Original Article

Genetic Variants in Haem Oxygenase-1 and Endothelial Nitric Oxide Synthase Influence the Extent and Evolution of Coronary Artery Atherosclerosis

(endothelial nitric oxide synthase (ENOS) / haem oxygenase-1 (HO-1) / coronary atherosclerosis / plaque burden / plaque composition)

A. KRÁL[1], T. KOVÁRNÍK[1], L. KRÁLÍK[2], H. SKALICKÁ[1], J. HORÁK[1], G. S. MINTZ[3], J. UHROVÁ[4], M. SONKA[5], A. WAHLE[5], R. DOWNE[5], M. ASCHERMANN[1], P. MARTÁSEK[2], A. LINHART[1]

[1]Second Department of Medicine – Department of Cardiovascular Medicine, [2]Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[3]Cardiovascular Research Foundation, New York, NY, USA
[4]Institute of Clinical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[5]Department of Electrical and Computer Engineering, The University of Iowa, Iowa City, IA, USA

Abstract. The genetic basis for atherosclerosis development and progression is poorly characterized. We aimed to assess the relationship between endothelial nitric oxide synthase *(ENOS)* 894 G/T, haem oxygenase-1 *(HO1)* dinucleotide-length promoter polymorphisms and coronary artery atherosclerotic involvement and its changes during statin therapy. Coronary angiography, intravascular ultrasound (IVUS), IVUS-derived virtual histology (VH) and genetic polymorphism analysis were performed at study entry. Patients were randomized 1 : 1 to standard or aggressive hypolipidaemic treatment, and a follow-up evaluation was performed after twelve months. Plaque magnitude was significantly higher in carriers of *HO1* risk variants when compared with carriers of the protective variants (< 25 GT repeats). Similarly, the total coronary atherosclerotic burden was significantly greater in *HO1* risk variant carriers than in *HO1* protective variant carriers. Both parameters did not differ with respect to the *ENOS* genotype. A higher prevalence of thin-cap fibroatheroma (TCFA) in *HO1* risk variant carriers was observed, compared with the *HO1* protective variant carriers. The prevalence of TCFA was not influenced by the *ENOS* genotype. Baseline plaque composition did not differ significantly with respect to both polymorphisms. Significant interactions between plaque composition changes and *ENOS* and *HO1* genotypes were observed during statin treatment. In conclusion, the protective *HO1* promoter polymorphism correlates with a lower coronary artery plaque burden, whereas the protective *ENOS* 894 G/T polymorphism seems to favourably influence changes of coronary artery plaque composition during statin therapy, but has no significant correlation to the magnitude of coronary atherosclerosis.

Received June 3, 2011. Accepted June 6, 2011.

The study was supported by a research grant of the Internal Grant Agency of the Ministry of Health of the Czech Republic, IGA NR 9214-3.

Corresponding author: Aleš Král, Second Department of Medicine – Department of Cardiovascular Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, U Nemocnice 2, 128 08 Prague 2, Czech Republic. Phone: (+420) 224 962 605; Fax: (+420) 224 912 154; e-mail: ales.kral@vfn.cz

Abbreviations: Asp – aspartic acid, CAD – coronary artery disease, CAG – coronary angiography, CO – carbon monoxide, ENOS – endothelial nitric oxide synthase, G – guanine, Glu – glutamic acid, HO-1 – haem oxygenase-1, IVUS – intravascular ultrasound, LDL – low-density lipoprotein, L-NAME – L-NG-nitroarginine methyl ester, NADPH oxidase – nicotinamide adenine dinucleotide phosphate oxidase, NO – nitric oxide, PAV – percent atheroma volume, PCI – percutaneous coronary intervention, PCR – polymerase chain reaction, TCFA – thin-cap fibroatheroma, T – thymidine, VH – virtual histology.

Introduction

Atherosclerosis is a chronic inflammatory process characterized by lipid accumulation in vessel walls. The genesis and progression of atherosclerosis are influenced by many factors, including genetic predisposition,

which is still insufficiently characterized. The role of the endothelial nitric oxide synthase (ENOS) in endothelial dysfunction and atherosclerosis development has been extensively studied in the past fifteen years (Förstermann, 2010). ENOS catalyses a reaction in which nitric oxide (NO), a potent vasodilator, inhibitor of leukocyte adhesion, thrombocyte adhesion and proliferation of vascular smooth muscle cells, is produced by endothelial cells (Tsutsui et al., 2010). Many common genetic variants of the *ENOS* gene have been identified (Wang and Wang, 2000). The most extensively studied one in relation to atherosclerosis is the exon 7 894 G/T or Glu298Asp polymorphism. In this polymorphism, guanine (G) is replaced by thymidine (T) at position 894 of the *ENOS* gene resulting in a substitution of glutamic acid (Glu) for aspartic acid (Asp) at position 298 of the ENOS protein. The ENOS Asp298 variant carriers present decreased enzymatic activity (Wang et al., 2000). The decrease has been attributed to selective proteolytic cleavage of the enzyme in endothelial and myocardial cells (Tesauro et al., 2000). However, other researchers have suggested that the apparent decrease in enzymatic activity might be due to artefacts in sample preparation (Fairchild et al., 2001). Many functional and clinical consequences have been identified in Asp allele carriers, including lower coronary basal blood flow (Naber et al., 2001), blunted endothelial-dependent vasodilation (Veldman et al., 2002), and increased risk of arterial hypertension and coronary artery disease (CAD) development (Li et al., 2010).

