

ARTICLE

# Association of the TGF- $\beta$ receptor genes with abdominal aortic aneurysm

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Abdominal aortic aneurysm (AAA) is a multifactorial condition. The transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway regulates vascular remodeling and mutations in its receptor genes, *TGFBR1* and *TGFBR2*, cause syndromes with thoracic aortic aneurysm (TAA). The TGF- $\beta$  pathway may be involved in aneurysm development in general. We performed an association study by analyzing all the common genetic variants in *TGFBR1* and *TGFBR2* using tag single nucleotide polymorphisms (SNPs) in a Dutch AAA case-control population in a two-stage genotyping approach. In stage 1, analyzing 376 cases and 648 controls, three of the four *TGFBR1* SNPs and nine of the 28 *TGFBR2* SNPs had a  $P < 0.07$ . Genotyping of these SNPs in an independent cohort of 360 cases and 376 controls in stage 2 confirmed association ( $P < 0.05$ ) for the same allele of one SNP in *TGFBR1* and two SNPs in *TGFBR2*. Joint analysis of the 736 cases and 1024 controls showed statistically significant associations of these SNPs, which sustained after proper correction for multiple testing (*TGFBR1* rs1626340 OR 1.32 95% CI 1.11–1.56  $P = 0.001$  and *TGFBR2* rs1036095 OR 1.32 95% CI 1.12–1.54  $P = 0.001$  and rs4522809 OR 1.28 95% CI 1.12–1.46  $P = 0.0004$ ). We conclude that genetic variations in *TGFBR1* and *TGFBR2* associate with AAA in the Dutch population. This suggests that AAA may develop partly by similar defects as TAA, which in the future may provide novel therapeutic options. *European Journal of Human Genetics* advance online publication, 12 August 2009; doi:10.1038/ejhg.2009.141

**Keywords:** abdominal aortic aneurysm; association study; *TGFBR1*; *TGFBR2*; transforming growth factor- $\beta$  pathway; vascular remodeling

## INTRODUCTION

Aneurysms of the abdominal aorta (AAA) affect 5–8% of men above 60 years of age.<sup>1</sup> In addition to male gender and age, other well-known risk factors of AAA are Caucasian race, smoking, the presence of other atherosclerotic conditions (myocardial infarction, cerebrovascular and peripheral arterial disease) and a positive family history.<sup>2</sup> AAA is therefore considered a multifactorial disorder in which both environmental and genetic factors contribute to its development.<sup>3</sup>

AAA develops through a not well-defined mechanism. Ultimately, vascular extracellular matrix (ECM) changes, accompanied by transmural inflammation, destructive remodeling of the elastic media, and depletion of medial smooth muscle cells, are observed.<sup>4</sup> This weakening of the vessel wall increases the risk of rupture with often a fatal outcome. Rupture rates increase with the size of the AAA, and therefore surgical intervention is considered when the AAA exceeds the 55 mm diameter threshold.<sup>5</sup>

Variations in genes that regulate ECM stability have been described to increase the susceptibility to AAA. Association has been reported with matrix-metalloproteinases<sup>6,7</sup> and their inhibitors (tissue inhibitors of metalloproteinases).<sup>7</sup> However, attempts to replicate these associations in different populations have led to conflicting results.<sup>8,9</sup>

In contrast, association of AAA with a sequence variant on 9p21 has consistently been replicated.<sup>10,11</sup> This variant was shown to associate with AAA, coronary artery disease (CAD), and intracranial aneurysms (IA) in different populations.<sup>10</sup> This variant did not predispose to the atherosclerotic conditions peripheral artery disease and large atherosclerotic or cardiogenic shock. Although atherosclerosis is a major determinant for development of CAD and AAA, it has no or a modest role in IA formation. Therefore, it was suggested that the variant on 9p21 influences another, shared mechanism in the development of CAD, AAA and IA, like vascular remodeling.

