

# THE ASSOCIATION BETWEEN SEROTONIN TRANSPORTER POLYMORPHISM, PLATELET SEROTONIN CONCENTRATION AND INSOMNIA IN NON-DEPRESSED VETERANS WITH POSTTRAUMATIC STRESS DISORDER

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## SUMMARY

**Background:** The role of serotonin transporter and its functional gene polymorphism (5-HTTLPR, serotonin transporter linked polymorphic region) was investigated in sleep disturbances in various mental disorders, with conflicting findings. Here, the association of particular sleep disturbances with 5-HTTLPR genotypes and platelet serotonin (5-HT) concentration was determined simultaneously in veterans with posttraumatic stress disorder (PTSD), who were subdivided into those with or without comorbid depression.

**Subjects and methods:** Croatian male, medication-free war veterans with PTSD (N=325), subdivided into those with or without comorbid depression, and subdivided further according to the various sleep disturbances, were evaluated using the Structured Clinical Interview, the Hamilton Rating Scale for Depression and the Clinician Administered PTSD Scale. Genotyping and platelet 5-HT concentration measurements were conducted using PCR and spectrofluorimetric methods, respectively.

**Results:** Nominally higher frequency of the 5-HTTLPR LL genotype compared to S carriers ( $p=0.026$ ;  $\chi^2$  test) and significantly higher platelet 5-HT concentration ( $p=0.001$ ; one-way ANOVA) were detected in non-depressed veterans with PTSD with early insomnia, compared to matched veterans without early insomnia.

**Conclusions:** Over-representation of the LL genotype of the 5-HTTLPR and higher platelet 5-HT concentrations were detected in veterans with PTSD who did not develop comorbid depression but had severe early insomnia. These results suggest that 5-HTTLPR genotypes and platelet 5-HT concentration are associated with early insomnia in non-depressed veterans with PTSD. Limitations of the study were the cross-sectional nature of the study, biallelic assessment of the 5-HTTLPR, and a lack of use of the specific sleep measurement scales. These results should be replicated in larger samples, validated on different populations, using specific sleep measurement scales and triallelic 5-HTTLPR assessment.

**Key words:** platelet serotonin - PTSD - comorbid depression - serotonin transporter polymorphism - sleep disturbances

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## INTRODUCTION

Altered serotonergic (5-hydroxytryptamine, 5-HT) function has been implicated in the vulnerability to develop posttraumatic stress disorder (PTSD) after traumatic experience (Kelmendi et al. 2016), but also in etiopathogenesis of depression (Adrien 2002) and sleep disturbances (Andretic et al. 2008, Hornung 2003, Landolt & Wehrle 2009, Monti 2011).

PTSD is defined with the re-experiencing, avoidance, hyper-arousal symptoms and negative alterations in cognition and mood (APA 2013), but also with nightmares and insomnia, sleep disturbances, possibly interfering with the fear extinction and compromising recovery (Germain et al. 2017, Lipinska et al. 2016, van Liempt 2012). PTSD is associated with a high rate of comorbidities, especially depression (Kessler et al. 1995), and insomnia is also a frequent symptom of

depression (Franzen and Buysse 2008). Serotonergic alterations might contribute to sleep disruptions in PTSD (Ursin 2002) and depression (Benca & Peterson 2008).

The 5HTT or SLC6A4 gene encodes a high-affinity, Na<sup>+</sup>-dependent, presynaptic 5-HT transporter (5-HTT) which regulates the activity of 5-HT and its concentration in the synapse, i.e. 5-HT signaling intensity and duration (Lesch et al. 1996). A common polymorphism in the promoter region of the 5-HTT gene is a 5-HTT linked polymorphic region (5-HTTLPR), resulting in short (S) allele and long (L) allele variants (Heils et al. 1996). The frequency of the S allele is 38%, whereas L allele is more common (62%) in Croatian population (Noskova et al. 2008). The S allele of the 5-HTTLPR is associated with reduced transcriptional activity compared to the L allele, resulting in reduced 5-HTT expression and function (Lesch et al. 1996).

