An association between rs7635818 polymorphism located on chromosome 3p12.3 and the presence of abdominal aortic aneurysm

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Short title: Gene variant rs7635818 and abdominal aortic aneurysm

Summary

BACKGROUND: The association between gene variant rs7635818 located on chromosome 3p12.3 and abdominal aortic aneurysm (AAA) was not unambiguously determined by the results of genome-wide association studies. The aim of our study was to examine this possible association in the Slovak population, with respect to the presence and severity of AAA.

PATIENTS AND METHODS: A cross-sectional study was conducted between August 2016 and March 2020. The study included 329 participans, 166 AAA patients and a control group of 163 subjects without confirmed AAA with comparable distribution of genders. The anteroposterior diameter of the abdominal aorta was determined by duplex ultrasonography. AAA was defined as subrenal aortic diameter ≥ 30 mm. DNA samples were genotyped using real-time polymerase chain reaction and subsequent high-resolution melting analysis in presence of unlabelled probe. Genetic models studying the possible association were adjusted to age, sex, smoking, arterial hypertension, diabetes mellitus, creatinine and body mass index (BMI) in multivariate analysis.

RESULTS: In the additive model, presence of each C-allele of rs7635818 polymorphism was associated with an almost 50% increase in probability of developing AAA (OR 1.49; 95% CI 1.06–2.08; p=0.020). Compared to GG homozygotes, CC homozygotes had more than two times higher risk of developing AAA (OR 2.23; 95% CI 1.14–4.39; p=0.020).

The risk of AAA was also in the recessive model higher for CC homozygotes compared to G-allele carriers (GC/GG) (OR 1.79; 95%CI 1.01–3.19; p=0.047).

The abdominal aortic diameter in CC homozygotes of the rs7635818 polymorphism was 7.66 mm greater compared to GG homozygotes (42.5±22.0 mm vs 34.8±21.3 mm; p=0.022) and 5.88 mm greater compared to G-allele carriers (GC/GG) (42.5±22.0 mm vs 36.6±21.0 mm; p=0.04) in univariate analysis.

CONCLUSIONS: C-allele variant in rs7635818 G>C polymorphism is associated with a higher probability of developing AAA in the Slovak population.

Key words: abdominal aortic aneurysm, polymorphism, contactin-3

Introduction

Abdominal aortic aneurysm (AAA), a potentially lethal disease, can be defined as an abdominal aortic diameter of 30 mm and more, or when maximum diameter is ≥ 50% greater than the suprarenal aortic diameter. The prevalence of AAA is from 1.3% to 3.3% in male population of 65-years and older, however, there is no data documenting real prevalence of AAA in Slovak population (Svensjö *et al*. 2011, Jacomelli *et al.* 2016, Grondal *et al.* 2015).

Although there is still a lack of knowledge regarding factors initiating AAA formation, an imbalance between synthesis and degradation of extracellular matrix, smooth muscle cell apoptosis, neovascularization and chronic aortic wall inflammation has been implicated in the AAA pathogenesis. There is an effort to identify an optimal biomarker that would reflect the AAA size and rupture risk. Animal models for AAA research are also used (Patelis *et al.* 2017).

The absence of effective medical therapy that slows AAA progression highlights the need for better understanding the factors influencing AAA pathogenesis. AAA shares several risk factors with atherosclerosis such as old age, smoking, male sex and family history of the disease. Chronic inflammatory and reparative processes in arterial wall are present in atherosclerosis as well (Slíva *et al*. 2019). Although the role of hypertension is still controversial, it is currently considered a risk factor for AAA (Wanhainen *et al.* 2019, Altobelli *et al.* 2018). Several studies have shown decreased risk of AAA in patients with diabetes mellitus, the reasons for which are unexplained (Raffort *et al.* 2018, Cosentino *et al.* 2020). It

is possible that metformin, the world's most commonly prescribed oral hypoglycemic agent, might be the AAA protective factor (Itoga *et al.* 2019).

