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On the role of NOS1 ex1f-VNTR in ADHD – allelic, subgroup, and meta-analysis

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On the role of *NOS1* ex1f-VNTR in ADHD – allelic, subgroup, and meta-analysis

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Abstract

Attention deficit/ hyperactivity disorder (ADHD) is a heritable neurodevelopmental disorder featuring complex genetics with common and rare variants contributing to disease risk. In a high proportion of cases, ADHD does not remit during adolescence but persists into adulthood. Several studies suggest that *NOS1*, encoding nitric oxide synthase I, producing the gaseous neurotransmitter NO, is a candidate gene for (adult) ADHD. We here extended our analysis by increasing the original sample, adding two further samples from Norway and Spain, and conducted subgroup and co-morbidity analysis. Our previous finding held true in the extended sample, and also meta-analysis demonstrated an association of *NOS1* ex1f-VNTR short alleles with adult ADHD (aADHD). Association was restricted to females, as was the case in the discovery sample. Subgroup analysis on the single allele level suggested that the 21-repeat allele caused the association. Regarding subgroups, we found that *NOS1* was associated with the hyperactive/impulsive ADHD subtype, but not to pure inattention. In terms of comorbidity, major depression, anxiety disorders, cluster C personality disorders and migraine were associated with short repeats, in particular the 21-repeat allele. Also, short allele carriers had significantly lower IQ. Finally, we again demonstrated an influence of the repeat on gene expression in human post-mortem brain samples. These data validate the role of NOS-I in hyperactive/impulsive phenotypes and call for further studies into the neurobiological underpinnings of this association.

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Introduction

Attention deficit / hyperactivity disorder (ADHD) is the most common neurodevelopmental disorder, with an estimated prevalence of around 5%. While a proportion of cases remits, a significant part of the patients do not fully recover and feature full or partial persistence of the disorder into adulthood, then termed adult ADHD (aADHD) (Faraone and others 2006). The childhood manifestation of the disorder is highly heritable – around 80% (Faraone and Khan 2006; Franke and others 2012) - and so is aADHD, when clinical diagnostic instruments are used (Larsson and others 2013). However, despite considerable efforts e.g. in consortia such as the Psychiatric Genomics Consortium, only few risk genes have been yet discovered and confirmed. This likely is due to the complex genetic architecture of ADHD involving common as well as rare variants, be it in the form of base pair mutations or copy number variants. This is even more true for the adult form of ADHD, where there are considerably less studies on risk genes (Franke and others 2012). Looking across the lifespan, it can be hypothesized that, in addition to genes predisposing to ADHD onset as such, genetic variance impacts on the persistence of the disorder (Chang and others 2013) – in fact, there is already one example where one allele of a polymorphism is associated with childhood ADHD, while the other allele is associated with adult ADHD (the gene encoding the dopamine transporter DAT (Franke and others 2010)). Finally, it is known that aADHD goes along with a high rate of both psychiatric (Jacob and others 2014a; Jacob and others 2014b; Jacob and others 2007) as well as somatic (Haavik and others 2010) comorbidities. Many of those comorbid phenomena have a heritable basis as well (e.g., (Lee and others 2013)), but only few risk variants for such comorbidity have been identified (e.g., (Cross-Disorder Group of the Psychiatric Genomics Consortium 2013)).

We have previously identified a highly polymorphic repeat in the promoter region of the gene encoding nitric oxide synthase 1 (*NOS1*), termed *NOS1* ex1f-VNTR, short alleles of which results in lower gene expression *in vitro*; supposedly such reduced expression is also present in the basal ganglia, as the specific splice form is predominantly expressed in this part of the brain (Freudenberg 2015; Reif and others 2006; Reif and others 2009; Rife and others 2009). The gene product, NOS-I, is the sole source for the gaseous neurotransmitter NO in the brain, which is also the second messenger of the NMDA receptor, and which is involved in learning, memory, behavioral control and emotional behaviors (Snyder and

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others 1998). Mice that lack fully functional Nos-I are more aggressive, impulsive, less anxious and cognitively impaired (Nelson and others 2006; Wultsch and others 2007). These data make *NOS1* an excellent candidate gene for ADHD and associated impulsive behaviors. Although we could not establish a role for *NOS1* ex1f-VNTR in childhood ADHD (potentially because of sample size limitations), we have earlier demonstrated that short (i.e, low-expression) variants of the *NOS1* repeat polymorphism are associated with aADHD as well as a wide range of impulsive behaviors including aggression, suicidality, and cluster B personality disorder (Reif and others 2009). The association with impulsive personality domains was sex-specific and found in females only: re-analysis of the case-control data reported in Reif and others (2009) revealed no significant association in males, but association of short alleles with aADHD in the females (OR=1.26, p=0.04; genotypic p-value assuming a recessive effect = 0.009).

We later replicated the association with impulsive traits in Estonian samples (Laas and others 2010), and also observed a gene × environment interaction (Reif and others 2011a). Also, emotional behaviors were found to be influenced by this polymorphism (Kurrikoff and others 2012). Further substantiating an association of *NOS1* with behavioral traits, brain activation during working memory (Kopf and others 2011) and impulsivity (Kopf and others 2012) tasks was shown to be altered by the presence of the short *NOS1* alleles. Unfortunately, the ex1f-VNTR is not tagged by single nucleotide polymorphisms (SNPs) in genome-wide association studies (GWAS), and no hypothesis-free approaches have yet confirmed the association with ADHD (although a SNP was found suggestively associated with quantitative ADHD symptoms in an early ADHD GWAS (Franke and others 2009)).

We here extended the original discovery aADHD sample from 383 to 987 patients, and added two additional samples from the IMpACT study group (www.impactadultadhdgenomics.org) for meta-analysis. In order to further characterize the polymorphism-related phenotype, we subsequently tested for an association with quantitative ADHD measures as well as comorbid conditions, based on the known pleiotropy of NO action. We also ran an exploratory analysis to see whether an individual short allele is associated with the traits in question. Finally, we tested whether the polymorphism indeed affects *NOS1* expression *in vivo* in human post-mortem brain specimens to functionally characterize the biological role effect of the ADHD risk factor.

