***NOTCH4* gene polymorphisms as potential risk factors for brain arteriovenous malformation development and hemorrhagic presentation**

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OBJECTIVE Arteriovenous malformations (AVMs) of the brain are a frequent and important cause of intracranial hemorrhage in young adults. Little is known about the molecular-genetic pathomechanisms underlying AVM development. Genes of the *NOTCH* family control the normal development of vessels and proper arteriovenous specification. Transgenic mice with constitutive expression of active NOTCH4 frequently develop AVMs. Here, the authors report a genetic association study investigating possible associations between *NOTCH4* gene polymorphisms and formation and clinical presentation of AVMs.

METHODS After PCR amplification and direct DNA sequencing or restriction digests, 10 single-nucleotide polymorphisms (SNPs) of the *NOTCH4* gene were used for genotyping 153 AVM patients and 192 healthy controls (i.e., blood donors). Pertinent clinical data were available for 129 patients. Uni- and multivariate single-marker and explorative haplotype analyses were performed to identify potential genetic risk factors for AVM development and for hemorrhagic or epileptic presentation.

RESULTS Eleven calculated haplotypes consisting of 3–4 SNPs (most of which were located in the epidermal growth factor–like domain of the *NOTCH4* gene) were observed significantly more often among AVM patients than among controls. Univariate analysis indicated that rs443198_TT and rs915895_AA genotypes both were significantly associated with hemorrhage and that an rs1109771_GG genotype was associated with epilepsy. The association between rs443198_TT and AVM bleeding remained significant in the multivariate regression analysis.

CONCLUSIONS The authors' results suggest *NOTCH4* SNPs as possible genetic risk factors for the development and clinical presentation of AVMs and a role of *NOTCH4* in the pathogenesis of this disease.

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KEY WORDS brain arteriovenous malformation; AVM; NOTCH4; single-nucleotide polymorphism; association study; vascular disorders

ARTERIOVENOUS malformations (AVMs) of the brain are vascular lesions that shunt arterial blood directly into a low-resistance system of draining veins without an intervening normal capillary bed. The prevalence of AVMs ranges from 15 to 18 per 100,000 adults,² with an approximate detection rate of 1 per 100,000 adults per year,⁶ which classifies these vascular malformations as rare diseases. The most common presentation of AVMs is intracerebral hemorrhage, which typically occurs in pa-

tients 20–40 years of age and affects approximately 50% of individuals with AVM.¹⁰ Moreover, 25%–40% of these patients present with epilepsy.³³ The risk for an intracerebral hemorrhage varies widely. Rates ranging from 0.9% in superficial lesions without a history of previous bleeding to 34% in deep-seated ruptured AVMs have been reported.³⁹ Treatment modalities include microsurgery, radiosurgery, and endovascular embolization, all of which carry sometimes substantial morbidity risks.⁴⁰

ABBREVIATIONS AVM = arteriovenous malformation; EGF = epidermal growth factor; SNP = single-nucleotide polymorphism.

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The etiology of AVMs is still poorly understood. Some data have suggested a role for a genetic component in the development of AVM.^{18,19} Of note, AVMs are a hallmark of hereditary hemorrhagic telangiectasia. This condition is caused by mutations in the *ENG* and *ACVRL1* genes and characterized by hereditary AVMs involving different organ systems, including the CNS.³² In addition, several case-control studies have found associations of single-nucleotide polymorphisms (SNPs) in various candidate genes with susceptibility to AVMs, the risk for hemorrhage, or both.^{16,17,23,28,36,41}

NOTCH4 is a member of the *NOTCH* gene family. Its members govern cellular differentiation and cell-fate determination and control the normal development of vessels and proper arteriovenous specification. NOTCH receptors and their ligands are expressed in arterial walls but not in those of the venous system,²⁰ and this arterial expression is both necessary and sufficient to induce that of ephrin-B2, considered to be a specific marker of arterial differentiation. The *NOTCH4* gene is located on chromosome 6p21.32. Its 30 exons (Fig. 1A) encode a 2003-amino-acids-long, single-pass transmembrane protein with ankyrin repeats in the intracellular and epidermal growth factor (EGF) repeats in the extracellular NOTCH4 domain. NOTCH4 binds to 2 different ligands: Delta and Serrate/Jagged.^{9,22} Ligand binding to the extracellular domain results in the proteolytic release of the intracellular domain and its translocation to the cell nucleus, which, in turn, initiates the expression of primary target genes belonging to the *HES* and *HER* gene families, which are involved in vascular development and proper arteriovenous differentiation.^{5,13,37}

