship between 5-HTTLPR alleles and platelet 5-HT concentration in the large groups of healthy male and female medication-free Caucasian subjects, free of neuro-psychiatric disorders.

The serotonin (5-hydroxytryptamine, 5-HT) transporter (5-HTT) is a protein that modulates the 5-HT activity, and alterations in 5-HT transmission are implicated in a number of physiologic and behavioral functions, altered behaviors and psychiatric disorders [16,30]. The 5-HTT terminates the synaptic action of 5-HT and it is the target of widely prescribed antidepressant drugs such as selective serotonin reuptake inhibitors (SSRI), [30]. Human

neuronal and platelet 5-HTT are identical and encoded by the same gene [15, 29]. The 5-HTT gene (*SLC6A4*) is positioned on chromosome 17, in location 17q11.1-q12; the *SLC6A4* gene spans 31 kb and consists of 14 exons, and it plays a major role in the regulation of the 5-HT synaptic function [10,15]. The transcriptional activity of the *SLC6A4* gene is modulated by a polymorphic repetitive element (5-HTT gene-linked polymorphic region, 5-HTTLPR) located upstream of the transcription start site. This functional polymorphism consists of a 44-base pairs insertion-deletion in the promoter region. There are several alleles within 5-HTTLPR, but the most frequent are short (*S*) and long (*L*) allele, composed of either 14- or 16-repeat units. The 5-HTTLPR short allele suppresses transcriptional activity of the promoter [14], and it is associated with a poor treatment response to SSRI [32].

The genotype-phenotype relationship, i.e. different effect of the 5-HTTLPR variants on 5-HTT function, was studied in different cell lines, regions of post mortem human brains, and in platelet 5-HT uptake experiments [14]. Neurons and platelets display structural and functional similarities, and therefore platelets have been proposed as a peripheral model of the central 5-HT neurons [1,11,34]. Platelet 5-HT concentration has been found to be altered in different neuro-psychiatric disorders, personality traits and behaviors [13,22,26,27], and it is significantly decreased after treatment with various SSRI [2,22]. It has been proposed that peripheral 5-HT platelet content can mirror the state of the central 5-HT system [3], and that baseline platelet 5-HT concentration might be used as a predictor of the antidepressant treatment response [5,21,22], while long allele of the 5-HTTLPR is related to a good response to antidepressants [32]. The reports on the association between 5-HTTLPR and platelet 5-HT concentration are inconsistent, showing either positive, negative or no association between 5-HTTLPR genotypes and increased, unaltered or decreased platelet 5-HT concentration [8,9,13,33]. To elucidate the functional relevance of the 5-HTTLPR variants, we studied 5-HTTLPR and platelet 5-HT concentration simultaneously in large groups of healthy male and

smaller groups of female Caucasian subjects of Croatian origin, free of neuro-psychiatric disorders.

A study included 434 male and 86 female unrelated, medication-free Caucasian healthy subjects (mainly medical staff and students) of Croatian origin, who were recruited from 2006 to 2009 from Clinical Hospital Centre Zagreb, Zagreb, Croatia, and who filled in the questionnaire answering the questions about their medical history, smoking and drinking habits. Male and female subjects were matched for age and body mass index (BMI), and they were  $40.036 \pm 12.175$  and  $43.047 \pm 13.189$  years old, with BMI of  $24.698 \pm 4.561$  and  $24.106 \pm 4.804$ . All individuals gave their detailed medical history, and they underwent complete physical, neurological and psychological examinations. Written informed consent was obtained from all participants, after explaining the aims and procedures of the study, under procedures approved by the Ethics committee of the Clinical Hospital Centre Zagreb, Zagreb, Croatia. All human studies have been carried out with the full cooperation of participants, adequate understanding, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Blood samples (8 ml) were drawn in a plastic syringe with 2 ml of acid citrate dextrose anticoagulant at 08.00 h. Platelet rich plasma (PRP) was obtained after centrifugation of whole blood at 984g for 3 min at room temperature and platelets were sedimented by further centrifugation of PRP at 5087g for 15 min in a refrigerated centrifuge. The platelet pellet was washed with saline and centrifuged again. The sedimented platelets were frozen and stored at  $-20^{\circ}\text{C}$  for a period not longer than 7 days before being assayed for 5HT concentration.

