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Alcohol-related phenotypes and platelet serotonin concentration

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Abstract

Alcohol dependence is a chronic relapsing mental disorder with heterogeneous and complex underlying biology. It is frequently associated with nicotine dependence, severity of alcohol dependence symptoms, and diverse alcohol-related phenotypes, including the presence of delirium tremens and withdrawal symptoms, early or late onset of alcohol abuse, aggression, suicidal behavior, and anxiety. While searching for peripheral biomarkers of altered serotonergic (5-HT) function in alcohol dependence and alcohol-related behaviors, we determined a peripheral biomarker, i.e., platelet 5-HT concentration in a large group of Caucasian subjects with alcohol dependence subdivided according to the presence of specific alcohol-related phenotypes and smoking status. Individuals with alcohol dependence (n = 661) of both sexes were evaluated using Structural Clinical Interview based on DSM-IV criteria, while platelet 5-HT concentration was determined using the spectrophotofluorimetric method. Smoking is significantly associated, while sex and age are not, with platelet 5-HT concentration. Severe alcohol dependence and lack of withdrawal symptoms were associated with significantly decreased platelet 5-HT concentration in alcohol-dependent non-smokers. In smokers, significantly lower platelet 5-HT concentration was found in patients with the late onset of alcohol abuse. These results suggested that platelet 5-HT concentration might be used as a peripheral marker of different alcohol-related phenotypes, after controlling for the effects of smoking and sex.

Highlights

- Smoking is associated with increased platelet 5-HT in alcohol-dependent patients.
- Sex does not affect platelet 5-HT concentration in alcoholism.
- Platelet 5-HT is related to withdrawal, severity, and age of onset of alcoholism.
- Delirium tremens, aggression, suicidality, and anxiety are not associated with platelet 5-HT.

Keywords
age at onset
alcohol dependence

platelet serotonin
severity
withdrawal

Introduction

Alcohol dependence is a chronic relapsing mental disorder with heterogeneous and complex underlying biology (Schuckit, 2009). There is still a lack of knowledge about the exact mechanism by which alcohol affects the brain, and how it exerts its action on pathways involved in positive and negative reinforcement, emotions, motivation, stress, reward, and arousal. Neurobiological underpinning is related to alcohol-induced changes of the neurotransmission modulated by dopamine, acetylcholine, opioid peptides, GABA, glutamate, glycine, adenosine, serotonin (5-hydroxytryptamine, 5-HT), corticotropin releasing hormone (CRH), neuropeptide Y, and others (Banerjee, 2014; Gilpin & Koob, 2008; Michalak & Biała, 2016). Chronic alcohol dependence is associated with depressive or anxiety symptoms (Ketcherside, Matthews, & Filbey, 2013), aggressive and suicidal behavior, impulsiveness, withdrawal symptoms with delirium tremens (i.e., a very severe withdrawal symptom), abstinence syndrome, frequent relapse, diverse severity, and different onset of alcohol abuse (American Psychiatric Association, 1994; Becker, 2008; Pettinati, Kranzler, & Madaras, 2003). Aggressive and impulsive behaviors are usually associated with early onset of alcohol abuse (Cloninger, 1987) and suicidal behavior (Gvion & Apter, 2011; Modesto-Lowe, Brooks, & Ghani, 2006; Sher, 2006). Presence and severity of these symptoms or behaviors worsen the clinical picture and lead to a poorer prognosis.

Serotonin is presumed to have an important role in the pathophysiology of alcohol dependence (Berger, Gray, & Roth, 2009; Johnson, 2004; Lovinger, 1997; Mukherjee, Das, Vaidyanathan, & Vasudeven, 2008; Ratsma, Van Der Stelt, & Gunning, 2002), but also in aggression and violence (Badawy, 2003; Bortolato et al., 2013; Klasen et al., 2019), impulsivity, and suicidal behavior (Ketcherside et al., 2013; Mann, 2013). Serotonin modulates brain pathways associated with addiction, such as prefrontal cortex and mesolimbic circuits (Ketcherside et al., 2013), regions involved in regulation of hedonic impulses, emotions, mood, anxiety, and impulse control (Pettinati et al., 2003). A small but

significant inverse relationship between aggressive behavior and the 5-HT system was confirmed in a meta-analysis (Duke, Bègue, Bell, & Eisenlohr-Moul, 2013). Previously, it was assumed that alcohol might induce aggressive behavior via a depletion of the central 5-HT levels (Badawy, 2003) or via reduction of the brainstem 5-HT transporter (5-HTT) (Heinz et al., 1998). However, the hypothesis of "5-HT deficiency in aggression" might be over-simplification, and it is proposed that 5-HT has numerous and pleiomorphic roles in the modulation of pathological aggression and suicidal behaviors (Bortolato et al., 2013). Impulsive aggressive behavior frequently develops in alcoholism, leading to suicidal behavior, and is presumably mediated by different homeostatic imbalances of the 5-HT system (Bortolato et al., 2013). Higher 5-HT1A and 5-HT2A receptor binding was found in postmortem brains of suicide victims with alcohol use disorder than in control subjects (Underwood et al., 2018). In male but not in female alcoholics after four weeks of abstinence, the cerebrospinal fluid (CSF) concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT, were inversely correlated with a reduction in 5-HTT availability (Heinz et al., 2002). In the postmortem brains of chronic alcoholics, reduced 5-HTT binding was detected in the dorsal amygdala, the anterior cingulate cortex, and striatum (Storvik, Tiihonen, Haukijärvi, & Tupala, 2006, 2007). However, recent findings detected higher 5-HTT binding in individuals with alcohol use disorders (Underwood et al., 2018). In chronic male alcoholics, decreased 5-HT synthesis was detected in the medial prefrontal cortex, a region responsible for planning, self-control, and moderating social behavior (Nishikawa et al., 2009). It was also decreased in the dorsal anterior cingulate gyrus, with negative correlations between regional 5-HT synthesis and higher alcohol consumption in the amygdala and orbitofrontal region, while increased 5-HT synthesis was found near the temporal gyrus and the occipital lobe (Nishikawa et al., 2009). All these data suggest that chronic alcohol dependence is associated with different abnormalities in 5-HT function,

synthesis, and concentration in different brain regions involved in the stress response, reward systems, emotion regulation, and motivation (Becker, 2008; Nishikawa et al., 2009).

