Intracranial aneurysm formation and eventual rupture leading to subarachnoid hemorrhage (SAH) is one of the most grave manifestations of vascular disease. Nitric oxide (NO) is a free radical of considerable interest in vascular pathobiology, with a multitude of effects, ranging from vasodilation, regulation of cellular respiration, and oxygen uptake to inflammation. Nitric oxide formation is catalyzed by endothelial, neuronal, and inducible isoforms of NO synthase (NOS), encoded by 3 separate genes. A considerable amount of evidence links the endothelial NO pathway to the pathogenesis and pathophysiology of SAH.

Only a few studies have investigated the association between SAH and variation in the endothelial NOS gene (NOS3). Those have focused on the effect of the common single nucleotide polymorphisms (SNPs) –922A > G, –786T > C, and 894G > T (Asp298Glu), as well as the intron-4 27-bp variable number of tandem repeats polymorphism (27-bp-VNTR). An initial association in a Caucasian population between SAH and the α-allele of 27-bp-VNTR has not been reproduced. Additionally, the 894T/T genotype was found to associate with SAH in a Turkish population. Genome-wide association studies did not find any association between NOS3 polymorphisms and SAH. However, these studies did not include the 27-bp VNTR polymorphism, and neither did they infer haplotypes, which in some cases have been shown to be of significance independent of individual SNPs.

In this case-control study we investigated the association between the above-mentioned NOS3 polymorphisms and haplotypes with aneurysmal SAH, while also testing for an interaction with well-established risk factors (sex, smoking, alcohol, arterial hypertension, and age).

**Methods**

The study was approved by the Regional Ethics Committee of Copenhagen and the Data Protection Agency, and written informed consent was obtained from all participants or their next of kin. Four hundred eight...
patients admitted to Neurointensive Care Unit at the University Hospital of Copenhagen (Rigshospitalet) with the diagnosis of spontaneous SAH were included. The inclusion period was between 2006 and 2011 and for a subset \((n = 61)\) between 2002 and 2004 as part of a randomized controlled trial.\(^2\) Fifty-six patients were excluded because the SAH etiology was determined to be non-neurysmal by angiography. In 16 patients, the severity of the hemorrhage led to death before angiography could be performed; these individuals were allowed into the analysis to also include the most severe phenotype. Of the remaining 352 patients, 333 were of Caucasian ethnicity as judged by reviewing names, addresses, social security numbers, and remarks in the patient charts, and these patients were included in the subsequent analysis. The 19 excluded patients originated from Greenland, Asia, Africa, and the Middle East. The control group consisted of 498 Danish patients undergoing nonvascular intracranial surgery (38%) or spine surgery (62%). A subpopulation of the participants were part of another genetic association study concerning the effects of the ACE I/D polymorphism, which found no association with SAH.\(^30\)

**Phenotypic Study Variables**

Information regarding smoking, alcohol use, and arterial hypertension was retrieved from the patient file. In controls this information was derived from the pre-anesthesia interview chart, whereas in patients the source was any sufficiently accurate description in the file, most often the admission chart. Given the limitations of collecting these data retrospectively, smoking was defined as ever (more than 1 pack-year) or never, alcohol abuse as ever (current or previous abuse of > 21 units per week) or never, and arterial hypertension was defined as yes (any condition necessitating lifelong antihypertensive medication or deliberate avoidance of antihypertensive medication against medical advice) or no.

**Purification of DNA and Genotyping**

DNA was purified from whole blood samples using the FlexiGene DNA kit (Qiagen, Hilden, Germany, cat. no. 51206). In 23 individuals (6 male, 17 female) only serum samples were available; DNA from these samples was purified using a previously described sodium iodine extraction method.\(^10\) As the DNA quality from this source is of a poorer quality, we controlled all statistical analyses (later) by omitting this subset. In the NO\(S\)3 promoter region the \(-922A \rightarrow G\) (rs 1800779) and the \(-786T \rightarrow C\) (rs2070744) along with the nonsynonymous exon-7 894G \(\rightarrow T\), Glu298Asp (rs2070744) along with the nonsynonymous exon-7 894G \(\rightarrow T\), Glu298Asp (rs2070744) along with the nonsynonymous exon-7 894G \(\rightarrow T\), Glu298Asp (rs2070744) along with the nonsynonymous exon-7 894G \(\rightarrow T\), Gla298Asp (rs1799983) were determined using TaqMan probes on an Applied Biosystem 7500 Fast Real Time PCR device according to the manufacturer's instructions. The PCR data were reviewed using the software supplied with the system (SDS version 1.3.1). The intron-4 27-bp-VNTR was genotyped as previously described by PCR followed by gel electrophoresis (forward primer: 5'-AGG CCC TAT GGT AGT GCC TTG-3'; reverse primer: 5'-TCT CTT AGT GCT GTG GTC AC-3'; MWG-Biotech AG).\(^2\) The 4 tandem repeats allele is denoted \(a\), whereas the 5 tandem repeats allele is denoted \(b\).

