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1 **Original Article**

2

3 ***OPRM1* A118G and serum β-endorphin interact with sex and digit ratio (2D:4D) to influence**

4 **risk and course of alcohol dependence**

5

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Abstract

Activation of mesolimbic mu-opioid receptor by its endogenous ligand, β-endorphin, mediates part of the rewarding effects of alcohol, yet there is controversial evidence surrounding the relationship between the functional mu-opioid receptor gene (*OPRM1*) A118G single nucleotide polymorphism and alcohol dependence risk. Some preclinical evidence suggests that sex and sex hormone-dependent prenatal brain organization may interact with the opioid system to influence alcohol drinking behavior. We genotyped 200 alcohol-dependent patients and 240 healthy individuals for the A118G variant and measured serum β-endorphin level at recruitment and during acute withdrawal. We then evaluated the association between these factors and alcohol dependence risk and outcome in the context of both sex and second-to-fourth digit length ratio (2D:4D) – a biomarker of prenatal sex hormone load. For the first time, the AA genotype was found to be associated with elevated alcohol-related hospital readmission risk, more readmissions, and fewer days until first readmission in male but not female patients. Upon accounting for 2D:4D, the G-allele predicted alcohol dependence and more readmissions (1 vs ≥2) in males, suggesting prenatal sex hormones interact with *OPRM1* to influence addiction pathology. Withdrawal β-endorphin level also correlated negatively with withdrawal severity in females but not in males, indicating β-endorphin might protect against withdrawal-induced stress in a sex-specific manner. Organizational effects of sex hormones may prime individuals for alcohol dependence by inducing permanent changes to the endogenous opioid system.

Keywords: mu-opioid receptor gene (*OPRM1*); A118G; β-endorphin; alcohol dependence; prenatal sex hormones

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52 **1. Introduction**

53 Activation of mesolimbic mu-opioid receptor (MOR) by its primary endogenous ligand, β-
54 endorphin (β-END), has been shown to mediate part of the rewarding effects of alcohol via stimulating
55 nucleus accumbens dopamine release (Heilig et al., 2011). For this reason, MOR and β-END are
56 considered high-interest pharmacological targets for the treatment and prevention of alcohol
57 dependence and relapse. The relatively common single nucleotide polymorphism (SNP) rs1799971
58 within exon 1 of the mu-opioid receptor gene (*OPRM1*), in which an adenine-guanine transition
59 (A118G) encodes for an asparagine-aspartic acid substitution (N40D), has been studied in the context
60 of alcohol dependence due to its strong yet opposing effects on receptor function and expression. The
61 G-allele has been reported to increase binding affinity for β-END three-fold (Bond et al., 1998) and
62 cause a four-fold increase in ventral striatal dopamine release following alcohol stimulation
63 (Ramchandani et al., 2011) but also reduce *OPRM1* mRNA and protein level in human post-mortem
64 brain tissue and transfected Chinese hamster ovary cells (Zhang et al., 2005).

65 Despite these potent functional effects, previous meta-analyses have been unable to establish a
66 clear association between *OPRM1* A118G and alcohol dependence in humans (Chen et al., 2012;
67 Schwantes-An et al., 2016). Several studies have found the G-allele to be associated with elevated
68 dorsal striatal cue-reactivity (Bach et al., 2015), craving (van den Wildenberg et al., 2007), and
69 subjective feelings of stimulation and happiness (Ray et al., 2004; 2013) following alcohol
70 consumption. However, epidemiological research and meta-analyses have linked both the A- and G-
71 allele to increased risk of alcohol dependence amongst European cohorts consisting of both sexes
72 (Bart et al., 2005; Schwantes-An et al., 2016). This inconsistency may be due to an inability or failure
73 to account for certain confounding factors.

74 There is evidence suggesting that sex-*OPRM1*, sex-β-END, and prenatal sex hormone-*OPRM1*
75 interactions may influence addiction pathology. Barr et al. (2007) found that the G-allele of the
76 primate equivalent of the A118G SNP significantly increases alcohol-induced stimulation, ethanol
77 consumption, alcohol preference, and percentage of days intoxicated in male but not female rhesus
78 macaques. In a humanized mouse model carrying this polymorphism, male GG mice self-administered
79 more nicotine than male AA mice, whereas there was no difference in female animals (Bernardi et al.,

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80 2016). A different mouse model containing a point mutation (A112G) equivalent to the human A118G
81 SNP also revealed male GG mice tend to have a higher conditioned place preference for a morphine-
82 paired environment than male AA mice (Mague et al., 2009). In humans, naltrexone, a MOR
83 antagonist, significantly and dose-dependently reduces the number of heavy drinking days in males
84 but not in females (Garbutt et al., 2005). However, few studies have investigated sex-specific *OPRM1*
85 genotype effects on alcohol dependence in a clinical cohort. Based on preliminary evidence, we
86 hypothesize that the A118G SNP as well as three additional *OPRM1* promoter variants relate more
87 strongly to alcohol dependence risk and outcome in males or females than in the combined cohort.
88 Furthermore, since male heavy drinker plasma β-END level has been shown to be higher than in
89 female heavy drinkers (Gianoulakis et al., 2003), peripheral β-END levels in patients, before and
90 during withdrawal, and in healthy individuals were investigated on a sex-specific basis.
91 Recent evidence has also revealed an interaction between prenatal testosterone level and
92 *OPRM1* influencing alcohol consumption in rodents (Huber et al., in press). In humans, elevated
93 exposure to androgens during the prenatal development window has been associated with increased
94 risk of alcohol dependence (Kornhuber et al., 2011; Lenz et al., 2017) and other behavioral disorders
95 such as video game addiction (Kornhuber et al., 2013). From a mechanistic standpoint, sex hormones
96 have been shown to induce permanent, organizational changes to the brain's reward system at the
97 neuronal and molecular level (Brown et al., 2015). Huber et al. (in press) found in mice that prenatal
98 treatment with flutamide, a potent androgen receptor antagonist, caused a significant reduction in adult
99 ventral striatal *OPRM1* RNA and a significant increase in alcohol self-administration. Given this
100 finding, we decided to also investigate prenatal sex hormone-*OPRM1* effects on alcohol dependence
101 and outcome, using the second-to-fourth digit length ratio (2D:4D) as a biomarker for prenatal sex
102 hormone load (Berenbaum et al., 2009; Zheng and Cohn, 2011).
103
104 **2. Methods**
105
106 *2.1 Cohort characteristics*