Platelet 5-HT concentration was determined in platelet pellets by the spectrofluorimetric method, as described previously [25]. The measurement of the 5-HT fluorescence was performed on a Varian Spectrophotofluorimeter Cary Eclipse, on an exciting wavelength of 345 nm and emitted wavelength of 485 nm. The detection limit of the method was 10.0 ng/ sample,

and intra- and inter-assay coefficients of variation were 3.66% and 8.69%, respectively. Platelet protein concentrations were measured by the method of Lowry et al. [17]

Genomic DNA was extracted from peripheral blood using a salting out method [19]. Polymerase chain reaction (PCR) amplification of polymorphic loci in the *SLC6A4* gene was performed in total volume of 15  $\mu$ L containing 150 ng DNA, 0.5  $\mu$ M of each specific primer (Sigma Aldrich, USA), 250  $\mu$ M of each dNTP (Invitrogen, USA), 0.5 units Taq polymerase (Qiagen, Germany) and 1X PCR buffer (67 mM Tris-HCl, pH 8.8, 6.7 mM MgCl<sub>2</sub>, 16.6 mM (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 0.01% Tween-20) (Qiagen, Germany). The primers used for amplification of 5-HTTLPR were 5'-GGCGTTGCCGCTCTGAATGC-3' and 5'-GAGGGACTGAGCTGGACAACCAC-3'. Cycle conditions consisted of an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, 30 seconds annealing at 55 °C, extension at 72°C for 1 minute as well. A final extension was carried out at 72°C for 5 minutes. PCR products were separated in 2% agarose gel, and visualized with ultraviolet light after ethidium bromide staining. All laboratory procedures were performed blind to subject status.

The results, expressed as means  $\pm$  standard deviations (SD), were evaluated with Sigma Stat 3.5 (Jandell Scientific Corp. San Raphael, California, USA) using one-way or two-way analysis of variance (ANOVA), followed by the Tukey's test, and Pearson's coefficient of correlation. The Hardy-Weinberg analysis was used to test the equilibrium of the population. The differences in the genotype and allele frequencies were evaluated using a  $\chi^2$  test. Bonferroni correction was used for multiple testing, using the total number of pair comparison (N=2) as correction factor. The level of significance was set to  $\alpha = 0.0025$ .

The sample of 434 male and 86 female medication-free Caucasian healthy subjects of Croatian origin were analyzed for the genotype and allele frequencies of 5-HTTLPR and platelet 5-HT concentration. The observed genotype distribution in male ( $\chi^2=0.03$ ; *d.f.*=1;

$P=0.983$ ) or female ( $\chi^2=0.27$ ;  $d.f.=1$ ;  $P=0.874$ ) subjects did not differ significantly from the expected Hardy–Weinberg equilibrium. No significant ( $\chi^2=4.827$ ;  $d.f.=2$ ;  $P<0.089$ ) differences were found in the genotype ( $L/L$ ,  $L/S$  or  $S/S$ ) frequency in 172 (39.6%), 201 (46.3%) and 61 (14.1%) male subjects, and in 43 (50.0%), 37 (43.0%) and 6 (7.0%) female subjects, respectively. The frequencies of long (62.8%) and short (37.2%) allele in male, and long (71.5%) and short (28.5%) allele in female carriers, were not significantly different after Bonferonni correction ( $\chi^2=4.383$ ;  $d.f.=1$ ;  $P<0.036$ ). Additional analysis of subjects according to the presence of homozygous  $S/S$  genotype versus the combined  $L/L$  and  $L/S$  genotypes ( $\chi^2=2.605$ ;  $d.f.=1$ ;  $P=0.107$ ), or the homozygous  $L/L$  genotype versus the combined  $S/S$  and  $L/S$  genotypes ( $\chi^2=2.769$ ;  $d.f.=1$ ;  $P=0.096$ ), revealed no significant differences in the frequency of these genotypes.