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Material and Methods

Genotyped samples

The discovery case-control sample, previously published in part (Reif and others 2009), consisted of 987 unrelated in– and outpatients (mean age 34.2 years, SD 10.1; 47% female) of self-reported central-European descent from the German chapter of the International Multicenter persistent ADHD CollaboraTion (IMpACT), who were referred to the Department of Psychiatry, University of Würzburg for diagnostic assessment and treatment of aADHD. All patients completed a semi-structured clinical interview according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders (Jacob and others 2007)). Inclusion criteria were onset before the age of 7 years, life-long persistence, current diagnosis and age of recruitment between 18 and 65 years. Childhood ADHD was retrospectively assessed with the DSM-IV symptom list for ADHD (17 items) that was used as a structured clinical interview, and additionally using the Wender-Utah Rating Scale (WURS; 61 items). Personality assessment was done using NEO PI-R (Costa 1992). Patients with a lifetime diagnosis of bipolar disorder (BPD), schizophrenia, significant neurologic co-morbidity, mental retardation, substance-induced disorders (before detoxification/withdrawal), or other somatic disorders, suggesting organic psychosis or with an intelligence quotient (IQ) below 80 were excluded from analysis. For a more detailed sample description, please refer to (Franke and others 2010). Information about clinical ADHD presentation, comorbid psychiatric disorders (other than those mentioned above) and dimensional traits are documented in Supplementary Table I. A comparison of *NOS1* ex1f-VNTR genotypes between published (Reif and others 2009) and recently recruited aADHD samples can be found in Supplementary Table II, this shows that allele frequencies between analysis waves do not differ. The control sample consisted of 2,019 unrelated healthy participants of Caucasian origin (mean age 27.2 years, SD 8.3; 62% female), all stemming from the same catchment area as the patient group. Of those, 385 (mean age 31.7 years, SD 10.8; 48% female) were anonymous blood donors, not screened for psychiatric disorders but free of medication. Further 1,634 healthy volunteers (mean age 26.4 years, SD 7.6; 65% female) were recruited and screened for psychiatric disorders. Assuming a 5% prevalence of ADHD, 19 individuals among the unscreened controls may be ADHD patients, which may

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affect the allele frequency of an associated polymorphism. Allele frequencies of the *NOS1* ex1f-VNTR however did not differ between screened and unscreened controls (p>>0.05).

Two further aADHD samples were obtained from IMpACT (Franke and others 2010; Halmoy and others 2010; Johansson and others 2008; Reif and others 2011b). The total replication sample included 710 aADHD patients (mean age 30.6 years, SD 11.5; 39% female) and 847 controls (mean age 48.7 years, SD 14.5; 45% female) of Norwegian (n_{cases}=416; $n_{controls}$=535) and Spanish (n_{cases}=294; $n_{controls}$=312) origin. All patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV criteria using standardized clinical interviews. Consensus eligibility criteria for this study across all study sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV, age at onset <7 years by retrospective diagnosis (which was confirmed by family members, wherever possible), life-long persistence, and current diagnosis. Norwegian controls were free of psychotropic medication, but not screened for psychiatric disorders; Spanish healthy volunteers were screened for and free of psychiatric disorders. Further information on replication samples can be obtained from Supplementary Table I. All patients and volunteers provided written informed consent after oral and written explanation about scope and aim of the investigation. The studies complied with the Declaration of Helsinki and were approved by the local ethical committees of the respective universities.

Genotyping of NOS1 ex1f-VNTR

The highly polymorphic *ex1f-VNTR* repeat, located in the upstream region of *NOS1* (GRCh 38: 117361839bp-117361898bp), consists mainly of 19 to 36 and in rare cases of 8 or 15 "GT" dinucleotide units. To facilitate genetic association studies, *NOS1* ex1f-VNTR alleles were dichotomized in short (8, 15 and 19-27 (GT) repeats, "S") and long (28-36 (GT) repeats, "L") alleles based on the historical median split; prevalent "S" and "L" alleles differ in promoter activation (Reif and others 2009) and the split was defined between rare alleles (Figure 1).

Genomic DNA for genotyping was extracted from venous blood of all participants, by a standard de-salting method. *NOS1* ex1f-VNTR alleles were identified using a polymerase chain reaction (PCR) amplification step followed by electrophoretic separation and product size determination on a DNA sequencer (CEQ8000; Beckman-Coulter, Krefeld, Germany) as

previously described in greater detail (Reif and others 2006). Primers and PCR conditions are available on request.

Statistical Analysis of NOS1 ex1f-VNTR

Statistical analysis of genotype data was performed with R version 2.15.2 (obtained from www.R-project.org). Genotypes of all three case and control samples did not deviate from Hardy-Weinberg equilibrium (HWE; χ^2 HWE p-value ≥ 0.05). To alleviate the possible effect of ethnic discrepancies, calculations were performed in each sample separately; for joint analyses, samples were subjected to meta-analysis (see below). The primary analysis carried out was a case-control comparison with allele counts (S vs. L). For better comparison with the results of Reif and others 2009 in second S allele homozygote counts (S/S vs. S/L+L/L, "recessive model") were analyzed post-hoc. Further post-hoc analyses were calculated for "21-repeat" allele counts ("21" vs. rest). These tests used 1-degree-of-freedom χ^2- tests. In case of fewer than five occurrences per cell of the contingency table Fisher's exact tests were used. We focused on the 21-repeat allele as it represents the only common (MAF > 5%) repeat length with significantly different frequencies between affected and unaffected individuals (see Supplementary Table III). Effect sizes were quantified with Cramer's phi.

Impact of genotypes on IQ and WURS sum scores were determined post-hoc with linear regression (R function lm) using genotype dosages (additive model) as independent and the trait of interest as dependent variable; both scores were also analyzed with a recessive model (SS vs. SL+LL) using grouped genotypes. ANOVA was used to examine interaction between genotype and sex effects (R function anova.lm). Effect size was quantified by Cohen´s f. P-values<0.05 indicated statistical significance.

Meta-analysis

To obtain maximal information regarding the *NOS1* ex1f-VNTR, we conducted a meta-analysis of the German, Norwegian and Spanish samples and thereby achieve a pooled sample power of 70% assuming a co-dominant model, a MAF of 0.05 (Power for Genetic Association version 2.0) and a relative risk of 1.25 to develop aADHD.