Haem oxygenase-1 (HO-1) is another enzyme that has gained much attention in the context of atherosclerosis. This enzyme catalyses a reaction in which the haem is degraded to free iron (Fe^{2+}), carbon monoxide (CO) and biliverdin, which is subsequently converted to bilirubin by the enzyme biliverdin reductase (Abraham and Kappas, 2008). CO and bilirubin are substances with vasodilatory, antioxidative, angiogenic, anti-proliferative and anti-inflammatory properties that contribute to the suppression of atherogenesis (Morita, 2005; Abraham and Kappas, 2008; Idriss et al., 2008). The level of transcription, and thus the enzymatic activity of this inducible enzyme is determined by the number of guanosine-thymidine (GT) dinucleotide repeats in the gene promoter. With an increasing number of dinucleotide repeats, the transcription of the gene decreases (Idriss et al., 2008). Multiple studies have identified a positive correlation between the presence of longer dinucleotide repeats in the gene promoter and the development of diseases with oxidative stress as the pathogenetic mechanism, including CAD (Kaneda et al., 2002; Idriss et al., 2008). Conversely, gene variants with a low number of GT repeats react to oxidative stress with increased transcriptional activity and seem to confer protection against the development of atherosclerosis (Morita, 2005).

The extent and severity of coronary atherosclerosis can be assessed by means of intravascular ultrasound (IVUS) (Mintz and Maehara, 2009). Atherosclerotic plaque composition can be assessed *in vivo* by means of IVUS-derived virtual histology (VH) (García-García et al., 2009). Very limited data are available on the relationship between the extent and character of atherosclerotic coronary artery involvement, the evolution of such an involvement and the gene variants affecting the atherosclerotic process (Pfohl et al., 1998).

We sought to evaluate the relationship between the *ENOS* exon 7 894 G/T polymorphism and the (GT) dinucleotide-length polymorphism of the *HO1* promoter and the extent and characteristics of coronary artery atherosclerotic involvement as assessed by IVUS and VH.

In addition, we sought to assess the influence of these polymorphisms on the changes in coronary atherosclerotic involvement during the course of hypolipidaemic statin-based therapy.

Material and Methods

Study population

Patients with chronic stable angina were included in the study. Patients with a history of an acute coronary syndrome were allowed to enter the study more than six weeks after symptom onset. The study was approved by local ethical committees in each of the four participating hospitals in the Czech Republic, and all patients signed informed consent for the study procedures. Patients already treated by statins as well as statin-naive patients were included. Randomization was performed after coronary angiography in the following 1 : 1 ratio:

1) Group A (aggressive): atorvastatin 80 mg once daily (O.D.) + ezetimibe 10 mg O.D.
2) Group S (standard): continuation of previous statin treatment (fluvastatin 80 mg, two patients – 5.3 %; atorvastatin 10 mg, eight patients – 21.1 %; atorvastatin 20 mg, 12 patients – 31.6 %; atorvastatin 40 mg, five patients –13.1 %; simvastatin 20 mg, 11 patients – 28.9 %; all statin doses O.D.). Medication was "open label" for statin prescription and blinded for the IVUS-VH analysis.

Catheterization and IVUS imaging

After coronary angiography (CAG) and percutaneous coronary intervention (PCI, when indicated), the operator selected a target vessel for IVUS. Only one coronary artery was investigated in each patient. The inclusion criteria were as follows:

1) native artery with plaque burden (PB) > 20 % by IVUS
2) stenosis ≤ 50 % of lumen diameter by angiography with no indication for either PCI or coronary artery bypass grafting (CABG)
3) plaque length > 30 mm by IVUS.

In cases with similar findings in more than one coronary artery, the artery with the largest PB was selected for analysis. An IVUS phased-array probe (Eagle Eye 20 MHz 2.9 F monorail), IVUS console, Gold standard

software, and automatic pullback (research pullback, model R-l00) were used (Volcano Corp., Rancho Cordova, CA). After administration of 200 μg of intracoronary nitro-glycerine, the IVUS probe was introduced into the selected coronary artery beyond a distal fiduciary point (well-defined side branch). Motorized pullback at 0.5 mm/s was performed through the rest of coronary artery. The proximal fiduciary point was the left main bifurcation in the left coronary artery and first branch or well-defined calcification in the right coronary artery. After twelve months, patients underwent repeated cardiac catheterization and IVUS of the same coronary artery with the same fiduciary points identified.

IVUS and VH-IVUS assessment

All measurements were performed in the catheterization laboratory of the General University Hospital of Charles University, Prague, Czech Republic and analysed by a single operator to ensure the precise fulfilment of all study criteria for plaque identification and plaque measurement. IVUS B-mode images were reconstructed from radiofrequency data (Volcano Therapeutics Inc., Rancho Cordova, CA), and contour detection was performed using cross-sectional views with semi-automatic contour detection software to provide a geometrical and compositional output (Rodriguez-Granillo et al., 2005). Manual planimetry was used in cases of non-adequate automatic software tracing (pc VH 2.1, Volcano Corp.). Each frame from the VH-IVUS loop was analysed. Volumes were calculated using Simpson's rule and then normalized for pullback length (Mintz and Maehara, 2009).