PTSD (Goenjian et al. 2012, Kilpatrick et al. 2007, Koenen 2007, Koenen et al. 2009, Lee et al. 2005, Zhao et al. 2017), depression (Gressier et al. 2016, Karg et al. 2011, Munafo et al. 2009), and sleep disturbances, such as primary insomnia (Deuschle et al. 2010), and changes in sleep quality (Barclay et al. 2011, Brummett et al. 2007, Hartman et al. 2014), have been reported to be associated with the 5-HTTLPR, but the findings are inconsistent (Gressier et al. 2013, Karg et al. 2011, Kolassa et al. 2010, Kovacic Petrovic et al. 2016, Lopez-Leon et al. 2008, Munafo et al. 2009, Navarro-Mateu et al. 2013, Pooler 2015, Risch et al. 2009). The S variant of 5-HTTLPR was associated with desynchronized circadian rhythm in healthy rotation shift workers (Sookoian et al. 2007), while 5-HTTLPR was also associated with the poor sleep quality in the general population (Barclay et al. 2011), but not in PTSD, as confirmed by the genome wide association study /GWAS/ (Pooler 2015).

Blood platelets have been previously studied as indirect, easy available peripheral index of central 5-HT function, since, as neurons, they also express 5-HTT (Camacho and Dimsdale 2000, Stahl 1985, Yubero-Lahoz et al. 2013). PTSD has been associated with both decreased (Guo et al. 2016, Li et al. 2016) or unchanged (Muck-Seler et al. 2003, Pivac et al. 2002) platelet 5-HT concentration. Peripheral 5-HT markers were reported to be related to different sleep disturbances in either general population or in psychiatric patients, but these findings are also conflicting. Lower (Kheirouri et al. 2016, Lechin et al. 2004, Nenadic Sviglin et al. 2011, Sookoian et al. 2007), as well as unaltered (Minakuchi et al. 2014, Pakalnis et al. 2009, Pivac et al. 2001) plasma/platelet/whole blood 5-HT concentrations in subjects with different sleep disturbances were also reported.

The aim of the present study was to determine the possible association of sleep disturbances with 5-HTTLPR variants and platelet 5-HT concentration in homogeneous group of male Caucasian veterans with combat related PTSD, subdivided into those with or without comorbid depression. The hypothesis of the study was that prominent sleep disturbances in veterans with PTSD, especially in a group with comorbid depression, will be significantly associated with the S allele of the 5-HTTLPR and with decreased platelet 5-HT concentration.

## SUBJECTS AND METHODS

### *Subjects and clinical measures*

The study included only male, medication free veterans (N=325) with chronic and current combat related PTSD, recruited in the Referral Centre for the Stress-related Disorders of the Department of Psychiatry in University Hospital Dubrava during 2001-2005, included in our previous study (Kovacic Petrovic et al.

2016). The subjects did not differ significantly according to the age, and were all Caucasians of the Croatian origin, soldiers serving in the Croatian armed forces between 1991-1995. They were exposed to the similar combat traumas during  $3.0 \pm 1.0$  years of service, and were evaluated  $6.1 \pm 2.7$  years after traumatic experience. PTSD, comorbid psychiatric disorders and various sleep disturbances were diagnosed (during 2001-2005) using the Structured Clinical Interview (SCID) for DSM-IV based on DSM-IV criteria (First et al. 1995), the Hamilton Depression Scale /HDRS/ (Hamilton 1960), and the Clinician Administered PTSD Scale /CAPS/ (Blake et al. 1995).

All participants completed a questionnaire previously described in details (Kovacic Petrovic et al. 2016). Exclusion criteria were also previously reported (Kovacic Petrovic et al. 2016). Veterans with PTSD with psychological, pharmacological treatment, SSRI treatment in the previous 6 weeks, a positive family history of psychiatric disorder, premorbid history of psychotic, mood, personality disorders, dementia, cognitive dysfunction, mental retardation, substance abuse, past or current alcohol or other substance abuse within 3 months, clinically significant abnormalities in electrocardiogram or laboratory findings and positive urine screen for illicit drugs and alcohol, acute or chronic physical illness, history of cardiovascular or neurological disorder, hypertension, diabetes or other metabolic and/or endocrine disorder were excluded. All study participants received detailed description of the study protocol, provided written informed consent, and patient anonymity was preserved. All studies were approved by the Ethics committee of the University Hospital Dubrava and were conducted in accordance with the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