Meta-analyses were performed with the R package metaphor version 0.5-7 (Viechtbauer 2009) using the "rma" command. As a measure for effect size, we calculated

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odds ratios (ORs) and applied the Q-statistic (Fleiss 1981; Lau and others 1997) to assess heterogeneity therein. Inconsistency across studies was quantified with the I^2 metric (I^2=(Q-df)/Q). The joint OR was determined as the weighted average of effect sizes entering the meta-analysis. When the Q-statistic showed no heterogeneity in the effect sizes, we applied fixed-effects models (Mantel and Haenszel 1959), where the weights correspond to the inversed variances of the study ORs. In the presence of significant ($p<0.05$) heterogeneity, we applied random-effects models (DerSimonian and Laird 1986). Here, weights are initially calculated as in the fixed-effects model, but are then down-weighted by the degree of variance of effect sizes.

Influence of NOS1 ex1f-VNTR on mRNA expression

The influence of *NOS1* ex1f-VNTR on mRNA expression was examined in human post-mortem brain (post-mortem intervals (PMI) from 28 h up to 111 h; mean PMI 54.8 h; SD 16.6) of 76 deceased individuals (mean age 48.6 years, SD 12.8; 24% female), all obtained from the Medical Research Council (MRC) Sudden Death Brain and Tissue Bank, Edinburgh. DNA and RNA from three brain regions (amygdala, forebrain, and midbrain) were isolated, using the MELT™ Total Nucleic Acid Isolation System (Applied Biosystem, AM Foster City, 1983) and stored at -80°C until use. RNA quality, measured with a Bioanalyzer (Agilent), revealed RNA integrity numbers (RIN) ranging from 2.4 to 8.1 (mean RIN 5.9; SD 0.94). Reverse transcription of 1 µg total RNA was done by using the iScript™ cDNA synthesis kit (Bio-Rad, München, Germany). cDNA was quantified in triplicates on a Bio-Rad CFX384 real-time PCR detection system, by applying the iQ™ SYBR green supermix from Bio-Rad and *NOS1*-specific QuantiTect Primer (QT00043372) from Qiagen in a 10 µl reaction volume. PCR conditions were 5 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C, followed by a melting curve analysis with a gradient of 65°C to 95°C of 0.5°C per 5 s. Raw data were normalized by mean efficiencies obtained from LinRegPCR (Ramakers and others 2003) and normalization factors based on the three (of six investigated) most stable housekeeping genes (*GAPDH*, *TBP*, *SDHA*), defined by the geNorm (Vandesompele and others 2002) software. The influence of *NOS1* ex1f-VNTR on mRNA expression was quantified with linear regression using additive and recessive (SS vs. SL+LL) genotype models with genotype and the sample RIN as independent and expression values as dependent (co-) variable. Effect sizes were quantified by Cohen´s f.

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Results

Association analysis of NOS1 ex1f-VNTR in three independent aADHD case-control samples

Due to different geographic origin of our study samples, allelic and recessive model associations were first determined separately in each sample; a meta-analysis was then conducted to assess the statistical significance of the pooled effect sizes with maximal statistical power (Table I and Figure 2, Supplementary Figure 1). When all three samples were analyzed jointly using a meta-analytic approach with a sample size totaling to 1,697 cases and 2,866 controls, short alleles were significantly associated with aADHD, however only when the analysis was restricted to females (P_{Fixed}=0.049; OR=1.14 [95% CI: 1.00-1.29]; Figure 2B) as it was the case in the discovery sample.

To test which of the 19 *NOS1* ex1f-VNTR alleles conveys the genetic predisposition towards aADHD, additional allelic case-control analyses were conducted for each of the 19 repeats, versus all other repeats (Supplementary Table III). In doing so, we found that the 21-repeat allele significantly increases the risk for aADHD in German females (p=0.023; Cramer's phi=0.039). Subsequent meta-analysis did however not confirm the genetic risk status of the 21-repeat allele both in the total sample (P_{Fixed}=0.116; OR=1.19 [95% CI: 0.96-1.46) as well as in its female subset (P_{Random}=0.620; OR=0.81 [95% CI: 0.34-1.90]; see Supplementary Table IV).

Association analysis of NOS1 ex1f-VNTR with aADHD clinical presentations

To examine whether *NOS1* ex1f-VNTR is specifically linked to one of the ADHD sub-phenotypes (inattentive (AD), hyperactive/impulsive (HD), or combined (AD+HD) presentation), we performed allelic and recessive model association analyses with all three ADHD clinical presentations by meta-analysis (Table II, Supplementary Table V, Supplementary Figures 2, 3, 4 and 5). In the allelic meta-analysis, short repeats were found to predispose to the hyperactive/impulsive (HD) (P_{Fixed}=0.032; OR=1.67 [95% CI: 1.07-2.62]) and the combined subtype (AD+HD) (P_{Fixed}=0.026; OR=1.18 [95% CI: 1.02-1.36]) in females.

In line with these findings, allelic case-control associations investigating the 21-repeat allele again revealed an increased risk for the AD+HD subtype in German females (p=0.030;

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Cramer's phi=0.039) and Spanish males (p=0.010; Cramer's phi=0.095), as well as in the HD subtype in the Norwegian sample (p=0.001; Cramer's phi=0.143). However, meta-analytic treatment did not result in a significant association (see Supplementary Table VI).

Association analysis of NOS1 ex1f-VNTR with comorbid disorders

In an exploratory manner, we assessed the impact of *NOS1* ex1f-VNTR on comorbid disorders, specifically Substance Abuse (Alcohol: SUDAlc and Drugs: SUDDrug), Major Depression (MD), Anxiety Disorder (AnxDis), Personality Disorders Cluster A (CL_A), B (CL_B) and C (CL_C), and Migraine (Migr), in the German, Norwegian and Spanish samples (see Tables III and IV and Supplementary Tables VII and VIII). In the allelic model, meta-analyses of all examined phenotypes revealed an association of *NOS1* ex1f-VNTR with comorbid MD, AnxDis and Migr, in all cases with the short allele being the risk factor. While AnxDis were associated with *NOS1* in the total sample (P_{Fixed}=0.024; OR=1.17 [95% CI: 1.02-1.34]), we observed a sex bias for MD (Total: P_{Fixed}=0.006; OR=1.18 [95% CI: 1.05-1.33]; males: P_{Fixed}=0.045; OR=1.19 [95% CI: 1.01-1.41]) and Migr (Total: P_{Fixed}=0.007; OR=1.27 [95% CI: 1.07-1.50]; females: P_{Fixed}=0.022; OR=1.31 [95% CI: 1.05-1.64]). Likewise, we also observed an association with Cl_C in males using the recessive model meta-analysis (P_{Fixed}=0.050; OR=2.72 [95% CI: 1.14-6.51]; See Supplementary Table VIII).