The primary IVUS endpoint for plaque magnitude assessment was the percent atheroma volume (PAV), which was calculated as ($\Sigma(EEM_{CSA}$-Lumen $_{CSA}$) divided by Σ EEM_{CSA}) x 100, where EEM_{CSA} was the external elastic membrane cross-sectional area, and Lumen $_{CSA}$ was the luminal cross-sectional area. The change of PAV was computed as $PAV_{follow\ up} - PAV_{baseline}$ for each patient (Nissen et al., 2006). To avoid differences in pullback length, baseline and follow-up studies were normalized to the same overall length (the mean of the two studies). The technical details of VH-IVUS as well as analysis recommendations have been well characterized elsewhere (García-García et al., 2009). VH-IVUS uses spectral analysis of IVUS radiofrequency data to classify plaque components into four categories: fibrous, fibrous-fatty, calcification, and necrotic core (Rodriguez-Granillo et al., 2005).

VH-IVUS analyses are reported in relative amounts (percentages of plaque) in this trial. The virtual histology-derived thin-cap fibroatheroma (VH-TCFA) was, as in previous studies, defined as a plaque with PAV > 40 % and consisting of more than 10 % of necrotic core in direct contact with the vessel lumen (García-García et al., 2009).

We utilized our own angiographic scoring system for the assessment of the atherosclerotic burden in coronary arteries. The score was calculated as a sum for all lesions with more than 20 % diameter stenosis found during CAG:

- 3 points: stenosis > 50 % in proximal third of artery
- 2 points: stenosis < 50 % in proximal third of artery, or stenosis > 50 % distal to proximal third of artery
- 1 point: stenosis < 50 % distal to proximal third of artery

Genetic analysis

ENOS polymorphism

Patient DNA was isolated from peripheral blood leukocytes using standard techniques. Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify exon 7 of the *ENOS* gene. Sample amplification was performed in an MJ Research DYAD 220 Peltier Thermal Cycler (Conquer Scientific, San Diego, CA). The following primers were used: forward primer ENOS7-sense, 5'-GAG ATG AAG GCA GGA GAC AGT-3' and reverse primer ENOS7-anti, 5'--TCC ATC CCA CCC AGT CAA T-3'. The mixture (final volume 25 μl) was incubated at 94 °C for 3 min, followed by 30 cycles (each 25 s at 94 °C, 40 s at 59 °C, and 40 s at 72 °C) at 72 °C for 10 min. Restriction analysis was performed by incubating three units of *Mbo*I restriction enzyme (Promega, Madison, WI) with the amplified DNA for 12 h overnight at 37 °C. The restriction products were separated by electrophoresis in a 3.8% agarose gel. The analysis of restriction products was performed after the addition of ethidium bromide.

The GG (Glu/Glu) variant was classified as being protective, whereas the GT (Glu/Asp) and TT (Asp/Asp) variants were classified as conferring risk. This dichotomization was chosen because of the low number of Asp homozygotes in our cohort. In addition, the presence of the T allele (including the Glu/Asp heterozygotes) has been associated with higher risk of coronary atherosclerosis (Gardemann et al., 2002).

HO1 polymorphism

After the DNA isolation (as described previously), the region of the *HO1* gene promoter containing a poly (GT)n repeat was amplified by PCR that included a fluorescently labelled sense primer (HMOX1_S 5-AGA-GCCTGCAGCTTCTCAGA-3) and an antisense primer (HMOX1_AS 5-ACAAAGTCTGGCCATAGGAC-3). All PCR products were generated in 25 μl volumes containing Plain Combi PP Master Mix (Top-Bio, Prague, Czech Republic), 1.6 pmol forward primer, 1.6 pmol reverse primer and 25 ng of template DNA. All amplifications were performed in a Dyad thermocycler (BIO-RAD, Hercules, CA) with the following protocol: a 5-min denaturation at 95 °C was followed by 30 cycles of 30 s at 95 °C, 30 s at 66 °C, 30 s at 72 °C and then a final extension at 72 °C for 5 min.

The PCR product sizes were determined using Li-cor 4200 (LI-COR Biosciences, Lincoln, NE) and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems,

Carlsbad, CA) DNA sequencers. We used IR700 labelled primers for Li-cor analysis and 6-FAM labelled primers for ABI analysis. The determination of fragment length was accomplished using SagaGT (LI-COR Biosciences) and Peak Scanner™ Software (Applied Biosystems). Selected samples were sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) automated DNA sequencer and then included as size markers in every electrophoresis run. We divided alleles according to the number of GT repeats in two subclasses based on higher induction of the *HO1* gene promoter by oxidative stress observed only in promoters with less than 25 (GT)n, class S (short) alleles, and lower induction in promoters with 25 or more (GT)n, class L (long) alleles, as described above (Kaneda et al., 2002; Morita, 2005). Homozygous class S and heterozygous class S carriers were grouped together and compared to homozygous class L carriers. This group classification was used because both the homozygous and heterozygous carriers of the class S allele demonstrate reduced inflammatory response and, thus, are classified as protective.

We analysed the influence of the *ENOS* and *HO1* promoter variants on the following parameters: 1) baseline angio score, 2) baseline PAV, 3) baseline plaque composition, 4) plaque composition changes during statin treatment and 5) changes of PAV during statin treatment. The influence of the risk *ENOS*/risk *HO1* genotype on these parameters was not tested due to the low number of patients with such a genotype.

Statistical analysis

Mean values ± standard deviations (or percentages) were calculated for all variables. The differences between the groups were compared using a χ^2 test. Statistical significance was calculated by Fischer's exact test for categorical variables and by Student's *t*-test for continuous variables. All data were analysed using JMP 3.2 statistical software (SAS Institute, Cary, NC). A P value of < 0.05 was considered statistically significant.