The transforming growth factor (TGF)  $\beta$ -pathway is an important regulator of vascular remodeling, and has effects on both ECM synthesis and ECM degradation.<sup>12</sup> Dysregulated TGF- $\beta$  signaling by mutations in the receptor genes, *TGFBR1* and *TGFBR2*, causes thoracic aortic aneurysm (TAA) syndromes, including Marfan syndrome type II (OMIM 154705),<sup>13,14</sup> Loeys–Dietz syndrome (OMIM 609192),<sup>14,15</sup> and thoracic aortic aneurysms leading to type A dissections.<sup>16,17</sup> Although these syndromes are clinically distinct, their phenotypes overlap, with TAA and aortic dissections as the common denominator. In families with TAA caused by mutations in the *TGFBR2* gene, family members had aneurysms at different locations

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of the vascular system. Most affected members had TAA of the ascending aorta but some also had aneurysms of the descending aorta, the carotid, brachiocephalic, subclavian or popliteal arteries whereas others had ruptured or unruptured IA.<sup>17,18</sup> Furthermore, detailed characterization revealed that half of the probands with Loeys-Dietz aortic aneurysm syndrome not only had TAA but also aneurysms at different locations, mainly consisting of AAA and aneurysms of head and neck.<sup>15</sup> It is therefore conceivable that dysregulated TGF- $\beta$  signaling has a role in aneurysm formation in general. Genetics variants in *TGFBRI* and *TGFB2* may influence their signaling capacity and thereby the quality of vascular remodeling induced by the TGF- $\beta$ .

In contrast to TAA syndromes, AAA is usually not caused by a single gene defect, but multiple genetic and environmental factors are thought to participate in its development. We hypothesized that variants in *TGFBRI* and *TGFB2* may contribute to AAA formation. To investigate this, we performed a genetic association study to analyze all the common *TGFBRI* and *TGFB2* variants using tag single nucleotide polymorphisms (SNPs) in a Dutch case-control population.

## MATERIALS AND METHODS

### Patient collection and controls

We included Dutch Caucasian cases with a proven AAA (> 30 mm) who visited their vascular surgeon from May 2007 until December 2007 in eight large centers in the Netherlands. Controls comprised healthy blood bank volunteers (828 samples) in whom an AAA was not excluded, and men between 60–80 years of age in whom an AAA was excluded by ultrasonography (196 control samples from age-matched controls out of an AAA-screening study).<sup>19</sup> The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, and by each local review board.

### Study design, SNP selection, and genotyping

Tag SNPs from the *TGFBRI* and *TGFB2* genes spanning the entire genes and 5 kb upstream and downstream of the genes were selected from the International HapMap Project using the aggressive tagger option of the program Tagger (Paul de Bakker, <http://www.broadinstitute.org/mpg/tagger/>, NCBI Build 35/UCSC hg17/May 2004) so that all SNPs with a minor allele frequency of 10% were captured with  $r^2 > 0.8$ . One tag SNP in *TGFB2* with low Illumina quality design scores was excluded. In total, five tag SNPs were derived for genotype analysis of *TGFBRI* and 30 tag SNPs for genotype analysis of *TGFB2*. SNP genotyping was performed using a two-stage genotyping approach. For stage 1, the tagging SNPs were genotyped in 376 Dutch Caucasian cases and 652 Dutch Caucasian controls (bloodbank volunteers) using the GoldenGate assay on an Illumina BeadStation 500 GX (Illumina Inc., San Diego, CA, USA). All tag SNPs were examined for their quality and one tag SNP in *TGFBRI* and two tag SNPs in *TGFB2* that had low signals were excluded. DNA samples with low signals for most SNPs were also excluded ( $n=4$ , all controls). For stage 2, all SNPs with arbitrary low  $P$ -values ( $P < 0.07$ ) in stage 1 were genotyped in a second independent cohort of 360 cases and 376 controls (180 bloodbank volunteers and 196 men aged 60–80 without an AAA). For these selected SNPs we obtained Taqman Assays on Demand (Applied Biosystems, Foster City, CA, USA), which were genotyped on an ABI7900HT instrument (Applied Biosystems). DNA samples with low signals were excluded (varying between 0.3 and 4% per assay).

### Statistical analysis

Association  $\chi^2$  with two-tailed  $P$ -values and Hardy-Weinberg equilibriums were calculated using the HAPLOVIEW program (available at <http://www.hapmap.org>). Differences in allele frequencies were assessed as an odds ratio (OR) with corresponding 95% confidence intervals (CI) and  $P$ -values, using the allele with the lower frequency in the controls as opposed to the allele frequency in cases as the reference allele. Significant associations in the joined analysis were corrected for multiple testing. We genotyped 32 SNPs in total, therefore associations with a  $P$ -value smaller than 0.0015 (0.05/32) were considered