In the search for peripheral biomarkers of altered serotonergic function in alcohol dependence and alcohol-related behaviors, platelets are a convenient model (Mammadova-Bach, Mauler, Braun, & Duerschmied, 2017), as they share similar processes (uptake, storage, 5-HTT, metabolism, release) as 5-HT neurons (Zhuang et al., 2018). Blood platelets were suggested (Camacho & Dimsdale, 2000; Goveas, Csernansky, & Coccaro, 2004; Zhuang et al., 2018) to be a limited peripheral surrogate model of the central 5-HT neurons.

In the studies that determined peripheral platelet 5-HT concentration, lower platelet 5-HT concentration (Nenadic-Sviglin et al., 2011; Pivac, Mück-Seler, Mustapić, Nenadić-Sviglin, & Kozarić-Kovacić, 2004; Pivac et al., 2008) and higher platelet monoamine oxidase type B (MAO-B) activity (Nedic Erjavec et al., 2014) were found in patients with alcohol dependence, compared to healthy controls. On the other hand, plasma tryptophan concentration, a precursor of 5-HT from the blood, did not differ between patients with alcohol dependence and healthy controls (Nishikawa et al., 2009). Chronic alcohol abuse induces thrombocytopenia (Silczuk & Habrat, 2020). Withdrawal syndrome and delirium tremens and/or seizures were significantly related to thrombocytopenia in alcohol-dependent individuals (Berggren et al., 2009), and with diminished central availability of tryptophan, suggesting reduced 5-HT function (van der Mast & Fekkes, 2000). Withdrawal is frequently associated with enhanced anxiety that is linked to a reduction in the 5-HT circuitry in alcohol-dependent subjects (Ketcherside et al., 2013). A lower ratio of tryptophan and large neutral amino acids in plasma were detected in alcoholic subjects with early onset of alcohol abuse, i.e., those who start dependent drinking before the age of 25 (Virkkunen & Linnoila, 1990). Early onset was associated with higher platelet 5-HT uptake (Javors, Tiourinine, & Prihoda, 2000) and reduced CSF 5-HIAA concentration (Fils-Aime et al., 1996). However,

no significant differences in platelet MAO-B activity were detected in smaller numbers of patients with alcohol dependence, subdivided according to those with and without delirium tremens, withdrawal symptoms, severity of alcohol symptoms, age at onset of alcohol abuse, aggression, suicidal behavior and attempt, and anxiety, controlled for the possible influence of gender, smoking, and alcohol-related liver disease (Nedic Erjavec et al., 2014).

In this study, we assessed platelet 5-HT concentration in a large group (n = 661) of male and female Caucasian subjects with alcohol dependence, subdivided according to the severity of alcohol symptoms and presence of diverse alcohol-related phenotypes (delirium tremens, withdrawal symptoms, age at onset of alcohol abuse, aggression, suicidal behavior, and anxiety) and smoking status. The hypothesis of the study was that platelet 5-HT concentration might be used as a peripheral marker of different alcohol-related phenotypes.

Methods

Subjects

We included 661 medication-free patients with alcohol dependence (538 males and 123 females), diagnosed by psychiatrists with expertise in addiction, using the Structured Clinical Interview for DSM-IV Disorders (SCID-IV) (First, Spitzer, Gibbon, & Williams, 2002), recruited in the Clinical Hospital Vrapce, Zagreb. They were inpatients admitted due to withdrawal syndrome or to acute intoxication with alcohol. Subjects were left overnight to sober, and the interview, clinical scales, and the sampling were done the next day. Clinical scales used were Hamilton Rating Scale for Depression (HAMD) (Hamilton, 1960), Hamilton Rating Scale for Anxiety (HAMA) (Hamilton, 1959), Clinical Global Impression (CGI) (Guy, 1976), and Brown-Goodwin Assessment for Lifetime Aggression (Brown-Goodwin scale) (Brown, Goodwin, Ballenger, Goyer, & Major, 1979). Based on the comprehensive psychiatric evaluation, interview, SCID, and different psychiatric scales, alcoholic patients were subdivided into patients with different alcohol-related phenotypes and

severity of alcohol dependence, as described in detail before (Nedic Erjavec et al., 2014). Current or a previous history of withdrawal, delirium tremens, and age of onset of alcohol abuse (early onset <25 years of age, late onset ≥25 years of age) were evaluated by the psychiatric interview. Severity of alcohol dependence was estimated according to CGI as mild (scores 1–4) and severe (scores 5–7). Additionally, the serum levels of liver enzymes, aspartate transaminase (AST), and alanine aminotransferase (ALT) were used as indicators of alcohol exposure levels. Current suicidal behavior was evaluated with the HAMD item 3, where a score greater than 0 meant that the suicidal behavior was currently present. Aggression was determined using the Brown-Goodwin scale, and patients were categorized into aggressive (≥8 scores) and non-aggressive (<8 scores) patients. Anxiety was determined using HAMA, and patients were subdivided into those with mild to moderate (<25 scores) and those with severe (≥25 scores) anxiety. The inclusion criteria were current alcohol dependency and voluntary participation. The exclusion criteria were current medication or psychotropic treatment, drug abuse, a severe alcoholic dementia, and comorbid PTSD. In addition to alcohol dependency, patients developed nicotine addiction and were subdivided according to the smoking status into 399 current smokers and 262 non-smokers. Their ages expressed as median (1st; 3rd quartile) were 49 (42; 55) for smokers and 52 (44; 60) for non-smokers.

