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Forearm vasodilator reactivity in healthy male carriers of the 3q22.3 rs9818870 polymorphism

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ABSTRACT

Background: A genome wide association study has identified a robust risk locus for cardiovascular disease on 3q22.3. However, the mechanisms by which the [C]/[T] polymorphism rs9818870 increases cardiovascular risk are unknown. This forearm blood flow (FBF) study addressed the question if the genetic association with cardiovascular disease in patients is preceded by incipient vasodilator impairment in young, healthy carriers of this new risk locus on chromosome 3.

Materials and methods: After a pre-screening of 74 subjects 17 male healthy volunteers homozygous/heterozygous for a single nucleotide polymorphism (SNP) risk allele on 3q22.3 and a control group of 17 healthy volunteers not carrying the allele were included into this case–control study.

Results: Forearm vascular endothelium-dependent and -independent vasodilator responses were in the normal range in both groups, although endothelium-dependent FBF reactivity to acetylcholine was significantly higher in SNP carriers of the risk allele.

Conclusion: The augmented endothelium-dependent vasodilation of the forearm resistance vasculature does not support the presence of endothelial dysfunction in young SNP carriers and indicates that other mechanisms are responsible for the strong association between coronary artery diseases and the rs9818870 polymorphism, located on 3q22.3.

Background

Cardiovascular disease including coronary artery disease (CAD) is a leading cause of mortality worldwide (Eurosurveillance editorial team, 2012). Besides well-known non-inheritable risk factors like diet or nicotine abuse, an increasing number of studies have convincingly demonstrated heritable components for CAD development (Coronary Artery Disease (C4D) Genetics Consortium, 2011; Samani et al., 2007; Schunkert et al., 2011). Twin studies have shown that genetic susceptibility accounts for approximately 30–60% of the inter-individual CAD risk-variation (Marenberg et al., 1994).

Genome wide association studies have identified distinct genomic regions with a strong gene–CAD association, e.g. a region on chromosome 9p21.3 (Helgadottir et al., 2007; McPherson et al., 2007; Samani et al., 2007). Homozygous carriers of the [C]-allele of the single nucleotide polymorphism (SNP) rs1333049 had a 30–70% increased risk for myocardial infarction as compared to subjects without the risk allele (Aschauer et al., 2010; Samani et al., 2007). The descriptive association has led to a number of functional studies in patients and healthy volunteers, which have elucidated the functional and molecular background of the rs1333049 variant. In particular, it was shown that rs1333049, possibly by its effect on CDKN2, increases arterial stiffness (Phababpha et al., 2013), influences carotid artery lumen diameter (Bella et al., 2013) and impairs arterial vasodilator resistance (Aschauer et al., 2010).

Recently, another susceptibility locus for CAD, obesity and dyslipidemia has been confirmed on chromosome 3q22.3 (Alshahid et al., 2013; Erdmann et al., 2009). Analysing genome wide data of more than 25,000 CAD patients (Ellis et al., 2011; Erdmann et al., 2009) a strong association could be reported for the susceptibility for CAD and SNP rs9818870. The underlying mechanism for the observed increase in CAD risk, however, also remains unclear. One potential explanation is offered by the location of the respective SNP in the 3′-UTR of the *MRAS* gene. It is known that the *M-ras* protein is a member of the ras superfamiliy of GTP-binding proteins, with an especially high expression in the cardiovascular system (Haas et al., 2012), which indicates a potential pivotal role in cardiovascular function. However, although the reported association was strong in patients, the implication of this polymorphism in healthy subjects has not been studied so far.

Based on previous findings that vasodilator resistance vessel function can be altered in healthy subjects with a specific genotype (Aschauer et al., 2010) the present study aimed to test if young male

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healthy volunteers display an impairment of endothelial function when risk allele carriers of the SNP on chromosome 3q22.3 (rs9818870) are compared to controls. The rationale for this study is derived from the fact that the endothelium plays a pivotal role in the pathophysiology of arteriosclerosis. Through the production of nitric oxide (NO), the endothelium modulates blood vessel tone and affects platelet adhesion and aggregation, thrombogenicity, and cell proliferation (Heitzer et al., 2001; Vita et al., 1990). Impaired endothelial function is an indicator for incipient atherosclerosis and can be used as a reliable marker (Flammer et al., 2012; Vita et al., 1990) that can be assessed with the forearm blood flow method (Flammer et al., 2012; Hokanson et al., 1975; Pleiner et al., 2002).

As an impaired endothelial function can be regarded as a predisposing factor for CAD development, the early identification of incipient atherosclerotic disease in asymptomatic healthy subjects could provide as an explanation for the reported gene–disease-association in carriers of the 3q22.3 rs9818870 polymorphism. Ultimately, if confirmed, such findings could be diagnostically employed to assess individual patients cardiovascular risk at an early stage.

Materials and methods

The study protocol was approved by the Ethics Committee of the Medical University of Vienna and complies with the Declaration of Helsinki, including current revisions and the ICH Good Clinical Practice guidelines. Written informed consent was provided by all subjects before inclusion. Reporting of the present results is in accordance with the EQUATOR-guidelines (Simera et al., 2010).

Study population

79 healthy male Caucasian subjects were screened for SNP rs9818870. Thereafter, a total of 17 subjects homozygous or heterozygous for the risk allele (either [T];[T] or [C];[T]), and 17 control subjects harbouring the [C];[C]-genotype were included in the study. All subjects were given a complete health examination including physical examination, ECG and laboratory screening on screening day. Blood pressure was measured non-invasively by employing a standard Riva Rocci method to exclude existing hypertension. All subjects were non-smokers and had no history or signs of arterial hypertension, altered lipid metabolism or any other cardiovascular risk factors (Table 1).