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107 The individuals included in this investigation were recruited between 2013 and 2014 for the
108 **N**eurobiology **of** **A**lco**h**olism (NOAH) study at the Universitätsklinikum Erlangen Department of
109 Psychiatry and Psychotherapy and the Klinikum am Europakanal Clinic for Psychiatry,
110 Psychotherapy, and Psychosomatic Medicine in Erlangen, Germany (Lenz et al., 2017). 200 early-
111 abstinent, alcohol-dependent patients (113 males and 87 females) admitted as inpatients for
112 withdrawal treatment were selected through an intensive screening process, and 240 healthy controls
113 (133 males and 107 females) were selected after two telephone screenings and an on-site interview, in
114 which individuals with prior psychiatric inpatient treatment and/or any psychiatric outpatient treatment
115 during the past ten years were excluded. Median age (years) for both patients and controls was 48
116 (interquartile ranges [IQR] 42/54 and 39/56) (Mann-Whitney *U* test [MWT], $p > 0.05$), and median
117 body mass index (kg/m^2) was 24.7 (IQR 22.1/28.2) and 26.5 (IQR 23.5/29.3) (MWT, $p > 0.05$),
118 respectively. In the patient group, median lifetime drinking (kg) and daily ethanol intake (g/d since
119 onset) were 483 (IQR 270/1,195) and 120 (IQR 58/240). Whole blood, behavioral scores, and other
120 parameters (Lenz et al., 2017) were collected at time of recruitment (day A) in patients and controls
121 and a median of five days later (day B) in patients only, during which they underwent withdrawal. The
122 German version of the Clinical Institute Withdrawal Assessment for Alcohol revised (CIWA-Ar) scale
123 was used to measure alcohol withdrawal severity (Stuppaeck et al., 1994). Blood samples were
124 collected in the morning for all individuals to minimize circadian effects on hormone level.
125 Individuals' hands were scanned using the HP Scanjet G4050 (Hewlett-Packard, Palo Alto, CA,
126 USA), and 2D:4D was calculated by three blinded raters, who measured the absolute lengths of the
127 second and fourth digits (GNU Image Manipulation Program, www.gimp.org). Lower 2D:4D
128 indicates higher prenatal androgen and lower estrogen load, and higher 2D:4D suggests the opposite.
129 Medical records for each patient were accessed 24-months after study recruitment to investigate the
130 number of alcohol-related hospital readmissions and days until first readmission.
131
132 *2.2 Blood preparation*

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133 After collection, blood samples were centrifuged (10 min, 2000 g at room temperature), and
134 serum was placed into storage at -80°C. Genomic DNA (gDNA) was extracted from whole blood
135 using the Gentra Puregene Blood Kit (Qiagen, Venlo, Netherlands) and stored at 4°C.
136
137 *2.3 Genotyping*
138 Genotyping for three *OPRM1* SNPs (rs1799971 [A118G], rs3798677, rs3798678) was
139 performed by high resolution melting (HRM) of polymerase chain reaction (PCR) products in the
140 Roche LightCycler 480 II (Roche Holding AG, Basel, Switzerland). The PCR primer sequences are
141 listed in Table 1. The reaction mixture consisted of 1x Rovalab buffer and 0.1 U Taq polymerase
142 (Rovalab GmbH, Teltow, Germany), 0.03 µl CyGreen (1:100 dilution, Enzo Life Sciences Inc.,
143 Farmingdale, NY, USA), 10 ng gDNA, 1.5-3.0 mM $MgCl_2$ (Table 1), 200 µM dNTPs (each), and 200
144 nM forward and reverse primers (Sigma-Aldrich, St. Louis, MO, USA) in a total volume of 10 µl.
145 PCR conditions were: 2 min at 95°C followed by 40 x (15s at 95°C, 30s at primer-specific annealing
146 temperatures [Tab. 1] 15s at 72°C). After PCR, product denaturation was performed at 95°C for one
147 minute, followed by rapid cooling to 40°C. A melting curve was generated by heating at 0.02°C/s
148 from 70°C to 90°C, and analysis was performed using the Roche LightCycler 480 Gene Scanning
149 Software v1.5. Genotypes for the three SNPs were confirmed by Sanger sequencing of gDNA samples
150 selected to act as standards (AA, AG, GG for each SNP).
151 Genotyping for the *OPRM1* CAn dinucleotide repeat polymorphism, described in Kranzler et
152 al. (1998), was performed by fragment length analysis of PCR products in an Applied Biosystems 48-
153 capillary array (Thermo Fisher Scientific, Waltham, MD, USA). The PCR primer sequences were the
154 same as for genotyping rs3798678 except the forward primer was fluorescently tagged with 6-
155 fluorescein amidite (Sigma-Aldrich, St. Louis, MO, USA). PCR reaction mixture and conditions were
156 the same as for the rs3798678 HRM-PCRs, except for the absence of the CyGreen intercalating dye.
157 Following PCR, products were diluted 1:20 in MilliQ water, and 1 µl of the dilution was mixed with
158 10 µl formamide (Sigma-Aldrich) and 0.5 ul GeneScan 500 ROX dye size standard (Thermo Fisher
159 Scientific) prior to fragment length analysis. CAn genotypes were confirmed by Sanger sequencing.