All participants signed written informed consent. The aims and procedures of the study were explained in detail and the study was approved by the Ethics committee of the Clinical Hospital Vrapce, Zagreb, Croatia. All human studies have been executed with the full cooperation of participants and adequate understanding and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Biochemical analysis

Blood samples (8 mL) were drawn in a plastic syringe with 2 mL of acid citrate dextrose anticoagulant in the morning. Platelet-rich plasma (PRP) was obtained after centrifugation of whole blood at 1810 × *g* for 3 minutes at room temperature, and platelets were precipitated by further centrifugation of PRP at 5030 × *g* for 15 minutes in a refrigerated centrifuge. The platelet pellet was washed with saline and centrifuged again. Obtained platelets were stored at −20 °C.

Platelet 5-HT concentration was determined in platelet pellets as described previously (Dvojkovic et al., 2020). Platelets were disrupted by sonication and platelet 5-HT concentration was measured using ortho-phthalaldehyde-enhanced fluorometry. In order to precipitate proteins, blank samples, 5-HT standards, and platelet sonicates were incubated with 10% $ZnSO_4$ and 1N NaOH for 5 minutes at room temperature and centrifuged. The supernatant was transferred into a new glass tube after centrifugation. 1% L-cysteine and 0.01% ortho-phthalaldehyde were added to all samples, which were then boiled for 10 minutes. The reaction was stopped by the addition of 1N NaOH. Fluorescence was measured in duplicate on a Varian Spectro-photofluorometer Cary Eclipse, with the excitation wavelength of 345 nm and emission wavelength of 485 nm. Platelet 5-HT concentration was corrected for the different platelet numbers by the platelet protein levels, measured by the method of Lowry and co-workers (Lowry, Rosenbrough, Farr, & Randall, 1951). The detection limit of the method was 10.0 ng/sample, with intra- and inter-assay coefficients of variation 3.66 and 8.69%, respectively.

Statistical analysis

Results were evaluated with Sigma Stat 3.5 (Jandel Scientific Corp., San Jose, California, United States). All tests used were two-tailed. The Kolmogorov-Smirnov test was used to determine the normality of the distribution. Because values of platelet 5-HT concentration deviated from normal distribution, they are shown as median and 1st (Q1) and

3rd (Q3) quartile or as violin plots. A multiple regression analysis was used to examine the association of platelet 5-HT concentration with age, sex, and smoking. A non-parametric Mann-Whitney U test was used to compare the values of platelet 5-HT concentration between the groups. Predictive value of platelet 5-HT concentration for alcohol-related phenotypes was tested by the Receiver operating characteristic (ROC) curves, controlled for smoking status. A chi-square test was used to evaluate the frequency of alcohol-related phenotypes between males and females or smokers and non-smokers with alcohol dependence. Standardized residuals (R) were calculated to determine a main contributor to significant differences (Field & Miles, 2012). To avoid issues with multiple testing (Leucht, Davis, Engel, Kane, & Wagenpfeil, 2007) and correction for multiple testing, all statistical tests were applied to cutoffs chosen *a priori*, and all alcohol-related phenotypes were defined *a priori* as described in Methods. All tests were two-tailed and α was set at 0.05. G*Power 3 Software (Faul, Erdfelder, Lang, & Buchner, 2007) was used for conducting power analyses, i.e., to determine *a priori* sample size and actual power. Statistical power was set to 0.800. For a χ^2 test (with expected small effect size = 0.2), the required sample size was 273 for df = 3. For multiple regression (with expected small effect size = 0.02, 2 predictors), the required sample size was 485. For the Mann-Whitney U test (with expected small effect size = 0.1), the required sample size was 614. As the study included 661 subjects, the sample size and the statistical power were appropriate to detect significant differences between the studied groups.

Results

Distribution of the particular alcohol-related phenotypes differed significantly (χ^2 test, df = 1) between male and female patients with alcohol dependence. Significant sex differences were found in the frequency of delirium tremens ($\chi^2 = 2.968$; $p = 0.027$; i.e., in 8% men vs. 2% women), early or late onset ($\chi^2 = 9.092$; $p < 0.001$; i.e., in 39% men vs. 19%

women), severe or mild aggression ($\chi^2 = 1.671$; $p < 0.001$; i.e., in 29% men vs. 12% women), and severe or mild anxiety symptoms ($\chi^2 = 8.324$; $p = 0.002$; i.e., in 21% men vs. 34% women). These significant differences were mostly induced by the major contribution of the female patients with delirium tremens (R = 1.90), female patients with early onset (R = 3.05), female patients with severe aggression (R = 3.01), and female patients with severe anxiety (R = 2.37). Frequency of other phenotypes such as withdrawal symptoms ($\chi^2 = 17.365$; $p = 0.085$), suicidal behavior ($\chi^2 = 1.636$; $p = 0.198$), and severity of alcohol dependence ($\chi^2 = 9.369$; $p = 0.240$) did not differ significantly between male and female patients with alcohol dependence. Severe withdrawal symptoms were detected in 24% of males and 17% of females; higher suicidal behavior was found in 26% of males and 32% of females, while severe alcohol dependence symptoms had 21% male and 26% female patients with alcohol dependence, respectively.