A two-way ANOVA analysis of 5-HTTLPR genotypes ( $L/L$  vs.  $L/S$  vs.  $S/S$ ) by gender (male vs. female) found no significant effect of gender or genotype, and no significant interaction between gender and genotype on platelet 5-HT concentration, Fig. 1.

Additional association analysis of platelet 5-HT concentration and 5-HTTLPR variants was varied out using one-way ANOVA to evaluate the possible relationship between platelet 5-HT concentration and/or the homozygous  $S/S$  genotype versus the combined  $L/L$  and  $L/S$  genotypes, or the homozygous  $L/L$  genotype versus the combined  $S/S$  and  $L/S$  genotypes. No significant effects of homozygous  $S/S$  genotype ( $F=1.401$ ;  $d.f.=3,516$ ;  $P=0.242$ ) or homozygous  $L/L$  genotype ( $F=1.690$ ;  $d.f.=3,516$ ;  $P=0.168$ ) on platelet 5-HT concentration was detected either in male or female healthy subjects.

There were no significant (Pearson's coefficient of correlation) correlations between age and platelet 5-HT concentration in male subjects with LL ( $r=0.189$ ;  $p=0.064$ ), LS ( $r=0.113$ ;  $p=0.240$ ), or SS ( $r=0.129$ ;  $p=0.347$ ) genotypes, or in female subjects with LL



( $r=0.220$ ;  $p=0.413$ ), LS( $r=-0.027$ ;  $p=0.909$ ), or SS ( $r=-0.485$ ;  $p=0.515$ ) genotypes, respectively.

The major finding of this study is a lack of significant association between platelet 5-HT concentration and 5-HTTLPR variants, suggesting that there is no functional relationship between 5-HTTLPR genotypes and platelet 5-HT concentration in large groups of healthy male and female medication-free Caucasian subjects, free of neuro-psychiatric disorders. This finding concurs with previous findings of no association between 5-HTTLPR variants and platelet 5-HT concentration [8,33], or whole blood 5-HT concentration [35], but is in contrast with a significant association found between *L/L* and *L/S* genotypes and increased blood 5-HT levels in mixed cohorts of patients with obsessive-compulsive disorder [9], or between a homozygous short allele of the 5-HTTLPR and increased platelet 5-HT concentration in female subjects [13]. These data were explained by the possible compensatory mechanism for poor 5-HTT function which may induce up-regulation of 5-HT synthesis in the enterochromaffin cells [13], or by the alterations in the 5-HT synthesis in the intestinal wall, 5-HT release and clearance by the lung, liver, kidney and capillary bed [9]. The discrepancies across studies might be due to many factors, i.e. population stratification, age, gender, small sample sizes [9,13], ethnic heterogeneity [24], differences in psychiatric disorders studied, clinical and biochemical characteristics, the differences in the method of platelet 5-HT determination, and adoption of different diagnostic criteria. Regarding clinical and biochemical characteristics that might have affected presented data, no significant association was detected between 5-HTTLPR variants and systolic or diastolic arterial blood pressure, waist circumference, BMI, plasma lipid levels, plasma fasting glucose, or insulin in healthy men [33], and in addition, our subjects were matched for BMI. In agreement with previous data [24,28], no significant differences were found between male and female carriers of *L/L*,

*L/S* or *S/S* genotypes, as well as long or short alleles. In line with previous findings suggesting lack of correlation between age and 5-HTT genotypes [33] or platelet 5-HT concentration [22], in our study age was not significantly correlated with platelet 5-HT concentration subdivided according to the 5-HTT genotypes.

Previous studies showed either no effect of 5-HTTLPR genotype on B<sub>max</sub> of platelet [<sup>3</sup>H]paroxetine binding, affinity for [<sup>3</sup>H]5-HT uptake or [<sup>3</sup>H] paroxetine binding in healthy male volunteers [8,23,28], or a dominant influence of the short allele on platelet 5-HTT V<sub>max</sub> [8,23], on binding availability of the 5-HTT in blood platelets [35], and more rapid platelet 5-HT uptake in healthy carriers of *L/L* genotype compared to *L/S* or *S/S* genotype [8,23]. Since basal platelet 5-HT concentration in healthy subjects is more strongly affected by the number or size of storage vesicles, or diffusion across the cell membrane [8], than by the 5HTTLPR variants, these data confirm that 5-HTTLPR variants are significantly associated with particular (i.e. density and binding availability of 5-HTT), but not with other (i.e. platelet 5-HT concentration) indices of platelet 5-HT system.

