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CLINICAL STUDY

Role of genetics in the development of cardiac allograft vasculopathy

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ABSTRACT
BACKGROUND: The association between genetic polymorphisms and early cardiac allograft vasculopathy (CAV) development is relatively unexplored. Identification of genes involved in the CAV process may offer new insights into pathophysiology and lead to a wider range of therapeutic options.
METHODS: This prospective study of 109 patients investigated 44 single nucleotide polymorphisms (SNPs) within the susceptibility loci potentially related to coronary artery disease, carotid artery intima-media thickness (cIMT), and in nitric oxide synthase gene. Genotyping was done by the Fluidigm SNP Type assays and Fluidigm 48.48 Dynamic Array IFC. The intima thickness progression (IT) was evaluated by coronary optical coherence tomography (OCT) performed 1 month and 12 months after heart transplantation (HTx).
RESULTS: During the first post-HTx year, the mean intima thickness (IT) increased by 24.0 ± 34.2 μm ($p < 0.001$) and lumen area decreased by –0.9 ± 1.8 mm2 ($p < 0.001$). The rs1570360 (A/G) SNP of the vascular endothelial growth factor A (VEGFA) gene showed the strongest association with intima thickness progression, even in the presence of the traditional CAV risk factors. SNPs previously related to carotid artery intima-media thickness rs11785239 (PRAG1), rs6584389 (PAX2), rs13225723 (LINC02577) and rs17477177 (CCDC71L), were among the five most significantly associated with IT progression but lost their significance once traditional CAV risk factors had been added.
CONCLUSION: Results of this study suggest that genetic variability may play an important role in CAV development. The vascular endothelial growth factor A gene SNP rs1570360 showed the strongest association with intima thickness (IT) progression measured by OCT, even in the presence of the traditional CAV risk factors *(Tab. 3, Fig. 3, Ref. 36).* Text in PDF *www.elis.sk*
KEY WORDS: cardiac allograft vasculopathy, optical coherence tomography, vascular endothelial growth factor A, intimal thickening, genetic polymorphism.

Introduction

Cardiac allograft vasculopathy (CAV) is one of the leading causes of allograft loss, and late mortality 5–10 years after heart transplantation (32.3 %), exceeding the contribution of malignancies (24.9 %) and infections (10.8 %) (1).

Even though a wide range of CAV risk factors (immune and non-immune) have been identified (2), there has been little or no advance in the development of therapeutics to prolong long-term graft survival. Modest advances in prevention of CAV have occurred through improved prophylaxis against severe cellular rejections, prevention, and treatment of cytomegalovirus (CMV) disease, and chronic treatment with statins (3, 4). More importantly, advantageous effects of m-TOR inhibitors have been recently reported in HTx patients (5, 6).

Finally, inhibitors of proprotein convertase subtilisin-kexin type 9 (PCSK9) are currently being extensively studied as to whether they can ameliorate CAV over the first year after HTx (7, 8).

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Tab. 1. Analysed susceptibility loci potentially related to coronary artery disease, carotid artery intima-media thickness, and in nitric oxide synthase gene.