Veterans with PTSD were subdivided according to the comorbid depression into 2 groups: 158 veterans without comorbid depression (in the further text designated as non-depressed veterans), and 167 veterans with comorbid depression (based on SCID), designated as depressed veterans. Additionally, depressed and non-depressed veterans were subdivided according to the sleep disturbances into those with or without insomnia and other sleep disturbances (nightmares, interrupted sleep) using HDRS and CAPS scales. Therefore, to evaluate whether participants have developed early, middle or late insomnia, groups were subdivided according to the items 4, 5 and 6 of the HDRS. Subjects were subdivided according to the HDRS item 4 into those without early insomnia (score 0), those with score 1 (occasional difficulty falling asleep, existing more than half an hour) and those with score 2 (nightly difficulty falling asleep) for symptoms of early insomnia. According to the HDRS item 5, veterans were also categorized into those without middle insomnia (having score 0), those with score 1

(being restless and disturbed during the night) or score 2 (waking during the night-and getting out of bed, except for purpose of voiding) of the HDRS item 5. According to the HDRS item 6, participants were further subdivided according to symptoms of late insomnia (the HDRS item 6) into those without late insomnia (score 0), those waking in early hours of the morning but going back to sleep (score 1) and veterans who were unable to fall asleep again after getting out of bed (score 2). Since PTSD is characterized with recurrent distressing dreams of the traumatic event and difficulties in falling or staying asleep, all participants were also subdivided according to the CAPS items B2 and D1. Therefore veterans were subdivided according to the CAPS item B2 into veterans without (score 0) and with (scores 3, 4, 5, 6, 7 and 8) recurrent distressing dreams of the event (the CAPS item B2). Additionally, veterans were subdivided according to the CAPS item D1 into veterans without (score 0) and with (scores 3, 4, 5, 6 and 7) difficulties in falling or staying asleep (the CAPS item D1).

#### **Genotyping of the 5-HTTLPR and determination of platelet 5-HT concentration**

Blood samples (8 ml) were collected during regular check-ups at 8.00 a.m. after fasting overnight, using plastic vacutainers with 2 ml of acid citrate dextrose anticoagulant. Genomic DNA was isolated from peripheral blood using a salting out method (Miller et al. 1988). Genotyping of the 5-HTTLPR was done with polymerase chain reaction (PCR), with primers used for amplification of 5-HTTLPR:

5'-GGCGTTGCCGCTCTGAATGC-3' and 5'-GAGGGACTGAGCTGGA-CAACCAC-3', as described previously (Noskova et al. 2008, Pivac et al. 2009).

Platelet 5-HT concentration was determined spectrophotometrically as described previously (Pivac et al. 2009) in platelet rich plasma, and measured by Varian Spectrophotofluorimeter Cary Eclipse, using an excitation wavelength of 345 nm and emission wavelength of 485 nm. The detection limit was 10.0 ng/ per sample. Intra- and inter-assay coefficients of variation were 3.66% and 8.69%, respectively. Platelet protein concentrations were measured in triplicates using the method of Lowry et al. (1951).

#### **Statistical analysis**

The results were expressed as numbers and percentages or median and 25th (Q1) and 75th (Q3) percentiles or mean  $\pm$  standard deviation (SD). The normality of the data was assessed using Kolmogorov-Smirnov test. The results obtained from 3 or more groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test, by two-way ANOVA or General Linear Model. Mann-Whitney test was used to evaluate differences between two groups when normality of the data

failed. The  $\chi^2$ -test with a Yates correction for continuity was used to assess the genotype frequency. Standardized residuals (R) were used to detect what category was responsible for the significant differences in  $\chi^2$ -test (Field et al. 2012). As we investigated 2 independent parameters (5-HTTLPR and platelet 5-HT), the level of significance was corrected to  $\alpha=0.025$ . All data were evaluated using SigmaStat 2.0 (Jandell Scientific Corp. San Raphael, California, USA) and Microsoft Excel. G\*Power 3 Software (Faul et al. 2007) was used to calculate *a priori* needed sample size and statistical power. For the 5-HTTLPR genotype frequency ( $\chi^2$ -test), expecting a small effect size=0.2, the required total sample size was 241 and the study included 325 subjects. For ANOVA, expecting a small effect size=0.25, the required total sample size was 292 and the study included 325 subjects. Power was set to 0.800. This calculation confirmed that study had required sample size and statistical power.

## **RESULTS**

Non-depressed veterans with PTSD (N=158, 48.6%) had significantly higher total CAPS scores and scores in the CAPS items B2 and D1 (describing recurrent distressing dreams of the traumatic event and difficulties in falling or staying asleep) than depressed veterans with PTSD. Veterans with PTSD with comorbid depression (N=167; 51.4%) had significantly higher HDRS total and HDRS item 6 (late insomnia) scores than veterans with PTSD without comorbid depression (Table 1).