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160 10% of all samples' genotypes for the polymorphisms were replicated with 100% accordance.
161 The four genetic variants were in Hardy-Weinberg equilibrium within the NOAH cohort (rs1799971
162 [A118G]: χ^2 = 0.180, p = 0.672; rs3798677: χ^2 = 1.521, p = 0.217; rs3798678: χ^2 = 1.521, p = 0.217;
163 CAn: χ^2 = 13.550, p = 0.809).
164

165 *2.4 β-END quantification by enzyme-linked immunosorbent assay (ELISA)*

166 Serum β-END was quantified using the Endorphin, beta (Human) EIA Kit by Phoenix
167 Pharmaceuticals, Inc. (Burlingame, CA, USA) following the manufacturer's guidelines. 25 µl of
168 original, unpurified serum were assayed in duplicate, and peptide concentrations were extrapolated
169 from a standard curve (7 dilutions from 30 ng/ml to 0.01 ng/ml), which was run on every 96-well plate
170 to minimize risk of interplate variation. The intra-assay and inter-assay coefficients of variation were
171 7% and 14%, respectively.
172

173 *2.5 Statistics*

174 Alpha (two-tailed) was set to 0.5. If not otherwise stated, we report median and IQR. For
175 group comparisons, we used MWTs, Kruskal-Wallis tests (KWTs), and Wilcoxon signed-rank tests.
176 The Spearman's method was employed to evaluate bivariate correlations and χ^2 tests with odds ratios
177 (OR) for differences in the frequencies. Log-rank [Mantel-Cox] tests were used for survival curves. To
178 increase statistical power, heterozygous subjects were combined with homozygous minor allele
179 individuals for SNP analysis (AA study subjects vs. G-allele carriers). In case of missing data points,
180 study subjects were excluded from the specific analyses, and the exact case number is stated. Data
181 were analyzed using IBM SPSS Statistics Version 21 for Windows (SPSS Inc., Chicago, IL, USA) and
182 Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA).
183

184 **3. Results**

185

186 *3.1 Trend for an association between OPRM1 A118G and alcohol dependence risk in females*

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187 The *OPRM1* A118G genotype (AA vs. AG/GG) did not predict risk of alcohol dependence in
188 the total cohort (χ^2 test; OR = 1.12 [95%-confidence interval (95%-CI) 0.71-1.76], χ^2 = 0.233, p =
189 0.629) or the male subgroup (OR = 0.81 [95%-CI 0.45-1.44], χ^2 = 0.528, p = 0.467). However, there is
190 a trend for AA females to have an elevated risk (OR = 1.91 [95%-CI 0.89-4.06], χ^2 = 2.848, p =
191 0.091).
192
193 *3.2 Sex-specific association between OPRM1 A118G and prospective alcohol-related hospital*
194 *readmission*
195 *OPRM1* A118G significantly predicted the outcome of male patients but not of female patients
196 over the 24-month period following study recruitment. In males, the AA genotype was significantly
197 associated with an elevated risk of alcohol-related hospital readmission (Males, readmitted: n(AA) =
198 62, n(AG/GG) = 14, non-readmitted: n(AA) = 21, n(AG/GG) = 16; females, readmitted: n(AA) = 38,
199 n(AG/GG) = 8, non-readmitted: n(AA) = 37, n(AG/GG) = 4; Fig. 1A), higher median number of
200 readmissions (Fig. 1B), and fewer mean days until first readmission (Fig. 1C). No significant
201 associations between genotype and outcome were observed in females (readmitted: n(AA) = 38,
202 n(AG/GG) = 8, non-readmitted: n(AA) = 37, n(AG/GG) = 4; Fig. 1A-B, D).
203
204 *3.3 No significant associations of rs3798677, rs3798678, and CAn polymorphisms with alcohol*
205 *dependence or outcome*
206 10 and 14-18 CAn repeats were detected within the NOAH cohort (Tab. 2). CAn allele length
207 and genotype were investigated for associations with alcohol dependence or outcome in the NOAH
208 cohort. Mean CAn allele length did not significantly predict risk of alcohol dependence or alcohol-
209 related hospital readmission (MWTs), nor was it significantly correlated with number of readmissions
210 or days until first readmission (Spearman-Rho) in both the total group and the male and female
211 subgroups. Similarly, no significant associations were observed between CAn genotype and alcohol
212 dependence or outcome (χ^2 tests, KWTs).
213 Promoter region SNPs rs3798677 (-73 bp relative to CAn) and rs3798678 (-3 bp relative to
214 CAn) had identical genotypes in the cohort (Tab. 1) and were also found to be in strong linkage