There is a strong genetic component to AAA, with first-degree relatives having a 2-times higher risk of developing the disease (Larsson *et al.* 2009). It is generally assumed that most AAAs have a polygenetic background where the small effect gene variants cause predisposition to the disease development. Differences in gene expression in the aneurysmal tissue and in the biopsy of healthy aortic tissue of the same patient were also found (Prucha *et al* 2019).

Genetic association between rs7635818 gene variant located on the short arm of chromosome 3 and AAA is not clear. It was reported in an early genome-wide association study (GWAS) of Elmore et al., but not replicated in the further two GWAS conducted by Gretarsdottir et al. and Jones et al. (Elmore *et al.* 2009, Gretarsdottir *et al.* 2010, Jones *et al.* 2009). The diverse results regarding this polymorphism association with AAA suggest importance for further study in this field (Smelser *et al.* 2010).

The aim of the present study was to examine the association between the gene variant rs7635818 and AAA risk in the Central European Caucasian population of Slovakia, and to evaluate the association between this polymorphism and the severity of AAA expressed by the diameter of abdominal aorta.

Methods

AAA patients enrolled in this study were outpatients and/or hospitalized patients at the Department of Angiology between August 2016 and March 2020. The antero-posterior measurements of abdominal aortic diameter were performed by duplex ultrasonography (Philips HD 15) in the axial view using curved array transducer in B-mode. Control patients

were selected from patients older than 60 years who were hospitalised at our institute and who needed ultrasonograhic examination of peripheral arteries (carotid arteries, limb arteries, renal arteries, right/left internal mammary arteries), and/or veins at our angiology outpatient clinic. Patients with confirmed carotid artery stenosis ≥ 50%, deep vein thrombosis, critical limb ischaemia were not included in the control group. Since international consensus concerning which boundaries to use during measurements of abdominal aortic diameter had not been reached by the beginning of the study, outer to outer measurement was used in order to prevent underestimation: the calipers were placed from the outer anterior aortic wall to the outer posterior aortic wall (Wanhainen *et al.* 2019). In AAA patients, measurements were performed in area of maximum subrenal abdominal aortic diameter. In control patients, aortic diameter measurements were performed below the level of the renal artery origin, 10–20 mm above the aortic bifurcation and a greater diameter was chosen. The definitive result was the mean of three measurements of maximum abdominal aortic diameter performed in systole. All diameters were measured by the same experienced sonographer (MM). AAA patients were defined as patients with subrenal aortic dilatation ≥ 30 mm. The control group of patients consisted of patients with aortic diameter < 25 mm.

Patients with known active malignancy, chronic inflammatory diseases, severe liver disease, chronic kidney disease stage 3–5, thoracic, iliac, femoral, popliteal aneurysm, history of aortic reconstructive surgery were excluded from the study.

Patients' anthropometric data was collected from their medical records. All patients were asked to complete a standardized health questionnaire on smoking habits and medical history. The history of chronic diseases among subjects was self-reported. Hypertension was defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mmHg, and/or current antihypertensive medication. Diabetes mellitus (DM) determination was based on self-reported usage of anti-diabetes medication, fasting glucose levels ≥ 7.0 mmol/l and/or

postprandial glucose levels ≥ 11.1 mmol/l. Dyslipidaemia was defined by the use of hypolipidemic medication, and/or by lipid levels out of range of recommended treatment goals (Catapano *et al.* 2016). Patients were classified as active smokers, ex-smokers and never smokers.