The 5-HTTLPR genotype distribution in PTSD veterans without comorbid depression ( $\chi^2=0.010$ ;  $df=1$ ;  $p=0.928$ ) or in veterans with PTSD with comorbid depression ( $\chi^2=0.575$ ;  $df=1$ ;  $p=0.450$ ) was in the expected Hardy-Weinberg equilibrium. The frequency of the 5-HTTLPR genotypes was 39.0% for the LL, 47.2% for the LS and 13.8% for the SS genotype in veterans with PTSD, and this distribution did not differ significantly ( $\chi^2=0.773$ ;  $df=2$ ;  $p=0.680$ ) from the genotypes frequencies in veterans with PTSD with comorbid depression: LL (34.5 %), LS (50.9%) and SS (14.8%). In addition, the frequency of the S carriers (the combined SS and SL genotypes) versus the homozygous LL genotype carriers ( $\chi^2=0.584$ ;  $df=1$ ;  $p=0.445$ ), i.e. in the dominant model (SS/LS vs. LL), was similar between veterans with PTSD with or without comorbid depression.

In the further analyses, veterans with PTSD with comorbid depression and veterans with PTSD without comorbid depression were subdivided into groups with or without sleep disturbances according to the items 4, 5 or 6 of the HDRS (i.e. early, middle and late insomnia), and items B2 and D1 of the CAPS (recurrent distressing dreams of the traumatic event and difficulties in falling or staying asleep).

**Table 1.** Clinical characteristic of patients with PTSD with or without comorbid depression

	PTSD (non-depressed veterans) N=158	PTSD with comorbid depression (depressed veterans) N=167
HDRS total scores	20.0 (16.0-26.0) <i>U</i> =28236.50; <i>p</i> <0.001	26.0* (23.0-30.0)
HDRS-4: early insomnia	1.0 (1.0-2.0) <i>U</i> =21872.50; <i>p</i> =0.054	1.0 (1.0-2.0)
HDRS-5: middle insomnia	1.0 (1.0-2.0) <i>U</i> =19757.00; <i>p</i> =0.836	1.0 (1.0-2.0)
HDRS-6: late insomnia	1.0 (1.0-2.0) <i>U</i> =23209.50; <i>p</i> =0.001	1.0* (1.0-2.0)
CAPS total scores	88.0 (70.0-101.0) <i>U</i> =6719.00; <i>p</i> <0.001	69.0* (61.0-73.0)
CAPS-item B2	6.0 (5.0-7.0) <i>U</i> =12440.50; <i>p</i> <0.001	5.0* (4.0-6.0)
CAPS-item D1	6.0 (5.0-6.0) <i>U</i> =16479.00; <i>p</i> =0.001	5.0* (4.0-6.0)

Data are expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles in parenthesis; \*significantly different scores vs. scores in veterans with PTSD without comorbid depression (Mann Whitney test); CAPS= Clinician Administered PTSD Scale; HDRS= Hamilton Depression Rating Scale; CAPS- item B2: recurrent distressing dreams of the event; CAPS- item D1: difficulties in falling or staying asleep

**Table 2.** Platelet 5-HT concentration and 5-HTTLPR genotypes (LL homozygotes and S carriers) in veterans with PTSD with or without comorbid depression with early insomnia (HDRS item 4)

HDRS item 4 (early insomnia)	0 scores	1 score	2 scores
<i>PTSD</i>			
Platelet 5-HT, mean (SD) <i>F</i> =11.692; <i>df</i> =2,155; <i>p</i> =0.001	0.89 (0.51)	0.91 (0.47)	1.31 (0.66)*, **
5-HTTLPR			
LL homozygotes, <i>n</i> (%)	4 (40.0)	32 (32.0)	26 (55.3) <sup>#</sup>
S carriers, <i>n</i> (%)	7 (60.0)	68 (48.0)	21 (44.7)
$\chi^2=7.277$ ; <i>df</i> =2; <i>p</i> =0.026			
<i>PTSD with comorbid depression</i>			
Platelet 5-HT, mean (SD) <i>F</i> =0.545; <i>df</i> =2,164; <i>p</i> =0.581	1.06 (0.47)	0.91 (0.47)	0.88 (0.47)
5-HTTLPR			
LL homozygotes, <i>n</i> (%)	3 (5.2)	34 (58.6)	21 (36.2)
S carriers, <i>n</i> (%)	3 (2.7)	63 (56.8)	45 (40.5)
$\chi=0.861$ ; <i>df</i> =2; <i>p</i> =0.650			