To evaluate the influence of age, sex, and smoking on platelet 5-HT concentration, multiple linear regression analysis was used, with platelet 5-HT concentration as the dependent variable, and age, sex, and smoking as independent variables. It revealed a significant model ($F_{(3,657)} = 10.824$; $p < 0.001$; $R_{adj}^2 = 0.043$) due to a significant effect of smoking ($ß_{smoking} = -0.203$; $p < 0.001$), but a lack of significant effect of age ($ß_{age} = -0.004$; $p = 0.908$) or sex ($ß_{sex} = -0.064$; $p = 0.097$) on platelet 5-HT concentration. To confirm these results, we compared platelet 5-HT data separately in male and female patients with alcohol dependence, as well as in smokers and non-smokers with alcohol dependence. There were no significant (Mann-Whitney test) differences (U = 29864.50; $p = 0.067$) in platelet 5-HT concentration (presented as median [Q1; Q3]) between female (0.72 [0.47; 0.93]) and male (0.76 [0.51; 1.08]) patients with alcohol dependence. In line with multiple regression finding, significant (U = 39246.0; $p < 0.001$) differences in platelet 5-HT were detected between smokers (0.84 [0.55; 1.15]) and non-smokers (0.68 [0.45; 0.89]) with alcohol dependence, as

smokers had significantly higher platelet 5-HT concentration than non-smokers. Therefore, in further analyses, male and female patients were merged into one group and were subdivided according to their smoking status and alcohol-related phenotypes. Demographic data are presented in Table 1. In patients with alcohol dependence subdivided into smokers and non-smokers, there was no significant difference in the distribution of men and women according to smoking status. Regarding the distribution of the alcohol-related phenotypes, significant differences were detected in the frequency of delirium tremens, age at onset of alcohol abuse, aggression, and suicidal behavior, while the distribution of withdrawal symptoms differed at the trend level between smokers and non-smokers. The main contributors to the significant differences were the number of non-smokers with delirium tremens (R = 1.82), the number of non-smokers with the early onset of alcohol abuse (R = 1.63), the number of non-smokers with current suicidal behavior (R = 1.52), and the number of non-smokers with severe aggression (R = 1.70). The distribution of patients with mild and severe alcohol dependence, as well as those with or without anxiety symptoms, did not differ significantly between smokers and non-smokers (Table 1). Results showing platelet 5-HT concentration in patients with alcohol dependence subdivided into smokers and non-smokers and according to alcohol-related phenotypes are shown in Table 2. In non-smokers, platelet 5-HT concentration was significantly decreased in patients with withdrawal symptoms compared to patients without these symptoms, and in patients with severe symptoms of alcohol dependence compared to those with mild symptoms of alcohol dependence. Multiple linear regression for AST ($F_{(3,626)}$ = 13.365; $p < 0.001$; $R_{adj}^2 = 0.056$) and ALT ($F_{(3,625)} = 14.076$; $p < 0.001$; $R_{adj}^2 = 0.059$) indicated significant effects of age, sex, and smoking to both, AST ($ß_{age} = -0.199$, $p_{age} < 0.001$; $ß_{sex} = -0.097$, $p_{sex} = 0.013$; $ß_{smoking} = 0.133$, $p_{smoking} = 0.001$) and ALT ($ß_{age} = -0.196$, $p_{age} < 0.001$; $ß_{sex} = -0.132$, $p_{sex} = 0.001$; $ß_{smoking} = 0.105$, $p_{smoking} = 0.008$) levels. As we found that plasma levels of AST and ALT strongly depend on age, sex, and smoking status,

correction for the effect of age and sex was performed by fitting the linear model of each liver enzyme dependent on age and sex and using the obtained standardized residuals to determine its correlation with platelet 5-HT levels for smokers and non-smokers separately. Spearman's correlation indicated significant negative correlation of platelet 5-HT concentration with AST ($\rho = -0.161$; $p = 0.002$) and ALT ($\rho = -0.151$; $p = 0.003$) levels in smokers, as well as with AST ($\rho = -0.211$; $p = 0.001$) and ALT ($\rho = -0.162$; $p = 0.010$) levels in non-smokers. Platelet 5-HT was also decreased in smokers with late onset compared to those with early onset of alcohol abuse. Within smokers, platelet 5-HT concentration did not differ between patients with or without delirium tremens, withdrawal, aggressive and suicidal behavior, severity of alcohol dependence, and anxiety. Platelet 5-HT concentration was higher at the trend level ($p = 0.058$) in aggressive smokers compared to non-aggressive smokers. Within non-smokers, similar platelet 5-HT concentration was found in patients with or without delirium tremens, withdrawal, age at onset of alcohol abuse, aggressive and suicidal behavior, and anxiety (Table 2). These results suggest that only withdrawal symptoms, age at onset, and severity of alcohol dependence were associated with platelet 5-HT concentration when corrected for smoking status. In order to demonstrate the predictive value of platelet 5-HT concentration controlled for smoking status, ROC curves were performed for the alcohol-related phenotypes that were shown to be associated with the platelet 5-HT concentration (Figure 1). Areas under the curves for platelet 5-HT were 0.409 ± 0.039 ($p = 0.024$, 95% CI: 0.333–0.485) for the withdrawal syndrome in non-smokers (n = 262), 0.356 ± 0.039 ($p = 0.001$, 95% CI: 0.279–0.433) for severity of alcohol dependence in non-smokers (n = 261) and 0.566 ± 0.030 ($p = 0.026$, 95% CI: 0.508–0.625) for age of onset of alcohol abuse in smokers (n = 396).