statistically significant after correction for multiple testing. With the AAA sample size from both stages, the study had 73% power to detect a susceptibility locus with a relative risk of 1.3, and 88% power to detect a susceptibility locus with a relative risk of 1.4, at a significance level of 0.05 assuming an additive model (genetic power calculator, SGDP statistical genetics group, <http://statgen.iop.kcl.ac.uk>). The population attributable risk (PAR) of the SNPs that remained significantly associated after correction for multiple testing was calculated using the following formula:  $PAR = 1 - 1 / (f_{wt} + (f_{het} * OR_{het}) + (f_{hom} * OR_{hom}))$ , where  $f_{wt}$ ,  $f_{het}$  and  $f_{hom}$  are the population frequencies of the wild-type, the heterozygous and the homozygous carriers, respectively. Similarly,  $OR_{het}$  and  $OR_{hom}$  are the odds ratios for heterozygous and homozygous carriers, respectively.

## RESULTS

The clinical data of the AAA cases analyzed in stages 1 and 2 are shown in Table 1.

In stage 1 no tag SNPs showed deviation from Hardy-Weinberg equilibrium. In this stage four tag SNPs of *TGFBRI* and 28 tag SNPs of *TGFB2* were successfully genotyped in 376 cases and 648 controls. The association data of these tag SNPs and corresponding 95% confidence intervals and  $P$ -values, are shown in the Supplementary Table online. Three of the four analyzed SNPs in *TGFBRI* and nine of the 28 analyzed SNPs in *TGFB2* showed arbitrary low  $P$ -values ( $P < 0.07$ ) (strongest association for *TGFBRI* SNP rs1571590,  $P=0.032$  and strongest association for *TGFB2* SNP rs3087465,  $P=0.002$ ) (Supplementary Table online and Table 2) and were selected for additional genotyping in an independent cohort of 360 cases and 376 controls.

In stage 2 there was also no evidence of a deviation from Hardy-Weinberg equilibrium. Association ( $P < 0.05$ ) for the same allele of one SNP in *TGFBRI* (rs1626340,  $P=0.007$ ) with AAA (Table 2) was shown. Combining both cohorts (stage 1 and stage 2) strengthened the association of this SNP as seen in stage 2 (OR 1.32, 95% CI 1.11–1.56,  $P=0.001$ ). This association remained statistically significant after correcting for multiple testing (ie,  $P < 0.0015$ , 0.05/32). The PAR of rs1626340 was estimated to be 10%. For the two remaining *TGFBRI* SNPs rs1571590 and rs10819634 association was shown on combined analyses of both cohorts (rs1571590 OR 1.28, 95% CI 1.08–1.52,  $P=0.005$  and rs10819634 OR 1.28, 95% CI 1.08–1.54,  $P=0.006$ ) (Table 2). The three SNPs that were associated with AAA in the joined analysis were in strong LD (Figure 1a). None of the constructed haplotypes had a stronger association with AAA than the independent SNPs (data not shown).

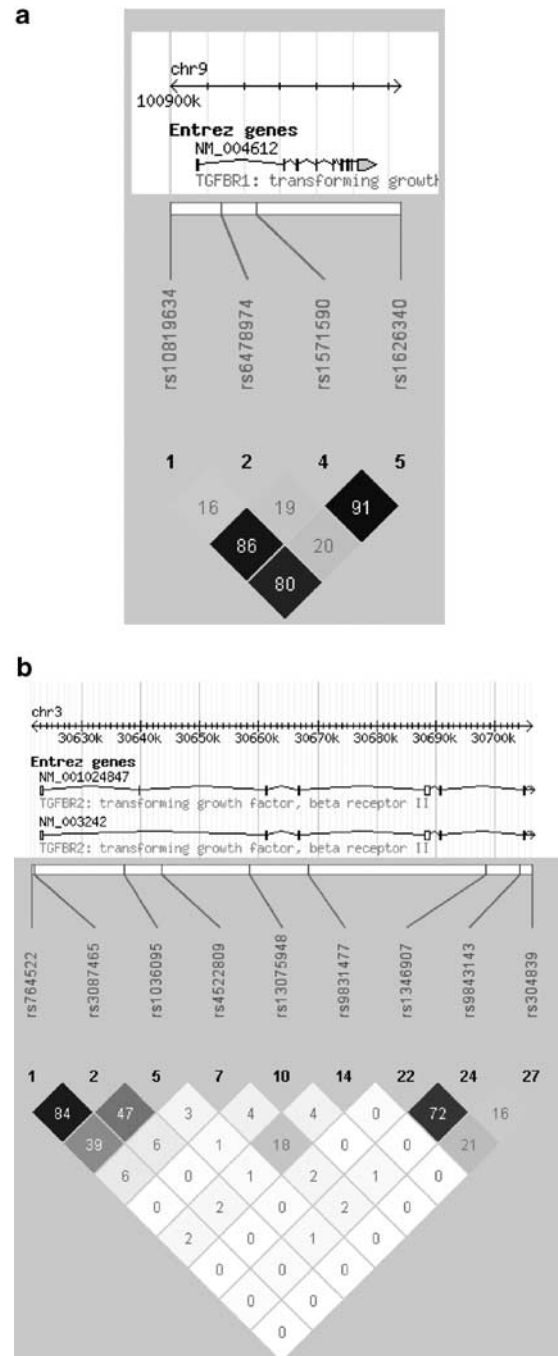
**Table 1 Clinical data of the analyzed cases with abdominal aortic aneurysms**

