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Associations of *NLRP3* and *CARD8* gene polymorphisms with alcohol dependence and commonly related psychiatric disorders: a preliminary study

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We investigated two functional polymorphisms in NLRP3 inflammasome genes (*NLRP3* rs35829419 and *CARD8* rs2043211) and their association with alcohol dependence and related anxiety, depression, obsession-compulsion, or aggression in 88 hospitalised alcohol-dependent patients, 99 abstinent alcohol-dependent participants, and 94 controls, all male Caucasian. Alcohol dependence-related psychiatric disorders were assessed with the Zung Depression and Anxiety scale, Buss-Durkee Hostility Inventory, Alcohol Use Disorders Identification Test, Brief Social Phobia Scale, Obsessive Compulsive Drinking Scale, and Yale-Brown Obsessive-Compulsive Scale. For genotyping we used the allele-specific quantitative polymerase chain reaction-based methods. The three groups differed significantly in *CARD8* rs2043211 distribution (P=0.049; chi-squared=9.557; df=4). The *NLPR3* rs35829419 polymorphism was not significantly associated with alcohol dependence. In hospitalised alcohol-dependent patients the investigated polymorphisms were not associated with scores indicating alcohol consumption or comorbid symptoms. In abstinent alcohol-dependent subjects homozygotes for the polymorphic *CARD8* allele presented with the highest scores on the Zung Anxiety Scale (p=0.048; df=2; F=3.140). Among controls, *CARD8* genotype was associated with high scores on the Obsessive Compulsive Drinking Scale (P=0.027; df=2; F=3.744). In conclusion, our results reveal that *CARD8* rs2043211 may play some role in susceptibility to alcohol dependence, expression of anxiety symptoms in abstinent alcohol-dependent subjects, and in obsessive compulsive drinking in healthy controls. However, further studies with larger cohorts are required to confirm these preliminary findings.

KEY WORDS: alcohol addiction; anxiety; genetic polymorphism; inflammasome; obsessive compulsive drinking

Chronic excessive alcohol consumption leads to neuroinflammation and may result in cognitive dysfunction and behavioural changes, as alcohol rapidly diffuses through the blood-brain barrier, alters neurotransmission, contributes to neurodegeneration, and impairs regeneration by activating microglia and astrocytes, but our understanding of the mechanisms by which alcohol triggers inflammation in the brain is still limited (1). What we do know is that peripheral endotoxemia induced by alcohol may lead to increased secretion of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β, IL-6, and interferon gamma (2). We also know that astrocyte activation may be mediated by the Toll-like receptor 4 pathway (TLR4), which activates downstream signalling molecules and cytokine secretion (1, 3). One study on mice has shown that ethanol directly triggers TLR4-mediated activation of the nucleotide-binding oligomerisation domain (NOD), leucine-rich repeats (LRR), and pyrin domain-containing protein 3 (NLRP3) inflammasome in glia cells (4). Another study on human and mouse cells suggests that the hyper-activation of the NLRP3 inflammasome may also be related to prolonged exposure to the products of ethanol metabolism (5).

The NLRP3 inflammasome is a cytoplasmic complex of intracellular sensors such as NOD-like receptors coupled with procaspase-1 and the apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (ASC) (6). Molecular damage triggers the assembly of the NLRP3 inflammasome leading to and IL-1β secretion and caspase-1 activation (7), which has been associated with early development of atherosclerosis in cerebral vessels and other heritable and acquired inflammatory diseases (8).

As *NLRP3* gene mutations may result in increased inflammasome activation and higher secretion of IL-1β (7), genetic variability of the genes coding for the NLRP3 inflammasome components was investigated in several diseases with an inflammatory component, such as Alzheimer's disease, atherosclerosis, inflammatory bowel

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disease, rheumatoid arthritis, and type 1 diabetes (9). One of the most commonly investigated single nucleotide polymorphisms (SNPs) coding for overactive NLRP3 inflammasome is the nonsynonymous gain-of-function polymorphism p.Q705K (rs35829419) in the *NLRP3* gene (10). Another polymorphism extensively studied is p.C10X (rs 2043211) in the *CARD8* gene, which codes for a non-functional protein and leads to loss of CARD8 inhibition of caspase-1. This polymorphic allele has been associated with increased cell death, although the actual role of CARD8 remains unclear (11). These two polymorphisms were not studied extensively in mental disorders, with a few exceptions (12, 13), even though it is known that alcohol dependence entails a 30–75 % higher risk of co-occurring mental disorders (14–16), including depression, anxiety, aggression, personality disorders, and dependence on other psychoactive substances (17–21).