In addition, the violin plots of platelet 5-HT in all alcohol-related phenotypes (withdrawal, delirium tremens, aggression, age of onset, suicidality, anxiety) and severity of

alcohol dependence are provided separately for smokers and non-smokers with alcohol addiction in Supplementary Figures 1–7.

Discussion

Major findings from this study are significantly lower platelet 5-HT concentration in non-smoking patients with alcohol dependence with present withdrawal symptoms, compared to non-smokers without these symptoms, in non-smokers with severe alcohol dependence compared to those with mild alcohol dependence, and in smokers with late onset compared to early onset of alcohol abuse. In general, non-smokers had significantly lower platelet 5-HT concentrations than smokers with alcohol dependence. This study found that platelet 5-HT concentration, corrected for the effect of smoking, was associated with the severity of alcohol dependence and specific alcohol-related phenotypes (i.e., withdrawal and age at onset) in male and female patients with alcohol dependence.

As sex is a major genetic modifier of disease pathophysiology and clinical presentation (Mauvais-Jarvis et al., 2020), we have included both male and female patients in our study. Sex-related differences were detected in particular alcohol-related phenotypes. In agreement with previous data (Sørensen et al., 2019; Soyka et al., 2006; Wojnar, Wasilewski, Matsumoto, & Cedro, 1997), in this study more male (8%) than female (2%) patients showed signs of delirium tremens and withdrawal symptoms (Sanvisens et al., 2020; Soyka et al., 2006). In the literature, alcohol-induced withdrawal was associated with earlier start of drinking (Sanvisens et al., 2020). Correspondingly, as reported previously (Wojnar et al., 1997), in our study more men (39%) had early onset of alcohol abuse when compared to women (19%). In agreement with the finding of more frequent anxiety disorders detected in women than in men with alcohol withdrawal symptoms (Wojnar et al., 1997), in our study more females (34%) had symptoms of severe anxiety compared to male patients (21%) with alcohol dependence. In social drinkers exposed to laboratory-induced aggression, alcohol

more strongly elevated aggression in men than in women (Giancola et al., 2009), which is in line with our findings of the more frequent aggression in male (29%) compared to female (12%) patients with alcohol dependence.

Alcohol and nicotine dependence contribute to similar mortality and morbidity. In the present study, the frequency of smoking was similar between male and female patients with alcohol dependence, as 62% of men and 55% of women smoked cigarettes. This distribution was also analogous to our previous data (Nenadic-Sviglin et al., 2011). Alcohol-related phenotypes developed differently in smokers and non-smokers with alcohol dependence, especially delirium tremens, age at onset of alcohol abuse, aggression, and suicidal behavior. Namely, non-smokers exhibit more frequent delirium tremens and earlier onset of alcohol abuse than smokers. As prior alcohol use predicts tobacco use, a more frequent finding of non-smokers than smokers with early onset agrees with the bidirectional association between alcohol and tobacco use in adolescence and young adulthood (Jackson, Sher, Cooper, & Wood, 2002). On the other hand, in line with the combined effect of current smoking on increased suicidal risk and ideation (Jung, 2019), current suicidal behavior and severe aggression were more frequently found in smokers compared to non-smokers.

Platelet 5-HT concentration might be affected by different medication (Dvojkovic et al., 2020; Li et al., 2015; Mück-Šeler & Pivac, 2011), age (Peitl, Getaldic-Svarc, & Karlovic, 2020), sex (Pivac et al., 2004, 2008), smoking (Nenadic-Sviglin et al., 2011), and different comorbidities (Peitl et al., 2020; Pivac et al., 2004). Our study was controlled for these confounders. Our previous studies (Nenadic-Sviglin et al., 2011; Pivac et al., 2004, 2008) and other (Bailly et al., 1990) studies, with smaller groups of patients with alcohol addiction, revealed that male and female alcoholics had significantly lower platelet 5-HT concentrations than healthy controls. Presence of the comorbid PTSD partly normalized the decreased platelet 5-HT values (Pivac et al., 2004, 2008), while a previous suicide attempt did not affect

platelet 5-HT concentration (Nenadic-Sviglin et al., 2011). Although alcohol dependence is frequently associated with comorbid depression, it did not significantly affect platelet 5-HT concentration (Nenadic-Sviglin et al., 2011). Since we included medication-free patients with alcoholism, the effect of medication on platelet 5-HT was excluded. In our previous study, we eliminated the possible effect of comorbid mental disorders (depression, schizophrenia, anxious disorder, anxious depressive disorder, acute stress reaction, personality disorder, adjustment disorders, mental retardation, organic mental disorders, dissociative disorder, persistent mood disorder, and phobic anxiety disorder) on platelet 5-HT concentration (Nenadic-Sviglin et al., 2011). In this study, we excluded comorbid PTSD and drug abuse. Therefore, the presence of comorbid mental disorders did not affect platelet 5-HT concentration. Although in a recent study, age was inversely and significantly correlated with platelet 5-HT concentration in patients with depression and in healthy subjects, but not in patients with schizophrenia (Peitl et al., 2020), multiple regression revealed no significant effect of age on platelet 5-HT in patients with alcohol dependence in our study. This lack of the influence of age on platelet 5-HT values is in agreement with our previous data (Nenadic-Sviglin et al., 2011; Svob Strac et al., 2019). Though some of our previous studies detected significant effects of sex on platelet 5-HT concentration (Pivac et al., 2004, 2008), presumably due to the smaller number of subjects included, while the other studies failed to detect these sex-related differences (Nenadic-Sviglin et al., 2011; Pivac et al., 2009; Svob Strac et al., 2019), this study, which included larger number of individuals with alcohol dependence, showed that sex was not a significant factor affecting platelet 5-HT concentration. However, as expected (Nenadic-Sviglin et al., 2011; Schmidt, Dufeu, Heinz, Kuhn, & Rommelspacher, 1997), smoking significantly increased platelet 5-HT concentration (Moffitt et al., 1998).