**Statistical Analysis**

Statistical analysis was performed with R version 2.15.1 Mac GUI (http://www.r-project.org/index.html). Haplotype estimation and testing for association with disease status was performed using the expectation-maximization algorithm implemented in the haplo.glm procedure of the R package Haplø Stats version 1.5.\(^27\) We estimated that the aforementioned study period would allow us to include about 350 patients giving us a similar or higher power than the majority of previous studies of SNPs and SAH. Categorical variables were analyzed using Fisher's exact test for contingency tables and logistic regression with backward stepwise selection of covariates for the multivariate model. Furthermore, the estimates of the final logistic regression model were controlled using a conditional logistic regression model with frequency matching for age and sex (R-package: Survival, clogit function; Therneau T: A package for survival analysis in S. R package version 2.36-14, 2012). Deviation from Hardy-Weinberg equilibrium was tested using the exact test in the genetics R-package. Missing data were handled by exclusion. All tests were 2-sided and considered statistically significant at the \(p < 5\%\) level; \(p\) values are presented uncorrected. The Bonferroni corrected significance level of the 4 individual SNP comparisons is \(\alpha_{\text{Bonferroni}} = 0.0125\).

**Results**

**Study Population Characteristics**

Three hundred thirty-three SAH patients were included along with 498 controls after applying the inclusion and exclusion criteria. The age range in cases was 22–87 years with a mean (± SD) of 56 ± 11 years; in controls the range was 18–89 years (mean 55 ± 15 years). Risk factors of the 2 study populations are presented in Table 1. Odds ratios for female sex, smoking, and hypertension were in good agreement with previous studies.\(^9\) Alcohol abuse was more strongly associated with SAH in our study population than in previous studies.\(^9\) Multiple aneurysms were found in 16% of the male patients and in 28% of the female patients (\(p = 0.04\); OR 2.0, 95% CI 1.0–4.2). All statistical analyses were repeated omitting the patients in whom angiography could not be applied due to early death (\(n = 16\)), and this did not change the overall results.

**Genotypes**

No genotype deviated significantly from Hardy-Weinberg equilibrium. We found a higher frequency of the \(b/b\)-genotype of the 27 bp-VNTR polymorphism in cases (77%) compared with controls (69%). The difference was borderline significant (\(p = 0.02\)) in a 2 × 2 table of the \(b/b\) versus \(b/a + a/a\) genotypes corresponding to an OR of 1.5 (95% CI 1.1–2.0). None of the other \(NO\(S\)3\) SNPs differed significantly between cases and controls (Table 2). When the population was stratified according to sex, the 27-bp-VNTR \(b/b\) genotype in male patients was associated with an OR for SAH of 2.8 (95% CI 1.5–5.6, \(p = 0.0005\)), whereas in the female subset there was no such association (Table 3). Removing the 16 patients who died before angiography led to the removal of 6 cases involving male patients with...
**NOS3 intron-4 VNTR polymorphism and SAH**

**TABLE 1: Risk factors in the study populations***

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cases</th>
<th>Controls</th>
<th>Univariate OR (95% CI)</th>
<th>p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (yrs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>22–87</td>
<td>18–89</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>56 ± 11</td>
<td>55 ± 15</td>
<td>2.3 (1.7–3.1)</td>
<td>4.3e-08</td>
</tr>
<tr>
<td>sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>233 (70)</td>
<td>253 (51)</td>
<td>3.7 (2.7–5.2)</td>
<td>2.2e-16</td>
</tr>
<tr>
<td>male</td>
<td>100 (30)</td>
<td>245 (49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ever</td>
<td>188 (56)</td>
<td>159 (32)</td>
<td>5.7 (3.2–10.7)</td>
<td>2.3e-10</td>
</tr>
<tr>
<td>never</td>
<td>89 (27)</td>
<td>282 (57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alcohol abuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ever</td>
<td>45 (14)</td>
<td>19 (4)</td>
<td>1.6 (1.1–2.2)</td>
<td>0.009</td>
</tr>
<tr>
<td>never</td>
<td>170 (51)</td>
<td>411 (83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>95 (29)</td>
<td>111 (22)</td>
<td>1.5 (1.1–2.0)</td>
<td>0.60</td>
</tr>
<tr>
<td>no</td>
<td>190 (57)</td>
<td>346 (69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>48 (14)</td>
<td>68 (14)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values represent numbers of patients (%) unless otherwise indicated. NA = information not available. † p value of Fisher’s exact test of 2 × 2 contingency tables omitting NA values or t-test for age.