However, up to date no human study has investigated the association between NLRP3 polymorphisms and alcohol dependence, and the aim of our study was to fill that gap by investigating the association between the above two polymorphisms, namely *NLPR3* rs35829419 and *CARD8* rs2043211, and alcohol dependence and symptoms of its usual comorbidities, i.e. anxiety, depression, and obsessive-compulsive or aggressive behaviour.

PARTICIPANTS AND METHODS

The study included only male participants (to exclude the influence of sex differences, see ref. 22) aged from 18 to 65 years from the Slovenian (Caucasian) population. The first group (group 1; N=88) included hospitalised alcohol-dependent patients who met the criteria of alcohol dependence of the 4th edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (23) and were no longer having major abstinence symptoms (after having spent at least two weeks in respective departments/units for addiction treatment). The second group (group 2; N=99) included abstinent alcohol-dependent individuals recruited from support group meetings who had maintained full abstinence for more than two years. Participants of either group were excluded if they had a history of abuse or dependence of psychoactive substances other than nicotine and other major mental or neurological disorders or significant medical illnesses in their medical records. The third group (group 3; N=94) included healthy controls recruited from blood donors, who answered a short structured clinical questionnaire through interview to exclude DSM-IV Axis I disorders or clinical problems with alcohol consumption.

All participants also gave information about their residence (rural or urban), partnership status (single or in partnership: either married or living in the extramarital union), and years of education and signed informed consent.

The following questionnaires were employed: Zung Depression (24) and Anxiety (25) scale and Brief Social Phobia Scale (BSPS) (26) to rate depression and anxiety symptoms, Alcohol Use Disorders Identification Test (AUDIT) (27) to define drinking habits and severity of alcohol problems and dependence, Yale-Brown Obsessive Compulsive Scale (YBOCS) (28) and Obsessive Compulsive Drinking Scale (OCDS) (29) to rate obsessive-compulsive traits, and Buss-Durkee Hostility Inventory (BDHI) (30) to rate symptoms of aggression and hostility. All the questionnaires were administered at the entry into the study by the same rater, who was blinded to the genotyping results.

The study was approved by the Slovenian National Medical Ethics Committee (approval No. 117/06/10 and 148/02/1011) and followed the latest version of the Declaration of Helsinki (31). Each participant received a code to protect all the personal and medical information. All biological and DNA samples were processed and analysed under these codes.

Blood sampling and DNA extraction

DNA was isolated from either 5 mL of whole blood collected by venepuncture from groups 1 and 3 (hospitalised patients and healthy controls, respectively) or from a buccal swabs collected from group 2 (abstinents). Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes as part of regular blood testing (group 1) or donation (group 3) and stored at +4 °C until extraction. For DNA extraction from blood samples we used the QIAamp Blood Mini Kit and for the extraction from buccal swabs the QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions (32).

NLRP3 rs35829419 (c.2113C>A, p.Gln705Lys) and *CARD8* rs2043211 (c.304A>T, p.Phe102Ile, p.Cys10Ter) were genotyped using fluorescence-based competitive allele-specific PCR (KASP) amplification combined with a KASP reporting system. Briefly, we added the universal KASP Master mix and SNP-specific KASP Genotyping Assay (KBioscience, Hoddesdon, Herts, UK) to the extracted DNA samples on transparent 96-well PCR plates. Thermal cycling reaction was performed according to the manufacturer's instructions and followed by end-point fluorescence detection. Genotyping was blind to any clinical data and was randomly repeated in 20 % of the samples to check for reliability. These repetitions confirmed initial findings in all such samples.

Statistical analysis

Pearson's chi-squared test was used to compare SNP frequencies between the three groups and their effects on categorical variables, i.e. total scores on applied questionnaires. One-way analysis of variance (ANOVA) was used to assess SNP effects and continuous variables in each of the three groups separately. The associations between each SNP and continuous variables (genotype-

phenotype associations) were compared between the groups with the factorial ANOVA. The level of statistical significance was set at 0.05. The study had sufficient power (0.80) to detect small-to-medium effect sizes (f2=0.021 and d=0.355). All statistics were run on the Statistica package, version 7.0 for Windows® (StatSoft Italia, Vigonza, Padua, Italy).