Several studies have already suggested a role for NOTCH signaling in AVM formation and progression to hemorrhage. Recent work with a mouse model with transgenic expression of constitutively active Notch4 in endothelial cells has demonstrated that upregulation of Notch4 signaling causes vascular abnormalities very similar to those observed in AVM.^{25–27} Conversely, normalization of the elevated Notch4 levels resulted in decreased AVM vessel size.²⁵ In addition, genome-wide expression profiling in peripheral blood revealed differential expression of some genes involved in NOTCH signaling in patients with ruptured and unruptured AVMs compared with healthy controls.⁴² Last, NOTCH4 activation in endothelial and smooth-muscle cells of human AVM was very recently demonstrated by immunohistochemistry.⁴⁴ Taken together, these studies suggest a role for NOTCH4 in the formation and phenotypes of AVMs. This observation prompted us to conduct a case-control study to investigate possible associations of genetic polymorphisms in the *NOTCH4* gene with disease susceptibility and relevant clinical features.

The present study investigated whether 10 SNPs in the *NOTCH4* gene in 153 AVM patients and 192 controls were potentially associated with AVM formation and other clinical findings. Specific *NOTCH4* SNP haplotypes were found to be associated with AVM susceptibility, and several single genetic markers could be linked to hemorrhagic (and epileptic) presentation. These data provide support for a role of the *NOTCH4* gene in the formation and progression of AVM. They also suggest that *NOTCH4*

genetic variants may have potential for use as new clinical biomarkers.

Methods

Study Cohort

In total, this study included 153 white patients with AVMs, 50% of whom were male. The median age at treatment was 39 ± 15.5 years (mean 41, range 9–70 years). All patients had undergone diagnostic digital subtraction angiography.³⁴ In 108 patients who underwent surgery, the diagnosis of AVM was confirmed histologically. Clinical and angiographic data were available for 129 patients (Table 1).

The control group consisted of 192 white healthy blood donors (96 men and 96 women), with a median age of 27 ± 5.5 years (mean 25, range 18–60 years). Before any blood donation, all candidate donors underwent a thorough clinical examination and were asked to fill out an extensive questionnaire about their own and family medical histories. In case of any medical conditions or signs and symptoms pointing to any significant disease (including recurrent headaches or epilepsy), cardiovascular risk factors, or hereditary diseases in either the individual or her or his family, the candidate donor was excluded from blood donation.

The study was approved by the ethics committee of the University of Bonn Medical Center. Informed consent was obtained from all patients and controls according to the Declaration of Helsinki.

Selection and Genotyping of *NOTCH4* Gene SNPs

Ten (2 exonic and 8 intronic) SNPs (rs434841 [A/G], rs3134931 [A/G], rs443198 [C/T], rs915895 [A/G], rs715299 [A/C], rs415929 [A/G], rs1109771 [A/G], rs3134798 [C/T], rs2071286 [A/G], and rs2071279 [G/T]) of the *NOTCH4* gene were selected from the HapMap project data (<http://hapmap.org>). We applied the SNPs Tagger algorithm¹¹ implemented in Haploview (dbSNP b126 on the National Center for Biotechnology Information b36 assembly),⁴ with the following characteristics: minor allele frequency < 2% in the white CEU population, pairwise selection, r^2 cutoff > 0.8, and maximum DNA segment size of 250 bp.

High-molecular-weight genomic DNA was isolated from leukocytes with a salting-out procedure.²⁴ All 10 polymorphisms were analyzed after PCR amplification, followed by restriction-enzyme digest analysis for 2 SNPs and direct sequencing of both DNA strands with the Big-Dye Terminator Cycle Sequencing V1.1 Ready Reaction kit and an automated ABI-3100 DNA sequencer (Applied Biosystems) for the remaining 8 SNPs. Primers were designed with the Primer3 software version 0.4.0 (Whitehead Institute for Biomedical Research). Details on the primers,³⁸ restriction enzymes, and PCR conditions used are available on request.