Alcoholism is related to withdrawal syndrome and delirium tremens and/or seizures. In our study, withdrawal symptoms were associated with reduced platelet 5-HT concentration in non-smoking patients with alcohol dependence, compared to non-smokers without these symptoms. A study investigating platelet 5-HT concentration in patients with alcohol withdrawal syndrome reported reduced platelet 5-HT concentration at the beginning of a 2-week abstinence period when compared to the concentration at day 12 of a detoxification program (Llinás, Caballero, Peñalver, & Valdés, 2014). On the other hand, another study (Bailly et al., 1990) did not find any differences in platelet 5-HT concentrations during and after the withdrawal period, but reported lower platelet 5-HT concentrations in patients with alcoholism, whether or not they are in withdrawal, in comparison to healthy control patients. Additionally, in alcohol-dependent patients, symptoms of withdrawal were associated with lower numbers of platelets (Berggren et al., 2009) and reduced central availability of tryptophan (van der Mast & Fekkes, 2000). In our study, this fact was taken into account and we corrected platelet 5-HT concentration according to the total platelet protein concentration.

Although increased anxiety frequently occurs with withdrawal symptoms in alcohol-dependent subjects (Ketcherside et al., 2013), our results showed that anxiety levels measured by HAMA were not associated with platelet 5-HT concentration in patients with alcohol dependence. In agreement with our data, platelet 5-HT content or plasma tryptophan were not correlated with symptoms of anxiety in depressed patients (Karege, Widmer, Bovier, & Gaillard, 1994). In patients with general anxiety disorder, higher platelet 5-HT concentration and higher 5-HT uptake capacity of 5-HTT were found, compared to healthy control subjects (Zhuang et al., 2018), but these did not differ from these values in depressed patients. The explanation for the discrepant results might be sought in different diagnoses and study designs, since our study included patients with alcohol dependence and compared patients with mild or severe anxiety, while the other study (Zhuang et al., 2018) included patients with

general anxiety disorder, who probably had higher anxiety levels than our patients, and compared them with healthy controls.

Lower platelet 5-HT concentration was found in non-smokers with severe alcohol dependence, evaluated using the CGI, compared to non-smokers with mild alcohol dependence. Additionally, platelet 5-HT concentration was in a weak negative correlation with AST and ALT serum levels, whose higher levels in patients with alcoholism normally indicate liver damage caused by higher alcohol exposure (Torruellas, French, & Medici, 2014). Those results are in agreement with the association between lower platelet 5-HT concentration and the severe symptoms of alcohol dependence evaluated by the CGI. As shown previously (Pivac et al., 2004), alcoholism is associated with reduced platelet 5-HT concentration and now we showed its association with more severe symptoms of alcohol dependence. This may be explained by the fact that alcohol use can cause hematological complications such as thrombocytopenia (Silczuk & Habrat, 2020). Not only alcohol, but also cigarette smoking, can affect platelets, with platelet count found to be increased in smokers (Ghahremanfard et al., 2015), possibly causing the finding that the association of the severity of alcohol dependence and platelet 5-HT concentration was found only in non-smokers. However, in order to minimize the effects of platelet count, in this study platelet 5-HT concentration was normalized by the platelet total protein levels.

Individuals who start dependent drinking early, before the age of 25, usually develop aggressive, impulsive (Cloninger, 1987), and suicidal behavior (Gvion & Apter, 2011; Modesto-Lowe et al., 2006; Sher, 2006). In this study, aggressive behavior was not associated with platelet 5-HT concentration. Aggression was related to lower platelet 5-HT concentration in personality disorder patients (Goveas et al., 2004), while significantly higher whole blood 5-HT was found in violent compared to non-violent male cohorts (Moffitt et al., 1998). Additionally, Limson and co-workers (1991) reported a negative correlation between a

serotonin metabolite, 5-hydroxyindoleacetic acid, measured in CSF and aggression evaluated using the same scale as in our study (Brown et al., 1979). Therefore, there are indices of increased and decreased peripheral 5-HT associated with aggression and/or violence, suggesting that differences might be due to the different methods evaluating aggression/violence, different diagnostic entities, and different indicators of 5-HT function.

In both smokers and non-smokers, presence of self-directed aggression (i.e., suicidal behavior) did not affect platelet 5-HT concentration, although lower platelet 5-HT concentration was reported to be associated with suicidal behavior in patients with major depression (Mück-Seler, Jakovljević, & Pivac, 1996; Pivac, Jakovljević, Mück-Seler, & Brzović, 1997; Ruljancic, Mihanovic, Cepelak, Bakliza, & Dodig Curkovic, 2013), bipolar depression (Giurgiuca et al., 2016), first episode of psychosis (Marcinko et al., 2007), or PTSD (Kovacic, Henigsberg, Pivac, Nedic, & Borovecki, 2008). Opposed to these data in other diagnostic categories (Rao, Hawellek, Papassotiropoulos, Deister, & Frahnert, 1998; Spreux-Varoquaux et al., 2001), in our study platelet 5-HT concentration, corrected for the effect of smoking, was not associated with suicidal behavior. The discrepancies might be explained by the fact that the effect of smoking was not evaluated in those previously reported studies. Present data confirmed our previous results showing that platelet 5-HT values did not differ in alcoholic patients subdivided according to the lifetime attempt of suicide, when results were controlled for the effect of smoking (Nenadic-Sviglin et al., 2011). Our present and previous (Nenadic-Sviglin et al., 2011) results do not support the use of platelet 5-HT as a peripheral marker of suicide attempt or suicidal behavior in alcoholism.