Blood samples for biochemical, hematological analyzes and for genomic DNA extraction were taken in the morning after 12 hours of fasting from the cubital vein**.** Whole blood for DNA analysis was collected into an evacuated tube containing K_2EDTA. DNA was extracted with a Wizard Genomic DNA Isolation kit (Promega, Co, Ltd, USA) according to the manufacturer´s instructions and resolved in DNA Rehydration Solution (10mM Tris, 1mM EDTA) from the kit. Spectrophotometer Nanodrop 2000 (Thermo Scientific) was used to quantify and assess the purity of DNA. The DNA samples were stored at -20°C until analyzed. Genotyping of polymorphism rs7635818 was performed using real-time polymerase chain reaction and subsequent high-resolution melting analysis in presence of an unlabelled probe on the Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The oligonucleotides (Forward-limit: 5′- GTGATTGATTAGACCAGGCTC -3′, Reverse-excess: 5′-TGTGGGATACAGTAGCGT-3′,
and Probe: 5′- TTGAATCTATCACTAACTCCTTCTCTAGGC –Phos) were purchased from Eurofins Genomics, Germany GmbH. Genotypes were identified using Eco^{TM} Software 4.1.

Other laboratory parameters (creatinine, LDL cholesterol, HDL cholesterol, fibrinogen) were measured in a local laboratory on the day of blood sampling by routine biochemical methods.

The study was performed with the approval of the Ethics Committee of the East Slovak Institute of Cardiovascular Diseases (Košice, Slovakia). Writen consent was obtained from all patients.

Categorical variables were described using counts and percentages and compared by chi-square test. Normality of continuous data was assessed using a one sample Kolmogorov-Smirnov test. Parametric data was presented as mean and standart deviation, non parametric data as a median with lower and upper quartile (25th, 75th percentile). For comparison of continuous variables between categorical groups Student *t*-test (for parametric distribution of variables) was used. Genotype and allele frequencies were compared using chi-square test. Binary logistic regression was used to estimate odds ratio (OR) and 95% confidence interval (CI). General linear models were used for testing of abdominal aortic diameter according to genotypes. Several genetic models were used for the evaluation of the association between the genotype and AAA. The additive genetic model assumes that there is a linear gradient in risk between the three genotypes and expresses OR per risk allele. The dominant genetic model compares C-allele carriers (GC/CC) to GG homozygotes. The recessive genetic model compares CC homozygotes to G-allele carriers (GG/GC). The co-dominant model compares CC homozygotes and GC heterozygotes to GG homozygotes separately. The adjustments in multivariate analyses were performed for AAA risk factors (age, hypertension, sex, smoking habit), AAA protective factor (diabetes mellitus), creatinine, and body mass index (BMI) due to their different distribution at univariate level ($p<0.1$). Differences were regarded as statistically significant at two-tailed p-values $p<0.05$, since this was a replication genetic study. Statistical analyzes were performed using SPSS statistics V17 (SPSS Inc., Chicago, IL, USA).

Results

During the study period, duplex ultrasonography measurements of abdominal aortic diameter were performed in 488 patients (206 patients with AAA and 282 patients without AAA) older than 60 years. Using exclusion criteria a total number of 329 participans (280 men

and 49 women) with mean age of 70.9±8.0 years were enrolled in the study, of which 166 subjects were patients with AAA and 163 were control subjects.

Baseline characteristics of AAA patients and the control group of patients are summarized in Table I. There was no significant deviation from Hardy-Weinberg equilibrium for tested polymorphism in AAA patients (p=0.976) and control patients (p=0.974). A clinical flowchart of the study is showed in Figure 1.

In the additive model, presence of each C-allele of rs7635818 polymorphism was associated with an almost 50% increase in probability of developing AAA (OR 1.49; 95% CI 1.06–2.08; p=0.020). Compared to GG homozygotes, CC homozygotes had more than two times higher risk of developing AAA (OR 2.23; 95% CI 1.14–4.39; p=0.020). In the recessive model, CC homozygotes had almost 80% higher risk of developing AAA compared to G-allele carriers (OR 1.79; 95%CI 1.01–3.19; p=0.047). The association between rs7635818 gene variant and AAA presence is shown in Table II.