RESULTS

Table 1 shows participant demographic characteristics of interest. Hospitalised and abstinent alcohol-dependent patients were significantly older (P<0.001; df=2; F=46.080) and had fewer years of education than controls (P<0.001; df=2; F=46.080). Significantly more abstinent alcohol dependents and controls were in partnership than hospitalised alcohol dependents (P=0.004; df=2; F=5.601).

Genotype distribution of *CARD8* did not deviate significantly from the Hardy-Weinberg equilibrium (HWE), but we found a deviation for *NLPR3* in controls (Table 2). The three groups significantly differed in the distribution of the *CARD8* rs2043211 genotypes (P=0.049; chi-squared=9.557; df=4) but not in the distribution of the *NLPR3* rs35829419 genotypes.

No significant association was found between the *NLPR3* genotypes and selected mental disorder symptoms (Table 3). On the other hand, *CARD8* rs2043211 genotypes were significantly associated with Zung Anxiety Scale scoring among abstinent alcohol-dependent participants (P=0.048; df=2, F=3.140). In this group, *CARD8* rs2043211 TT genotype carriers reached the highest mean scores on the Zung Anxiety Scale (Table 4). In controls the *CARD8* rs2043211 genotype was associated with OCDS scoring (P=0.027; df=2; F=3.744). However, because control candidates with clinical problems with alcohol consumption were excluded from the study and because OCDS measures obsessive-compulsive symptoms related to alcohol drinking, this result may be considered a chance finding that has no clinical importance.

DISCUSSION

We found that *CARD8* rs2043211 was associated with the risk of alcohol dependence, but found no such association for *NLPR3* rs35829419. However, our findings are limited only to males. A recent study on rodents (22) reported NLPR3 inflammasome-dependent differences in alcohol consumption between male and female mice, but there are no reports of similar kind in humans.

Another significant association we did observe is the one between *CARD8* rs2043211 and anxiety in abstinent alcohol-dependent participants. This finding supports the

Table 1 Socio-demographic characteristics of study participants

Characteristics	Hospitalised alcohol-dependent patients (n=88)	Abstinent alcohol-dependent participants (n=99)	Controls (n=94)	p-value
Age (years)	45.8±10.0	49.1±8.1	34.4±11.7	0.003
Education (years)	11.3±2.2	11.7±2.4	12.7±1.9	0.001
Single	38 (43 %)	22 (22 %)	24 (25 %)	0.004
In partnership	50 (56 %)	77 (78 %)	70 (75 %)	
Rural residents	45 (51 %)	48 (48 %)	36 (38 %)	0.293
Urban residents	43 (49 %)	51 (52 %)	58 (62 %)	

Means ± standard deviations are given for continuous or the number of participants for categorical variables. Partnership means marital or extramarital union. p-value is shown for the comparison between all three groups

Table 2 *NLPR3* rs35829419 and *CARD8* rs2043211 genotype distribution by groups

Genotypes	Hospitalised alcohol-dependent patients (n=88)	Abstinent alcohol-dependent participants (n=99)	Controls (n=94)	p-value[1]	Merged alcohol-dependents (n=185)	p-value[2]
NLPR3						
CC	78 (88 %)	87 (88 %)	87 (93 %)	0.838	165 (88 %)	0.500
AC	9 (11 %)	11 (11 %)	6 (6 %)		20 (11 %)	
AA	1 (1 %)	1 (1 %)	1 (1 %)		2 (1 %)	
CARD8						
AA	34 (39 %)	52 (53 %)	45 (48 %)	**0.049**	86 (46 %)	0.055
AT	44 (51 %)	42 (42 %)	34 (36 %)		86 (46 %)	
TT	9 (10 %)	5 (5 %)	15 (16 %)		14 (8 %)	

p-[1] comparison between all three groups. p-[2] comparison between the merged groups of alcohol-dependents and controls. Bolded p-values are statistically significant (p<0.05)

Table 3 Associations between *NLPR3* rs35829419 genotypes and alcohol-related mental disorders