Gene	Protein	OMIM acc ID	SNP
	Nitric oxide synthase		
NOS1	Nitric oxide synthase 1	163731	rs2682826, rs1879417
GSTP1	Glutathione S-transferase, PI	134660	rs1695
NOS2	Nitric oxide synthase 2	163730	rs2297518
	Genes associated with CAD/risk factors of CAD		
LDLR	LDL receptor	606945	rs6511720
–	Noncoding region 2q36.3	n.a.	rs2943634
MTHFD1L	Methylenetetrahydrofolate dehydrogenase 1-like, nadp(+)-dependent	611427	rs6922269
CDKN2B-2/ANRIL	Antisense noncoding RNA in the ink4 locus	613149	rs10757274 rs1333049
IL-6	Interleukin 6	614752	rs1800795
CILP2	Cartilage intermediate layer protein 2	612419	rs16996148
CX37 / GJA4	Gap junction protein, 37-kd; connexin 3	121012	rs1764391
MTHFR	n(5,10)-methylenetetrahydrofolate	236250	rs1801133 rs1801131
FTO	FTO alpha-ketoglutarate-dependent dioxygenase	610966	rs17817449
C5orf67	Unknown	n.a.	rs9686661
SORT1	Sortilin	602458	rs646776
APOE	Apolipoprotein E	107741	rs429358 rs7412
APOA5	Apolipoprotein A5	606368	rs662799, rs964184
CBFA2T3	Core-binding factor, alpha subunit 2, translocated to, 3	603870	rs602633 rs844396
LINC02577		n.a.	rs13225723
CYP2R1	Cytochrome p450, subfamily IIR, polypeptide1	608713	rs10741657 rs1562902
SLC2A13	Solute carrier family 2 (facilitated glucose transporter), member 13	611036	rs515291
SFXN2	Sideroflexin 2	615570	rs2902548
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9	607768	rs6957201
	Genes associated with carotid artery intima-media thickness		
KCNK5	Potassium channel, subfamily 1k member 5	603493	rs10947789
VEGFA	Vascular endothelial growth factor a	192240	rs3025039, rs1570360
SOD2	Superoxide dismutase 2	147460	rs5746136
FOXO3	Forkhead box o3a	602681	rs2802292
VTI1A	Vesicle transport through interaction with t-snares 1a	614316	rs11196033
EBF1	Early b-cell factor 1	164343	rs36071027
PRMT9	Protein arginine methyltransferase 9	616125	rs11413744
LINC01717	Long intergenic non-protein coding RNA 1717	n.a.	rs201648240
SIRT1	Sirtuin 1	604479	rs3758391
SIRT3	Sirtuin 3	604481	rs12363280
SIRT6	Sirtuin 6	606211	rs107251
NRXN1	Neurexin I	600565	rs933585
ZHX2	Zinc finger and homeodomain protein 2	609185	rs11781551
SLC22A3	Solute carrier family 22 (extraneuronal monoamine transporter), member 3;	604842	rs2048327
BMPR1B	Bone morphogenic protein receptor, type ib	603248	rs3796433
PRAG1	Peak1-related kinase-activating pseudokinase 1	617344	rs11785239
CCDC71L	Coiled-coil domain containing 71 Like	n.a.	rs17477177
ADAMTS7	A disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 7	605009	rs3825807
PAX2	Paired box gene 2	167409	rs6584389
PPCDC	Phosphopantothenoylcysteine decarboxylase	609854	rs1867148
BMPR1B	Bone morphogenetic protein receptor	603248	rs4456963
AIG1	Androgen induced gene 1	608514	rs6907215

Nonetheless, despite all efforts in consistent prevention and treatment, a large proportion of HTx patients still experience a significant CAV progression even during the first year after the index procedure (9). Hence, the real genesis of this disease remains elusive. Therefore, identification of genes involved in the process of CAV may provide a novel insight into pathophysiology and lead to new therapeutic options. Only scarce data have ever been published about the possible role of genetic predisposition for CAV development (10).

Therefore, we aimed to evaluate whether genotyping of selected susceptibility loci potentially related to coronary artery disease, carotid artery intima-media thickness progression (IMT), and nitric oxide synthase, performed in HTx recipients, may disclose new possible pathways in prevention and treatment of CAV.

Methods

Between October 2014 and March 2018, 109 consecutive subjects who survived the first 12 months after HTx, from the Heart Centre at IKEM, Prague, and the Centre of Cardiovascular and Transplantation Surgery, Brno, Czech Republic were enrolled. The study (clinical trial NCT02503566) complies with the Declaration of Helsinki and was approved by the respective ethics committees. This project was supported, in part, by NIH NIBIB grant R01-EB004640. All HTx recipients ≥ 18 years of age were deemed eligible for inclusion into the study provided they were able and willing to give their informed consent. Exclusion criteria included kidney disease stage ≥ IV (glomerular filtration < 30 mL/min), unfavourable post-HTx clinical conditions such as severe nosocomial sepsis with prolonged antibiotic treatment during the first month, ongoing need for circulatory support using a ventricular assist device, and acute allograft failure.