Results

Patient population

Between November 2005 and April 2009, 107 Caucasian patients were enrolled in the trial. Overall, 98 patients returned for follow-up imaging. The final IVUS and VH analysis was performed in 89 patients. Data from nine patients were unsuitable for VH-IVUS analysis primarily because of problems with the ECG signal. Of these 89 patients, 70 were enrolled in the genetic sub-study. From the genetic sub-study population, 32 patients were assigned to the "aggressive" treatment group and 38 were assigned to the "standard" treatment group. Baseline patient clinical characteristics are summarized in Table 1.

Table 1. Baseline patient characteristics in both treatment groups

	Aggressive	Standard	P
Male	27 (84.4 %)	24 (63.2 %)	ns
Age	63.5 ± 9.0	65.5 ± 11.5	ns
Arterial hypertension	26 (81.3 %)	33 (86.8 %)	ns
Diabetes mellitus	9 (28.1 %)	9 (23.7 %)	ns
Hyperlipoproteinaemia	22 (68.8 %)	28 (73.7 %)	ns
Active smokers	22 (68.8 %)	23 (60.5 %)	ns
History of MI	16 (50.0 %)	12 (31.6 %)	ns
Statin naive	13 (40.6 %)	13 (34.2 %)	ns
Betablockers	21 (65.6 %)	26 (68.4 %)	ns
ACE inhibitors	22 (68.8 %)	28 (73.7 %)	ns

Treatment assignment versus distribution of genetic variants

The distribution of protective and risk *ENOS* and *HO1* variants did not differ between the treatment groups. ENOS 298 Asp/Asp homozygotes and Glu/Asp heterozygotes formed 40.6 % of the patients from the aggressive group and 60.5 % of the patients in the standard group (P = 0.14). The risk-related polymorphism of the *HO1* gene promoter was found in 43.8 % of the patients from the aggressive group and in 36.8 % of the patients from standard group (P = 0.62).

Treatment assignment versus baseline plaque magnitude and composition

Baseline plaque magnitude expressed as PAV did not differ with respect to treatment assignment (PAV group A: 46.7 ± 6.2 vs. PAV group S: 46.4 ± 7.0, P = 0.81).

Patients randomized to group A had a higher proportion of calcified tissue in plaques at baseline than did patients in group S but did not differ significantly in the proportion of other plaque components at baseline (see Table 2).

Baseline plaque composition

The differences in baseline plaque composition according to *ENOS* and *HO1* variants are summarized in Table 3. We did not observe significant differences in baseline plaque composition with respect to both polymorphisms. A statistically non-significant trend for a higher proportion of necrotic core in plaques in the risk *HO1* variant carriers was noted. There was a higher prevalence of TCFA in *HO1* risk variant carriers, although this result was of borderline statistical significance (*HO1* risk: 70.0 % vs. protective: 46.2 %, P = 0.047).

Table 2. Baseline plaque composition vs. treatment assignment

Plaque tissue in %	Aggressive	Standard	P
Fibrous	54.4 ± 8.3	58.2 ± 9.2	0.08
Fibro-fatty	20.2 ± 9.7	22.3 ± 9.0	0.3
Dense calcium	9.9 ± 7.1	6.1 ± 4.6	0.009
Necrotic core	15.3 ± 8.9	11.6 ± 7.3	0.06

Table 3. Baseline plaque composition according to ENOS and HO1 polymorphisms

Plaque tissue in %	Protective *ENOS*	Risk *ENOS*	Protective *HO1*	Risk *HO1*
Fibrous	56.0 ± 9.0	57.4 ± 8.1	57.3 ± 8.3	55.8 ± 8.9
	P = 0.54		P = 0.51	
Fibro-fatty	20.8 ± 8.9	22.0 ± 10.2	22.9 ± 10.4	18.9 ± 7.4
	P = 0.65		P = 0.12	
Necrotic core	14.2 ± 8.3	13.1 ± 8.8	12.1 ± 7.9	16.3 ± 9.1
	P = 0.61		P = 0.07	
Dense calcium	8.7 ± 7.6	7.3 ± 5.2	7.4 ± 6.8	8.8 ± 5.5

ENOS genotype had no influence on the prevalence of TCFA in our population (*ENOS* risk: 50.0 % vs. protective: 65.6 %, P = 0.19).

Baseline PAV and angio score

The percent atheroma volume (PAV) was significantly higher in carriers of *HO1* promoter risk variant (*HO1* risk 49.3 % ± 6.6 vs. protective 45.0 % ± 5.5, P = 0.004) but did not differ with respect to the *ENOS* genotype (*ENOS* risk 46.7 % ± 6.8 vs. protective 46.7 % ± 5.9, P = 0.98). Similarly, the total coronary atherosclerotic burden, as expressed by the angio score, was significantly greater in *HO1* risk variant carriers (*HO1* risk 9.4 ± 4.0 vs. protective 6.9 ± 3.2, P = 0.005), but did not differ with respect to the *ENOS* genotype (*ENOS* risk 8.1 ± 3.9 vs. *ENOS* protective 7.8 ± 3.6, P = 0.69, Fig. 1).