The abdominal aortic diameter in CC homozygotes of the rs7635818 polymorphism was 7.66 mm greater compared to GG homozygotes (42.5±22.0 mm vs 34.8±21.3 mm; p=0.022) and 5.88 mm greater compared to G-allele carriers (GC/GG) (42.5±22.0 mm vs 36.6±21.0 mm; p=0.040) in univariate analysis. In multivariate analysis a similar trend for greater abdominal aortic diameter in C-allele carriers was observed, but did not reach the level of statistical significance in any model after multivariate adjustements. The association between examined polymorphism and AAA diameter is documented in Table III.

Discussion

The main finding of the present study was a replication of a previously observed association between polymorphism rs7635818 and AAA risk in the Slovak population. The present study found that CC homozygosity of the rs7635818 polymorphism increases the

likelihood of developing AAA. The severity of AAA expressed by the diameter of abdominal aorta was also higher in CC homozygotes reflecting in a difference of 7.66 mm between CC homozygotes and GG homozygotes in univariate analysis. Our study is the first study analyzing the association of this polymorphism with AAA in the Central European Caucasian population of Slovakia.

Previous genetic discoveries in AAA have pointed to inflammation and immune function (*IL6R* [interleukin 6 receptor] and *CDKN2BAS1/ANRIL* [also known as CDKN2B-AS1, CDKN2B antisense RNA 1]), low-density lipoprotein metabolism (*SORT1* [sortilin 1] and *LDLR* [low-density lipoprotein receptor]), cell growth and survival (*DAB2IP* [DAB2 interacting protein]) as important mediators of AAA development. A recently published meta-analysis of GWAS identifies four disease-specific risk loci on chromosomes 1 (*SMYD2*), 13 (*LINC00540*), 20 (*PCIF1/MMP9/ZNF335*) and 21 (*ERG*). The exact function of AAA associated polymorphisms in pathogenesis of AAA is the subject of ongoing research (Jones *et al.* 2017).

Our study is the replication of the GWAS results of Elmore et al. who included participants from the USA, Canada and Belgium. In this study, C-allele carriers (CC/CG) had significantly higher risk of developing AAA in comparison with GG homozygotes (OR 1.33; 95% CI 1.10–1.61; p=0.0028) and an even stronger association with AAA was observed in a subset of smokers (Elmore *et al.* 2009*)*.

A further GWAS including patients from Iceland and the Netherlands did not find a significant association between rs7635818 and AAA in their discovery samples (Gretarsdottir *et al.* 2010). Neither did a study in the New Zealand Caucasian population focusing on the 3p 12.3. locus replicate the reported association between rs7635818 variant and AAA (Jones *et al.*

2009). Our study expands on previously published studies regarding this polymorphism and presented data may be included in future metaanalyses.

Ethnic variations of polymorphism rs7635818 regarding AAA have not been studied. This may be explained by the relatively rare occurrence of AAA. In addition, genetic studies and especially GWAS requires a large sample size, so they are often multicentric with limited population stratification.

The mechanism whereby rs7635818 gene variant may affect AAA risk is not elucidated so far. Examined polymorphism rs7635818 is not likely the genetic variant that is functionaly responsible for increased AAA risk (Elmore *et al.* 2009). Investigated polymorphism rs7635818 is situated in the intergenic region upstream of the *CNTN3* (Contactin-3) gene transcription start site, which encodes a lipid-anchored cell adhesion molecule contactin-3 (Chatterjee *et al*. 2019). Polymorphism that is near a functional gene variant can serve as marker for the functional variant. Three polymorphisms located within intron 2 of *CNTN3* gene (the nearest gene to examined polymorphism) and with high linkage disequilibrium to polymorphism rs7635818 (not in all ethnic groups) showed suggestive association with AAA. However, confirmed associations were not significant after adjustment for multiple testing (Jones *et al.* 2009).