Mental disorders	*NLPR3* genotype	Hospitalised alcohol-dependent patients		Abstinent alcohol-dependent participants		Controls	
		Mean score±SD	p-value	Mean score±SD	p-value	Mean score±SD	p-value
Obsession (YBOCS)	CC	4.2±4.6	0.742	1.8±2.2	0.661	1.7±1.5	0.498
	AC	3.6±4.4		1.3±2.5		1.0±0.0	
	AA	1.0		1.0		1.0	
Compulsion (YBOCS)	CC	2.9±3.1	0.787	1.4±1.8	0.810	1.3±0.9	0.644
	AC	3.2±3.3		1.1±0.3		1.0±0.0	
	AA	1.0		1.0		1.0	
Social phobia (BSPS)	CC	12.9±11.0	0.555	12.1±10.1	0.353	10.5±7.2	0.615
	AC	8.8±10.9		16.5±11.1		8.7±1.3	
	AA	10.0		18.0		5.0	
Obsessive-compulsive drinking (OCDS)	CC	18.9±11.1	0.681	3.2±2.3	0.757	3.5±1.7	0.926
	AC	15.8±13.2		3.7±3.3		3.3±0.8	
	AA	14.0		3.0		4.0	
Depression (Zung)	CC	35.9±11.0	0.881	31.1±7.4	0.653	23.0±3.8	0.311
	AC	34.9±11.5		29.3±6.1		21.8±2.4	
	AA	31.0		27.0		28.0	
Anxiety (Zung)	CC	34.9±8.4	0.535	30.1±6.9	0.886	22.6±3.1	0.742
	AC	31.9±7.1		31.1±5.6		22.7±3.4	
	AA	31.0		29.0		25.0	
Aggression (BDHI)	CC	24.3±10.8	0.853	20.5±10.3	0.735	14.8±8.8	0.220
	AC	26.0±7.2		19.6±7.4		10.2±4.1	
	AA	21.0		13.0		25.0	
Alcohol dependence – severity (AUDIT)	CC	23.0±6.8	0.069				
	AC	28.4±4.0					
	AA	23.0					

AUDIT – Alcohol Use Disorders Identification Test; BDHI – Buss-Durkee Hostility Inventory; BSPS – Brief Social Phobia Scale; OCDS – Obsessive Compulsive Drinking Scale; YBOCS – Yale-Brown Obsessive Compulsive Scale

hypothesis that inflammasome could contribute to the development of psychiatric disorders associated with alcohol dependence (33–35). To the best of our knowledge, this is the first such finding, as no human study conducted so far reported a direct association between *CARD8* and anxiety in alcohol dependents.

The limitation of our study is a relatively small but ethnically homogeneous sample. The advantage is that all the questionnaires were applied by the same rater.

In conclusion, our findings point to a link between the genes of the innate immune system and alcohol dependence. However, further studies with larger cohorts are required to confirm these preliminary findings.

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Conflicts of interest

The authors declare no conflict of interest. Our data were in part presented as abstract at the conference of the International Society for the Study of Xenobiotics.

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Table 4 Associations between *CARD8* rs2043211 genotypes and alcohol-related mental disorders

Mental disorder	CARD8 genotype	Hospitalised alcohol-dependent patients		Abstinent alcohol-dependent subjects		Controls	
		Mean score±SD	p-value	Mean score±SD	p-value	Mean score±SD	p-value
Obsession (YBOCS)	AA	3.6±4.0	0.244	1.4±1.9	0.203	1.5±1.3	0.591
	AT	2.7±5.1		2.0±2.0		1.8±1.7	
	TT	2.2±2.2		2.8±4.0		1.8±1.3	
Compulsion (YBOCS)	AA	2.9±3.5	0.481	1.3±1.9	0.960	1.2±0.6	0.406
	AT	3.0±2.9		1.5±1.3		1.4±1.2	
	TT	1.7±1.7		1.4±0.9		1.3±0.6	
Social phobia (BSPS)	AA	10.2±8.8	0.291	11.7±8.2	0.567	10.9±7.7	0.695
	AT	12.7±11.5		13.9±12.3		10.2±7.0	
	TT	16.0±10.3		13.2±10.8		9.1±4.4	
Obsessive-compulsive drinking (OCDS)	AA	16.2±11.6	0.161	3.5±2.7	0.401	3.5±1.4	**0.027**
	AT	18.9±10.9		2.9±2.0		3.9±2.0	
	TT	24.1±10.8		2.5±0.9		2.6±0.8	
Depression (Zung)	AA	34.1±8.3	0.560	30.0±6.7	0.418	23.5±4.3	0.201
	AT	36.6±11.7		31.5±8.0		22.1±3.3	
	TT	37.1±16.3		33.6±6.5		23.4±3.7	
Anxiety (Zung)	AA	32.9±6.2	0.320	29.1±5.6	**0.048**	23.0±3.5	0.477
	AT	35.5±9.1		30.8±7.4		22.1±2.7	
	TT	36.1±10.6		36.4±9.5		22.5±2.5	
Aggression (BDHI)	AA	26.0±8.7	0.534	19.8±8.3	0.758	15.0±9.0	0.970
	AT	23.3±11.6		20.7±11.9		14.2±9.3	
	TT	23.4±11.6		23.0±10.3		14.3±6.5	
Alcohol dependence – severity (AUDIT)	AA	23.4±6.5	0.818				
	AT	23.9±7.2					
	TT	22.3±5.4					