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215 disequilibrium with A118G (rs3798677 AA/G-allele carriers vs. A118G AA/G-allele carriers; χ^2 =
216 8.287, p = 0.004). Similar to the CAn promoter variant, rs3798677 and rs3798678 did not significantly
217 predict dependence or outcome and were also not associated with the number of readmissions or days
218 until first readmission (χ^2 tests, MWTs, Log-rank [Mantel-Cox] tests).
219
220 *3.4 Serum β-END level declines during acute withdrawal in male and female patients and relates to*
221 *withdrawal severity*
222 At baseline, there was no significant difference in median serum β-END level between
223 patients and controls in both males and females. During a median of five days withdrawal, patient β-
224 END concentrations declined significantly to a level that was also significantly lower than in control
225 subjects (Fig. 2A). Moreover, there was a significant negative correlation between withdrawal serum
226 β-END level (day B) and CIWA-Ar withdrawal severity score in female patients (Fig. 2B) but not in
227 males (Spearman-Rho, n = 82, ρ = -0.097, p = 0.385). No significant associations between patient
228 serum β-END level and outcome were detected, except for one finding that day B β-END level was
229 correlated with days until first readmission in males (Spearman-Rho, n = 94, ρ = -0.232, p = 0.025).
230
231 *3.5 A118G-2D:4D interaction influences risk and outcome of alcohol dependence*
232 To test whether AA study subjects differ from G-allele carriers with regard to the effect of
233 prenatal sex hormone load on risk of alcohol dependence, we sex- and genotype-specifically
234 normalized patients' 2D:4D values to the mean 2D:4D and its standard deviation of the control group
235 (= [each patient's 2D:4D value – mean controls' 2D:4D value] / standard deviation of controls' 2D:4D
236 values). Male G-allele carrier patients differed significantly from AA male patients with regard to the
237 normalized 2D:4D values (Fig. 3A), meaning a significantly stronger 2D:4D deviation between
238 patients and controls in the G-allele carrier group than in the AA group. No significant 2D:4D-A118G
239 interaction was observed in females (Fig. 3B). Subsequently, we explored whether similar interactions
240 are also relevant to the patients' outcome. We previously reported a significant negative correlation
241 between 2D:4D values and number of alcohol-related hospital readmissions during the 12-month
242 follow-up in individuals with at least one readmission (Lenz et al., 2017). Thus, we sex- and genotype-

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243 specifically normalized 2D:4D values of each patient with at least two readmissions to the mean
244 2D:4D and standard deviation of patients with only one alcohol-related readmission during the follow-
245 up (= [2D:4D value of each patient with one readmission – mean 2D:4D values of patients with at
246 least two readmissions] / standard deviation of 2D:4D values of patients with at least two
247 readmissions). In support of the previous finding, male G-allele carrier patients deviated significantly
248 from AA male patients with regard to the normalized 2D:4D values (Fig. 3C), meaning a significantly
249 stronger 2D:4D deviation between patients with one readmission and patients with at least two
250 readmissions in the G-allele carrier group than in the AA group. No significant 2D:4D-A118G
251 interaction with respect to alcohol-related readmission was found in females (Fig. 3D). These two
252 observations suggest that in males, higher prenatal androgen (and lower estrogen) load interacts with
253 the *OPRM1* A118G G-allele to elevate risk and worsen outcome of alcohol dependence.
254

255 **4. Discussion**

256 Over the last 30 years, the proportion of neuroscience studies including both sexes has grown
257 substantially, yet approximately 80% of such publications still fail to analyze results by sex despite
258 clear sex differences in the risk and pathology of most major neuropsychiatric disorders, including
259 alcohol dependence (Beery and Zucker, 2011). The present study aimed to investigate sex-specific
260 *OPRM1* and β-END effects on alcohol dependence risk and outcome as well as prenatal sex hormone-
261 *OPRM1* interactions.

262 In the NOAH cohort, the *OPRM1* A118G SNP did not predict alcohol dependence in either
263 males or females but related sex-specifically to outcome. To our knowledge, this is the first
264 publication to demonstrate that the AA genotype is associated with increased risk of alcohol-related
265 hospital readmission, more readmissions, and fewer days until first readmission in male patients and
266 therefore may be relevant for predicting relapse in a clinical setting. In previous naltrexone studies,
267 Oslin et al. (2003) and O'Malley et al. (2008) found that placebo-treated AA alcohol-dependent
268 patients were more likely to relapse than G-allele carriers, but this association did not achieve
269 statistical significance – perhaps because a large proportion of the two study cohorts consisted of
270 females. Schacht et al. (2017) also did not observe a significant difference in percentage of heavy

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271 drinking days between AA individuals and G carriers, although the duration of their study (10 months)
272 was shorter than ours (24 months). Considering the G allele has been shown to strongly increase MOR
273 binding affinity for β-END, we hypothesize the AA genotype elevates readmission risk by
274 hyposensitizing MOR, causing dependent patients to consume more alcohol in order to overcome
275 intrinsic reward deficit (Kleinjan et al., 2015).

276 Interestingly, we found that the A118G polymorphism does predict alcohol dependence in
277 male patients upon accounting for 2D:4D, suggesting *OPRM1* and sex hormones interact in the
278 prenatal state to influence addiction risk in adult life. Additionally, 2D:4D and *OPRM1* A118G
279 interact to predict more readmissions (1 vs $\geq$2) in male patients; the group of patients with no recorded
280 alcohol-related readmissions was excluded because it does not account for possible hospital
281 readmissions outside the Erlangen area. The hypothesized interaction between prenatal sex hormone
282 load and MOR signaling has recently been confirmed in a rodent study, in which intraperitoneal
283 injection of pregnant dams with the potent androgen receptor antagonist flutamide resulted in
284 significantly reduced ventral striatal *OPRM1* RNA expression and reduced alcohol consumption in the
285 adult male offspring (Huber et al., in press). Other studies indirectly corroborate this finding. Prenatal
286 stress, which is associated with children's 2D:4D, has been shown to influence adult pain sensitivity in
287 a sex-specific manner (Butkevich et al., 2007), and prenatal exposure to cigarette smoke – another
288 contributor to prenatal androgen load (Lenz et al., 2017) – reduces adult ventral striatal reward
289 response (Müller et al., 2013) and also interacts with the *OPRM1* rs2281617 SNP to elevate adolescent
290 dietary fat preference (Lee et al., 2015). Our findings in combination with these studies suggest
291 organizational sex hormone signaling may induce lasting changes to the endogenous opioid system
292 and mesolimbic reward pathway.