Haplotype Analysis

Recent work has suggested that a multimarker (haplotype) analysis may improve genotyping efficiency and refine the association signal.¹¹ We therefore performed an explorative haplotype study and calculated all possible haplotypes consisting of up to 4 SNPs. This analysis re-

FIG. 1. A: The location of the *NOTCH4* gene on Chromosome 6 and the distribution of all the SNPs investigated in the present study throughout the gene. The rectangles (1–30) represent the gene exons. **B:** The 3 SNPs that were present in all haplotypes associated with increased susceptibility to AVM. Interestingly, they are located in a part of the gene coding for the Ca²⁺-binding region of the EGF-like domain of the *NOTCH4* protein (not shown). **C:** Calculated haplotype frequencies of the *NOTCH4* SNPs that were associated with increased AVM susceptibility (uncorrected $p \leq 1 \times 10^{-15}$). LCI = left 95% CI; Pat. = patients; RCI = right 95% CI.

sulted in calculated haplotype frequencies of 1% in some cases. This low haplotype frequency, along with the limited number of patients and controls in this study, precluded further analysis of more complex haplotypes.

Genotype–Phenotype Correlations

Because the data from the association study suggested a link between *NOTCH4* gene variants and AVM biology, we also looked for possible correlations between

TABLE 1. Patient characteristics

Characteristic	No. of Patients (n = 129)
Mean age in yrs (median $\pm$ SD [range])	41 (39 $\pm$ 15.5 [9–70])
M/F ratio	65:64
Epilepsy	
Present	56
Absent	72
Not determined	1
Hemorrhage	
Present	64
Absent	65
AVM-associated aneurysm	
Present	17
Absent	112
Deep drainage	
Present	49
Absent	80
Location	
Superficial	74
Deep	31
Not determined	24
Supratentorial	116
Infratentorial	13
AVM size	
≥ 3 cm	58
< 3 cm	71
Spetzler-Martin grade	
I	19
II	59
III	43
IV	7
V	1
Treatment	
Surgery only	93
Embolization & surgery	15
Radiosurgery only	5
Embolization & radiosurgery	16

NOTCH4 SNP genotypes and clinical parameters. Hence, we investigated all SNPs as potential predictors of hemorrhagic presentation or presentation with epilepsy.

Biostatistical Analysis

Hardy-Weinberg equilibrium and linkage disequilibrium were estimated with Haploview software V4.0.⁴ All investigated SNPs were in Hardy-Weinberg equilibrium among cases and controls. The Cochran-Armitage trend test was applied to compare genotype frequencies among AVM patients, AVM patient subsets, and healthy controls. Statistical significance of correlations among clinical parameters and of genotype–phenotype correlations was assessed with standard methods, including multivariate logistic regression analyses. All tests were 2 sided, and

a *p* value < 0.05 was considered statistically significant. The FAMHAP software V19.0¹⁴ was used to calculate and compare haplotype frequencies consisting of up to 4 SNPs in patients versus controls and of up to 3 SNPs in patient subsets.

Results

Clinical and Angiographic Data

Relevant clinical and demographic data were retrieved for 129 patients and included the following parameters: clinical presentation (bleeding, epilepsy, or other), maximum AVM diameter, presence or absence of AVM-associated aneurysms or deep venous drainage, and AVM location and Spetzler-Martin grade³⁴ (Table 1). Sixty-four (50%) patients had a history of AVM bleeding, and symptomatic epilepsy was recorded in 56 (43%) patients. One hundred eight (84%) patients had surgery for AVM, 15 of whom had undergone AVM embolization before the operation. Five patients underwent only radiosurgery, and 16 patients underwent a combination of radiosurgery and embolization. Nineteen of the patients had Spetzler-Martin Grade I AVMs, 59 Grade II, 43 Grade III, 7 Grade IV, and 1 Grade V.

NOTCH4 SNP Haplotypes Are Associated With Susceptibility to AVMs

In total, 153 AVM patients and 192 healthy individuals were genotyped for 10 different *NOTCH4* SNPs. To determine if any of the *NOTCH4* SNPs were associated with susceptibility to the disease, we performed a case-control analysis comparing the SNP genotype distributions in the patient and control groups. No statistically significant correlations were detected, and only a trend for an association was observed for the rs3134798 SNP (*p* = 0.1).