HDRS=Hamilton's Rating Scale for Depression; 5-HT=serotonin; 5-HTTLPR=5-HT transporter linked polymorphic region; LL homozygotes=LL homozygous genotype; S carriers=the combined SL and SS genotype; PTSD=posttraumatic stress disorder. \**p*=0.015 vs. platelet 5-HT concentration in veterans with PTSD with 0 scores; \*\**p*=0.001 vs. platelet 5-HT concentration in veterans with PTSD with 1 score on the HDRS item 4 (Tukey's test); <sup>#</sup>*p*=0.026 (a trend) vs. S carriers with 2 scores of insomnia; *R*=1.73 for the carriers of the LL homozygous genotype in veterans with PTSD with 2 HDRS scores

Groups of veterans with PTSD with and without comorbid depression were subdivided according to the dominant model into the 5-HTTLPR LL homozygotes and S carriers, and according to the HDRS item 4 (early insomnia). The frequency of the LL homozygotes and S carriers differed nominally (*p*=0.026) between non-depressed veterans with PTSD with or without early insomnia (Table 2), due to a slightly higher frequency of the LL homozygous genotype in the group with 2 scores of insomnia symptoms (*R*=1.73). In the group of depressed veterans with PTSD, the frequency of the LL

homozygous genotype and S carriers did not differ significantly (*p*=0.581) when veterans were subdivided according to scores 0, 1 and 2 on the item 4 (early insomnia) of the HDRS (Table 2).

General linear model (*R*<sup>2</sup>=0.045) revealed no significant effect of diagnosis, i.e. PTSD vs. PTSD + comorbid depression (*F*=1.251; *df*=1; *p*=0.265), smoking (*F*=3.455; *df*=1; *p*=0.064), and 5-HTTLPR genotype (*F*=0.081; *df*=1; *p*=0.776), or their three-way interaction (*F*=1.647; *df*=4; *p*=0.163), on platelet 5-HT concentration.

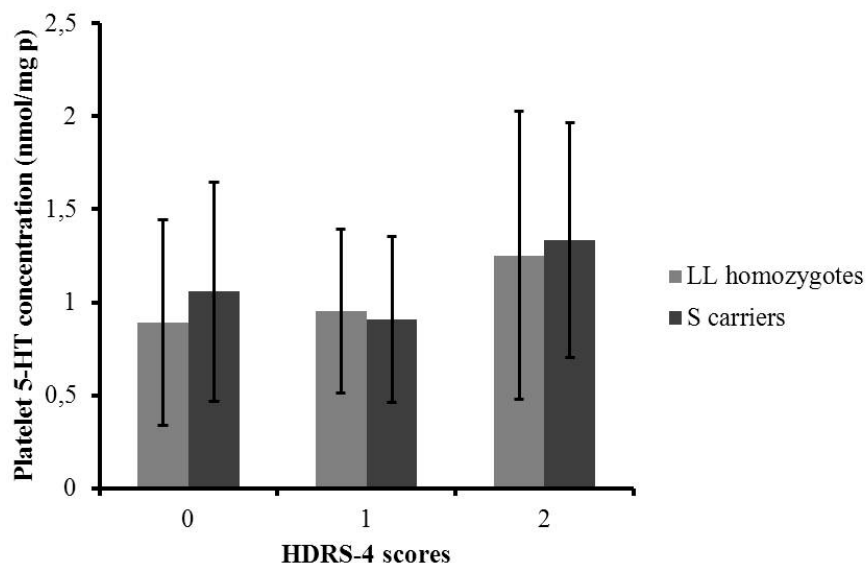


Figure legend: The results are expressed as means  $\pm$  standard deviations; 5-HT=platelet serotonin; 5-HTTLPR=5-HT transporter linked polymorphic region; HDRS=Hamilton's Rating Scale for Depression; LL homozygotes=LL homozygous genotype; S carriers=the combined SL and SS genotypes

**Figure 1.** Platelet 5-HT concentration subdivided according to 5-HTTLPR variants and scores in early insomnia (HDRS item 4) in non-depressed veterans with PTSD

Significant differences (Table 2) in platelet 5-HT concentration ( $p=0.001$ ) were found in non-depressed veterans with PTSD who were subdivided according to the item 4 of the HDRS (i.e. early insomnia symptoms). Namely, veterans with 2 scores of early insomnia had significantly ( $p=0.015$ ) higher platelet 5-HT concentrations than veterans without early insomnia (score 0) and significantly ( $p=0.001$ ) higher platelet 5-HT concentration than veterans with 1 score (Table 2). Opposed to these results, when early insomnia was determined by the item 4 of the HDRS in the group of depressed veterans with PTSD, a lack ( $p=0.581$ ) of association between platelet 5-HT concentration and early insomnia was found (Table 2).