293 The functional effects of the A118G polymorphism may explain how 2D:4D and *OPRM1*
294 A118G interact to influence alcohol dependence. Previously, the G-allele was shown to confer both
295 activational and inhibitory effects on mesolimbic reward signaling in that it increases receptor binding
296 affinity for β-END but also impairs *OPRM1* transcription, making it an unreliable predictor of alcohol
297 dependence. However, after integrating 2D:4D values in our analyses, the G-allele becomes
298 significantly associated with elevated risk, as prenatal androgens have been shown to increase *OPRM1*

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299 RNA expression irrespective of genotype and thus eliminate the protective, inhibitory effects of the G-
300 allele on receptor expression (Huber et al., in press). This 2D:4D-*OPRM1* A118G interaction may also
301 explain why the G-allele tends to protect against dependence risk in our cohort of female study
302 subjects (OR = 1.91, p = 0.091) – females are exposed to far lower amounts of prenatal sex hormones
303 than males and therefore should retain the protective, inhibitory effects of the G-allele. Additional *in*
304 *vitro* and animal experiments will be required to establish the exact mechanism of the prenatal sex
305 hormone-*OPRM1* interaction, although androgen- and estrogen-induced epigenetic modifications in
306 the form of DNA methylation may be a promising starting point (Ghahramani et al., 2014).

307 In addition to *OPRM1* variation, we measured serum β-END level in healthy subjects and
308 alcohol-dependent patients at baseline and during early alcohol withdrawal. At time of recruitment,
309 patients' serum β-END did not significantly differ from controls' but strongly declined during
310 withdrawal in both males and females, which is consistent with previous studies in males (Esel et al.,
311 2001; Marchesi et al., 1996) and reveals this effect to be unrelated to sex. This finding indicates that
312 both male and female patients may depend on alcohol to maintain a homeostatic serum β-END level.

313 At this time, it is unclear how β-END relates to withdrawal. In the NOAH cohort, β-END
314 level was not associated with follow-up outcome in either sex, except for one questionable finding that
315 may be a false positive. The lack of association may be due to several reasons. Previous studies have
316 found peripheral β-END and brain β-END to be under independent regulation, so our serum
317 measurements may not accurately represent the effects of withdrawal on brain β-END processing
318 (Veening et al., 2012). Also, in our analyses, we have compared β-END level during acute withdrawal
319 with long-term outcome when it is unclear whether β-END is stable or dynamic throughout the
320 withdrawal period. β-END level after longer withdrawal intervals may prove to be a stronger predictor
321 of long-term outcome.

322 Interestingly, in a study completed by Racz et al. (2008), female mice tended to be more
323 affected by β-END deficiency with regard to physical withdrawal severity than male mice – an
324 observation which validates our finding that CIWA-Ar withdrawal severity score correlates negatively
325 with serum β-END concentration during withdrawal in females but not in males. Both β-END
326 deficiency and female sex contribute to a higher functioning HPA axis (Goel et al., 2014). Thus,

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327 endogenous opioid production and biological sex may converge on the HPA axis to influence
328 withdrawal-induced stress – an interaction that further highlights sex differences in the etiology of
329 alcohol dependence and outcome. Sex hormone signaling may also exert direct effects on β-END
330 production. A previous study from our group found that the number of CAGn repeats within exon 1 of
331 the androgen receptor (AR) gene correlates negatively with promoter methylation of the β-END
332 precursor gene – pro-opiomelanocortin – and that this methylation is partly responsible for the
333 association between the AR CAGn variant and alcohol craving (Muschler et al., 2014). Understanding
334 these sex effects is of high clinical interest, as it may shed light on the mechanisms of current
335 withdrawal treatments, such as acamprosate, which has been found to both improve withdrawal
336 symptoms and reverse withdrawal β-END deficiency (Hammarberg et al., 2009; Zalewska-Kaszubska
337 and Czarnecka, 2005).

338 There are several limitations to our approach. The associational study design prohibits us from
339 establishing a causal role for prenatal sex hormones in influencing *OPRM1*. Our assessment of 2D:4D-
340 genotype interactions in predicting outcome is limited by the low number of G-carriers who
341 readmitted to the hospital. We also have not corrected for multiple hypothesis testing. Furthermore,
342 prenatal sex hormone load is measured indirectly using 2D:4D as a biological proxy, although it is
343 quite reliable (Zheng and Cohn, 2011).