Lower platelet 5-HT was found in smokers with late onset compared to early onset of alcohol abuse, in agreement with data from our previous study in all alcoholic patients (Svob Strac et al., 2019). However, when patients were controlled for the effect of smoking, difference in platelet 5-HT was not confirmed, since the only difference was induced by

smoking and not by early or late onset of alcohol abuse (Svob Strac et al., 2019). In the present study, non-smokers with early and late onset of alcohol abuse had similar platelet 5-HT concentrations, and the differences that were found in smokers disappeared, presumably because non-smokers had much lower platelet 5-HT than smokers. These data do not agree with the lower ratio of tryptophan in early-onset alcoholics (Virkkunen & Linnoila, 1990). Higher platelet 5-HT uptake (Javors et al., 2000) and reduced CSF 5-HIAA concentration (Fils-Aime et al., 1996) was detected in early-onset compared to late-onset alcoholics, suggesting possible reduced 5-HT turnover or decreased 5-HT function in the brain. Early-onset alcoholics had reduced levels of 5-HTT compared to late-onset patients (Storvik, Haukijärvi, Tupala, & Tiihonen, 2008), suggesting defects in 5-HT functioning in early development. The reason for these discrepancies is not clear at present; however, these previous studies included much smaller numbers of cases and did not control peripheral 5-HT indices according to the smoking status, and/or other alcohol-related phenotypes.

Platelet 5-HT has numerous and complex roles in the periphery, interacting with the tissue microenvironment to regulate platelet and immune cell functions (Mammadova-Bach et al., 2017). Although platelets and platelet 5-HT were recently disputed as a model for psychiatric disorders (Li et al., 2015), this study included a very small number of subjects (87 depressed patients and 32 healthy controls) and compared human platelet data with brain data from the rat model of chronic unpredictable mild stress. Therefore, it is difficult to accept their conclusion that “platelet 5-HT does not correlate with 5-HT levels in emotion-related brain regions”. Since 99% of the 5-HT in the body is found and stored in platelets, and 5-HT uptake, storage, and metabolism in platelets resemble those in neurons, platelets were suggested to be a marker showing brain biochemical changes that develop in the presence of anxiety (Camacho & Dimsdale, 2000), while platelet 5-HT was suggested to be a candidate

biomarker for monitoring clinical status in alcoholism (Llinás et al., 2014) or bipolar depression (Giurgiuca et al., 2016).

A few limitations of the study should be acknowledged. Platelet 5-HT concentration was assessed in only one time point, whereas a longitudinal study would have been more informative. Only Caucasian subjects were included, which prevents the generalization of the results. Strengths are a large number (n = 661) of homogeneous patients with alcohol-related phenotypes, a detailed evaluation of phenotypes done by psychiatrists with a huge expertise in addiction, adequate sample size and statistical power, inclusion of male and female subjects, and controlling of platelet 5-HT concentration for smoking status and other confounding variables. As alcohol dependence is a complex entity, this study evaluated its phenotypes according to the proposed research domain criteria (Insel et al., 2010).

This study revealed that platelet 5-HT has a predictive value in the case of withdrawal syndrome, and predictive value in the severity of alcohol dependence in non-smokers with alcohol dependence and age of onset of alcohol abuse in smokers. We have found also a negative correlation of platelet 5-HT concentration with liver enzymes, AST and ALT levels, as indicators of alcohol exposure, in smokers, as well as in non-smokers with alcohol dependence. In conclusion, lower platelet 5-HT concentration, controlled for the effects of smoking and sex, is associated with withdrawal symptoms, severity of alcohol dependence, and age at onset of alcohol abuse in patients with alcohol dependence.

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Disclosure

The authors report no conflicts of interest in this work.

Contributions

Nela Pivac developed the original idea. Gordana Nedic Erjavec, Matea Nikolac Perkovic, and Dubravka Svob Strac managed the experimental work and determined platelet 5-HT and platelet protein concentrations. Gordana Nedic Erjavec and Lucija Tudor did the statistical evaluation of the data. Nela Pivac and Gordana Nedic Erjavec did the data analysis and interpretation, performed the literature search, and wrote the first draft of the article. Suzana Uzun, Oliver Kozumplik, and Ninoslav Mimica explained the research goals and described protocol, inclusion/exclusion criteria in detail to the patients, insured participant adherence, and selected and evaluated patients. Senka Repovecki and Jasminka Bektic Hadzic did the sampling of patients. All authors have read and approved the final version and have contributed substantially to the design, performance, analysis, and reporting of this study.

Figure legends

Figure 1. Predictive value of platelet 5-HT concentration in the case of withdrawal syndrome, severity of alcohol dependence in non-smokers, and age of onset of alcohol abuse in smokers.

Supplementary Figure 1. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and presence of delirium tremens.

Supplementary Figure 2. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and presence of withdrawal symptoms.

Supplementary Figure 3. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and onset of alcohol dependence.

Supplementary Figure 4. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and suicidality.

Supplementary Figure 5. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and presence of aggression.

Supplementary Figure 6. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and severity of alcohol dependence.

Supplementary Figure 7. The distribution of platelet 5-HT concentration among alcohol-dependent subjects divided according to smoking status and anxiety.