No significant differences in the frequency of the 5-HTTLPR genotypes or in platelet 5-HT concentration were detected in depressed and non-depressed veterans with PTSD, when groups were subdivided into those without and with sleep disturbances according to the HDRS scores (items 5 and 6) and using the CAPS scores (items B2 and D1), respectively. These non-significant results are available on request.

To elucidate the interaction between higher platelet 5-HT concentration and higher frequency of the LL homozygous genotype in early insomnia, non-depressed veterans with PTSD were subdivided according to their 5-HTTLPR genotype and early insomnia scores (Figure 1). Two-way ANOVA was performed with genotype (S carriers vs. LL homozygotes) and HDRS item 4 scores, set as categorical predictors, and platelet 5-HT concentration as dependent variable. HDRS item 4 score had significant effect on platelet 5-HT concentration ( $F=1.982$ ;  $df=2$ ;  $p=0.002$ ), while genotype

( $F=0.277$ ;  $df=1$ ;  $p=0.600$ ) and genotype x HDRS item 4 score interaction ( $F=0.147$ ;  $df=2$ ;  $p=0.864$ ) did not significantly contribute to the platelet 5-HT concentration differences. These results revealed that higher scores of early insomnia, and not 5-HTTLPR genotype, contributed to the significant difference in platelet 5-HT concentration.

## DISCUSSION

The main findings from the present study were: 1) a slight association between the 5-HTTLPR polymorphism and early insomnia, i.e. nominally higher frequency of the LL genotype of the 5-HTTLPR compared to S carriers, in non-depressed PTSD veterans with early insomnia; 2) a significant association between platelet 5-HT concentration and early insomnia (i.e. significantly higher platelet 5-HT concentration in non-depressed veterans with early insomnia compared to those without early insomnia); 3) similar distribution of the 5-HTTLPR genotypes and similar platelet 5-HT concentrations in depressed veterans with PTSD, subdivided into those with or without various sleep disturbances.

To the best of our knowledge, this is the first study to investigate in parallel the association of 5-HTTLPR and platelet 5-HT concentration with sleep disturbances in veterans with PTSD, with or without comorbid depression. Although we did not confirm our hypotheses, as sleep disturbances complicated with comorbid depression in PTSD were not related to more frequent presence of the S allele or to lower platelet 5-HT concentration, our results partially confirmed the involvement

of 5-HT system in sleep regulation (Gottesmann 2004; Hornung 2003; Ursin 2002). Namely, higher frequency of LL homozygotes, responsible for the higher expression levels of 5-HTT, increased 5-HTT transcription efficiency and elevated 5-HT uptake (Lesch et al. 1996), compared to S carriers, was found in non-depressed veterans with PTSD with early insomnia. This might suggest that the LL genotype of the 5-HTTLPR might present a risk factor for early insomnia in PTSD. This finding agrees with the increased frequency of the L allele compared to S allele found in general population with the poor sleep quality (Barclay et al. 2011). To our knowledge, there are no studies investigating the role of 5-HTTLPR in veterans with PTSD, with or without comorbid depression, and different sleep disturbances. Only one study found a lack of significant association between symptoms of sleep disturbances and specific alleles of different genes, determined by GWAS, in PTSD (Pooler 2015). In contrast, compared to L allele, the S allele of the 5-HTTLPR was associated with poor sleep quality in other diagnostic categories, such as chronically stressed adult primary caregivers for a spouse or parent with dementia (Brummett et al. 2007), or was significantly more prevalent in rotating workers who worked shifts more than 60 months and had desynchronized circadian rhythm and disturbed sleep and wakefulness patterns, resulting in sleep problems (Sookoian et al. 2007). The S allele was more frequent in the insomnia group of healthy workers of the Chinese origin (Huang et al. 2014), or was over-represented in patients who had a diagnosis of chronic primary insomnia and psychophysiological insomnia, according to DSM-IV and International Classification of Sleep Disorders (Deuschle et al. 2010). The significant association of the S allele with primary insomnia in Caucasian population was restricted to female subjects and to allelic, but not genotypic distribution (Deuschle et al. 2010). The explanation for the discrepant results might be sought in the different diagnostic groups, various number of subjects included in the studies, differences in the assessment of sleep quality and in phases of sleep, choice of the phenotype studied, ethnic and gender differences, and different levels of stress exposure. Since the 5-HTTLPR SS genotype was significantly associated with fluoxetine-induced insomnia and agitation in depressed patients (Perlis et al. 2003), the differences in the literature findings might also be due to the use of un-medicated subjects (present study) vs. subjects receiving antidepressant treatment. Other possible explanation is that some of veterans with early insomnia might have developed sleep apnea, which has been associated with the more frequent presence of the L allele of the 5-HTTLPR in Chinese Han population (Yue et al. 2008) and in healthy older Caucasians (Schroder et al. 2014). Besides, increased stress-reactivity and hyper-arousal symptoms are predisposing factors to insomnia (Deuschle et al. 2010, Harvey et al.