344 Previous studies report controversial results regarding the role of the *OPRM1* A118G SNP in
345 alcohol dependence. Here we show that upon analyzing findings by sex, AA genotype significantly
346 predicts alcohol-related hospital readmission, number of readmissions, and days until first
347 readmissions in male patients. Additionally, upon accounting for sex and 2D:4D – a biomarker for
348 prenatal sex hormone load – the *OPRM1* G-allele significantly predicts alcohol dependence and
349 outcome. Serum β-END level during withdrawal was also found to relate sex-specifically to outcome,
350 as it correlates negatively with CIWA-Ar withdrawal severity in females but not in males. Since this
351 study is limited by its associational design, future work is needed to assess whether prenatal sex
352 hormones influence alcohol dependence and outcome by directly influencing *OPRM1* and β-END or
353 whether prenatal sex hormones and the opioid system are independent predictive factors of risk.
354 However, given the existing wealth of information on the cross-regulation between opioid and sex

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355 hormone signaling, the observations collected here support a novel mechanism of addiction
356 pathogenesis.
357

358 **Declarations**

359 *Ethics approval and consent to participate*

360 The NOAH study was previously approved by the Ethics Committee of the Medical Faculty of
361 the Friedrich-Alexander University Erlangen-Nürnberg (NOAH study ID 81_12) and was in
362 accordance with the sixth revision of the Declaration of Helsinki, set forth by the World Medical
363 Association (Seoul, 2008), as well as the International Conference on Harmonization Guidelines for
364 Good Clinical Practice (1996).
365

366 *Conflict of Interest*

367 The authors do not declare any personal or financial conflicts of interest.
368

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372 had no role in the study design, data collection, analysis, decision to publish, or preparation of the
373 manuscript.
374

375 *Authors' contributions*

376 JBG, CM, and BL designed the study. JBG performed the *OPRM1* variant genotyping, and
377 CM and BL performed the serum β-END ELISAs. CW and BL checked patients' medical records for
378 alcohol-related hospital readmission. JBG and BL performed the statistical analyses and wrote the first
379 draft of the paper. All authors contributed in a substantial way to the project, provided feedback on the
380 manuscript, and approved the final version for publication.
381

382 *Acknowledgment*

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390

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549 **Figures**

550

551 **Table 1.** RefSNP numbers and PCR conditions for the three SNPs genotyped in this study. rs3798677
552 and rs3798678 are silent variants located within the *OPRM1* promoter.

553

RefSNP		rs1799971		rs3798677		rs3798678	
Nucleotide		*c.*A118G		A>G		A>G	
Amino acid		p.N40D		-		-	
Primers	**Fwd**	CCCGGTTCCTGGGTCAACT		ATTAGCCCCAAAAGAGATG		GTTGAGCAATATGAAGGCCATGATG	
	Rev	GACCGCATGGGTCGGACAG		TTTCATAAGTTCACAATAGAGTCAA		GGAGGAAGGACACATTTTAGAATCCAG	
Size (bp)		58		65		84-100	
[$MgCl_2$] (mM)		2.0		3.0		2.5	
T_A (°C)		63		60		58	
		Patients	Controls	Patients	Controls	Patients	Controls
Absolute genotype frequencies (m/f)	**NN**	83/75	103/82	72/61	79/84	72/61	79/84
	Nn	28/12	28/24	34/24	49/18	34/24	49/18
	nn	2/0	2/1	7/2	5/5	7/2	5/5

554
555 Fwd forward, Rev reverse, T_A PCR annealing temperature, m male, f female
556

557 **Table 2.** Absolute *OPRM1* CAn genotype frequencies.

Genotype	**Absolute genotype frequencies**	
	Patients (m/f)	**Controls (m/f)**
1015	0/0	1/0
1016	1/0	0/0
1017	1/0	1/0
1416	1/1	1/0
1417	0/0	0/1
1515	8/3	11/4
1516	10/10	10/8
1517	27/17	34/18
1518	5/3	5/3
1616	1/2	5/5
1617	15/16	18/20
1618	1/0	5/4
1717	32/25	34/34
1718	11/10	7/9
1818	0/0	1/1

558
559 m male, f female
560

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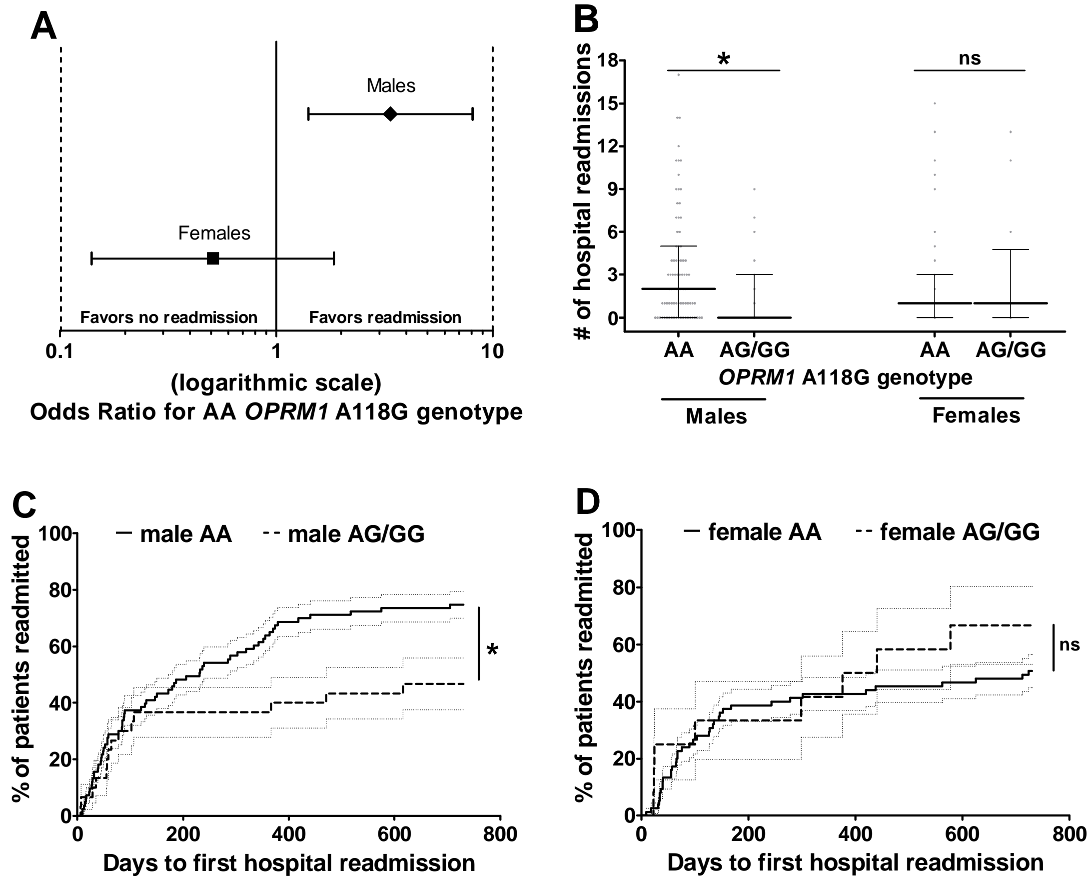