Next, as outlined in *Methods*, calculated haplotype frequencies were compared between the 2 groups. After Bonferroni correction for multiple testing, this analysis revealed that 11 haplotypes were significantly more common among the AVM patients (uncorrected *p* $\leq 1 \times 10^{-15}$), suggesting that these haplotypes were associated with greater susceptibility to AVM. Interestingly, the haplotypes did not map randomly to the *NOTCH4* gene. Rather, all of them contained at least 2 of 3 SNPs (rs1109771 [A/G], rs415929 [A/G], and rs715299 [A/C]), which were all localized to the region of the gene coding for the Ca²⁺-binding part of the EGF-like domain of the NOTCH4 protein (Fig. 1B and C).

Genotype–Phenotype Correlations

Univariate analysis indicated that the homozygous rs443198_TT and rs915895_AA genotypes were statistically significantly associated with a greater risk for hemorrhage than their heterozygous reference genotypes (Table 2). Univariate analysis also revealed that clinical parameters that were significantly correlated with AVM bleeds in the present series included an AVM size of < 3 cm and no epilepsy at presentation. A multivariate stepwise regression analysis including rs443198_TT, rs915895_AA, AVM size < 3 cm, and no epilepsy at presentation as covariates confirmed rs443198_TT and no epilepsy at pre-

TABLE 2. Univariate analysis of clinical and genetic factors influencing the risk for AVM hemorrhage

Characteristic	No. of Patients (%) [*]		OR	95% CI	p Value
	Hemorrhage	No Hemorrhage			
Sex					
Male	30 (46)	35 (54)	0.76	0.38–1.51	0.429
Female	34 (53)	30 (47)			
Mean age at Dx ± SD in yrs	39.7 ± 13.6	41.9 ± 17.5	1.01	0.99–1.03	0.437
AVM location					
Supratentorial	56 (48)	60 (52)	0.58	0.18–1.89	0.369
Infratentorial	8 (62)	5 (38)			
Deep location					
Yes	18 (58)	13 (42)	1.54	0.66–3.60	0.316
No	35 (47)	39 (53)			
Aneurysm					
Present	6 (35)	11 (65)	0.51	0.18–1.47	0.211
Absent	58 (52)	54 (48)			
Deep drainage					
Present	25 (51)	24 (49)	1.10	0.54–2.23	0.802
Absent	39 (49)	41 (51)			
Epilepsy					
Absent	46 (64)	26 (36)	3.73	1.78–7.82	0.001†
Present	18 (32)	38 (68)			
AVM size					
<3 cm	41 (58)	30 (42)	2.08	1.03–4.21	0.042†
≥3 cm	23 (40)	35 (60)			
Spetzler-Martin grade					
I	12 (63)	7 (37)	2.86	0.52–15.77	0.178
II	26 (44)	33 (56)	1.31	0.29–6.01	0.467
III	23 (54)	20 (46)	1.92	0.41–9.05	0.626
IV or V	3 (38)	5 (62)			
rs_443198					
CC vs TT	9 (75)	3 (25)	0.46	0.11–1.91	0.096
TT vs CT	29 (58)	21 (42)	2.12	1.01–4.49	0.010†
CT	26 (39)	40 (61)			
rs_915895					
GG vs AA	6 (75)	2 (25)	0.47	0.09–2.55	0.996
AA vs AG	31 (58)	22 (42)	2.14	1.03–1.44	0.022†
AG	27 (40)	41 (60)			

Dx = diagnosis.

^{*} Row percentages are shown.

† Statistically significant p value.

sensation as independent variables significantly associated with hemorrhage (Table 3).

Statistically significant predictors of epileptic presentation included the rs1109771_GG genotype, AVM size ≥

TABLE 3. Multivariate regression analysis of 4 variables significantly associated with hemorrhage in the univariate analysis

Variable	OR	95% CI	p Value
No epilepsy	4.65	1.83–8.70	0.001*
AVM size <3 cm	NS	NS	>0.05
rs_443198_TT	2.11	0.95–4.67	0.028*
rs_915895_AA	NS	NS	>0.05

NS = not significant.

^{*} Statistically significant p value.

3 cm, nonhemorrhagic presentation, and supratentorial versus infratentorial location ($p < 0.001$) (Table 4). When we performed multivariate stepwise regression analysis with rs1109771_GG, AVM size ≥ 3 cm, and presentation as covariates, only nonhemorrhagic presentation remained an independent predictor for epilepsy (Table 5).