Genetic testing

Single nucleotide polymorphism (SNP) genotyping was performed using Fluidigm SNP Type assays and Fluidigm 48.48 Dynamic Array IFC (integrated fluidic circuit) for Genotyping on the Fluidigm BioMark platform. The assays are based on allele-specific polymerase chain reaction (PCR) detection system and include locus-specific primers and specific target amplification (STA) primers. The data were analysed using the BioMark SNP Genotyping Analysis software. All assays were custom-prepared and further details are available upon request from corresponding author. DNA analysis included 44 SNPs selected within the susceptibility loci potentially related to coronary artery disease, carotid artery intima-media thickness (IMT), and in nitric oxide synthase gene (Tab. 1).

OCT method

Coronary OCT imaging was performed at 1 month (M1) and 12 months (M12) after HTx as part of routine surveillance cardiac catheterization using a commercial intracoronary OCT system (ILUMIEN/DRAGONFLY OPTIS, St. Jude Medical). A 54 mm-long segment of each HTx patient's left anterior descending (LAD) artery, located within a proximal 100-mm segment, was imaged using automated pullback at 18 mm/sec and 10 frames/mm. Where the LAD artery exhibited unfavourable anatomical characteristics (small diameter, extreme tortuosity, muscle

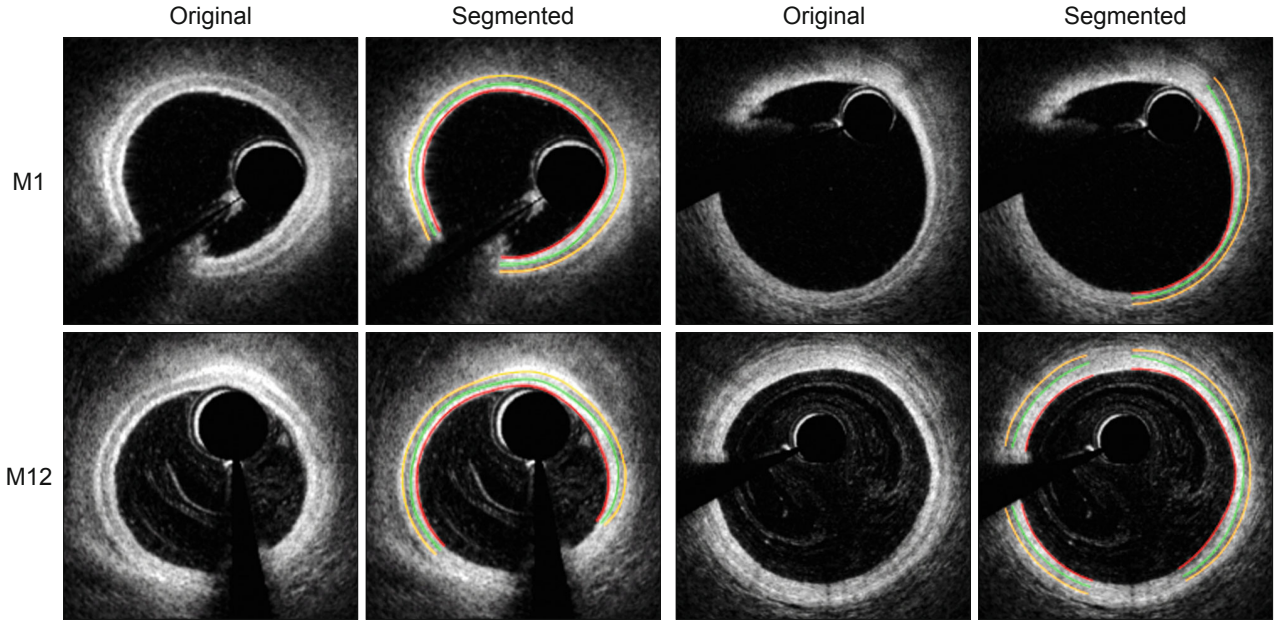

Fig. 1. Automated OCT segmentation of coronary wall layers for both M1 and corresponding M12 frames: lumen (red), outer intima (green) and outer media (orange).

bridge), the proximal segment of the left circumflex (LCx) or the right coronary artery (RCA) was imaged. The proximal fiduciary point was the left main bifurcation in the left coronary artery and first branch or well-defined calcification in RCA or LCx. After 12 months, patients underwent repeat cardiac catheterization and OCT of the same coronary artery with the same fiduciary points identified.

