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**Molekulární podklady endotelové dysfunkce: genetické varianty  
endotelové syntázy oxidu dusnatého a hemoxygenázy 1**

**Molecular basis of endothelial dysfunction: endothelial nitric  
oxide synthase and heme oxygenase 1 genetic variations**

**PhD thesis**

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Prague, 2015

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## Summary

Endothelial dysfunction is a pathologic state characterized by an altered equilibrium among vasodilatory and antithrombotic mediators and vasoconstrictive and prothrombotic mediators produced by the vascular endothelium. Multiple factors induce impaired production or increased consumption nitric oxide (NO), the key mediator of vascular homeostasis, produced by the nitric oxide synthase enzymes (NOS). Endothelial dysfunction represents one of the initial steps in the development of atherosclerosis, a chronic inflammatory disease of the vascular wall. The inducible enzyme heme oxygenase 1 (HO-1) represents one of the main cellular defense mechanisms against increased oxidative stress and decreased NO bioavailability accompanying endothelial dysfunction and atherosclerosis. We studied the genetic determinants of endothelial dysfunction and atherosclerosis by evaluating the association of the G894T endothelial NOS (eNOS) polymorphism and the HO-1 (GT)<sub>n</sub> promoter polymorphism with coronary artery atherosclerosis severity and risk profile and their evolution during hypolipidaemic treatment. In addition, we searched for genetic variations in exons 25 and 26 of eNOS gene, encoding the C-terminal part of the protein, deemed crucial for proper enzyme function and the 3' - untranslated region crucial for eNOS mRNA stability. We did not find an association of the eNOS G894T polymorphism with the extent and risk profile of coronary atherosclerosis, nevertheless we observed its association with atherosclerotic plaque composition changes during hypolipidaemic therapy. We described a novel variant (G3911A) in the noncoding mRNA of exon 26, a finding warranting further research of its possible impact on eNOS posttranscriptional regulation. The risk HO-1 promoter polymorphism was found to positively correlate with coronary artery atherosclerotic burden and high-risk coronary plaque features.

**Key words:** endothelial dysfunction, atherosclerosis, nitric oxide, endothelial nitric oxide synthase, heme oxygenase 1, polymorphism

## Souhrn

Endoteliální dysfunkce je patologický stav charakterizovaný narušenou rovnováhou mezi vazodilatačními a antitrombotickými mediátory a vazokonstriktivními a protrombotickými mediátory produkovanými cévním endotelem. Řada faktorů vede k narušení produkce nebo zvýšené konzumci oxidu dusnatého (NO), produkovaného syntázami oxidu dusnatého (NOS), který je klíčovým mediátorem cévní homeostázy. Endoteliální dysfunkce představuje jeden z počátečních kroků v rozvoji aterosklerózy, chronického zánětlivého onemocnění cévní stěny. Inducibilní enzym hem oxygenáza 1 (HO-1) představuje jeden z hlavních buněčných obranných mechanismů proti zvýšenému oxidativnímu stresu a snížené dostupnosti NO, které jsou průvodními jevy endoteliální dysfunkce a aterosklerózy. Studovali jsme genetické determinanty endoteliální dysfunkce a aterosklerózy pomocí hodnocení vlivu G894T polymorfizmu endotelové NOS (eNOS) a (GT)<sub>n</sub> polymorfizmu promotoru HO-1 na rozsah a rizikový profil koronární aterosklerózy a jejich vývoje během hypolipidemické léčby. Dále jsme hledali genetické varianty v exonech 25 a 26 genu eNOS kódujících C-terminální část proteinu, která je považovaná za klíčovou pro správnou funkci enzymu a 3' nepřepisovanou oblast klíčovou pro stabilitu eNOS mRNA. Nenalezli jsme asociaci eNOS G894T polymorfizmu s rozsahem a rizikovým profilem koronární aterosklerózy, nicméně jsme pozorovali jeho vliv na změny složení aterosklerotických plátů během hypolipidemické léčby. Objevili novou variantu (G3911A) v nekódující mRNA exonu 26, nález zasluhující další výzkum pro jeho možný vliv na posttranskripční regulaci eNOS. Rizikový polymorfismus HO-1 promotoru pozitivně koreloval s rozsahem koronární aterosklerózy a rizikovými charakteristikami koronárních plátů.