After backward multivariate logistic regression from a model containing main and 2-way interaction effects of the b/b genotype, 9 cases involving females with the b/b genotype, and 1 case involving a female patient with the a/b genotype. This would change the p value to 0.002 for the b/b versus a/b + a/a model in men and 0.92 in women.

**Multivariate Model**

After backward multivariate logistic regression from a model containing main and 2-way interaction effects of the 27-bp-VNTR polymorphism (b/b vs b/a + a/a), sex, smoking, arterial hypertension, and age, a reduced model as presented in Fig. 1 was derived. In particular, no significant interactions between the 27-bp-VNTR b/b genotype and smoking, hypertension, or age were found. According to this model, male carriers of the b/b genotype have an OR of 2.7 (95% CI 1.2–6.5, p = 0.02) for SAH regardless of age, smoking, or hypertension status, while female b/b carriers have an OR of 1.0 (95% CI 0.6–1.6, p = 0.98). We did not include alcohol abuse in the model, as the amount of missing data in the case group was considerable (35%). As an alternative statistical approach, we controlled the estimates using conditional logistic regression. Using frequency matching (post hoc stratification) of cases and controls to age and sex, the effect of the b/b genotype in male patients was estimated to an OR of 2.5 (95% CI 1.1–5.0, p = 0.02). Thus, the data suggest that the increased risk for aneurysmal SAH conferred by the 27-bp-VNTR b/b-genotype in men is of similar magnitude as that conferred by either arterial hypertension or smoking (Fig. 1).

**Haplotypes**

Estimated haplotype frequencies are presented in Table 4 and are close to previously described estimates, except that we did not find the 786C-4 b -894G haplotype with frequency in our population. The haplotype logistic regression analysis revealed that the effect of the intron 4 b/b genotype was not due to a single haplotype containing the b allele, but rather a significantly lower frequency of the G-C-a-G haplotype in male cases (p = 0.01), corresponding to an OR of 0.3 (95% CI 0.1–0.8) as presented in Table 4. Since all a alleles belong to a single haplotype (G-C-a-G) it is not possible to discriminate between haplotype and genotype specific effects with regard to this allele.

**Discussion**

In this case-control study we found that the b/b genotype of the intron-4 27-bp VNTR polymorphism was
associated with aneurysmal SAH and that the univariate association was driven by an interaction with sex. Carriers of the $b/b$ genotype were much more prevalent among male patients than among female patients, corresponding to an OR of 2.8 for SAH in men and an OR of 1.1 in women. This effect was robust to adjustment for 2-way interaction of smoking, alcohol, arterial hypertension, and age with genotype in multivariate logistic regression models. Furthermore, haplotype analysis with adjustment for the same confounders revealed that no single haplotype containing the $b$ allele was responsible for the observed genotype effect. Taken together, the results indicate that the intron-4 27-bp-VNTR polymorphism by itself, independent of arrangements in haplotypes, accounts for increased risk of aneurysmal SAH in men but not in women.

In the multivariate model (Fig. 1) we included interactions between smoking and hypertension ($p = 0.07$), age and smoking ($p = 0.08$), and age and hypertension ($p = 0.001$), which may appear to decrease the effects of smoking and hypertension with advancing age (OR per decade 0.79 and 0.54, respectively). These effects are due to smoking and hypertension being much more prevalent in SAH patients (cases) of younger age than in similar controls, whereas the distribution of these 2 risk factors differs less between SAH patients (cases) and controls of higher age. This suggests that individuals vulnerable to aneurysm formation and subsequent rupture may tend to develop SAH at a younger age if exposed to smoking and hypertension. However, the choice of control group may obviously bias this finding. In summary, the multivariate risk factor model (Fig. 1) shows that the effect of the $b/b$ genotype of the 27-bp-VNTR in males is of the same magnitude as smoking and arterial hypertension, both well-known risk factors for aneurysmal SAH.