Single allele meta-analysis of comorbid MD with the 21-repeat (Total: P_{Fixed}=0.019; OR=1.38 [95% CI: 0.95-1.97]); males: P_{Fixed}=0.015; OR=1.61 [95% CI: 1.112.34]) reflected the association results of the S allele, which points to the 21-repeat allele being the main driver for the genetic risk of the S allele. Also in comorbid Migr, the 21-repeat allele was significantly associated with the co-morbid disorder, caused mainly by association in males (Migr: P_{Fixed}=0.039; OR=1.84 [95% CI: 1.07-3.16]; See Supplementary Table IX).

In the following exploratory analysis, we examined the impact of *NOS1* ex1f-VNTR on WURS and IQ in the German sample. The S allele has no significant effect on the WURS sum score (p>0.05). Also, no association with IQ scores was found with the allelic model. Grouped genotypes (SS: 109.2 ± 12.6 vs. SL+LL: 111.7 ± 14.1) revealed however a significantly decreased IQ score ($beta_{Total}$=-2.57; $beta_{Female}$=-3.77) for homozygous S-allele carriers in the total sample ($p_{recessive}$=0.040; Cohen´s effect-size (f^2)=0.007) and in females ($p_{recessive}$=0.030; Cohen´s f^2=0.015).

Functional effect of NOS1 ex1f-VNTR on gene expression

Quantification of mRNA in human post-mortem amygdalae revealed a significant increase of total *NOS1* expression values (Total: $beta_{allelic}$=0.47; $beta_{recessive}$=0.73; Male: $beta_{allelic}$=0.55; $beta_{recessive}$=0.93) for the short risk allele of *NOS1* ex1f-VNTR in the total MRC sample (N=59; $p_{allelic}$=0.030; Cohen´s effect-size (f^2)=0.108; $p_{recessive}$=0.040; Cohen´s f^2=0.097) and the male (N=45; $p_{allelic}$=0.039; Cohen´s f^2=0.132; $p_{recessive}$=0.036; Cohen´s f^2=0.135), but not the female subset (N=14; $p_{allelic}$=0.531; $p_{recessive}$=0.733). Neither in forebrain (N=64; p_{best}=0.196) nor in midbrain (N=55; p_{best}=0.329) *NOS1* ex1f-VNTR affected mRNA expression (Supplementary Figure 6). Unfortunately, no other brain regions were available for further analysis.

Discussion

The present study aimed to corroborate the association of *NOS1* ex1f-VNTR with aADHD (Reif and others 2009) and related phenotypes (Freudenberg 2015). The association with aADHD in the original study was specific to females, but no attempt at replication has been published to date. We almost tripled the size of the original discovery sample (Reif and others 2009) and furthermore added two independent samples for meta-analysis. Overall, our data confirmed a sex-specific association of *NOS1* ex1f-VNTR with aADHD, especially with clinical presentations featuring hyperactive/impulsive symptoms and also with comorbid AnxDis, MD, CL_C and Migr.

In reporter gene assays, short repeat lengths consistently resulted in lower gene expression as shown by two independent groups (Reif and others 2009; Rife and others 2009). In contrast, we here detected increased *NOS1* expression in the amygdala in short allele carriers. However, despite it is well known that *NOS1* is expressed in the amygdala, it is unclear which alternative first exon (and hence alternative promoter) drives expression in this structure. As the effect of the *NOS1* ex1f-VNTR genotype may be highly tissue-specific, and also as we measured total *NOS1* driven from alternative promoters, our data might represent compensatory up-regulation of total *NOS1* in response to decreased *NOS1* ex1f expression either in the amygdala as such or elsewhere. Also, since both cell lines used for the reporter gene assays (HeLa and Sk-n-Mc (Reif and others 2009; Rife and others 2009))

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were originally derived from cancer tissue of female donors, whereas the post-mortem brain sample was mainly composed of male specimens (76%), one may speculate that the observed discordance of S allele effects reflects the sex differences found in the association studies (see below). Further studies in larger samples of human tissue are clearly warranted to clarify the molecular effect of the *NOS1* ex1f-VNTR.

Association of NOS1 with adult ADHD

Our initial finding (Reif and others 2009), namely that short *NOS1* ex1f-VNTR alleles are associated with aADHD in females, was successfully replicated in the German sample. A validation using Spanish and Norwegian samples did however not succeed; differences in the genetic background due to the ethnic composition of the samples as well as sample size constraints (sample sizes were not even half as large as the German sample) provide plausible explanations for the failure to replicate. Meta-analysis of *NOS1* ex1f-VNTR of three samples however confirmed the sex-specific association; since sex distribution in samples was almost balanced, power considerations may rather not explain the observed differences between males and females. Hence, biological mechanisms might form an alternative explanation, and in this context it is noteworthy that a *cis*-acting estrogen binding site is present directly upstream of the *NOS1* ex1f-VNTR, so that effects of sex hormones on *NOS1* expression seem plausible. Sex-specific associations with *NOS1* ex1f-VNTR were previously observed in an independent sample representing the general population (Kurrikoff and others 2012). Moreover, in murine models for aggression, effects of *Nos1* deficiency were shown in both sexes, however in the opposing direction: male mice became more (Chiavegatto and others 2001) and female mice became less aggressive (Gammie and Nelson 1999). The latter observation was confirmed by a selective breeding approach, where mice selected for high maternal aggression were found to have increased *Nos1* expression (Gammie and others 2007). Sex effects thus should be explicitly addressed in further studies on *NOS1*'s role in behavior and psychopathology.