**Klíčová slova:** endoteliální dysfunkce, ateroskleróza, oxid dusnatý, endotelová syntáza oxidu dusnatého, hem oxygenáza 1, polymorfismus

## Abbreviations

HO-1 – heme oxygenase 1

eNOS – endothelial nitric oxide synthase

nNOS – neuronal nitric oxide synthase

iNOS – inducible nitric oxide synthase

CO – carbon monoxide

NO – nitric oxide

sGC – soluble guanylate cyclase

ADMA – asymmetric dimethylarginine

BH<sub>4</sub> – tetrahydrobiopterine

CaM – calmoduline

FAD – flavin adenin dinucleotide

FMN – flavin mononucleotide

cGMP – cyclic guanosine monophosphate

hsp90 – heat shock protein 90

ROS – reactive oxygen species

RNS – reactive nitrogen species

O<sub>2</sub><sup>-</sup> – superoxide

ONOO<sup>-</sup> – peroxynitrite

PCR – polymerase chain reaction

bp – base pair

SNP – single nucleotide polymorphism

Asp – aspartate

Glu – glutamate

CAG – coronary angiography

IVUS – intravascular ultrasound

VH – virtual histology

PAV – percent atheroma volume

VH-TCFA – virtual histology derived thin cap fibroatheroma

CAD – coronary artery disease

PAD – peripheral artery disease

FRS – Framingham risk score  
NAPDH - nicotinamide adenine dinucleotide phosphate  
NADPH oxidase – nicotinamide adenine dinucleotide phosphate oxidase  
mRNA – messenger RNA  
DNA – deoxyribonucleic acid  
MAPK – mitogen activated protein kinase  
Phosphatidylinositol 3-kinase – PI3K  
Nrf2 – nuclear factor-E2 related factor-2  
NFκB – nuclear factor kappa B  
VSMC – vascular smooth muscle cell  
LDL – low density lipoprotein  
ox-LDL – oxidized low density lipoprotein  
HDL – high density lipoprotein  
ApoA – apolipoprotein A  
ApoE – apolipoprotein E  
TGF – transforming growth factor  
VEGF – vascular endothelial growth factor  
TNFα – tumor necrosis factor alpha  
3'-UTR – 3' untranslated region  
ICAM-1 – intercellular adhesion molecule-1  
VCAM-1 – vascular cell adhesion molecule-1

# **1. INTRODUCTION**

## 1.1. Endothelial dysfunction and atherosclerosis

Endothelial dysfunction is a pathologic state characterized by an imbalance among vasodilatory and antithrombotic mediators and vasoconstrictive and prothrombotic mediators produced by the vascular endothelium. It is defined by decreased anticoagulation properties of the endothelium, increased expression of adhesive molecules and increased cytokine and reactive oxygen species production (ROS) by the endothelia. Multiple mechanisms including biochemical or physical injury coupled with genetic predisposition can lead to impaired production or dysregulation of pivotal mediators of vascular homeostasis, chiefly nitric oxide (NO), a gaseous molecule produced by the nitric oxide synthase (NOS) enzymes. Many other vasoactive substances, such as prostacyclin, bradykinin, thromboxane, endothelin, angiotensin-II, reactive oxygen species (ROS) and other free radicals, are produced by the endothelial cells and other cell types to control the function of the endothelia, vascular smooth muscle cells (VSMC) and of circulating blood cells (Furchgott et al., 1999; Ignarro et al., 1999). Altered production of vasoprotective agents, i.e., endothelial dysfunction, is associated with all major cardiovascular risk factors, such as hypertension, hypercholesterolemia, smoking and diabetes mellitus and importantly represents a mechanism leading to the development and progression of atherosclerosis (Schachinger et al., 2000; Halcox et al., 2002; Ignarro and Napoli, 2004; Bugiardini et al., 2004; Lerman and Zeiher, 2005; Li and Forstermann, 2013). In addition, endothelial dysfunction is predictive of future cardiovascular disease and cardiovascular events (Seals et al., 2014). Fundamentally, endothelial dysfunction is characterized by a loss of NO bioavailability, as will be discussed further (Pae et al., 2010). The heme oxygenase (HO) family of enzymes catalyzes the degradation of heme to biliverdin (which is subsequently converted to bilirubin by the enzyme biliverdin reductase), carbon monoxide (CO) and free iron ( $\text{Fe}^{2+}$ ) (Abraham and Kappas, 2008). The inducible form, HO-1 is designated as one of the main cellular defense mechanisms against increased oxidative stress and decreased NO bioavailability accompanying endothelial dysfunction and atherosclerosis (Abraham and Kappas, 2008; Pae et al., 2010).