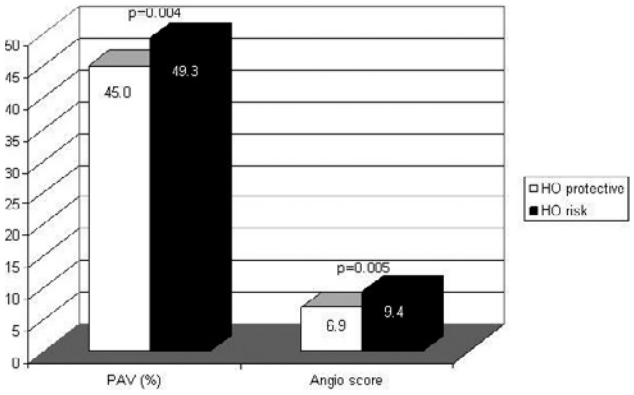

Fig. 1. Baseline percent atheroma volume and angio score according to the *HO1* polymorphism

Treatment assignment versus changes in PAV and plaque composition during follow-up

The type of treatment (aggressive vs. standard) did not significantly influence changes in PAV (PAV change aggressive group: -0.33 ± 2.70 vs. standard group: 1.28 ± 4.20, P = 0.06) or changes in plaque composition (data not shown).

Changes of plaque composition and PAV during follow-up

A significant interaction between the *ENOS* and *HO1* variants and changes in plaque composition was observed, the results are summarized in Table 4. The type of *HO1* and *ENOS* genotype did not significantly affect changes of PAV during follow-up in our patients (PAV change in % protective *ENOS*: 0.77 ± 4.10 vs. risk *ENOS*: 0.56 ± 3.10, P = 0.8; PAV change in % protective *HO1*: 0.81 ± 4.0 vs. risk *HO1*: 0.21 ± 4.0, P = 0.35).

Discussion

HO-1 and coronary atherosclerosis

The fundamental finding of our study is that patients with the *HO1* risk promoter polymorphism had greater coronary artery atherosclerotic burden (as expressed as a higher angio score) and more pronounced atherosclerotic plaques in their coronary arteries (as expressed as higher percent atheroma volume). Furthermore, we observed a trend toward a higher proportion of necrotic tissue in the plaques of the *HO1* risk variant carriers.

Table 4. Changes of plaque composition according to ENOS and HO1 polymorphisms

Plaque tissue change in %	Protective *ENOS*	Risk *ENOS*	Protective *HO1*	Risk *HO1*
Fibrous	-0.3 ± 6.3	-2.4 ± 7.6	-0.7 ± 8.2	-2.3 ± 4.8
	P = 0.22		P = 0.34	
Fibro-fatty	-1.0 ± 10.4	-5.1 ± 9.7	-5.7 ± 10.9	0.9 ± 7.4
	P = 0.09		P = 0.006	
Necrotic core	1.3 ± 7.0	3.9 ± 6.7	4.1 ± 7.4	0.4 ± 5.8
	P = 0.1		P = 0.03	
Dense calcium	0.5 ± 6.0	3.7 ± 5.3	2.8 ± 6.8	1.3 ± 3.9
	P = 0.02		P = 0.29	

Another significant finding was the higher prevalence of TCFA plaques in these patients. These results are important because higher proportions of necrotic tissue inside plaques and the presence of the TFCA type of plaque are related to the development of unstable plaques (Virmani et al., 2006).

Tabas et al. (2009) demonstrated that the formation and progression of the plaque necrotic core is caused by apoptosis of foam cells in conjunction with defective phagocytosis of the dead cells. This finding is in accordance with the observed reduction of plaque necrotic core size in LDL receptor-deficient mice being secondary to decreased macrophage activation and apoptosis induced by interleukin 10 *(IL10)* over-expression (Pinderski et al., 2002). Similarly, the structural integrity of the TCFAs is directly linked to the survival of vascular smooth muscle cells (VSMC) of the thin-fibrous cap, which prevents exposure of plaque content to the haemostatic system. HO-1, through CO and its further signalling pathways, inhibits apoptosis in VSMC, macrophages and foam cells, thus attenuating key processes that contribute to plaque destabilization (Tabas et al., 2009; Larsen et al., 2010). In animal studies, *HO1* induction was associated with a significant increase in cap thickness and a reduction in the necrotic core size and plaque lipid accumulation, contributing to plaque stabilization (Cheng et al., 2009). In contrast, *HO1* knockout mice exhibited severely reduced numbers of VSMC due to increased apoptosis (Yet et al., 2003). On the other hand, CO concurrently inhibits VSMC migration and proliferation (Morita et al., 1997; Rodriguez et al., 2010), and hence the essential processes of atherosclerosis development (Abraham and Kappas, 2008). Furthermore, CO exerts an anti-inflammatory effect on plaque macrophages and endothelia through multiple pathways including decreased TNF-α and increased IL-10 production (Yet et al., 2003).

Additional mechanisms of the anti-atherosclerotic effect of HO-1 exerted through bilirubin include the scavenging of reactive oxygen species (ROS), regulation of cellular redox state, modulation of secondary inflammatory processes through several pathways, including inhibition of NADPH oxidase and protein-kinase C (PKC) and prevention of oxidant-mediated cell death (Kushida et al., 2002; Abraham and Kappas, 2008; Larsen et al., 2010). Moreover, bilirubin inhibits LDL oxidation, a key process in atherogenesis, and thereby reverses the decreased ENOS activity caused by oxidized LDL (Kawamura et al., 2005; Larsen et al., 2010).

Through these pleiotropic effects, HO-1 seems to confer protection against the progression of the atherosclerotic process as well as destabilization of advanced plaques. The anti-atherosclerotic effect of HO-1 was demonstrated in a study by Juan et al. (2001). In the study, the group demonstrated that adenovirus-mediated *HO1* gene transfer inhibits atherosclerosis in apolipoprotein E (ApoE)-deficient mice, an atherosclerosis-prone model of mice. Importantly, HO-1 deficiency has been described in humans. The deficiency leads to an accelerated atherosclerosis characterized by the presence of fatty streaks and fibrous plaques in early childhood (Yachie et al., 1999).