OCT image interpretation

For each frame of all OCT pullbacks, luminal, intimal-layer, and medial-layer surfaces were automatically segmented using a fully three-dimensional LOGISMOS graph-based approach developed at the University of Iowa (11, 12), as previously documented (9, 13). Boundaries were identified as OCT brightness changes depicting tissue interfaces between adjacent wall layers. Automatically identified borders were efficiently edited using our Just-Enough-Interaction method adapted for the OCT segmentation environment (14, 15) (Fig. 1). This technique allows segmentation errors to be efficiently corrected in a 3D fashion on a regional basis if/as needed, an alternative to contour-by-contour/frame-by-frame manual retracing. Furthermore, portions of the OCT-imaged wall that were not analysable due to, e.g., guidewire shadow, excessive focal atherosclerosis, blood artifacts, etc., were excluded automatically using a deep learning network. This highly accurate, multilayer model ensures quantitative CAV analysis of every OCT frame of the imaged vessel for both the baseline and follow-up image pullbacks. After identifying corresponding vascular landmarks, baseline and follow-up pullback pairs were co-registered, enabling location-specific and fully three-dimensional comparisons of layer-based changes using quantitative indices.

Statistical analysis

Numerical variables are described as mean ± standard deviation or median and interquartile ranges (IQR), where appropriate, categorical variables as frequency (percent). The coronary morphological changes from M1 to M12 were evaluated using paired Student's t test. Categorical variables, presented as counts and percentages, were compared using Fisher's exact test.

Due to a unique sample with a low sample size and numerous candidate genes, as well as to account for complex interactions between genes, we have used machine learning methods to analyze the association of candidate genes with IT progression over time. The dataset was randomly divided at the patient level into the training and validation datasets. The training set was used for hyperparameters tuning, with 10-fold cross validation and a grid search. The best combination of hyperparameters was selected by the root-mean-square-error. The final model was applied to the validation dataset. Of the three machine learning methods used (support vector machine, random forest, and Bayesian regression), the random forest showed the best performance and is reported in the results section. To identify the most influential genes in the model, we used the feature importance function. To compute the feature importance for a single variable, the model prediction loss (mean absolute error increase-MAE) was measured before and after shuffling the values of the variable. By shuffling the variable values, the association between the feature and the outcome did not hold anymore. The larger the increase in prediction error, the more important the variable was. The shuffling was repeated to achieve

Tab. 2. Patient characteristics.

	n=109
Age	50.8 (±12.7)
Sex (men)	78 % (85)
Ischaemic cardiomyopathy before HTx	20 % (22)
VAD before HTx	25 % (27)
CMV infection	9 % (10)
Cellular reject severe	9 % (10)
Humoral rejection	4 % (4)
Cold ischaemia time	134.4 (±52.4)
Donor sex (men)	68 % (74)
Donor age	42.1 (±11.6)
BMI M1 / M12	26.1 (±4.4) / 28.6 (±4.8)
LVEF M1 / M12	61.9 (±4.2) / 61.2 (±4.8)
Cholesterol M1 / M12	4.8 (±1.2) / 4.2 (±1.2)
HDL M1 / M12	1.5 (±0.5) / 1.2 (±0.4)
LDL M1 / M12	2.5 (±0.9) / 2.2 (±0.9)
TAG M1 / M12	1.5 (±1.0) / 1.8 (±1.1)
Creatinine M1 / M12	90.8 (±37.4) / 113.8 (±39.3)
HBA1c M1 / M12	39.3 (±8.0) / 46.7 (±15.7)
Glycaemia M1 / M12	5.5 (±1.5) / 6.8 (±2.7)
hsTnT M1 / M12	145.9 (±160.8) / 29.4 (±34.2)
Aspirin M1 / M12	62 % (68) / 71 % (77)
Tacrolimus M1 / M12	97 % (106) / 96 % (105)
Statin M1 / M12	80 % (87) / 84 % (92)
Mycophenolate mofetil M1 / M12	95 % (104) / 87 % (95)
mTOR M1 / M12	0 % (0) / 10 % (11)
Beta blocker M1 / M12	55 % (60) / 74 % (81)
Ivabradine M1 / M12	2 % (2) / 16 % (17)
Prednisone M1 / M12	100 % (109) / 83 % (90)