Genotyping

DNA for sequence analysis was derived from EDTA-anticoagulated whole-blood based on a modified salting-out precipitation method using Gentra Puregene Blood kits (Qiagen, Hilden, Germany). Discrimination of genotypes was subsequently carried out on an ABI 7900HT sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) by means of the polymerase-chain reaction (PCR)-based 5′ nuclease assay (Hui et al., 2008). For this, sequence specific oligonucleotides containing specific fluorophores at their 5′-ends bind their respective allele, which is located within a PCR-amplification sequence, during the annealing phase of each PCR-cycle. Initially, the fluorophores' signals are quenched by specific molecules attached to the 3′-ends of the oligonucleotides. During strand elongation, this fluorophore gets separated from the quenching molecule by the proofreading-activity of Taq-polymerase. Consequently, its typical fluorescence signal can be detected. Hence, end-point-measurement of sequence-specific fluorescence indicates the presence of the corresponding allele.

In short, the PCR was carried out in a total volume of 10 μL containing 5 μL of TaqMan® Genotyping Mastermix (Applied Biosystems), 1× TaqMan® SNP genotyping assays containing sequence specific oligonucleotides (assay ID: C_26070086_10, Applied Biosystems) and ≈10 ng of genomic DNA. During the reaction, an initial DNA strand denaturation step for 10 min at 95 °C was followed by 40 cycles of alternating denaturation for 15 s at 95 °C and annealing/elongation steps for 1 min at 60 °C. End-point-fluorescence was measured using SDS 2.4 sequence detection software (Applied Biosystems).

Table 1
Characteristics of subjects with [C];[C] or [C];[T]/[T];[T] genotype regarding the rs9818870 sequence variant. Data are presented as median (interquartile range).

	[C];[C] genotype (n = 17)	[C];[T]/[T];[T] genotype (n = 17)
Age (years)	25 (21–28)	25 (21–29)
BMI (kg m^{-2})	23 (22–25)	23 (21–25)
Systolic blood pressure (mm Hg)	120 (113–129)	127 (118–135)
Diastolic blood pressure (mm Hg)	64 (53–69)	72 (63–82)
Heart rate (bpm)	73 (65–79	71 (65–78)
Glucose (mg dL^{-1})	87 (82–93)	88.0 (84–93)
Creatinine (mg dL^{-1})	0.99 (0.92–1.07)	0.99 (0.86–1.09)
Blood urea nitrogen (mg dL^{-1})	14.4 (12.1–17.2)	16.1 (13.1–18.7)
Triglyceride (mg dL^{-1})	112 (67–164)	99 (63–102)
Cholesterol (mg dL^{-1})	186 (158–234)	170 (145–180)
Bilirubin (mg dL^{-1})	0.7 (0.5–0.9)	0.9 (0.7–1.2)
Leukocytes (g L^{-1})	5.7 (4.7–6.6)	5.5 (4.9–5.8)

Forearm blood flow measurement

FBF was measured in both arms, as described previously (Benjamin et al., 1995; Hokanson et al., 1975; Pleiner et al., 2002). Strain gauges, placed on the forearms, were connected to plethysmographs (EC-6; D.E. Hokanson, Inc., Bellevue, WA, USA) to measure changes in forearm volume in response to inflation of venous congesting cuffs. Drug effects were expressed as percentage change in the ratio of FBF in the intervention to the control arm where pre-dose ratio was defined as 100%. Flow measurements were recorded for 9 s at 30 s intervals during drug infusions and the upper arm cuff repeatedly inflated. The early linear increase of the flow curves was used for calculation of FBF and was expressed as min^{-1} 100 mL^{-1} forearm volume.

Experimental protocol

Subjects abstained from alcohol and stimulating beverages containing xanthine derivatives 12 h before the study day. After an overnight fast and 7–8 h of sleep FBF measurements were conducted in a quiet room with an ambient temperature of 22 °C. During the examinations, subjects were lying in a supine position. A fine-bore (27-gauge) needle (Sterican, Braun, Melsungen, Germany) was inserted into the brachial artery of the non-dominant arm for local drug administration. After a 20 min resting period, baseline FBF was recorded over 5 min, and the response to the endothelium-dependent dilator acetylcholine (ACh; 25, 50 and 100 nmol min^{-1} each for 3 min; Clinalfa, Läufelfingen, Switzerland) was measured. After a 15 min washout period to allow restoration of baseline blood flow, FBF was measured in response to the endothelium-independent dilator glycerol trinitrate (GTN; 4, 8 and 16 nmol min^{-1}, each for 3 min; Perlinganit® (UCB-Group, Schwarz Pharma AG/Germany) or Nitropohl® (POHL-BoskampGmBH/Germany)).

Statistical analysis

A sample size calculation for the FBF measurements was performed with the assumption of an alpha level of 0.05 and a beta level of 0.2 and a standard deviation of approximately 20% of repeated FBF measurements as shown in previous studies. Consequently, a sample size of 17 patients per group had the statistical power of 80% to detect a difference of 20% between groups.

Linear mixed models were calculated for ACh or GTN on the factors SNP and dose level. The models also included interaction terms for SNP and dose level. The correlations of repeated measurements within a patient, as well as different variances at the three dose levels, were accounted for by allowing for unstructured covariance matrices in the