According to our findings, *HO1* promoter polymorphisms play an important role in atherosclerosis development, progression and perhaps in plaque type formation as well. The blunted induction of gene transcription in *HO1* promoter risk variant carriers resulting in increased oxidative stress (Morita, 2005) presumably accounts for the increased susceptibility of these individuals to the development of more profound coronary atherosclerosis, such as what we have observed in our population. Our results are in accordance with previous studies (Kaneda et al., 2002). Moreover, other current findings as well as our results suggest that adequate HO-1 activity may help to prevent the transformation of a lesion to a high-risk plaque by impeding accretion of the necrotic core and by promoting smooth muscle cell survival in the fibrous cap (Yet et al., 2003; Cheng et al., 2009; Larsen et al., 2010).

The observed influence of the *HO1* promoter variants on changes in plaque composition is somewhat puzzling. We observed an unfavourable type of plaque transformation in patients with the protective *HO1* variant, characterized by a decrease of fibro-fatty tissue and an increase of necrotic core proportion during statin therapy. Paradoxically, the *HO1* risk variant was associated with an increase of fibro-fatty tissue and a significantly lower increase in the necrotic core proportion when compared with the protective variant.

The more favourable plaque composition changes observed in patients with the *HO1* risk promoter variants might possibly be caused by statin therapy. Statins are known to induce *HO1* expression (Chen et al., 2006). We speculate that attenuated induction of *HO1* expression could be one of the principal contributors to the chronic inflammatory process inside advanced atherosclerotic plaques in *HO1* risk variant carriers. This insufficient expression might be reversed by statin treatment, thus possibly leading to the favourable plaque composition changes observed in the present study. It is important to note that almost 40 % of our patients were statin naive prior to trial enrolment, and all statins utilized in our trial have been demonstrated to induce *HO1* expression (Chen et al., 2006). We further hypothesize that the atherosclerotic process in carriers of the protective *HO1* promoter variants might be driven primarily by other mechanisms not as susceptible to statin therapy. These other mechanisms might possibly explain the "unfavourable" plaque composition changes observed during statin treatment in these patients.

ENOS and coronary atherosclerosis

In our population, the *ENOS* 894 G/T polymorphism had no impact on the severity of coronary artery atherosclerotic involvement as quantified by the angio score, baseline plaque magnitude or plaque composition. Multiple studies, including the work by Colombo et al. (2003), have demonstrated a relationship between this

ENOS polymorphism and the presence and severity of CAD, whereas other researchers have not confirmed such a relationship (Jaramillo et al., 2010; Li et al., 2010). Previous studies, however, did not utilize IVUS and VH for the precise evaluation of the extent and character of the coronary artery atherosclerotic involvement.

In recent years, substantial evidence demonstrating that alterations in NO synthesis promote atherosclerosis in experimental animals has been presented. ApoE-deficient mice treated with L-NAME (L-NG-nitroarginine methyl ester), an inhibitor of nitric oxide synthases, experienced significant progression of aortic atherosclerosis (Kauser et al., 2000). This finding strongly suggests that decreased endogenous NO production plays an important role in the progression of atherosclerosis in mice. Likewise, long-term inhibition of ENOS by administration of L-NAME to rats resulted in induction of coronary inflammation and atherosclerosis (Tomita et al., 1998). The importance of ENOS in vasculoprotection was further demonstrated in a study by Kuhlencordt et al. (2001), who utilized an *ApoE*/*ENOS* double-knockout mice model.

Multiple *ENOS* gene polymorphisms with variable clinical impacts, including the *ENOS* 894 G/T polymorphism (Wang and Wang, 2000), have been identified in humans. The ENOS 298Asp/Asp, and possibly Glu/Asp variants, which seem to result in decreased NO synthesis (Veldman et al., 2002) have been correlated with an increased risk of coronary atherosclerosis in most human studies (Gardemann et al., 2002; Li et al., 2010). Although we did not find a relationship between the presence of the *ENOS* (298 Asp/Asp and Glu/Asp) risk variants and the magnitude of coronary atherosclerosis and baseline plaque composition, there still was an observed association between this *ENOS* polymorphism and atherosclerotic plaque composition changes during statin treatment. In patients with the *ENOS* protective variant (Glu/Glu), we found a significantly lower increase in plaque-calcified tissue as well as a trend toward smaller progression of the necrotic core during statin therapy when compared with carriers of the risk variants. To the best of our knowledge, our findings represent the first data on the possible influence of the *ENOS* 894 G/T polymorphism on plaque composition changes during statin treatment.

Statin treatment has been shown to reduce levels of oxidized LDL (Tavridou et al., 2010), resulting in enhanced ENOS activity (Kawamura et al., 2005). Furthermore, atorvastatin has been shown to stimulate the expression of *ENOS* in human endothelial cells (Dulak et al., 2005). We speculate that the effects of statins might be more pronounced in the wild-type (Glu/Glu) ENOS enzyme carriers and, therefore, the greater up-regulation of ENOS activity could contribute to the favourable changes in plaque composition observed in these patients. However, the extent to which these observations are affected by statin treatment remains to be elucidated by future investigations.

Changes in plaque burden

Likely due to the relatively short duration of follow-up and only modest percentage changes in plaque volumes, similar to those observed in other IVUS studies (Nissen et al., 2006), no significant correlation between *HO1* and *ENOS* variants and changes of PAV was observed in our patient population.