Atherosclerosis is a chronic inflammatory disease of the vessel wall resulting from an excessive inflammatory fibroproliferative response to various forms of insult to the endothelium and VSMC characterized by accumulation of lipids and fibrous elements (Ross, 1993; Araujo et al., 2012). Atherosclerotic lesions primarily arise at vessel bifurcations, characterized by disturbed blood flow, probably owing to the loss of the atheroprotective effect of laminar shear stress (Gimbrone et al., 2000). Under these circumstances, the

endothelia increase the expression of adhesion and chemotactic molecules, acquire increased permeability for macromolecules including low-density lipoprotein (LDL), thus modifying the composition of the subendothelial extracellular matrix. The entry and subsequent retention of LDL particles in the vessel wall is considered to be a crucial initial mechanism of atherogenesis (Skålén et al., 2002). The trapped LDL particles are subject to initial mild oxidation (generating minimally modified LDL-mmLDL), which promotes their atherogenic effects via stimulation of pro-inflammatory cytokine production, e.g., interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF- $\alpha$ ) by macrophages (Miller et al., 2005; Choi et al., 2009; Maiolino et al., 2013). Activated macrophages further promote LDL oxidation through myeloperoxidase and ROS (Parhami et al., 1993). Completely oxidized LDL particles (oxLDL) are internalized by macrophages via scavenger receptors, thus inciting their transformation into foam cells (Henriksen et al., 1983), which represent the hallmark of the atherosclerotic plaque. Additionally, oxLDL elicit VSMC, macrophage and endothelial cell apoptosis with ensuing necrotic core development. Lastly, oxLDLs have been shown to up-regulate the expression and induce activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, a family of ROS generating enzymes (Rueckschloss et al., 2001). Indeed, NADPH oxidase and xanthine oxidase-mediated oxidative stress is involved in the development of endothelial dysfunction and vascular inflammation underlying atherosclerosis (Ignarro and Napoli, 2004; Yang and Ming, 2006; Drummond et al., 2011). VSMC represent the second cellular line crucial for the development of atherosclerotic lesions. VSMC, originating from the tunica media, upon stimulation by various stimuli, e.g., oxLDLs and platelet-derived growth factor (PDGF), migrate to the subendothelial space, where they proliferate. Furthermore, VSMC produce extracellular matrix molecules, and co-form the fibrous cap overlying the atherosclerotic plaque (Maiolino et al., 2013). In summary, endothelial dysfunction is involved in atherosclerotic lesion formation and progression by promoting early, as well as late mechanisms of atherosclerosis, including up-regulation of adhesive molecules, increased cytokine secretion, increased cellular permeability, enhanced LDL oxidation, platelet activation and VSMC proliferation and migration (Hadi et al., 2005; Abraham and Kappas, 2008).

## 1.2. Nitric oxide synthase isoforms and structure

The nitric oxide synthase family comprises of three enzyme isoforms, the neuronal (nNOS, NOS I), the inducible (iNOS, NOS II) and endothelial NOS (eNOS, NOS III). The nNOS gene located on chromosome 12 (12q24) consists of 29 exons and spans 240 kb. The nNOS is abundantly expressed in the central and peripheral nervous system, where the nNOS-derived NO serves as a neurotransmitter. Furthermore, the nNOS is expressed in the myocardium, skeletal muscle, smooth muscle and VSMCs (Forstermann and Sessa, 2012). Importantly, nNOS-derived NO plays a significant role the regulation of vascular tone in the periphery (Melikian et al., 2009). The eNOS gene consists of 26 exons, spans 21 kb and is located on chromosome 7 (7q35-36) encoding a 1203-aminoacid 133-kDa protein. The eNOS gene is predominantly expressed in the endothelium and cardiomyocytes. NO produced by the eNOS is one of the major determinants of vascular tone, inhibitor of vascular adhesion molecule production and VSMC proliferation. The eNOS and nNOS represent the constitutive enzyme isoforms, whereas the iNOS is an inducible enzyme. The iNOS gene located on chromosome 17 (17q11.2–q12) comprising of 26 exons has a genomic size of 37 kb. INOS is predominantly expressed in the immune system cells, however, under certain circumstances, i.e., elevated levels of pro-inflammatory mediators, can be expressed in virtually all tissues (Roman et al., 2002; Balligand et al., 2009). Once induced, iNOS produces sustained high levels of NO (Raman et al., 1999; Forstermann and Sessa, 2012).