Despite considerable sample size, our data is not definitive as the strength of association is not very pronounced; also, the effect in the German sample was the most significant, suggesting that the association of *NOS1* with aADHD follows the "winner's curse". However, the finding that short alleles convey risk across all published studies (Freudenberg 2015) and also in the present dataset supports the hypothesis that psychiatric

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disorders are influenced by *NOS1* ex1f-VNTR and hence *NOS1* might constitute one example of risk genes that underlie the frequently observed comorbidity of psychiatric disorders. The *NOS1* ex1f-VNTR effect on examined phenotypes was generally modest as it usually is in polygenic traits (Freudenberg 2015). Also, the rather crude distinction in short and long alleles (originally merely based on statistical reasons, i.e. the median repeat length) might obscure biological effects. A more detailed analysis of all individual alleles suggested the 21-repeats to be associated with the phenotypes in question, at least in the German sample. Although here, respective calculations yielded stronger significance levels and thus greater effect sizes as compared to the short/long dichotomized polymorphism, discordant results were obtained from the Norwegian and Spanish samples, thus demanding further and larger samples to detail if the genetic risk is indeed conveyed by the 21-repeat allele.

aADHD subtype and comorbid disorders

As the IMpACT samples are well characterized and broadly phenotyped, this provided the opportunity to run exploratory analyses on dimensional traits and co-morbid conditions (although these findings have to be considered with caution until replicated). A plethora of pharmacological and genetic studies in rodents suggest a role of NOS-I in anxiety and mood regulation (Gao and others 2014; Gao and others 2013; Gregers 2011; Pereira and others 2013; Yazir and others 2012). Accordingly, there is (more scarce) evidence that mood disorders are influenced by *NOS1*: a SNP in exon 29, for example, increased the risk for recurrent depressive disorder in a preliminary study (Galecki and others 2011), and the *NOS1* ex1f-VNTR S allele went along with significantly higher depressiveness in a population-representative sample (Kurrikoff and others 2012). To our best knowledge, there are no other studies on *NOS1* ex1f-VNTR and depression or anxiety in humans despite the wealth of preclinical non-genetic human data suggesting an involvement of NOS-I in depression and anxiety. In the context of aADHD, as tested in the present study, both affective and anxiety disorders seem to be linked to short alleles of *NOS1* ex1f-VNTR indicating that *NOS1* may also have a role in emotional behaviors in humans. Regarding substance use disorders, a role for *NOS1* could especially be assumed given data from knockout mice (Spanagel and others 2002); however, no effect could be found in our human sample. This of course does not rule out a role for NOS-I in alcohol abuse, but argue against a major contribution through a common genetic variant in the gene. Also, we could not replicate our previous finding (Reif

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and others 2009) that short *NOS1* alleles are associated with Cluster B personality disorders, although a trend towards an association was seen in the recessive model (corresponding to the analysis strategy of the original discovery) meta-analysis of the total sample. The reason for this likely is sample size (only 126 patients were included here, in contrast to 322 patients in the initial study).

Previously we have presented evidence for comorbidity between ADHD and migraine (Fasmer and others 2011). The association between *NOS1* and migraine in ADHD patients is interesting (Table III) with regard to the abundant data showing the involvement of NO in migraine pathophysiology (de O. S. Mansur and others 2012). The present findings therefore suggest that *NOS1* may be a link between these two disorders.

The exploratory ADHD subtype analysis might guide follow-up studies. In line with our prior hypothesis that NOS-I has a role especially in hyperactivity and motor impulsivity (Reif and others 2009), we here demonstrate preliminary evidence that short alleles are associated with hyperactive/impulsive subtypes of ADHD, but not pure inattentive adult ADHD. The neurobiological rationale for this might be that NOS-I modulates striatal function by its influence on dopaminergic neurotransmission. Thus, it seems to be especially worthwhile to test *NOS1* ex1f-VNTR's influence on impulsive phenotypes mediated by the striatum and whether this interacts with the dopamine system. Finally, we here also found a negative effect of the short allele on general IQ. Keeping in mind previously found influences of *NOS1* on verbal IQ (Donohoe and others 2009), one may hypothesize a specific role of NOS-I in verbal intelligence which is currently under investigation in our laboratory along with neuroimaging experiments in order to identify the neural correlates thereof.

Taken together, the present study strengthens the notion that short alleles – potentially the 21-repeat allele – of *NOS1* ex1f-VNTR are associated with aADHD in a sex specific manner; its role likely is complex with effects on hyperactivity/impulsivity, stress-related disorders, migraine and IQ. NOS-I thus might be a molecular link between these phenotypes, explaining at least a little genetic part of the observed co-morbidity of ADHD with these phenotypes. This hypothesis could be directly tested in further studies, and also, functional studies are warranted to elucidate the neurobiological pathways that link genetic variation to neural systems and behavior.

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Disclosure/Conflict of Interest

None of the authors reported any biomedical financial interests or potential conflicts of interest.

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Figures (2)

Figure 1: Chromosomal position of the *NOS1* ex1f-VNTR according to NCBI´s genome built GRCh 38 and distribution of the repeat length variants in German, Norwegian and Spanish samples.

Figure 2: Forest plots of *NOS1* ex1f-VNTR recessive and allelic model in the total sample **(A)**, as well as the female **(B)** and the male **(C)** subset.

Tables (4)

Table I: Association results for the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish samples, followed by meta-analysis. Table shows allele and genotype counts for cases and controls, as well as χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) and recessive (SS vs. SL+LL) and models in the total sample, and their sex-specific subsets. Further, total cases and control counts are given for the allelic and recessive models as well as p-values for heterogeneity and odds ratios plus p-values of the fixed effect model. Bold face indicates significant p-values ($p<0.05$).

Table II: Association results of aADHD clinical presentations (inattentive (AD), hyperactive (HD), and combined (AD+HD) type ADHD) for the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish sample, followed by a meta-analysis. Along allele counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Table III: Association results of *NOS1* ex1f-VNTR with comorbid axis 1 disorders (Substance Abuse (Alcohol: SUDAlc and Illicit Drugs: SUDDrug), Major Depression (MD), Anxiety Disorder (AnxDis)) in the German, Norwegian and Spanish samples, followed by meta-analysis. Along allele and genotype counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Table IV: Association results of *NOS1* ex1f-VNTR with comorbid co-morbid axis 2 and somatic disorders (Personality Disorder Cluster A (CL_A), B (CL_B) and C (CL_C), Migraine (Migr)) in the German, Norwegian and Spanish sample, followed meta-analysis. Along allele and genotype counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Figures (6)

Supplementary Figure 1: Funnel plots of *NOS1* ex1f-VNTR recessive and allelic model in the total sample **(A)**, as well as the female **(B)** and the male **(C)** subset.

Supplementary Figure 2: Forest plots of allelic *NOS1* VNTR meta-analysis on aADHD clinical presentations **(A)** inattentive (AD), **(B)** hyperactive (HD), and **(C)** combined (AD+HD) type in the total sample and its sex specific subsets.

Supplementary Figure 3: Funnel plots of allelic *NOS1* VNTR meta-analysis on aADHD clinical presentations **(A)** inattentive (AD), **(B)** hyperactive (HD), and **(C)** combined (AD+HD) type in the total sample and its sex specific subsets.