Study limitations

The main limitation of our study was the relatively limited population size. Nevertheless, given the complexity of the performed coronary artery involvement evaluation and the necessity of an invasive follow-up examination, the study sample size is fairly reasonable to allow at least preliminary conclusions on the relationship between the assessed gene variants and character of coronary atherosclerotic involvement.

In conclusion, the present study is the first to demonstrate the influence of the *HO1* promoter dinucleotide-length polymorphism on the extent of coronary artery involvement as assessed by CAG and IVUS. The protective *HO1* promoter variant seems to correlate with a lower coronary plaque burden and possibly a lower necrotic core proportion in coronary plaques but does not prevent a negative type of plaque composition shift in terms of a decrease of fibro-fatty and an increase of necrotic core proportion during statin therapy. A possible explanation for this observation may be the enhanced *HO1* gene expression induced by statins. The enhanced expression may correct the deficient expression in risk *HO1* promoter variant carriers, an effect not plausible in the protective variant carriers who demonstrate sufficient gene expression. Based on our results, we speculate that the protective *ENOS* 894 G/T polymorphism favourably influences changes of plaque composition during statin therapy, but apparently has no relationship to the severity of coronary artery atherosclerotic involvement and baseline plaque composition. To the best of our knowledge, no study to date has evaluated these relationships. Larger-scale studies are necessary to confirm these preliminary findings. Our findings contribute to the recognition of the genetic background of atherosclerosis development and the elucidation of possible causes of variable clinical responses to hypolipidaemic therapy.

References

Abraham, N. G., Kappas, A. (2008) Pharmacological and clinical aspects of heme oxygenase. *Pharmacol. Rev*. **60**, 79-27.

Chen, J. C., Huang, K. C., Lin, W. W. (2006) HMG-CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways. *Cell. Signal.* **18**, 32-39.

Cheng, C., Noordeloos, A. M., Jeney, V., Soares, M. P., Moll, F., Pasterkamp, G., Serruys, P. W, Duckers, H. J. (2009) Heme oxygenase 1 determines atherosclerotic lesion pro-

gression into a vulnerable plaque. *Circulation* **119**, 3017-3027.

Colombo, M. G., Paradossi, U., Andreassi, M. G., Botto, N., Manfredi, S., Masetti, S., Biagini, A., Clerico, A. (2003) Endothelial nitric oxide synthase gene polymorphisms and risk of coronary artery disease. *Clin. Chem.* **49**, 389-395.

Dulak, J., Loboda, A., Jazwa, A., Zagorska, A., Dörler, J., Alber, H., Dichtl, W., Weidinger, F., Frick, M., Jozkowicz, A. (2005) Atorvastatin affects several angiogenic mediators in human endothelial cells. *Endothelium* **12**, 233-241.

Fairchild, T. A., Fulton, D., Fontana, J. T., Gratton, J. P., McCabe, T. J., Sessa, W. C. (2001) Acidic hydrolysis as a mechanism for the cleavage of the Glu(298)→Asp variant of human endothelial nitric-oxide synthase. *J. Biol. Chem*. **276**, 26674-26679.

Förstermann, U. (2010) Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch.* **459**, 923-939.

García-García, H. M., Mintz, G. S., Lerman, A., Vince, D. G., Margolis, M. P., van Es, G. A., Morel, M. A., Nair, A., Virmani, R., Burke, A. P., Stone, G. W., Serruys, P. W. (2009) Tissue characterisation using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. *EuroIntervention* **5**, 177-189.

Gardemann, A., Lohre, J., Cayci, S., Katz, N., Tillmanns, H., Haberbosch, W. (2002) The T allele of the missense Glu(298)Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. *Atherosclerosis* **160**, 167-175.

Idriss, N. K., Blann, A. D., Lip, G. Y. (2008) Hemoxygenase-1 in cardiovascular disease. *J. Am. Coll. Cardiol*. **52**, 971-978.

Jaramillo, P. C., Lanas, C., Lanas, F., Salazar, L. A. (2010) Polymorphisms of the NOS3 gene in Southern Chilean subjects with coronary artery disease and controls. *Clin. Chim. Acta.* **411**, 258-262.

Juan, S. H., Lee, T. S., Tseng, K. W., Liou, J. Y., Shyue, S. K., Wu, K. K., Chau, L. Y. (2001) Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **104**, 1519-1525.

Kaneda, H., Ohno, M., Taguchi, J., Togo, M., Hashimoto, H., Ogasawara, K., Aizawa, T., Ishizaka, N., Nagai, R. (2002) Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. *Arterioscler. Thromb. Vasc. Biol.* **22**, 1680-1685.

Kauser, K., da Cunha, V., Fitch, R., Mallari, C., Rubanyi, G. M. (2000) Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice. *Am. J. Physiol. Heart Circ. Physiol*. **278**, H1679-1685.

Kawamura, K., Ishikawa, K., Wada, Y., Kimura, S., Matsumoto, H., Kohro, T., Itabe, H., Kodama, T., Maruyama, Y. (2005) Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. *Arterioscler. Thromb. Vasc. Biol*. **25**, 155-160.

Kuhlencordt, P. J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T. H., Hajjar, R., Picard, M. H., Huang, P. L. (2001) Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double knockout mice. *Circulation* **104**, 448-454.

Kushida, T., LiVolti, G., Goodman, A. I., Abraham, N. G. (2002) TNF-α-mediated cell death is attenuated by retrovirus delivery of human heme oxygenase-1 gene into human microvessel endothelial cells. *Transplant. Proc*. **34**, 2973-2978.