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Table 1. The frequency of severity of alcohol dependence and alcohol related phenotypes in patients with alcohol dependence subdivided according to smoking status

	Smokers N (%)	Non-smokers N (%)
Sex (male/female)	331 (83.0) / 68 (17.0)	207 (79.0) / 55 (21.0)
	χ^2=1.63; P=0.202	
Delirium tremens (yes/no)	20 (5.0) / 379 (95.0)	26 (9.9) / 236 (90.1)
	χ^2=5.89; **P=0.015**	
Withdrawal (yes/no)	82 (20.6) / 317 (79.4)	71 (27.1) / 191 (72.9)
	χ^2=3.81; P=0.051	
Age of onset of alcohol abuse (early/late)	154 (38.9) / 242 (61.1)	75 (29.0) / 184 (71.0)
	χ^2=6.79; **P=0.009**	
Aggressive behavior (yes/no)	130 (33.0) / 264 (67.0)	39 (15.2) / 218 (84.8)
	χ^2=25.70; **P<0.001**	
Suicidal behavior (yes/no)	122 (30.7) / 276 (69.3)	59 (22.5) / 203 (77.5)
	χ^2=5.25; **P=0.022**	
Severity of alcohol dependence (severe/mild)	305 (76.6) / 93 (23.4)	210 (80.5) / 51 (19.5)
	χ^2=1.35; P=0.245	
Anxiety (yes/no)	91 (22.8) / 308 (77.2)	66 (25.2) / 196 (74.8)
	χ^2=0.50; P=0.481	

Results are presented as numbers and percentages; Test statistics used= χ^2 test; significant results are in bold.

Table 2. Platelet 5-HT concentration in subjects with alcohol dependence subdivided into smokers and non-smokers and according to the severity of alcohol dependence and presence of different alcohol related phenotypes

Alcohol dependence-related phenotype		Platelet 5-HT concentration	
		Smokers	Non-smokers
Delirium tremens	Yes	0.58 (0.37; 1.03)	0.65 (0.40; 0.91)
	No	0.85 (0.56; 1.16)	0.68 (0.45; 0.89)
		U=4700.50; P=0.070	U=3144.50; P=0.835
Withdrawal	Yes	0.78 (0.49; 1.12)	0.54 (0.42; 0.78)
	No	0.85 (0.57; 1.16)	0.69 (0.47; 0.91)
		U=13910.00; P=0.327	U=8010.00; **P=0.024**
Age of onset of alcohol abuse	early	0.90 (0.57; 1.26)	0.68 (0.44; 0.95)
	late	0.79 (0.52; 1.12)	0.68 (0.45; 0.89)
		U=16158.50; **P=0.026**	U=6773.50; P=0.817
Aggressive behavior	Yes	0.90 (0.58; 1.20)	0.69 (0.43; 0.92)
	No	0.81 (0.52; 1.12)	0.66 (0.45; 0.88)
		U=15144.00; P=0.058	U=4055.50; P=0.647
Suicidal behavior	Yes	0.88 (0.57; 1.20)	0.71 (0.42; 0.92)
	No	0.83 (0.53; 1.14)	0.67 (0.45; 0.87)
		U=16119.50; P=0.498	U=5722.00; P=0.603
Severity of alcohol dependence	Mild	0.83 (0.56; 1.09)	0.81 (0.60; 0.94)
	Severe	0.85 (0.55; 1.18)	0.63 (0.41; 0.87)
		U=14140.00; P=0.965	U=6898.50; **P=0.001**
Anxiety	Yes	0.87 (0.55; 1.16)	0.68 (0.44; 0.88)
	No	0.84 (0.55; 1.15)	0.67 (0.45; 0.89)
		U=13776.50; P=0.806	U=6443.00; P=0.963

Platelet 5-HT concentration values are presented as median (Q1; Q3); Test statistics used= Mann Whitney U test; significant results are in bold.

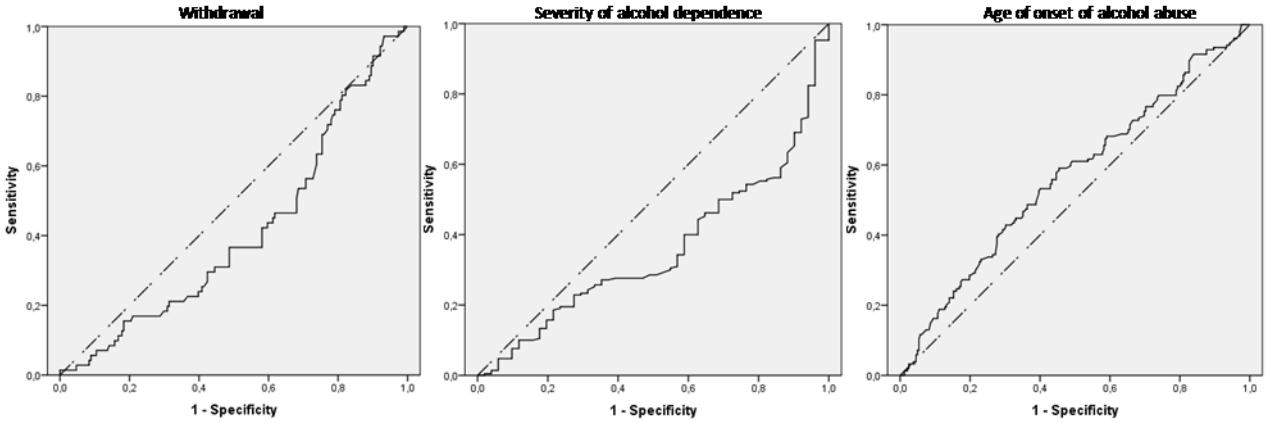

Figure 1. Predictive value of platelet 5-HT concentration in case of the withdrawal syndrome and severity of alcohol dependence in non-smokers and age of onset of alcohol abuse in smokers.

Supplementary Figures 1-7: The violin plots of platelet 5-HT in all the studied alcohol related phenotypes for smokers and non-smokers separately