Following the same rationale as outlined above for the susceptibility study, we also performed explorative haplotype analyses to look for genotype–phenotype correlations of *NOTCH4* haplotypes with clinical presentation. Because of the smaller sample size (129 patients versus a total of 345 cases and controls in the susceptibility study), we restricted this analysis to haplotypes consisting of up to 3 SNPs. Several statistically significant associations were observed, none of which remained significant after correction for multiple testing.

Discussion

Brain AVMs represent an important cause for neurological impairment due to intracerebral bleeding or sometimes symptomatic epilepsy in young adults.^{32,33} Prevention of AVM hemorrhages (or rehemorrhages) remains the primary goal of AVM treatment, and clinical decision making often consists of balancing the presumed risk of (further) bleeds against the risks of AVM treatment. A better understanding of the biology of the disease could lead to the identification of novel biomarkers for the prediction of the patients' future clinical course (i.e., for hemorrhage prediction) or even ultimately result in the development of new treatment strategies. Studying the genetic factors involved in a certain disease may identify genes and biological mechanisms underlying its pathogenesis. Following this rationale, we and others have investigated genetic polymorphisms of candidate genes for possible associations with susceptibility to AVMs and with specific clinical features of the disease such as hemorrhagic presentation.^{17,18,23,28,32,35}

In the present work, we tested whether SNPs in the *NOTCH4* gene are potential genetic risk factors for the development of AVMs, AVM bleeds, or AVM-associated epilepsy. Our results suggest a role of the *NOTCH4* gene in AVM biology. First, they suggest an association between *NOTCH4* gene SNPs and increased susceptibility to AVMs. Second, our results from the multivariate analysis also linked certain *NOTCH4* gene variants to key clinical features such as hemorrhage. We note that *NOTCH4* is a highly plausible candidate AVM gene. Murphy et al.

TABLE 4. Univariate analysis of clinical and genetic factors in AVM patients influencing the risk for presenting with epilepsy

Characteristic	No. of Patients (%) [*]		OR	95% CI	p Value
	Epilepsy	No Epilepsy			
Sex					
Male	31 (48)	33 (52)	1.46	0.73–2.96	0.286
Female	25 (39)	39 (61)			
Mean age at Dx $\pm$ SD in yrs	41.0 $\pm$ 13.6	40.9 $\pm$ 16.9	1.00	0.98–1.02	0.986
Hemorrhage					
Absent	46 (64)	26 (36)	3.73	1.78–7.82	0.001†
Present	18 (32)	38 (68)			
Deep location					
Present	9 (29)	22 (71)	0.42	0.17–1.04	0.060
Absent	36 (49)	37 (51)			—
Aneurysm					
Present	9 (53)	8 (47)	1.53	0.55–4.26	0.414
Absent	47 (42)	64 (58)			—
Deep drainage					
Present	25 (51)	24 (49)	1.61	0.78–3.31	0.193
Absent	31 (39)	48 (61)			
AVM location					
Infratentorial	0 (0.00)	13 (100)	NA	NA	<0.001†
Supratentorial	56 (48)	59 (52)			
AVM size					
<3 cm	25 (36)	45 (64)			
$\geq$ 3 cm	31 (53)	27 (47)	2.07	1.02–4.21	0.045†
Spetzler-Martin grade					
I	6 (32)	13 (68)	0.28	0.05–1.56	0.152
II	24 (41)	34 (59%)	0.42	0.09–1.94	0.549
III	21 (48)	22 (52)	0.57	0.12–2.70	0.717
IV or V	5 (62)	3 (38)			
rs_1109771					
AA vs GG	10 (37)	17 (63)	0.44	0.16–1.18	0.364
GG vs AG	23 (58)	17 (42)	2.34	1.03–5.29	0.034†
AG	22 (37)	38 (63)			

* Row percentages are shown.

† Statistically significant p value.