All NOS isoforms share a similar structure, a N-terminal oxygenase domain connected via a calmodulin (CaM)-binding amino acid sequence to a reductase domain containing two flavins FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) and a NADPH amino acid binding sequence. The oxygenase domain represents the catalytic part of the enzyme. The NOS enzymes catalyze a reaction in which L-arginin is oxidized to NO and L-citrullin. NADPH, molecular oxygen and the cofactors  $Zn^{2+}$  and tetrahydrobiopterin ( $BH_4$ ) are essential for proper enzyme function. Functional NOS share a homodimer structure, in which NADPH bound to the reductase domain transfers electrons via the two flavins, FAD and FMN, across the dimer to the prosthetic heme group of the opposite oxygenase domain, converting ferric heme to ferrous that binds oxygen actively (Raman et al., 1999; Balligand et al., 2009). NO synthesis by the NOS enzymes takes places in two steps. Firstly, NOS hydroxylates L-arginine to  $N^{\omega}$ -hydroxy-L-arginine. In the second reaction phase, NOS oxidizes  $N^{\omega}$ -hydroxy-L-arginine to L-citrulline and NO (Raman et al., 1999; Forstermann and Sessa, 2012). The active dimeric form of eNOS is stabilized by heme, L-arginine and  $BH_4$ . In

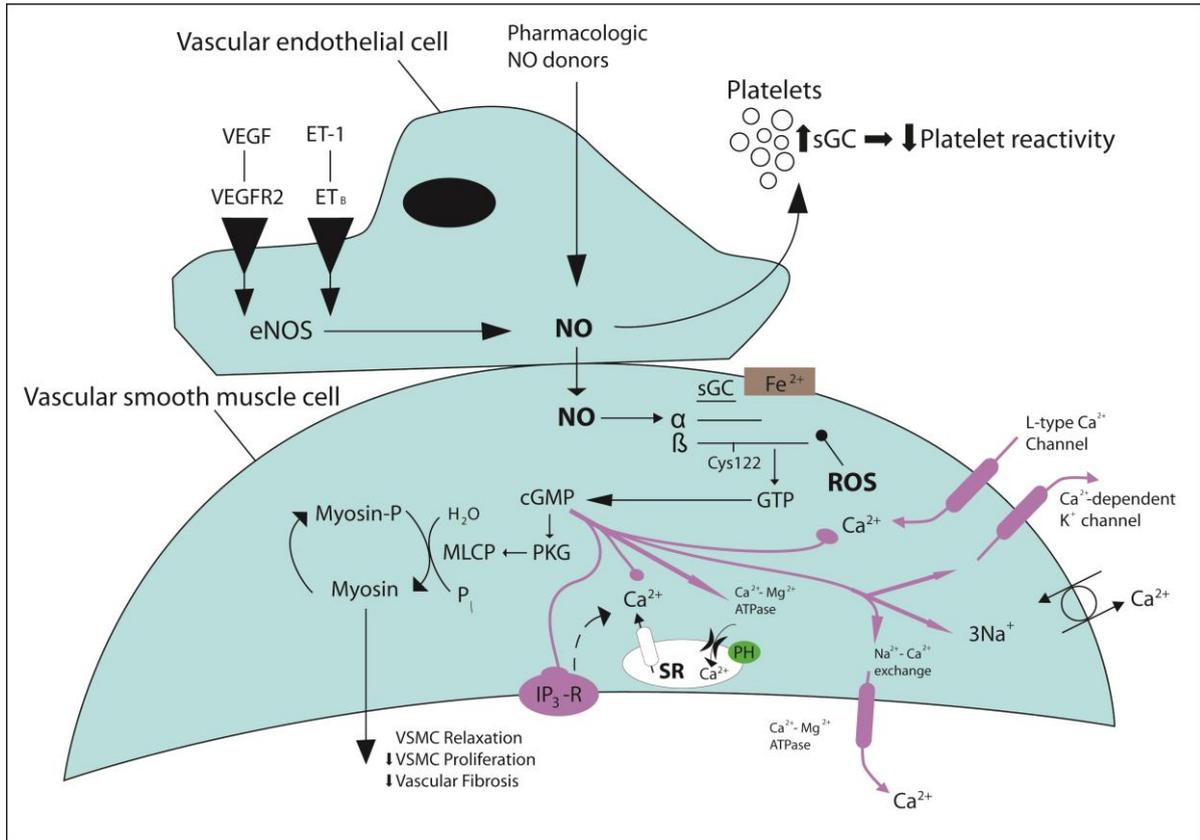
the constitutive NOS isoforms (cNOS), this transport is enabled by the binding of CaM to the CaM binding sequence after an increase in intracellular calcium ion concentration due to a variety of stimuli, whereas in iNOS, CaM is tightly bound to the enzyme and therefore does not serve as a regulator of enzyme function (Raman et al., 1999; Balligand et al., 2009). NOS isoforms differ in catalytic activity, regulation and cellular localization. The cNOS isoforms produce low concentrations of NO upon stimulation. In contrast, iNOS induced by pro-inflammatory mediators produces high concentrations of NO, thus serving an important role in host defense against pathogens and tumor cells, but also contributing to several pathologic states including septic shock, tissue damage in chronic inflammatory processes and acceleration of atherosclerosis in animal models (Kuhlencordt et al., 2001a; Ricciardolo et al., 2004; Lange et al., 2009). As mentioned above, BH<sub>4</sub> is an essential enzyme cofactor, and in its absence, the eNOS and nNOS isoforms transfer electrons to molecular oxygen, leading to production of the deleterious superoxide radical, as will be further discussed in extent (Vasquez-Vivar et al., 1998). The C-terminal parts of the reductase domain of NOS determine the rate of reduction equivalent transfer to the heme part of the protein (Roman et al., 2000 a, b; Jáchymová et al., 2005). These C-termini slow electron transfer between the two flavins, enabling tight electron coupling, thereby suppressing superoxide production (Raman et al., 1999). In endothelial dysfunction, electron coupling is altered, giving rise to an increased amount of superoxide radicals (Schulz et al., 2008; Yang et al., 2009). The eNOS has the longest C-terminus of the three isoforms-42 amino acids, compared to 21 amino acids in the iNOS and 33 amino acids in the nNOS, **figure 1**. The NO synthesis rate is inversely related to the length of the C-terminal part of each isoform, hence is highest in iNOS and lowest in eNOS (Raman et al., 1999). Due to its dominant role in the development of endothelial dysfunction and atherosclerosis, the function and regulation of the eNOS isoform will be discussed in detail.