We have presented preliminary female data to show that 5-HTTLPR variants do not affect significantly platelet 5-HT concentration in female healthy subjects, and in addition, our female group was substantially larger than female groups used in previous studies [9,13,23]. Peripheral 5-HT marker was used since lower baseline platelet 5-HT concentration [5,21,22] and long allele of the 5-HTTLPR [32] were proposed to be markers of good response to SSRI. Although we expected to find a relationship between platelet 5-HT concentration and 5-HTTLPR variants, our results did not confirm this association.

In conclusion, our results showed the lack of genotype-phenotype relationship between platelet 5-HT concentration and 5-HTTLPR variants, and this study represents a replication of the previous data [8,33,35], obtained on a larger sample of healthy subjects. Our failure to demonstrate a functional relationship between 5-HTTLPR variants and platelet 5-

HT concentration in healthy subjects might suggest that genotype-induced changes in 5-HTT transcription and consequent alterations in platelet 5-HT concentration might be differently regulated in general population sample as opposed to subjects with psychiatric disorders.

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## Legend of the Figure

Fig. 1. Analysis of the effect of 5-HTTLPR polymorphism on platelet 5-HT concentration, expressed as nmol/mg proteins. Each column represents mean  $\pm$  SD. Numbers in parentheses represent number of subjects per group. 5-HTTLPR=serotonin transporter gene-linked polymorphic region; 5-HT=serotonin. Two-way ANOVA (gender and genotype): between gender ( $F=0.122$ ;  $d.f.=1,514$ ;  $P=0.727$ ), between genotypes ( $F=1.000$ ;  $d.f.=2,514$ ;  $P=0.369$ ), interaction ( $F=2.031$ ;  $d.f.=2,514$ ;  $P=0.132$ ).

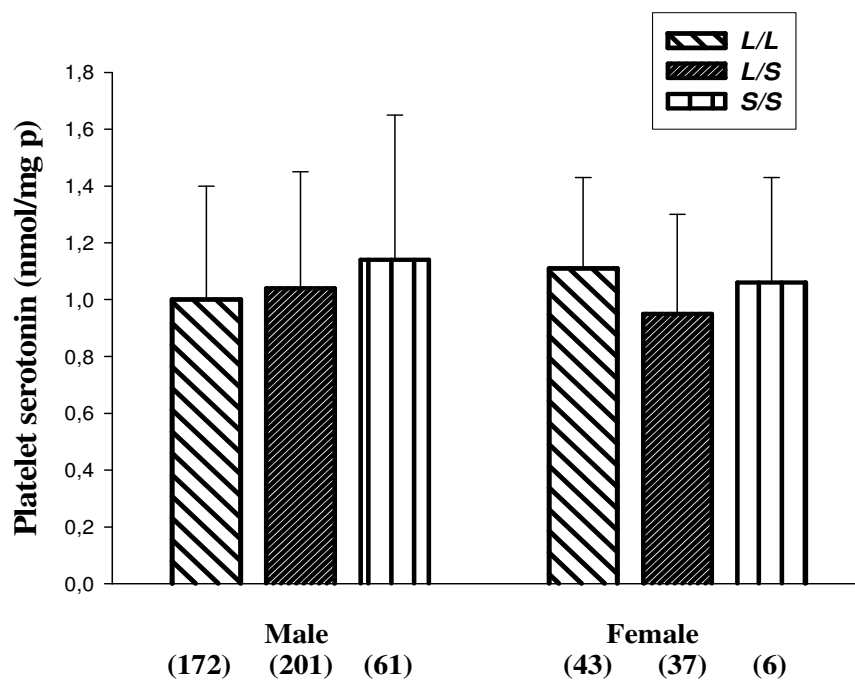


Fig 1.