TABLE 5. Multivariate regression analysis of 3 variables significantly associated with epileptic presentation in the univariate analysis

Variable	OR	95% CI	p Value
Hemorrhage	0.27	0.13–0.56	0.001*
AVM size $\geq$ 3 cm	NS	NS	>0.05
rs_1109771_GG	NS	NS	>0.05

* Statistically significant p value.

genes with roles in inflammatory pathways.^{1,8,12,17,28} In contrast, genes involved in angiogenesis have so far received surprisingly little attention.^{30,41} Genes with a known role in angiogenesis that have been reported to be associated with AVM formation include *ALK1*,^{29,32} *ANGPTL4*,²³ and *VEGF*.⁷ Interestingly, the VEGF and ALK1 pathways involved in vascular development interact with the NOTCH pathway.^{21,31} Most recently, Zhang and coworkers proposed an AVM pathogenesis pathway in which NOTCH signaling is located downstream of AVM-causative genes like *ALK1* and *VEGF*.⁴³

We did not detect statistically significant correlations between single *NOTCH4* SNPs and AVM formation, which may be well explained by the relatively small size of our patient cohort. However, it has been shown that haplotype-based tagging can outperform single-marker tests and can help to both increase the association efficiency and refine the association signal.¹¹ Therefore, we also investigated whether calculated haplotypes consisting of up to 4 SNPs were possibly associated with AVM. We note that this approach was somewhat exploratory; however, this analysis linked specific haplotypes to an increased risk for developing AVM (Fig. 1B and C). In addition, we did find a single-marker association between the *NOTCH4* rs443198_TT SNP genotype and a key clinical feature, that is, presentation with hemorrhage. Of note, in the multivariate regression analysis, rs443198_TT remained a statistically significant predictor of a hemorrhagic presentation. Interestingly, rs443198_TT has not been previously reported to be associated with hemorrhage in other diseases, an observation that points to a possible specific involvement of this *NOTCH4* SNP in AVM-associated hemorrhage.

The *NOTCH4* SNPs linked to AVM biology in our analyses did not map randomly to the gene, supporting the contention that our results are not chance findings. All of the haplotypes linked to an increased risk for AVM formation contained at least 2 of 3 SNPs (rs715299, rs415929, and rs110977), all of which map to the Ca²⁺-binding sites of the NOTCH4 EGF-like receptor domain (Fig. 1B and C), which is known to play an important role in receptor-ligand interactions. Therefore, any genetic or structural variance in this region might have functional consequences. The rs443198 SNP, which is linked to clinical presentation of hemorrhage, maps relatively closely to rs715299 and was also present in several haplotypes associated with AVM formation. rs443198 is located in the coding part of exon 3 and is predicted to result in an (albeit synonymous) amino acid change or in an alternative splice site.¹⁵

have shown that expression of constitutively active *Notch4* leads to the development of AVMs in mice.^{26,27} Moreover, normalizing Notch4 signaling by turning off constitutive expression of the *Notch4* gene in mice reduces AVM vessel size.²⁵ Using immunohistochemistry, ZhuGe and colleagues have recently shown upregulation and activation of NOTCH4 in endothelial and smooth-muscle cells in human AVMs.⁴⁴

A number of studies have investigated genetic polymorphisms for possible associations with AVM development; however, many of these investigations have focused on

Limitation of the Study and Further Investigations

Our study has significant limitations. First, the small sample size in our study could have led to false-positive associations and confounder effects. Second, our conclusions are based in part on calculated haplotype frequencies. This approach was somewhat exploratory, the results of which may not be as robust as the results of a case-controlled comparison of SNP genotype distributions. The results of our study suggest but do not provide conclusive evidence for a significant link between NOTCH4 and AVM disease. Nevertheless, we believe that the data presented here, along with previous preclinical investigations in animal models, support a role for NOTCH4 in the formation and presentation of AVM. We propose that validation of our results via a confirmatory analysis of an independent and, if possible, larger AVM patient cohort will be a crucial next step.

Conclusions

Our results point to a role of the NOTCH4 gene in the formation and progression of AVM. Our data suggest that some NOTCH4 gene SNPs are linked to the risk for developing an AVM and also to AVM clinical parameters such as hemorrhage and epilepsy. We note the limitations of our study, most notably its small sample size, which may allow for differences in the cohort substructure to go undetected, leading to false-positive associations or confounder effects. Nevertheless, we believe that our data clearly warrant follow-up studies and, if possible, confirmation in a larger patient cohort.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Delev, Simon. Acquisition of data: Delev, Pavlova, Grote, Boström, Höllig. Analysis and interpretation of data: Delev, Pavlova, Simon. Drafting the article: Delev, Simon. Critically revising the article: Pavlova, Schramm, Oldenburg. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Delev. Statistical analysis: Fimmers. Administrative/technical/material support: Delev, Pavlova, Grote, Höllig, Schramm, Oldenburg, Simon. Study supervision: Delev, Simon.

Supplemental Information

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