	Stage 1 (N=376)	Stage 2 (N=360)
Male sex – no. (%)	337 (89.6)	326 (90.6)
Mean age – year ( $\pm$ SD)	72.1 (7.5)	71.3 (7.3)
Diameter AAA – mm ( $\pm$ SD)	57.1 (15.3)	57.6 (16.0)
Ruptured AAA – no. (%)	13 (3.5)	22 (6.1)
Surgical intervention – no. (%)	240 (63.8)	216 (60.0)
<i>Smoking status – no. (%)</i>		
Currently	107 (28.5)	108 (30.0)
Previously	242 (64.4)	229 (63.6)
Never	27 (7.1)	23 (6.4)
Hypertension – no. (%)	227 (60.4)	216 (60.0)
Other cardiovascular disease – no. (%)	233 (62.0)	214 (59.4)
Family history of AAA – no. (%)	87 (23.1)	79 (21.9)

**Table 2** The analysis of the *TGFBR1* and *TGFBR2* genes in abdominal aortic aneurysm cases and controls

Gene	SNP	Associated allele	Stage 1: frequency allele $P < 0.07$ (%)			Stage 2: frequency allele $P < 0.07$ in stage 1 (%)			Stage 1 + 2: allele frequencies combined (%)					
			Cases (n=376)	Controls (n=648)	OR (95% CI)	P-value	Cases (n=360)	Controls (n=376)	OR (95% CI)	P-value	Cases (n=736)	Controls (n=1024)	OR (95% CI)	P-value
<i>TGFBR1</i>	rs10819634	A	18.5	15.3	1.25 (1.05–1.67)	0.066	20.2	16.4	1.28 (0.98–1.69)	0.064	19.3	15.7	1.28 (1.08–1.54)	0.006
	rs1571590	C	20.6	16.8	1.28 (1.02–1.61)	0.032	21.9	18.6	1.23 (0.94–1.59)	0.123	21.2	17.4	1.28 (1.08–1.52)	0.005
	rs1626340*	A	21.7	18.4	1.23 (0.98–1.54)	0.069	24.0	18.2	1.43 (1.10–1.82)	0.007	22.8	18.3	1.32 (1.11–1.56)	0.001
	rs764522	A	21.7	17.3	1.32 (1.05–1.67)	0.014	20.6	18.3	1.16 (0.89–1.49)	0.271	21.1	17.6	1.25 (1.05–1.49)	0.01
	rs3087465	A	25.1	19.4	1.39 (1.12–1.72)	0.002	22.5	19.9	1.16 (0.90–1.49)	0.239	23.9	19.6	1.28 (1.10–1.52)	0.002
<i>TGFBR2</i>	rs1036095*	C	26.6	21.1	1.35 (1.10–1.67)	0.005	24.6	19.9	1.32 (1.02–1.69)	0.035	25.7	20.6	1.32 (1.12–1.54)	0.001
	rs4522809*	A	58.6	51.8	1.32 (1.10–1.58)	0.003	56.4	51.1	1.24 (1.01–1.53)	0.042	57.6	51.5	1.28 (1.12–1.46)	0.0004
	rs13075948	C	75.1	70.0	1.29 (1.05–1.58)	0.015	69.8	69.6	1.01 (0.81–1.26)	0.924	72.5	70.3	1.12 (0.96–1.29)	0.152
	rs9831477	A	46.5	41.0	1.25 (1.05–1.50)	0.014	43.1	40.9	1.09 (0.89–1.35)	0.42	44.9	41.0	1.17 (1.03–1.35)	0.021
	rs1346907	A	47.3	42.0	1.23 (1.03–1.49)	0.020	47.3	45.1	1.10 (0.88–1.35)	0.402	47.3	43.1	1.18 (1.03–1.35)	0.014
	rs9843143	A	53.3	48.5	1.22 (1.02–1.46)	0.034	53.0	52.4	1.02 (0.83–1.26)	0.8287	53.2	49.9	1.14 (0.99–1.46)	0.059
	rs304839	C	20.7	17.9	1.25 (1.00–1.57)	0.052	19.7	19.2	1.03 (0.79–1.34)	0.824	20.2	18.0	1.16 (0.98–1.37)	0.096