In a population of American Caucasians ($n = 51$) Khurana et al. reported an association between the $a$ allele and SAH of OR 4 in a multivariate model, which is contradictory to our results. However, in another study, the same group found the $b/b$ genotype to be more frequent in patients with unruptured aneurysms than in those with ruptured aneurysms (80% vs 50%). Ozüm et al. reported an association between the 894 T/T genotype (corresponding to Glu298Glu) and SAH in a Turkish population ($n = 53$). Others have found no association between any of the polymorphisms ($-786T > C$, 894G > T, and intron 4 $b/a$) or related haplotypes with SAH in German and Japanese populations. Thus, our data, although not necessarily in contradiction with the 2 negative association studies, point in another direction. Importantly, marked differences in both genotype and haplotype frequencies of NOS3 exist between ethnicities, which could explain some of the discrepancies between studies.

The etiology behind the sex differences in SAH patients remains unknown. Earlier studies indicate that women have higher blood flow velocities, especially in
the posterior circulation, and that autoregulation of cerebral blood flow may function differently in men and women under some circumstances such as orthostatic stress.\(^ {22,23} \) Also, there are anatomical differences concerning the angulation of arterial bifurcations in the circle of Willis, suggesting that shear stress on the vessel wall is larger in women.\(^ {21} \) Furthermore, female SAH patients tend to have more ruptured aneurysms located at the internal carotid artery than men, as well as more frequently harboring multiple aneurysms.\(^ {31} \) Altogether, these lines of evidence suggest differences in the pathogenesis of intracranial aneurysms between the sexes. However, none of the traditional cardiovascular risk factors are, to our knowledge, known to interact with sex to increase the risk of SAH. Concerning the peripheral endothelial function, the female population of the Framingham Offspring Study were observed to have higher brachial artery flow-mediated dilation than men (3.3% vs 2.4%) in the population,\(^ 5 \) and in the same cohort NOS3 polymorphisms located near intron 4 were associated with brachial artery flow-mediated dilation in men.\(^ {14} \) Similarly, the digital reactive hyperemia index is lower in men.\(^ {22} \)

Recent studies show that the 27-bp-VNTR is the origin of small interfering RNA and that the resulting 27-bp-nucleotide acts as a posttranscriptional negative feedback regulator of endothelial NOS.\(^ {33} \) Furthermore, in cell cultures the 27-bp-VNTR repeats of length 4 (the b allele) have been shown to produce more 27-nt-siRNA and inhibit endothelial NOS mRNA levels more than the 4-repeat variant.\(^ {34} \) Experimental rodent models of aneurysm formation have suggested that regulation of endothelial and neuronal NOS protect against aneurysm formation, whereas inducible NOS promotes aneurysm growth.\(^ {1,11,25} \) Thus, a biological pathway for the functionality of this genetic variant may exist, though how this translates to aneurysm formation in men more than in women is of yet a matter of speculation.

**Limitations**

The present choice of control group of surgical patients may be considered a weakness, but given that no such group is ideal, it reflects the background population well with regard to age, sex, geographic origin, and risk factor distribution.\(^ 8 \) Also, there are differences between cases and controls in the gathering of vascular risk factor data, which most certainly lead to some bias in our results, probably tending to cause overestimation of effects. This is frequently seen when comparing case-control to cohort studies of SAH.\(^ 9 \) However, this does not hamper our main result regarding the effect of 27-bp-VNTR polymorphism on SAH risk, as univariate and multivariate methods yielded almost identical effect estimates. In addition, genotyping was independent of sex. Whether genetic risk factors could be shared between SAH and the variety of nonvascular neurosurgical conditions represented within the control group is speculative, but the control group’s genotype distribution resembles that of control groups in other studies.\(^ {19} \) Major strengths of this study include ethnic homogeneity and the well-defined phenotype, although the main weakness of this study is that the sex-specific association of the b/b genotype was not prespecified.

**Conclusions**

We report a sex-specific association of the b/b genotype of the intron-4 b/a polymorphism with aneurysmal SAH (OR 2.8, 95% CI 1.5–5.6). The potential increase in risk of male carriers is comparable to that of arterial hypertension or smoking. The data indicate that an NOS3 genotype-environment interaction is involved in the pathogenesis of SAH in men but not in women.

**Acknowledgment**

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**Disclosure**

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Author contributions to the study and manuscript preparation include the following. Conception and design: Olsen, Staalsø, Rommer. Acquisition of data: Staalsø, Edsen, Kotinis, Springborg. Analysis and interpretation of data: Olsen, Staalsø, Rommer. Drafting the article: Olsen, Staalsø. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Olsen. Statistical analysis: Staalsø. Study supervision: Olsen.
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