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Supplementary Figure 4: Forest plots of recessive *NOS1* VNTR meta-analysis on aADHD clinical presentations **(A)** inattentive (AD), **(B)** hyperactive (HD), and **(C)** combined (AD+HD) type in the total sample and its sex specific subsets.

Supplementary Figure 5: Funnel plots of recessive *NOS1* VNTR meta-analysis on aADHD clinical presentations **(A)** inattentive (AD), **(B)** hyperactive (HD), and **(C)** combined (AD+HD) type in the total sample and its sex specific subsets.

Supplementary Figure 6: Genotype-specific *NOS1* ex1f expression in different brain regions.

Supplementary Tables (9)

Supplementary Table I: Demographic overview

Supplementary Table II: Comparison of the *NOS1* ex1f-VNTR genotypes between the former (Reif and others, 2009) and recent aADHD samples using allelic (S-alleles vs. L-alleles) and recessive (SS vs. SL+LL) models. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table III: Distribution of the 19 *NOS1* ex1f-VNTR-repeat alleles

Supplementary Table IV: Association results for the 21-repeat allele of *NOS1* ex1f-VNTR in the German, Norwegian and Spanish samples, followed by meta-analysis. Along allele counts for cases and controls, the $\chi 2$- and the p-value of the calculated allelic (21-repeat-alleles vs. all other alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed or random effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table V: Association results of aADHD clinical presentations (inattentive (AD), hyperactive (HD), and combined (AD+HD) type ADHD) for the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish sample. Along allele counts for cases and controls, the $\chi 2$- and the p-value of the calculated recessive (SS vs. SL+LL) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table VI: Association results of the three aADHD clinical presentations (inattentive (AD), hyperactive (HD), combined (AD+HD)) for the 21-repeat allele of the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish sample, followed by a meta-analysis. Along allele counts for cases and controls, the $\chi 2$- and the p-value of the calculated allelic (21-repeat alleles vs. all other alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed or random effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table VII: Association results of *NOS1* ex1f-VNTR with comorbid axis 1 disorders (Substance Abuse (Alcohol: SUDAlc and Illicit Drugs: SUDDrug), Major Depression (MD), Anxiety Disorder (AnxDis)) in the German, Norwegian and Spanish samples, followed by meta-analysis. Along allele and genotype counts for cases and controls, the $\chi 2$- and the p-value of the calculated recessive (SS vs. SL+LL) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table VIII: Association results of *NOS1* ex1f-VNTR with comorbid co-morbid axis 2 and somatic disorders (Personality Disorder Cluster A (CL_A), B (CL_B) and C (CL_C), Migraine (Migr)) in the German, Norwegian and Spanish sample, followed by meta-analysis. Along allele and genotype counts for cases and controls, the $\chi 2$- and the p-value of the calculated recessive (SS vs. SL+LL) model in the total sample, as well as their gender specific subsets are shown. Further, total

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cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

Supplementary Table IX: Association results of the 21-repeat allele of *NOS1* ex1f-VNTR with comorbid disorders (major depression (MD), anxiety disorders (AnxDis), migraine (Migr) for in the German, Norwegian and Spanish sample, followed by meta-analysis. Along allele counts for cases and controls, the $\chi 2$- and the p-value of the calculated allelic (21-repeat alleles vs. all other alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed or random effect model are given. Bold face indicates significant p-values ($p<0.05$).

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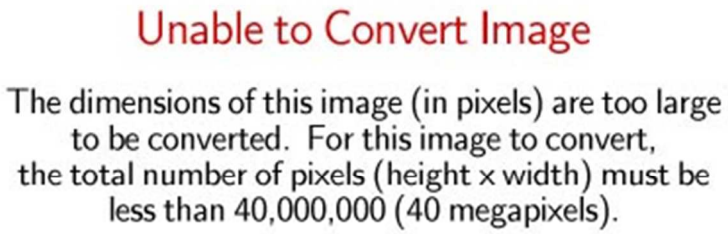

Figure 1: Chromosomal position of the NOS1 ex1f-VNTR according to NCBI´s genome built GRCh 38 and distribution of the repeat length variants in German, Norwegian and Spanish samples.

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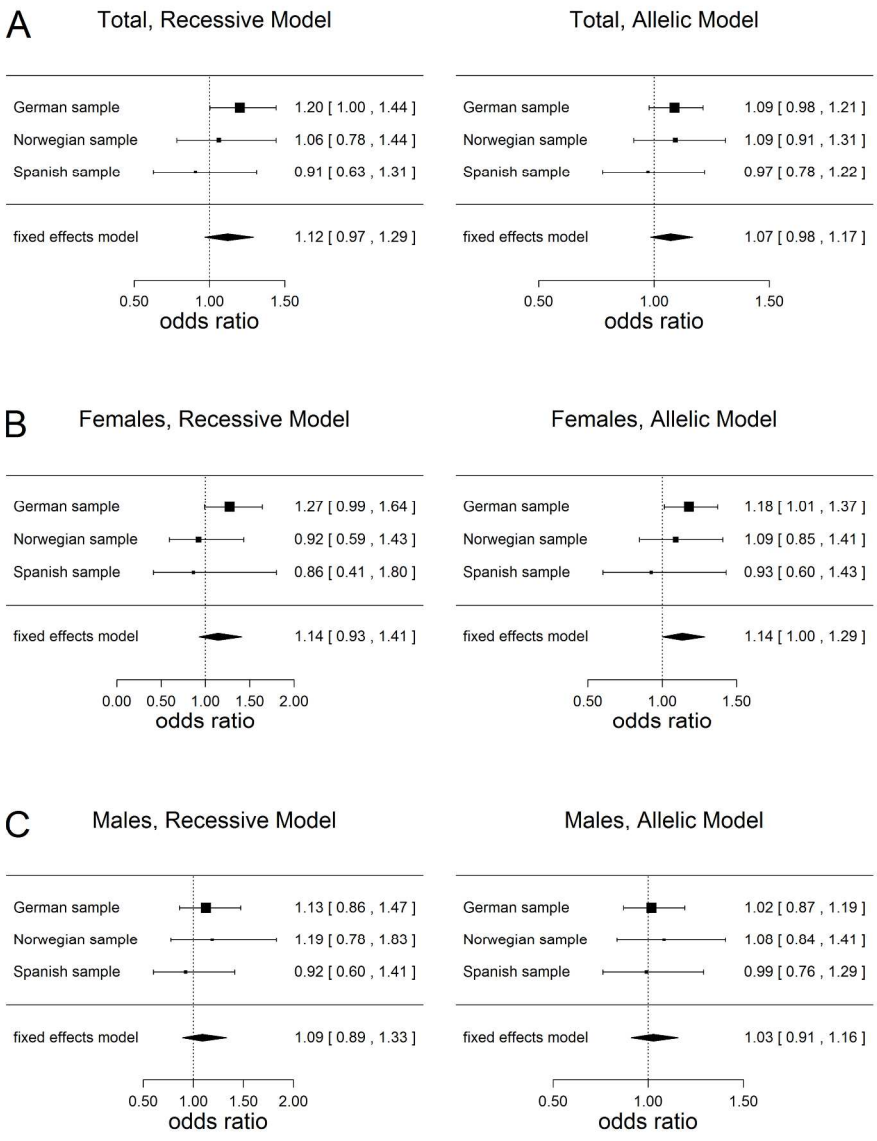