2014). In agreement, in our study, non-depressed veterans with PTSD had significantly higher arousal symptoms (measured with the CAPS) compared to depressed veterans with PTSD, and these symptoms are presumably associated with higher stress levels. The relationship between the S allele and stress is not simple or straight forward, since the stress level influences insomnia, and in healthy Chinese workers with low, but not high level of job stress, the S allele was protective against insomnia (Huang et al. 2014). In Chinese patients with PTSD, the S allele was related to elevated stress sensitivity, and 5-HTTLPR polymorphism influenced the relationship between stress and PTSD (Zhao et al. 2017). Since we have not evaluated stress level, at present we are not able to confirm these results. The effect of 5-HTTLPR on sleep is sex dependent (Deuschle et al. 2010, Hartmann et al. 2014, Huang et al. 2014), and this possible influence was excluded since the present study included only male Croatian army soldiers, while other studies included participants of both genders (Brummett et al. 2007, Deuschle et al. 2010). In students with a persistent short sleep pattern, an over-representation of the 5-HTTLPR SS genotype was detected in groups who reported shorter sleep and higher depressed mood scores (Carskadon et al. 2012). As we did not detect any significant differences in the frequency of the 5-HTTLPR genotypes between veterans with PTSD who have developed comorbid depression, subdivided according to various sleep disturbances, our results did not confirm that sleep deficits induced by insomnia, stress or lifestyle choices might interact with genetic vulnerability, and result in higher risk of depressive symptoms (Carskadon et al. 2012). Comparable to our results, 5-HTTLPR genotypes frequencies did not differ in depressed patients with or without paroxetine induced hyper-insomnia (Murata et al. 2013).

Present study detected the highest peripheral, platelet 5-HT concentration in non-depressed veterans with PTSD, who had the most prominent symptoms of early insomnia, compared to veterans without early insomnia. This result is in line with the more frequent LL genotype found in early insomnia in the same group, since LL genotype is responsible for the higher 5-HT reuptake, more circulating 5-HT which is taken up from the blood into platelets and consequently more 5-HT stored in platelets. This finding contrasts the findings of the reduced platelet 5-HT concentration in the smaller group of different Caucasian veterans with PTSD and early insomnia (Pivac et al. 2010), in shift workers with sleep problems (Sookoian et al. 2007), or in alcoholic patients with insomnia (Nenadic Sviglin et al. 2011). In addition, reduced plasma 5-HT levels in autistic children with sleep disordered breathing problems (Kheirouri et al. 2016), lower free and platelet 5-HT levels in healthy volunteers during sleep vs. wake (Lechin et al. 2004), and decreased 5-HT uptake levels in subjects with sleep bruxism, the subgroup of sleep related movement dis-