Mouse iNOS	RYHEDIFG	AVFSYGAKKGSALEEPKATR	= 21 =
RAT nNOS	RYHEDIFG	VTLRTYEVTNRLRSESI AFIEESKKDADEVFSS	= 33 =
Bovine eNOS	RYHEDIFG	LTLRTOEVT SRIRTOSFSLOERHLRGAVPWAFDPPGPDTPGP	= 42 =

**Figure 1.** C-terminal parts of three mammalian NOS isoforms. (Adapted from Fig. 2 of Jáchymová et al., 2005)

### 1.3. Nitric oxide - mechanisms of action in the vasculature

Upon stimulation by various agonist stimuli, e.g., acetylcholine, shear stress, endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and bradykinin, the eNOS produces NO, which regulates multiple aspects of vascular function via activation of the primary NO receptor, the soluble guanylate cyclase (sGC). Furthermore, NO initiates nitrosation reactions with iron-sulphur-centered proteins or proteins with reactive thiols (S-nitrosylation) (Sessa, 2004; Maron and Michel, 2012). Upon activation, the sGC produces cGMP (cyclic guanosine monophosphate), which functions as a second messenger to stimulate protein kinase G (PKG)-dependent activation of myosin light chain phosphatase (MLCP), thereby promoting VSMC relaxation, leading to vasodilatation, and inhibition of thrombocyte reactivity. Alternatively, cGMP may modulate VSMC relaxation by decreasing intracellular levels of  $Ca^{2+}$  via inhibition of  $Ca^{2+}$  mobilization through L-type  $Ca^{2+}$  channels, through stimulation of the inositol triphosphate-3 receptor (IP3-R) or by decreasing intracellular  $Ca^{2+}$  levels directly via activation of ion exchangers. In thrombocytes, cGMP mediated decrease of  $Ca^{2+}$  influx inhibits activation of the glycoprotein IIbIIIa, which is essential for thrombocyte aggregation (Harmon et al., 2012; Maron and Michel 2012), **figure 2.**