BMI – body mass index; CMV – cytomegalovirus; hsTnT - highly sensitive troponin T; HDL – high-density lipoprotein; HTx – heart transplant; HBA1c – glycated haemoglobin; LDL – low-density lipoprotein; LV – left ventricle; M – month; mTOR – mammalian target of rapamycin; M1 – 1 month after HTx; M12 – 12 months after HTx; TAG – triacylglycerols; VAD – ventricular assist device

Table 3. Optical coherence tomography findings of CAV.

	M1 after HTx	M12 after HTx	Change from M1 to M12	p
Mean luminal area (mm^2)	8.7±2.5	7.9±2.5	-0.9±1.8	< **0.001**
Mean intimal thickness (μm)	107.5±45.1	131.5±56.1	24.0±34.2	< **0.001**
Mean medial thickness (μm)	83.9±21.4	85.3±20.4	1.4±12.5	0,237
Mean intimal/medial ratio	1.4±0.3	1.6±0.5	0.3±0.3	< **0.001**

HTx – heart transplant; M – month

more accurate results, since the permutation feature importance tends to be quite unstable. Results are presented as the feature importance plot with MAE.

We used the Tidymodels, RandomForest and iml packages for R (R: A language and environment for statistical computing. Vienna, Austria).

Traditional risk factors for CAV development that were included into statistical analysis were as follows: patient age, donor age, ischemic cardiomyopathy before HTx, CMV infection, severe cellular rejection, humoral rejection, cold ischemia time, body mass index (BMI) M1/M12, total cholesterol M1/M12, high-density lipoprotein (HDL) M1/M12, low-density lipoprotein (LDL) M1/M12, triacylglycerols (TAG) M1/M12, glycated haemoglobin (HBA1c) M1/M12, glycemia M1/M12 and high-sensitive troponin (hsTnT) M1/M12.

Results

In total, 109 out of 113 originally recruited patients were enrolled into the study (4 patients died prior to M12 follow-up visit). In this cohort, 95 left anterior descending (LAD), 5 left circumflex (LCx), and 9 right coronary (RCA) arteries were analysed. Patients´ characteristics are shown in Table 2.

In the 109 M1 / M12 pairs of registered OCT pullbacks, the median analysable angular range was 274° (IQR 252° to 295°) per frame. The average overlapping pullback length was 36.8 ± 8.4 mm, resulting in a total of 40,152 registered frame pairs being analysed for all 109 patients.

During the first post-HTx year, the mean intima thickness increased by 24.0 ± 34.2 μm ($p < 0.001$) and lumen area decreased by –0.9 ± 1.8 mm^2 ($p < 0.001$), while there was no change in media thickness ($p = 0.237$) (Tab. 3).

Figure 2 displays ten genes with the highest impact on IT progression in the random forest model. Among these, the SNP rs1570360 (A/G) in the vascular endothelial growth factor-A (VEGFA) gene showed the strongest association with intima thickness progression. Another four most significant SNPs were rs11785239 (PRAG1), rs6584389 (PAX2), rs13225723 (LINC02577) and rs17477177 (CCDC71L).

The strongest clinical risk factors associated with early CAV development were LDL cholesterol and total cholesterol levels at 1 month after HTx. Even after inclusion of the classical risk factors of CAV progression into statistical analysis, rs1570360 (VEGFA) remained significantly associated with CAV progression (Fig. 3).

Discussion

The main findings of this study can be summarised as follows: (1) Out of all studied SNPs, the rs1570360 (A/G) located in the promotor region of Vascular endothelial growth factor A (VEGFA) gene showed the strongest association with intima thickness (IT) progression measured by OCT, (2) the rs1570360 remained associated with intimal thickness progression even in the presence of the traditional CAV risk factors, (3) SNPs previously associated with carotid artery intima-media thickness- rs11785239 (PRAG1), rs6584389 (PAX2), rs13225723 (LINC02577) and rs17477177 (CCDC71L) were reported among the five most significantly associated polymorphisms related to IT progression; nevertheless no significant association with IT progression was found in the presence of traditional CAV risk factors.