\*Significant association after correction for multiple testing.



**Figure 1** Block-based haplotype analysis using HAPLOVIEW ( $R^2$  displayed) of all tag SNPs tested in stage 1 of *TGFBR1* (a) and the tag SNPs with  $P < 0.07$  in stage 1 of *TGFBR2* (b).

In stage 2, association was observed for the same allele of two of the nine *TGFBR2* SNPs (rs4522809,  $P=0.042$ ; rs1036095,  $P=0.035$ ) (Table 2). Combining both cohorts strengthened the association of these two SNPs as seen in stage 2 (rs4522809 OR 1.28, 95% CI 1.12–1.46,  $P=0.0004$  and rs1036095 OR 1.32, 95% CI 1.12–1.54,  $P=0.001$ ). These associations remained statistically significant after correcting for multiple testing (ie,  $P < 0.0015$ , 0.05/32). The PARs of rs4522809 and rs1036095 were estimated to be 3 and 6%, respectively. No association was shown for SNPs rs1346907, rs764522, rs3087465,

and rs9834177 in stage 2, but on combined analyses of both cohorts association of these SNPs was statistically significant (rs1346907 OR 1.18, 95% CI 1.03–1.35,  $P=0.014$ ; rs764522 OR 1.25, 95% CI 1.05–1.49,  $P=0.01$ , rs3087465 OR 1.28, 95% CI 1.10–1.52,  $P=0.002$ , rs9831477 OR 1.17, 95% CI 1.03–1.35,  $P=0.021$ ) (Table 2). For the remaining SNPs rs13075948, rs9843143, and rs304839 no association in stage 2 nor in the combined cohorts was shown (Table 2). The nine *TGFBR2* SNPs that were tested in stage 2 were scattered throughout the gene and there was no obvious LD between the SNPs (Figure 1b). Therefore, no haplotypes were constructed.

## DISCUSSION

By analyzing genetic variants in the *TGFBR1* and *TGFBR2* genes, involved in the development of TAA and possibly involved in the development of aneurysms in general, we identified SNPs in *TGFBR1* and *TGFBR2* that associate with AAA in the Dutch population.

A common feature of AAA is fragmentation of the elastic laminae and smooth muscle cell loss.<sup>4</sup> This may be the result of defective vascular remodeling, which under physiological circumstances induces adaptive changes within the vessel wall upon hemodynamic stress or as a response to vascular injury. Vascular remodeling includes a tightly regulated balance between degradation and rebuilding of the ECM. A pathological shift towards excessive ECM degradation results in loss of vascular wall integrity, which may precede aneurysm formation.<sup>12</sup> One of the upstream regulators of vascular remodeling is the TGF- $\beta$  signaling pathway, which has been extensively studied in light of TAA syndromes.<sup>12</sup> TGF- $\beta$  transduces its signals by two transmembrane receptors, which are encoded by the *TGFBR1* and *TGFBR2* genes.<sup>20</sup> Mutations in these genes are responsible for Marfan syndrome type II (OMIM 154705),<sup>13</sup> Loeys-Dietz syndrome (OMIM 609192)<sup>14,15</sup> and thoracic aortic aneurysms leading to type A dissections (TAA; OMIM 608967).<sup>16,17</sup> Marfan syndrome type I is clinically similar to Marfan type II syndrome, but it is caused by mutations in the fibrillin-1 gene.<sup>21</sup> Fibrillin-1 encodes the structural ECM protein fibrillin-1, which is thought to regulate the availability of active TGF- $\beta$  by binding to TGF- $\beta$  in its latent form.<sup>22</sup> In mouse models of Marfan syndrome type I increased TGF- $\beta$  activity was observed in different organs, including aortic tissue.<sup>23</sup> Similarly, aortic tissues of Loeys-Dietz syndrome patients showed increased TGF- $\beta$  signaling activity.<sup>24</sup> Paradoxically, mutations in the receptor genes are primarily located within its intracellular kinase domain, resulting in kinase inactivity.<sup>12</sup> This is believed to impair intracellular signaling. Apparently, fine-tuning of TGF- $\beta$  signaling is required for optimal vascular wall structure maintenance. In light of this, it is interesting to note that in AAA expression of TGF- $\beta$ 1 was found to be upregulated,<sup>25</sup> whereas with gene therapy in rats the already formed AAA was shown to stabilize after overexpression of TGF- $\beta$ 1.<sup>26</sup>