**Figure 2. Pleiotropic effects of eNOS-derived NO in the vascular wall.**

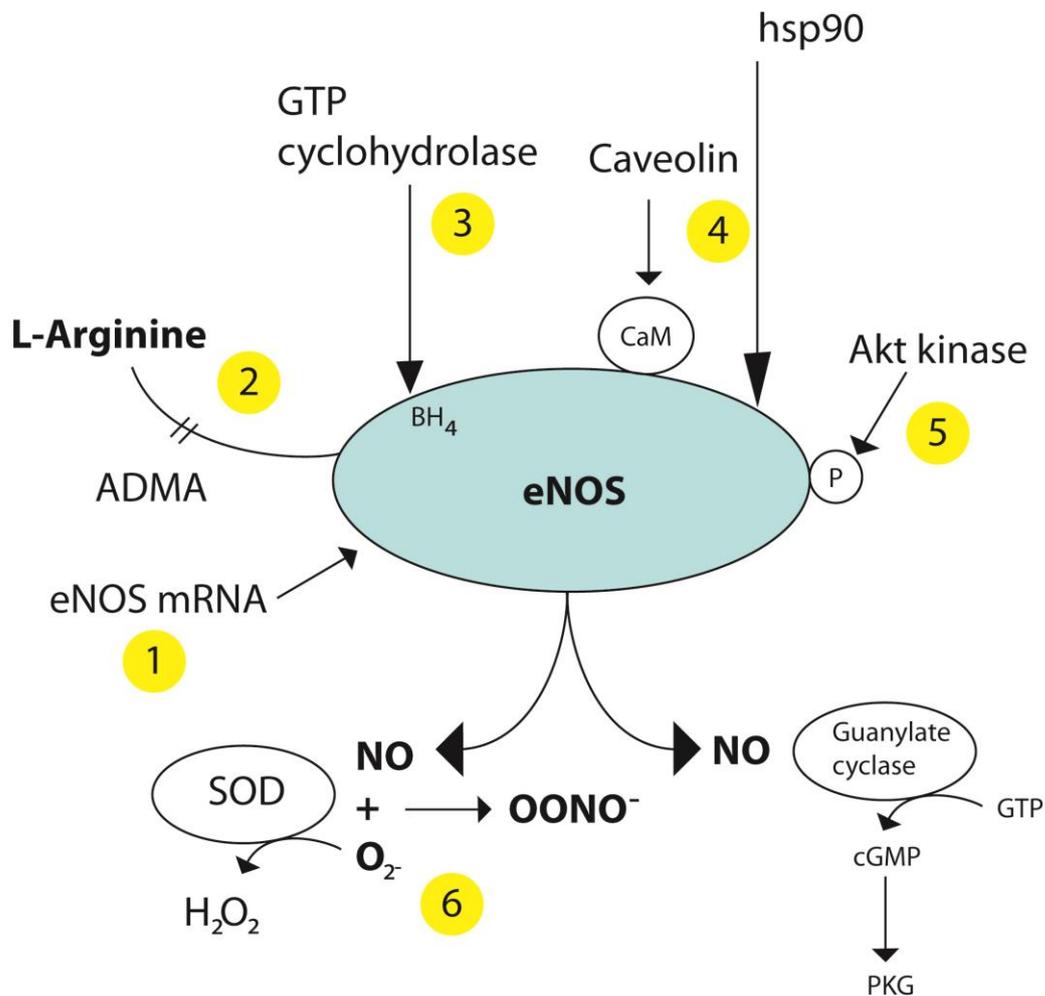
Activation of eNOS occurs in response to various stimuli, e.g., acetylcholine, shear stress or via receptor-mediated activation by ET-1, VEGF, or bradykinin. Increased levels of reactive oxygen species (ROS) adversely affect normal eNOS-NO signaling through multiple mechanisms including oxidation of functional cysteine residues of sGC. Arrows indicate agonist effects, circular heads indicate inhibitory effects.

cGMP-cyclic guanosine monophosphate; ET-1-endothelin 1; ET<sub>B</sub>-endothelin receptor type B; GTP- guanosine triphosphate; IP<sub>3</sub>-R- inositol triphosphate-3 receptor; MLCP-myosine light chain phosphatase; NO-nitric oxide; PKG-protein kinase G; ROS-reactive oxygen species; sGC-soluble guanylate cyclase; SR-sarcoplasmatic reticulum; VEGF-vascular endothelial growth factor; VEGFR2- vascular endothelial growth factor receptor 2, VSMC- vascular smooth muscle cell. (Adapted from Fig. 4 of Maron and Michel, 2012)