Contactin-3, a translation product of *CNTN3* gene, is primarily expressed in the nervous system especially in frontal lobe, occipital lobe, cerebellum and amygdala, although products derived from *CNTN3* transcripts were also detected in aortic tissue, including both normal and AAA tissue (Elmore *et al.* 2009). There is little knowledge about contactin-3 functions in nervous and arterial system under physiological or pathological conditions (Elmore *et al.* 2009, Chatterjee *et al.* 2019). Contactin-3 belongs to the group of adhesion molecules, transmembrane or membrane-linked glycoproteins that mediate the connections between cells or the attachment

of cells to substrate such as stroma or basement membrane. Cell adhesion molecules are involved in inflammation, cell proliferation, migration, differentiation, cell apoptosis; they are essential for the maintenance of tissue architecture integrity and guarantee the functionality of the organs. Taking into account the wide function of these molecules, examined polymorphism rs7635818 may participate in processes associated with metabolism of extracellular matrix, smooth muscle cell functions or aortic inflammation that belongs to mechanisms essential in AAA pathogenesis. Better understanding of contactin-3 function in the aortic wall is necessary and could uncover its possible role in the pathogenesis of an AAA.

By recognizing the genes and gene polymorphisms present in the pathogenesis of AAA, we would be able to estimate a genetic risk of AAA in an individual patient. Results of the present study may increase the interest of polymorphism rs7635818 function. No studies to date have examined the relationship between rs7635818 polymorphism and *CNTN3* expression. Tissue studies with quantitative analysis of contactin-3 mRNA levels may help to determine whether this gene variant can affect expression of *CNTN3* gene and to explain the biological role of contactin-3 in aorta. This may partially help to elucidate the pathogenesis of AAA.

The present study also has some limitations due to the small number of patients, since AAA belongs to the group of relatively rarely occuring diseases. The number of women that were included in our study was small which is typical for AAA studies. AAA patients had higher creatinine compared to the control group. The association between AAA and chronic kidney disease in known, but common pathophysiological pathways (matrix metalloproteinases activation, chronic inflammation and neoangiogenesis) are explained insufficiently (Matsushita *et al.* 2018). Our AAA patients had higher body mass index that may reflect greater muscle mass and participate in elevation of creatinine. CT-angiography, which is performed as an important diagnostic tool in AAAs before surgery and may be repeated, could also cause worsening of renal functions. We did not differentiate between familial and sporadic AAA cases

as our older patients do not have knowledge of their parents' or siblings' diseases. Duplex ultrasonography examination has a limitation in intraobserver variability, and it might underestimate aortic size when compared with computed tomography, but it is a reliable, non-invasive, safe, and cost-effective method for assessing aortic size (Sprouse *et al.* 2003, Chiu *et al.* 2014).

Conclusions

The present study has confirmed an association between gene variant rs7635818 located on the short arm of chromosome 3 and abdominal aortic aneurysm in the Slovak population. The carriership of each C-allele of the rs7635818 polymorphism was associated with an increased risk of developing abdominal aortic aneurysm by approximately 50%. Further studies are needed to assess the exact biological role of this gene variant. Better understanding of abdominal aortic aneurysm pathogenesis may help to develop prevention strategies based on the key biological pathways, as well as to identify possible targets for medical treatment.

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TABLE I. Baseline characteristics of AAA patients and the control group of patients.

	AAA pacients N = 166	Control patients N = 163	p-value
Basic information			
Men, No. (%)	147 (88.6%)	133 (81.6%)	0.952
Age, years¡	72.5±7.76	69.2±7.86	<0.01
Abdominal aortic diameter, mm¡	57,0±12,4	18,4±2,81	<0.01
Active smokers, No. (%)	58 (34.9%)	67 (41.1%)	0.299
Ex-smokers, No. (%)	76 (45.8%)	82 (50.3%)	0.412
Body mass index, kg/m²¡	27.9±4.28	26.6±4.75	0.014
Comorbidities			
Coronary artery disease, No. (%)	94 (56.6%)	80 (49.1%)	0.189
COPD, No. (%)	32 (19.3%)	26 (16.0%)	0.429
Type 2 diabetes mellitus, No. (%)	45 (27.1%)	44 (27.0%)	0.981
Hypertension, No. (%)	151 (91.0%)	152 (93.3%)	0.442
Dyslipidaemia, No. (%)	142 (85.5%)	148 (90.8%)	0.140
Treatment			
Statins, No. (%)	109 (65.7%)	106 (65.0%)	0.552
ACE inhibitors/ angiotensin II receptors blockers, No. (%)	116 (69.9%)	121 (74.2%)	0.656
Calcium chanels blockers, No. (%)	70 (42.2%)	63 (38.7%)	0.402
Beta blockers, No. (%)	114 (68.7%)	84 (51.5%)	0.001
Laboratory parameters			
Creatinine (µmol/l)*	91.7 (75.9; 107.0)	76.2 (67.2; 92.4)	<0.01
LDL cholesterol (mmol/l)¡	2.92±1.07	3.02±1.01	0.483
HDL cholesterol (mmol/l)*	1.08 (0.98; 1.24)	1.17 (0.96; 139)	0.354
Fibrinogen (g/l) *	3.55 (2.97; 4.13)	3.50 (3.10; 4.50)	0.114