CAV-related graft failure is associated with the majority of patient mortality within 5 to 10 years, surpassing the contributions of malignancies and infections (1). Early rapid development of CAV is reported in a high proportion of HTx patients, occurring

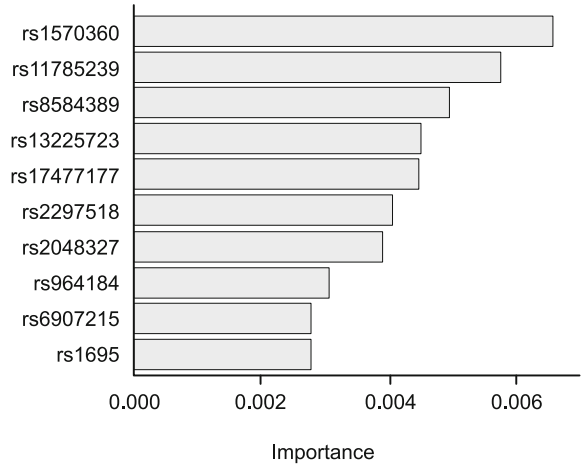

Fig. 2. Relative importance of the ten most influential genes associated with intima thickness progression. The higher the feature importance (higher loss in mean absolute error), the higher the influence of a gene on IT progression.

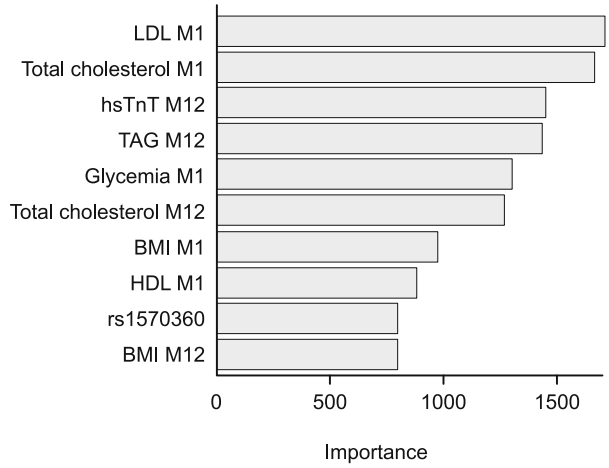

Fig. 3. Relative importance of the most influential risk factors associated with intima thickness progression.

as early as within one year after the index procedure (9). Hence, an early identification of such patients is of utmost importance. Optical coherence tomography (OCT) enables the thin intimal layer to be clearly differentiated from the tunica media, a crucial factor in determining early progression of CAV (9). Using our highly automated 3D-OCT analysis, after registering the overlapping baseline and follow-up pullbacks, a total of 40152 registered frame pairs were analysed in 109 patients. This approach is the most detailed quantitative OCT method available for assessing early changes in cardiac vasculature (9, 16). During the first post-HTx year, the mean intima thickness significantly increased by 24.0 ± 34.2 μm ($p < 0.001$).

The aim of this study was to identify genes possibly involved in CAV development, which could lead to new targets for prevention and therapy of CAV. VEGFA as one of the most important factors of vasculogenesis has many properties that could play a central role in pathophysiology of CAV development. Such as its ability to induce migration of vascular smooth muscle cells, to stimulate expression of ICAM-1, VCAM-1, E-selectin and matrix metalloproteinases or enhancing macrophage infiltration (17–20). The studied SNP rs1570360 (A/G) is located in the promoter region at –1154bp from the transcriptional start site (TSS) of the VEGFA gene (21), which is located on chromosome 6p21.3. The location of this SNP causes a change in transcription binding motifs and can influence expression of VEGFA gene (22). Tambur AR et al (23) was first to investigate the connection between VEGF polymorphisms and early CAV development. When diagnosed by IVUS, patients carrying the rs1570360 SNP had a significantly increased risk of early CAV development.