Figure 2: Forest plots of NOS1 ex1f-VNTR recessive and allelic model in the total sample (A), as well as the female (B) and the male (C) subset.
270x361mm (300 x 300 DPI)

Table I: Association results for the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish samples, followed by meta-analysis. Table shows allele and genotype counts for cases and controls, as well as χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) and recessive (SS vs. SL+LL) and models in the total sample, and their sex-specific subsets. Further, total cases and control counts are given for the allelic and recessive models as well as p-values for heterogeneity and odds ratios plus p-values of the fixed effect model. Bold face indicates significant p-values (p<0.05).

	Total		Females		Males	
	S/L	SS/SL+LL	S/L	SS/SL+LL	S/L	SS/SL+LL
German Sample						
Cases	956/1018	233/754	464/454	112/347	492/564	121/407
Controls	1870/2168	413/1606	1162/1342	253/999	708/826	160/607
χ2-value	2.39	3.90	4.61	3.52	0.05	0.78
P-value	*0.122*	***0.048***	***0.032***	*0.061*	*0.827*	*0.378*
Norwegian Sample						
Cases	422/410	97/319	199/199	41/158	223/211	56/161
Controls	519/551	119/416	283/309	65/231	236/242	54/185
χ2-value	0.92	0.15	0.46	0.13	0.37	0.64
P-value	*0.338*	*0.695*	*0.498*	*0.718*	*0.544*	*0.423*
Spanish Sample						
Cases	290/298	71/223	76/84	17/63	214/214	54/160
Controls	312/312	81/231	83/85	20/64	229/227	61/167
χ2-value	0.06	0.26	0.12	0.15	0.00	0.13
P-value	*0.813*	*0.607*	*0.730*	*0.695*	*0.948*	*0.716*
Total:						
Cases	1668/1726	401/1296	752/744	175/573	1002/1084	246/797
Controls	2701/3031	613/2253	1528/1736	338/1294	1173/1295	275/959
Heterogeneity:						
Q (df = 2)	0.83	1.93	1.19	2.18	0.25	0.81
P-value	0.66	0.38	0.552	0.336	0.884	0.668
Cochran-Mantel-Haenszel Metaanalysis:						
Fixed effect	1.07	1.12	1.14	1.14	1.03	1.09
P-value	*0.116*	*0.135*	***0.049***	*0.233*	*0.699*	*0.418*

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Table II: Association results of aADHD clinical presentations (inattentive (AD), hyperactive (HD), and combined (AD+HD) type ADHD) for the *NOS1* ex1f-VNTR in the German, Norwegian and Spanish sample, followed by a meta-analysis. Along allele counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values ($p<0.05$).

	Total			Females			Males		
	AD	**HD**	**AD+HD**	**AD**	**HD**	**AD+HD**	**AD**	**HD**	**AD+HD**
German Sample									
Cases (S/L)	214/244	83/67	659/707	94/90	34/26	336/338	120/154	49/41	323/369
Controls (S/L)	1870/2168	1870/2168	1870/2168	1162/1342	1162/1342	1162/1342	708/826	708/826	708/826
χ2-value	0.03	4.73	1.53	1.51	2.48	2.53	0.52	2.35	0.05
P-value	*0.866*	***0.030***	*0.216*	*0.219*	*0.115*	*0.112*	*0.471*	*0.125*	*0.819*
Norwegian Sample									
Cases (S/L)	65/71	29/27	225/209	27/35	11/5	130/122	38/36	18/22	95/87
Controls (S/L)	519/551	519/551	519/551	283/309	283/309	283/309	236/242	236/242	236/242
χ2-value	0.02	0.23	1.38	0.41	2.74	1.01	0.10	0.28	0.42
P-value	*0.876*	*0.632*	*0.241*	*0.523*	*0.098*	*0.314*	*0.751*	*0.595*	*0.517*
Spanish Sample									
Cases (S/L)	93/89	10/12	187/197	25/29	2/2	49/53	68/60	8/10	138/144
Controls (S/L)	312/312	312/312	312/312	83/85	83/85	83/85	229/227	229/227	229/227
χ2-value	0.07	0.18	0.16	0.16	-	0.05	0.34	0.23	0.11
P-value	*0.794*	*0.675*	*0.688*	*0.691*	*1.000*	*0.828*	*0.561*	*0.631*	*0.735*
Total:									
Cases (S/L)	372/404	122/106	1071/1113	146/154	47/33	515/513	226/250	75/73	556/600
Controls (S/L)	2701/3031	2701/3031	2701/3031	1528/1736	1528/1736	1528/1736	1173/1295	1173/1295	1173/1295
Heterogeneity:									
Q (df = 2)	0.09	1.62	1.21	0.97	1.34	1.95	0.94	2.28	0.51
P-value	0.958	0.445	0.546	0.615	0.511	0.377	0.625	0.318	0.776
Cochran-Mantel-Haenszel Metaanalysis:									
Fixed effect	1.01	1.29	1.07	1.11	1.67	1.18	0.99	1.14	1.02
P-value	*0.884*	*0.071*	*0.181*	*0.442*	***0.032***	***0.026***	*0.935*	*0.505*	*0.803*

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Table III: Association results of *NOS1* ex1f-VNTR with comorbid axis 1 disorders (Substance Abuse (Alcohol: SUDAlc and Illicit Drugs: SUDDrug), Major Depression (MD), Anxiety Disorder (AnxDis)) in the German, Norwegian and Spanish samples, followed by meta-analysis. Along allele and genotype counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values (p<0.05).