562

563 **Figure 1. Effect of *OPRM1* A118G genotype on prospective alcohol-related hospital readmission**
564 **during the 24-month follow-up**

565 (A) A118G AA genotype is associated with elevated readmission risk in males (OR = 3.37 [95%-CI
566 1.41-8.07], $\chi^2 = 7.863$, p = 0.005) but not in females (OR = 0.51 [95%-CI 0.14-1.85], $\chi^2 = 1.063$, p =
567 0.303). (B) AA males had a higher median number of readmissions than male G-allele carriers (AA: 2
568 [IQR 0-5], AG/GG: 0 [IQR 0-3], Mann-Whitney U test, $U = 869.0$, p = 0.012), whereas genotype did
569 not predict number of readmissions in females (AA: 1 [IQR 0-3], AG/GG: 1 [0-5], MWT, $U = 399.5$,
570 p = 0.509). Data are presented as median ± IQR. (C) AA males had fewer mean days until first
571 readmission than male G-allele carriers (AA: 313.3 ± 30.6, AG/GG: 457.7 ± 57.9, Log-rank [Mantel-
572 Cox] test, $\chi^2 = 5.562$, p = 0.018). (D) There was no difference in days until first readmission between
573 AA females and female G-allele carriers (AA: 447.7 ± 35.9, AG/GG: 398.8 ± 83.0, Log-rank [Mantel-
574 Cox] test, $\chi^2 = 0.913$, p = 0.339). C and D show survival curves ± SEM. OR odds ratio, 95%-CI 95%-
575 confidence interval. *p < 0.05, ns not significant.

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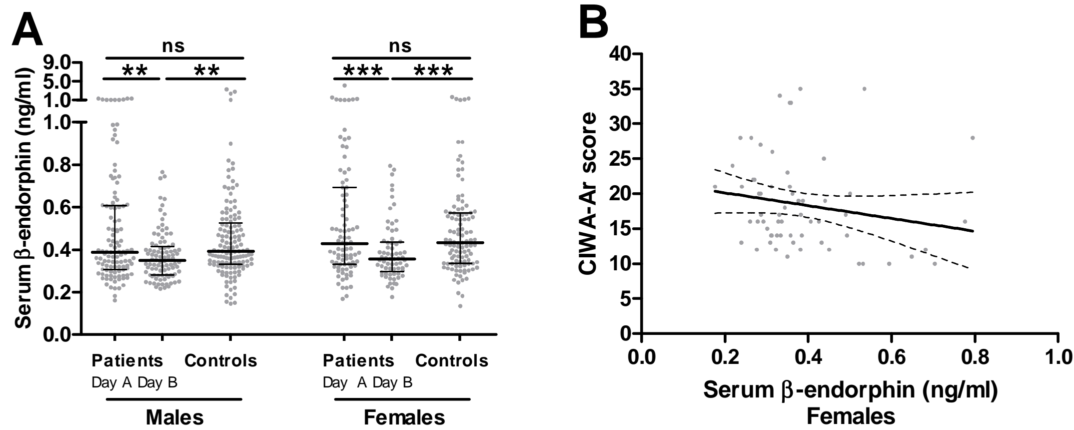

576

577 **Figure 2. Sex-specific serum β-END level in study subjects and correlation of day B β-END level**
578 **with withdrawal severity in female patients**

579 (A) There was no difference in serum β-END level between patients and controls at baseline (male
580 patients: 0.388 ng/ml [IQR 0.307-0.607], male controls: 0.392 ng/ml [IQR 0.330-0.526], Mann-
581 Whitney U test, $U = 7507.0$, $p = 0.989$; female patients: 0.428 ng/ml [IQR 0.331-0.692], female
582 controls: 0.433 ng/ml [IQR 0.335-0.574], Mann-Whitney U test, $U = 4638.0$, $p = 0.966$). During
583 withdrawal of a median of 5 days, patient serum β-END level declined (Wilcoxon signed-rank tests,
584 male patients: $z = -3.2$, $p = 0.001$, female patients: $z = -3.5$, $p < 0.001$) to a lower level than in control
585 subjects (male patients: 0.348 ng/ml [IQR 0.280-0.415], Mann-Whitney U test, $U = 4627.0$, $p = 0.001$;
586 female patients: 0.356 ng/ml [IQR 0.297-0.435], Mann-Whitney U test, $U = 2525.0$, $p < 0.001$). Data
587 are shown as median ± IQR. Two outliers – one male and one female control – above 10 ng/ml β-END
588 level are not shown. (B) Serum β-END level on day B correlated significantly with severity of alcohol
589 withdrawal syndrome, quantified by the CIWA-Ar score (Spearman-Rho, $n = 62$, $\rho = -0.282$, $p =$
590 0.026). Dotted lines represent the 95% confidence intervals of the best-fit from a linear regression
591 analysis. CIWA-Ar Clinical Institute Withdrawal Assessment for Alcohol revised scale, IQR
592 interquartile range, $^{**}p = 0.001$, $^{***}p < 0.001$, ns not significant.