orders (Minakuchi et al. 2014) compared to corresponding controls were also reported. On the other hand, our findings of unaltered platelet 5-HT concentration in depressed PTSD veterans, with or without different sleep disturbances agree with unchanged platelet 5-HT in chronic renal patients with and without sleep disturbances and depressive symptoms (Pivac et al. 2001), unaltered platelet 5-HT concentration detected in different but much smaller groups of depressed or non-depressed veterans with PTSD with or without sleep disturbances (Muck-Seler et al. 2003), and with similar whole blood 5-HT content between adolescents with migraine with or without parasomnias, daytime sleepiness, night awakenings, and sleep onset delays (Pakalnis et al. 2009). The reasons for these discrepant findings might be explained by the much lower number of patients used in previous studies, different sleep disturbances and different measurement instruments used. Our results suggest that early insomnia in PTSD (without comorbid depression) was associated with higher platelet 5-HT concentration, presumably because early insomnia induces sleep disturbances, occasional or nightly difficulties in falling asleep, and therefore veterans were more disturbed, angrier, more aggressive or experienced more hyper-arousal symptoms than veterans without early insomnia, as shown in their higher CAPS total and subscale scores compared to depressed veterans. This would be in line with the higher platelet 5-HT concentration found in PTSD veterans with psychotic features (Pivac et al. 2006) or in aggressive violent offenders (Zhou et al. 2006). In line with our previous findings (Pivac et al. 2009, Kovacic Petrovic et al. 2016), but in contrast to data obtained on the much smaller groups of shift workers, where SS genotype carriers had lower platelet 5-HT levels than L carriers (Sookoian et al. 2007), platelet 5-HT concentration was not affected by the 5-HTTLPR genotype. These results suggest that increased platelet 5-HT and more frequent LL genotype found in early insomnia are independent findings. Non-depressed veterans with PTSD, with severe early insomnia, carriers of the LL genotype, had higher platelet 5-HT concentration compared to S carriers, implying that higher scores of early insomnia symptoms, and not 5-HTTLPR genotype, contributed to the increased platelet 5-HT concentration. The differences in diagnoses, number of subjects involved, methods used, phenotypes investigated, but also in statistical power might have contributed to these differences.

Limitations of the study should be acknowledged: the study was cross-sectional, the assessment was done for bi-allelic and not tri-allelic (the A/G SNP; rs25531) 5-HTTLPR (Lipsky et al. 2009), specific sleep measurement scales were not used to assess sleep disturbances, and the subjects were ethnic Croatian males, thus the findings may not be generalizable. Two recent meta-analyses showed completely opposite findings regarding the bi- or tri-allelic approaches when deter-

mining the association between PTSD and 5-HTTLPR (Navarro-Mateu et al. 2013, Zhao et al. 2017). The advantages of the present study were evaluation of the two measures of 5-HT system in ethnically homogenous Caucasian population of Croatian origin with shared trauma, inclusion of medication-free male war veterans with combat related PTSD, with or without comorbid depression, clinical measures of PTSD, depression and sleep disturbances obtained with SCID and psychometric scales, required sample size and optimal statistical power, and platelet 5-HT concentration controlled for smoking, comorbid depression, 5-HTTLPR genotypes and various sleep disturbances.

## CONCLUSIONS

Our results have shown that early insomnia was associated with slightly higher frequency of the 5-HTTLPR LL genotype compared to the S carriers, and with the significantly higher platelet 5-HT concentration in veterans with PTSD who did not develop comorbid depression. The LL genotype, responsible for the higher 5-HT reuptake and reduced 5-HT availability in the synaptic cleft, might be associated with disturbed prefrontal control in PTSD, and more hyper-arousal symptoms in PTSD (Grabe et al. 2009). Veterans with PTSD who developed comorbid depression had lower PTSD symptoms, similar frequency of the 5-HTTLPR genotypes and similar platelet 5-HT concentration when subdivided according to various sleep disturbances. This study provides the first data from the ethnically homogenous population for the further meta-analyses and should be validated and replicated in different groups, ethnicities and in both gender. Since only early insomnia was associated with 5-HTTLPR and platelet 5-HT concentration, further studies are necessary to confirm and clarify the role of 5-HT in sleep disturbances in PTSD.

### **Contribution of individual authors:**

Zrnka Kovacic Petrovic evaluated subjects with PTSD, did the diagnostic procedures and evaluated veterans using psychiatric scales.

Tina Peraica evaluated veterans with PTSD and evaluated them using the clinical scales.

Gordana Nedic Erjavec & Matea Nikolac Perkovic did the genotyping and determined platelet serotonin concentration.

Lucija Tudor analyzed the results and did the statistical evaluation of results.

Nela Pivac, Zrnka Kovacic Petrovic & Gordana Nedic Erjavec wrote the first draft of the MS. Nela Pivac wrote the final version.

Dubravka Svob Strac & Gordana Nedic Erjavec did the proof reading.

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