	Total				Females				Males			
	SUDALc	SUDDrug	MD	AnxDis	SUDALc	SUDDrug	MD	AnxDis	SUDALc	SUDDrug	MD	AnxDis
German Sample												
Cases (S/L)	125/127	201/199	370/354	189/181	49/41	82/68	198/188	113/105	76/86	119/131	172/166	76/76
Controls (S/L)	1870/2168	1870/2168	1870/2168	1870/2168	1162/1342	1162/1342	1162/1342	1162/1342	708/826	708/826	708/826	708/826
χ2-value	1.03	2.27	5.67	3.10	2.26	3.88	3.21	2.37	0.03	0.18	2.49	0.82
P-value	0.309	0.132	***0.017***	*0.078*	0.133	0.049	*0.073*	*0.123*	0.854	0.671	*0.115*	*0.365*
Norwegian Sample												
Cases (S/L)	91/105	108/110	289/261	289/261	28/34	36/34	141/135	141/135	63/71	72/76	148/126	148/126
Controls (S/L)	519/551	519/551	519/551	519/551	283/309	283/309	283/309	283/309	236/242	236/242	236/242	236/242
χ2-value	0.29	0.08	2.37	2.37	0.16	0.33	0.81	0.81	0.23	0.02	1.50	1.50
P-value	0.593	0.780	*0.124*	*0.124*	0.692	0.566	*0.368*	*0.368*	0.630	0.878	*0.220*	*0.220*
Spanish Sample												
Cases (S/L)	57/69	100/110	106/98	91/85	0/8	11/13	37/37	27/33	57/61	89/97	69/61	64/52
Controls (S/L)	312/312	312/312	312/312	312/312	83/85	83/85	83/85	83/85	229/227	229/227	229/227	229/227
χ2-value	0.95	0.36	0.24	0.16	-	0.11	0.01	0.34	0.14	0.30	0.33	0.91
P-value	0.329	0.551	*0.627*	*0.69*	**0.007**	0.743	*0.932*	*0.558*	0.711	0.586	*0.565*	*0.341*
Total:												
Cases (S/L)	273/301	409/419	765/713	569/527	77/83	129/115	376/360	281/273	196/218	280/304	389/353	288/254
Controls (S/L)	2701/3031	2701/3031	2701/3031	2701/3031	1528/1736	1528/1736	1528/1736	1528/1736	1173/1295	1173/1295	1173/1295	1173/1295
Heterogeneity:												
Q (df = 2)	2.27	1.81	0.40	0.37	5.78	1.20	0.38	1.41	0.29	0.50	0.11	0.03
P-value	0.322	0.405	0.819	0.831	0.055	0.550	0.825	0.495	0.865	0.781	0.945	0.984
Cochran-Mantel-Haenszel Metaanalysis:												
Fixed effect	1.00	1.07	1.18	1.17	1.05	1.26	1.17	1.15	0.96	0.99	1.19	1.19
P-value	*0.992*	*0.366*	**0.006**	***0.024***	*0.841*	*0.090*	*0.062*	*0.160*	0.775	0.975	***0.045***	0.082

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Table IV: Association results of *NOS1* ex1f-VNTR with comorbid co-morbid axis 2 and somatic disorders (Personality Disorder Cluster A (CL_A), B (CL_B) and C (CL_C), Migraine (Migr)) in the German, Norwegian and Spanish sample, followed meta-analysis. Along allele and genotype counts for cases and controls, the χ2- and the p-value of the calculated allelic (S-alleles vs. L-alleles) model in the total sample, as well as their gender specific subsets are shown. Further, total cases and control allele counts, p-values for heterogeneity and odds ratios plus p-values of the fixed effect model are given. Bold face indicates significant p-values (p<0.05).

	Total				Females				Males			
	CL_A	CL_B	CL_C	Migr	CL_A	CL_B	CL_C	Migr	CL_A	CL_B	CL_C	Migr
German Sample												
Cases (S/L)	7/5	101/97	23/25	161/147	0/2	44/50	11/15	103/83	7/3	57/47	12/10	58/64
Controls (S/L)	1870/2168	1870/2168	1870/2168	1870/2168	1162/1342	1162/1342	1162/1342	1162/1342	708/826	708/826	708/826	708/826
χ2-value	0.70	1.68	0.05	4.09	-	0.01	0.17	5.59	-	2.93	0.61	0.09
P-value	0.404	0.196	0.824	**0.043**	0.502	0.939	0.677	**0.018**	0.202	0.087	0.433	0.767
Norwegian Sample												
Cases (S/L)	-	-	-	115/109	-	-	-	64/68	-	-	-	51/41
Controls (S/L)	-	-	-	519/551	-	-	-	283/309	-	-	-	236/242
χ2-value	-	-	-	0.60	-	-	-	0.02	-	-	-	1.13
P-value	-	-	-	0.440	-	-	-	0.887	-	-	-	0.287
Spanish Sample												
Cases (S/L)	2/4	25/29	16/8	43/23	-	2/6	4/2	21/11	2/4	23/23	12/6	22/12
Controls (S/L)	312/312	312/312	312/312	312/312	83/85	83/85	83/85	83/85	229/227	229/227	229/227	229/227
χ2-value	-	0.27	2.57	5.49	-	-	-	2.83	-	0.00	1.87	2.66
P-value	0.686	0.602	0.109	**0.019**	1.000	0.280	0.682	0.092	0.686	0.977	0.171	0.103
Total:												
Cases (S/L)	9/9	126/126	39/33	319/279	0/2	44/50	11/15	188/162	9/7	80/70	24/16	131/117
Controls (S/L)	2701/3031	2701/3031	2701/3031	2701/3031	1528/1736	1528/1736	1528/1736	1528/1736	1173/1295	1173/1295	1173/1295	1173/1295
Heterogeneity:												
Q (df = 2)	1.26	1.11	1.42	2.77	0.53	1.62	0.84	2.92	2.37	0.93	0.32	1.77
P-value	0.261	0.292	0.233	0.250	0.769	0.443	0.658	0.232	0.306	0.627	0.850	0.413
Cochran-Mantel-Haenszel Metaanalysis:												
Fixed effect	1.10	1.13	1.30	1.27	0.53	0.94	0.99	1.31	1.41	1.27	1.63	1.22
P-value	*0.980*	*0.397*	*0.327*	**0.007**	*0.544*	*0.840*	*0.874*	***0.022***	0.662	*0.184*	0.182	0.165

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