Categorical data expressed as counts with percentages; *non parametric data expressed as median (25^{th}, 75^{th} percentiles); ¡parametric data gives as mean ± standart deviation; AAA: abdominal aortic aneurysm; ACE: angiotensin-converting enzyme; COPD:chronic obstructive pulmonary disease

TABLE II. The association between rs7635818 polymorphism and abdominal aortic aneurysm presence.

Genetic models of 3p12.3 rs7635818 polymorphism	AAA patients N = 166	Control patients N = 163	OR (95%CI) p-value	OR (95%CI) p^{adj}-value
Co-dominant				
GG	40 (24.1%)	56 (34.4%)	1.0	1.0
GC	81 (48.8%)	81 (49.7%)	1.40 (0.84–2.33); p=0.195	1.41 (0.81–2.45); p=0.222
CC	45 (27.1%)	26 (15.9%)	2.42 (1.29–4.55); p=0.006	2.23 (1.14–4.39); p=0.020
Dominant				
GG	40 (24.1%)	56 (34.4%)	1.0	1.0
GC/CC	126 (75.9%)	107 (65.6%)	1.65 (1.02–2.67); p=0.041	1.62 (0.96–2.73); p=0.070
Recessive				
GG/GC	121 (72.9)	137 (84.0%)	1.0	1.0
CC	45 (27.1%)	26 (16.0%)	1.96 (1.14–3.37); p=0.015	1.79 (1.01–3.19); p=0.047
Additive	-------	-------	1.54 (1.13–2.11); p=0.007	1.49 (1.06–2.08); p=0.020

AAA = abdominal aortic aneurysm, OR = odds ratio, ^{adj} adjustment including age, hypertension, sex, smoking habit, diabetes mellitus, creatinine and body mass index

TABLE III. The association between polymorphism rs7635818 and abdominal aortic aneurysm diameter.

Genetic models of 3p12.3 rs7635818 polymorphism	Patients N=329	Mean aortic diameter±standard deviation (mm)	Mean difference (95%CI) (mm)	p-value	p^{adj}-value
Co-dominant					
GG	96 (29.2%)	34.8±21.3	0.00		
GC	162 (49.2%)	37.7±20.9	2.84 (-2.55–8.22)	0.301	0.365
CC	71 (21.6%)	42.5±22.0	7.66 (-1.11–14.2)	0.022	0.076
Dominant					
GG	96 (29.2%)	34.8±21.3	0.00		
GC/CC	233 (70.8%)	39.1±21.3	4.30 (-0.78–9.39)	0.097	0.174
Recessive					
GG/GC	258 (78.4%)	36.6±21.0	0.00		
CC	71 (21.6%)	42.5±22.0	5.88 (0.27–11.48)	0.040	0.126
Additive	----------	----------	3.75 (0.52–6.99)	0.024	0.077

[adj] after adjustment including age, hypertension, sex, smoking habit, diabetes mellitus, creatinine and body mass index

FIGURE 1. Clinical flowchart of the study.