The rs1570360 has previously been associated with higher VEGF mRNA and protein production (24, 25). VEGFA over expression in the allograft and elevated VEGFA plasma or serum levels play a role in CAV development, and they could predict subsequent CAV development, as is demonstrated in two studies on paediatric heart transplant patients. Compared to the patients without CAV, the VEGF-A levels were significantly raised even before the CAV was detectable by IVUS or angiography (26). Daly et al (27) concluded that elevated plasma VEGF-A concentration above 90 pg/mL is associated with a higher risk of moderate to severe CAV development within 5 years posttransplant. When VEGF-A was combined with VEGF-C and PF-4 (platelet factor-4) levels, it allowed for a sensitive and specific diagnosis of mild established CAV (28).

Reinders et al (29) discovered that mononuclear cell infiltration and both acute and chronic rejection induce VEGF expression. Patients with persistent intragraft overexpression of VEGF in endomyocardial biopsy (EMB) samples during the first posttransplant year were more likely to develop CAV. Another study suggested that localization of VEGF protein to the vascular endothelium at any time posttransplant results in a 2.5-fold increase in the risk of developing CAV (30). In animal studies, development of intimal thickening correlated with intragraft VEGF protein expression, while blockade of VEGFR signalling pathway resulted in significantly reduced arteriosclerotic lesions. VEGF inhibition was also shown to reduce the severity and incidence of CAV in mouse cardiac transplantation models (31). In addition, VEGF administration caused neointimal thickening after vascular injury in dogs (32).

Recently, rs1570360 was investigated for its association with a risk of coronary heart disease (CHD) development (33). Polymorphisms of the VEGF-A gene have a connection to many other vascular diseases. Recently, studies have found association of the rs1570360 polymorphism with extracranial internal carotid artery stenosis and ischemic stroke (34), or acute cerebral infarction (35).

So-called ´traditional´´ risk factors of CAV development can be categorised as immune and non-immune. They include clinical variables such as old age, ischaemia–reperfusion injury, obesity, hypertension, diabetes mellitus, hypercholesterolemia, CMV infection, cellular or humoral rejection, or presence of anti-HLA donor specific antibodies (DSA) (3, 4). Results of our recent study did not, however, identify a direct association between presence of DSA and IT progression in an early period after HTx (36). The most robust clinical risk factors associated with early CAV development in current cohort of patients were LDL cholesterol and total cholesterol levels at 1 month after HTx. The crucial role of hyperlipidaemia in the early CAV development was already confirmed in our other previous work (9). After taking into consideration other non-immune risk factors into statistical analysis, rs1570360 (VEGFA) remained significantly associated with CAV progression. This confirms it´s possible role in CAV development, hence future analyses focusing on VEGFA might be of major interest.

Remaining four most significant SNPs (PRAG1, PAX2, LINC02577 and CCDC71L) have been previously linked with carotid artery intima-media thickness (cIMT) or carotid plaque development. Nevertheless, none of them remained statistically significant in terms of CAV progression after addition of traditional CAV risk factors into statistical analysis.

Conclusion

Genetic variability may play an important role in CAV development. Vascular endothelial growth factor A gene showed the strongest association with intima thickness (IT) progression measured by OCT. Association with intimal thickness progression remained even in the presence of the traditional CAV risk factors. Further identification of risk alleles associated with accelerated CAV may be useful in CAV risk stratification and post-transplant management individualisation.

Study limitations

1. OCT imaging results were only available at M1 after HTx. Thus, we were unable to differentiate between donor-transmitted coronary artery disease and *de novo* progression of CAV during the first post-HTx month. Due to the additional need to administer angiographic contrast and the associated risk of renal function deterioration, only one vessel per patient was examined by OCT. Due to the limited depth of OCT tissue penetration (1.5 to

3 mm), layered coronary structures, especially the external elastic lamina, were not always visible in some patients with extensive donor-transmitted coronary disease, thus impacting our ability to precisely detect changes in the coronary vasculature.

2. Our analysis is limited by low number of patients on the one hand, and high number of candidate genes on the other hand, which precluded the usage of traditional statistical methods. While we used novel machine learning methods, our conclusions should be considered only preliminary and exploratory.

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