Our findings that variations within the TGF- $\beta$  receptor genes, which may result in different functional activity or expression, associate with AAA suggest that the TGF- $\beta$  pathway has a role in AAA pathogenesis, similar to that seen in TAA. This may offer novel therapeutic options, because aortic root dilatation in Marfan mouse models could be prevented by administration of TGF- $\beta$  antagonists.<sup>27</sup> It is unlikely that aneurysm formation is preventable in AAA by inhibiting a single pathway, because in contrast to the TAA syndromes, AAA is considered as a complex disease. Not a single gene defect, but multiple genetic and environmental factors contribute to its pathogenesis. However, targeting different molecular players as well as aiming for efficient risk factor reduction (ie, smoking cessation, tension control) may prove to be worth while in future therapies.

The three SNPs that were found to associate significantly with AAA after correction for multiple testing together had a PAR of 19%. The PAR of rs10757278, previously found to be associated with AAA in a part of the current cohort, was estimated to be 26%.<sup>10</sup> Therefore, these SNPs combined can explain almost half of the increased genetic risk.

A previous study analyzing one polymorphism of the *TGFBR1* gene (9A6A) in 201 Italian AAA patients and 252 controls failed to show an association with AAA.<sup>28</sup> In the presence of the angiotensin-converting enzyme DD genotype they did show that the *TGFBR1* 6A allele affected susceptibility to AAA. The Italian study only analyzed one sequence variant in *TGFBR1*, whereas we selected tag SNPs on the basis of known patterns of LD to capture the maximum coverage of the gene. This difference in approach may explain the discrepant results of the two studies. Furthermore, the power of the previous study may have been too small to detect the association.

A recent study reported no association of tag SNPs of *TGFBR1* and *TGFBR2* with AAA in 640 cases and 1071 controls.<sup>29</sup> This is an interesting finding, because those AAA cases were identified from population-based screening, and had much smaller AAAs (36.1 *versus* 57.3 mm in our cohort). This suggests that the *TGFBR1* and *TGFBR2* SNPs we found to be associated with AAA predispose to a kind of AAA that proceeds to a severe form, often requiring surgery. In our group over 60% of the patients had a surgical intervention. In addition, genetic heterogeneity may explain part of the discrepant results.

In complex conditions as AAA, the contribution of a single gene variation is expected to be modest, and low OR are expected to be found. Therefore, substantial sample sizes are needed to reach statistical significant values. As we used a two-stage approach, our sample size in stage 1 was not appropriate to select only those SNPs with a  $P<0.05$ . We therefore selected the SNPs in stage 1 with arbitrary low  $P$ -values;  $<0.07$ . Association of some of these selected SNPs in the second stage, and associations of other SNPs on combined analyses of both stages, suggests that they are true associations. Moreover, three SNPs remained significantly associated after proper correction for multiple testing of the joint analysis, which strongly indicates that they are true associations. The ultimate proof of these associations will be consistent replication in other patient populations and the identification of the functional variants, which can be achieved by sequencing risk haplotypes in a succeeding study. Recently, TGF- $\beta$  receptor genes were directly sequenced in patients with familial forms of intracranial aneurysms.<sup>30</sup> No mutations in *TGFBR1* and *TGFBR2* were identified, but novel variants were discovered in the co-receptor genes  $\beta$ -glycan (*TGFBR3*) and endoglin (*ENG*).

A shortcoming of our study is that we were not able to control for confounding, because no information of the common AAA risk factors, like hypertension and smoking, was available in our control group. Demographically, the cases and controls are similar because they are both from Dutch origin, and genome-wide association data of the control group did not reveal population stratification.

In conclusion, we identified several SNPs in the *TGFBR1* and *TGFBR2* genes that associate with AAA. These are intriguing findings that give new insights in AAA pathogenesis and in the future may provide novel therapeutic options.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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