Larsen, K., Cheng, C., Duckers, H. J. (2010) Regulation of vulnerable plaque development by the heme oxygenase/carbon monoxide system. *Trends Cardiovasc. Med.* **20**, 58-65.

Li, J., Wu, X., Li, X., Feng, G., He, L., Shi, Y. (2010) The endothelial nitric oxide synthase gene is associated with coronary artery disease: a meta-analysis. *Cardiology* **116**, 271-278.

Mintz, G. S., Maehara, A. (2009) Serial intravascular ultrasound assessment of atherosclerosis progression and regression. State-of-the-art and limitations. *Circ. J*. **73**, 1557-1560.

Morita, T., Mitsialis, S. A., Hioke, H., Liu, Y., Kourembanas, S. (1997) Carbon monooxide controls the proliferation of hypoxic smooth muscle cells. *J. Biol. Chem*. **272**, 32804-32809.

Morita, T. (2005) Heme oxygenase and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1786-1795.

Naber, C. K., Baumgart, D., Altmann, C., Siffert, W., Erbel, R., Heusch, G. (2001) ENOS 894T allele and coronary blood flow at rest and during adenosine-induced hyperemia. *Am. J. Physiol. Heart Circ. Physiol.* **5**, H1908-1912.

Nissen, S. E., Nicholls, S. J., Sipahi, I., Libby, P., Raichlen, J. S., Ballantyne, C. M. (2006) Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: The ASTEROID trial. *J. Am. Med. Assoc*. **295**, 1556-1565.

Pfohl, M., Athanasiadis, A., Koch, M., Clemens, P., Benda, N., Häring, H. U., Karsch, K. R. (1998) Insertion/deletion polymorphism of the angiotensin I-converting enzyme gene is associated with coronary artery plaque calcification as assessed by intravascular ultrasound. *J. Am. Coll. Cardiol*. **31**, 987-991.

Pinderski, L. J., Fischbein, M. P., Subbanagounder, G., Fishbein, M. C., Kubo, N., Cheroutre, H., Curtiss, L. K., Berliner, J. A., Boisvert, W. A. (2002) Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient mice by altering lymphocyte and macrophage phenotypes. *Circ. Res*. **90**, 1064-1071.

Rodriguez, A. I., Gangopadhyay, A., Kelley, E. E., Pagano, P. J., Zuckerbraun, B. S., Bauer, P. M. (2010) HO-1 and CO decrease platelet derived growth factor-induced vascular smooth muscle cell migration via inhibition of Nox1. *Arterioscler. Thromb. Vasc. Biol*. **30**, 98-104.

Rodriguez-Granillo, G. A., Aoki, J., Ong, A. T., Valgimigli, M., Van Mieghem, C. A., Regar, E., McFadden, E., De Feyter, P., Serruys, P. W. (2005) Methodological considerations and approach to cross-technique comparisons

using *in vivo* coronary plaque characterization based on intravascular radiofrequency data anlaysis: insights from the integrated Biomarker and Imaging Study (IBIS). *Int. J. Cardiovasc. Intervent*. **7**, 52-58.

Tabas, I., Seimon, T., Timmins, J., Li, G., Lim, W. (2009) Macrophage apoptosis in advanced atherosclerosis. *Ann. N. Y. Acad. Sci*. **1173**, E40-45.

Tavridou, A., Efthimiadis, A., Efthimiadis, I., Manolopoulos, V. G. (2010) Simvastatin-induced changes in circulating oxidized low-density lipoprotein in different types of dyslipidemia. *Heart Vessels* **25**, 288-293.

Tesauro, M., Thompson, W. C., Rogliani, P., Qi, L., Chaudhary, P. P., Moss, J. (2000) Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. *Proc. Natl. Acad. Sci. USA* **6**, 2832-2835.

Tomita, H., Egashira, K., Kubo-Inoue, M., Usui, M., Koyanagi, M., Shimokawa, H., Takeya, M., Yoshimura, T., Takeshita, A. (1998) Inhibition of NO synthesis induces inflammatory changes and monocyte chemoattractant protein-1 expression in rat hearts and vessels. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1456-1464.

Tsutsui, M., Shimokawa, H., Otsuji, Y., Yanagihara, N. (2010) Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases. *Pharmacol. Ther.* **128**, 499-508.

Veldman, B.A., Spiering, W., Doevendans, P. A., Vervoort, G., Kroon, A. A., de Leeuw, P. W., Smits, P. (2002) The Glu298Asp polymorphism of the NOS 3 gene as a determinant of the baseline production of nitric oxide. *J. Hypertens.* **20**, 2023-2027.

Virmani, R., Burke, A. P., Farb, A., Kolodgie, F. D. (2006) Pathology of the vulnerable plaque. *J. Am. Coll. Cardiol*. **47**, C13-18.

Wang, X. L., Sim, A. S., Wang, M. X., Murrell, G. A., Trudinger, B., Wang, J. (2000) Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity. *FEBS Lett*. **1**, 45-50.

Wang, X. L., Wang, J. (2000) Endothelial nitric oxide synthase gene sequence variations and vascular disease. *Mol. Genet. Metab.* **70**, 241-251.

Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., Koizumi, S, (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J. Clin. Invest.* **103**, 129-135.

Yet, S. F., Layne, M. D., Liu, X., Chen, Y. H., Ith, B., Sibinga, N. E., Perrella, M. A. (2003) Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J*. **17**, 1759-1761.