Bolded p-values are statistically significant (p<0.05). AUDIT – Alcohol Use Disorders Identification Test; BDHI – Buss-Durkee Hostility Inventory; BSPS – Brief Social Phobia Scale; OCDS – Obsessive Compulsive Drinking Scale; YBOCS – Yale-Brown Obsessive Compulsive Scale

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Povezave genskih polimorfizmov NLRP3 in CARD8 z odvisnostjo od alkohola in pogosto povezanimi psihiatričnimi motnjami: preliminarna raziskava

Raziskovali smo dva funkcionalna polimorfizma v genih NLPR3 inflamasoma (*NLRP3* rs35829419 in *CARD8* rs2043211) in njuno vlogo pri pojavnosti odvisnosti od alkohola ali izražanju anksiozne, depresivne, obsesivno-kompulzivne ali agresivne sopojavne simptomatike ob odvisnosti od alkohola. Vključili smo tri skupine kavkazijskih moških: 88 hospitaliziranih bolnikov in 99 abstinirajočih preiskovancev z odvisnostjo od alkohola ter 94 kontrol. Za oceno sopojavne simptomatike smo uporabili Zungovo lestvico depresivnosti in anksioznosti, Buss-Durkeejevo lestvico sovražnosti, AUDIT (angl. Alcohol Use Disorders Identification Test), Kratko lestvico socialne fobije, Lestvico obsesivno-kompulzivnega pitja in Yale-Brownovo lestvico obsesivnosti in kompulzivnosti. Za genotipizacijo smo uporabili alelno specifično kvantitativno polimerazno verižno reakcijo (PCR). Porazdelitev *CARD8* rs2043211 se je med preiskovanimi skupinami statistično pomembno razlikovala (p=0,049; hi-kvadrat=9,557; df=4). Polimorfizem *NLPR3* rs35829419 ni bil pomembno povezan z odvisnostjo od alkohola.. Pri preiskovancih hospitaliziranih zaradi odvisnosti od alkohola preiskovani polimorfizmi niso bili statistično povezani z večjo porabo alkohola ali s sopojavno simptomatiko. Genotip *CARD8* je bil statistično značilno povezan z oceno po Zungovem vprašalniku anksioznosti pri abstinirajočih preiskovancih, kjer so homozigoti za polimorfni alel dosegali višje rezultate (p=0,048; df=2; F=3,140). Med kontrolami je bil genotip *CARD8* povezan z ocenami na lestvici obsesivno kompulzivnega pitja (p=0,027; df=2; F=3,744). Naši rezultati nakazujejo nova dognanja, da *CARD8* rs2043211 lahko igra določeno vlogo pri pojavnosti odvisnosti od alkohola, izražanju anksiozne simptomatike pri abstinirajočih preiskovancih z odvisnostjo od alkohola in pri obsesivno kompulzivnem pitju pri zdravih kontrolah. Naše preliminarne izsledke pa je potrebno ovrednotiti z nadaljnjimi raziskavami na večjih vzorcih.

KLJUČNE BESEDE: anksioznost; genetski polimorfizem, inflamasom; obsesivno kompulzivno pitje; zasvojenost z alkoholom