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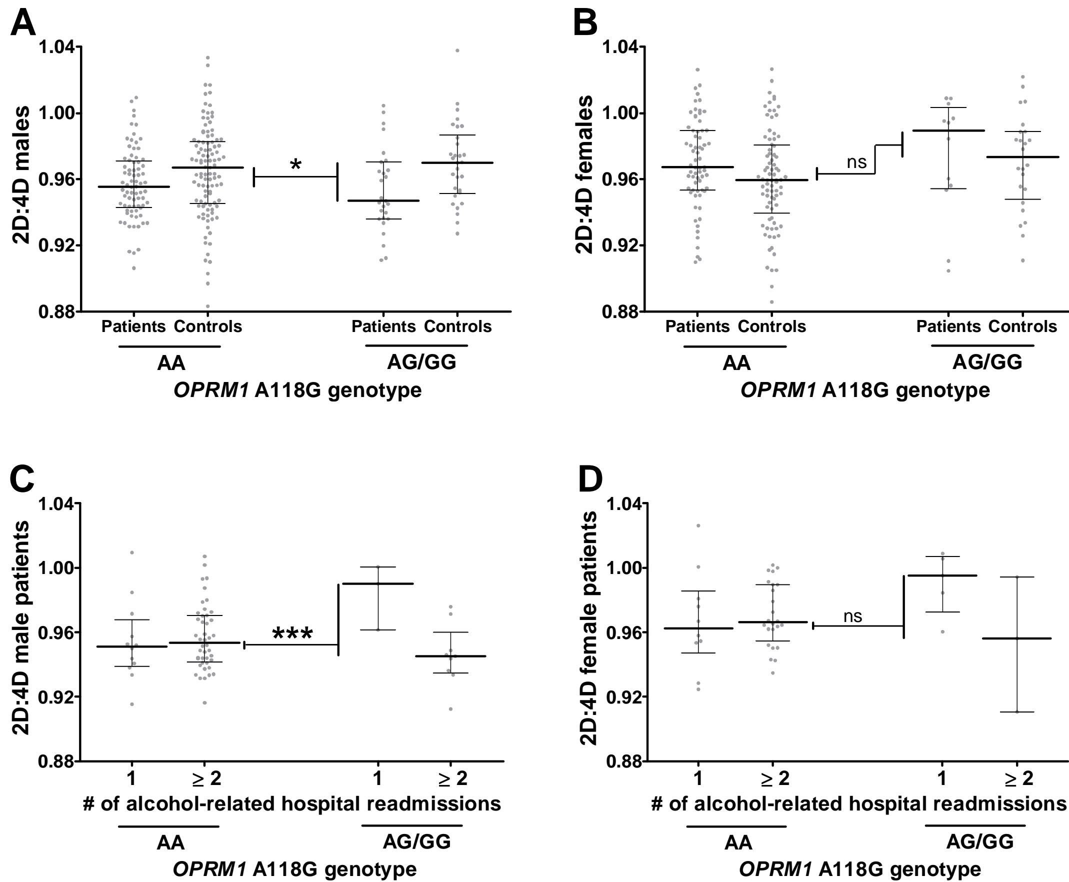

596

597

598 **Figure 3. Sex-specific interaction of *OPRM1* A118G genotype with 2D:4D in risk and course of**
599 **alcohol dependence**

600 (A) We sex- und genotype-specifically normalized patients' 2D:4D values to the mean 2D:4D and
601 standard deviation of the control group and found that male G-allele carrier patients deviated
602 significantly from AA males with regard to the normalized 2D:4D values (normalized 2D:4D;
603 AG/GG: n = 27, median = -0.941, AA: n = 76, median = -0.331, Mann-Whitney *U* test, *U* = 742.0, p =
604 0.033). (B) This difference was not observed in females (normalized 2D:4D; AG/GG: n = 12, median
605 = 0.639, AA: n = 67, median = 0.240, Mann-Whitney *U* test, *U* = 375.0, p = 0.712). (C) 2D:4D value
606 of each patient with at least two readmissions was sex- and genotype-specifically normalized to the
607 mean 2D:4D and standard deviation of patients with one readmission; these normalized values
608 differed significantly between G-allele carriers and AA study subjects in males (normalized 2D:4D;
609 AG/GG: n = 9, median = -1.922, AA: n = 44, median = -0.020, Mann-Whitney *U* test, *U* = 23.0, p <
610 0.001) but not in females (D; normalized 2D:4D; AG/GG: n = 3, median = -1.771, AA: n = 24, median

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611 = -0.025, Mann-Whitney *U* test, $U = 14.0$, $p = 0.090$). Data shown as median ± interquartile range. *p
612 < 0.05, *** $p < 0.001$, ns not significant.
613