In addition, cGMP signaling is likely responsible for the inhibitory effect of NO on VSMC proliferation through multiple mechanisms (Forstermann and Sessa, 2012). S-nitrosylation contributes to the anti-proliferative actions and ion channel modulation exerted by NO in the vascular wall (Feil et al., 2003; Miranda et al., 2003). Furthermore, S-nitrosylation is responsible for the inhibition of apoptosis through inactivation of caspase-3 and caspase-8 (Sessa, 2004). Moreover, NO decreases endothelial expression of adhesion molecules, which are implicated in cellular migration, proliferation, atherogenesis, and thrombosis, such as ICAM-1, VCAM-1, P-selectin,  $\beta$ -1 integrin and monocyte chemoattractant protein 1 (MCP-1) (Kubes et al., 1991; Zeiher et al., 1995; Bombeli et al., 1998; Chamorro-Jorganes et al., 2011; Ponnuswamy et al., 2012). NO also inhibits leukocyte adhesion to the vessel wall by interfering with the binding ability of the leukocyte adhesion molecule CD11/CD18 to the endothelial cell surface or by suppressing CD11/CD18 expression in leukocytes (Arndt et al., 1993). Collectively, these effects serve to maintain normal vascular tone by inducing vessel relaxation, inhibiting vascular cellular proliferation and fibrosis and preventing immune cell infiltration of the vascular wall (Maron and Michel, 2012).

#### **1.4. eNOS regulation**

The bioactivity of eNOS derived NO is fundamentally determined by the balance between the synthesis and degradation rate of the molecule. NO bioavailability is regulated at the level of eNOS gene expression, the eNOS enzymatic activity and lastly at the level of NO degradation. Dysregulation of any of these mechanisms can cause endothelial dysfunction (Stocker and Keaney, 2004; Madamanchi et al., 2005), as will be discussed further, **figure 3**.



**Figure 3. Regulation of eNOS activity and possible mechanisms of endothelial dysfunction.**

1. changes in eNOS mRNA or protein levels; 2. decreased substrate availability; 3. decreased cofactor availability; 4. altered sub-cellular localization and protein-protein interactions; 5. altered phosphorylation; 6. decreased NO bioavailability due to increased ROS generation. ADMA-asymmetric dimethylarginine; BH<sub>4</sub>-tetrahydrobiopterine; CaM-calmoduline; cGMP-cyclic guanosine monophosphate; GTP-guanosine triphosphate; H<sub>2</sub>O<sub>2</sub> – hydrogen peroxide; hsp90-heat shock protein 90; NO–nitric oxide; O<sub>2</sub><sup>-</sup> superoxide; ONOO<sup>-</sup>peroxynitrite; PKG-protein kinase G; SOD-superoxide dismutase. (Adapted from Fig. 1 of Liu and Huang, 2008)

#### **1.4.1. Regulation of eNOS gene transcription**

Laminar shear stress has been identified as a key signal regulating eNOS transcription in isolated endothelia, endothelial progenitor cells (EPC) and intact arteries (Ranjan et al., 1995; Woodman et al., 2005; Tao et al., 2006; Balligand et al., 2009). In addition, this has been demonstrated in vivo, as exercise training (causing increased hemodynamic shear stress) increases eNOS gene expression, leading to improved NO-mediated endothelial functions (Woodman et al., 1997). Human eNOS transcription in response to shear stress is nuclear factor kappa B (NFκB)-dependent. In a healthy endothelium with adequate NO production, shear stress activates NFκB and promotes eNOS expression with a consecutive increase in NO production. Increased NO levels ultimately nitrosylate the p50 subunit of NFκB, leading to its inhibition, and thus terminate NFκB-dependent eNOS transcription. In a dysfunctional endothelium with decreased NO bioavailability, a deleterious, sustained activation of NFκB may result from the failure of NO to adequately inhibit NFκB, thereby promoting pro-inflammatory gene transcription (Grumbach et al., 2005). This interaction between shear stress, NFκB activation, NO production and subsequent NFκB inhibition represents a negative-feedback loop responsible for the short-term increases in eNOS transcription in response to shear (Grumbach et al., 2005). These findings emphasize the crucial role of NO in modulation of the endothelial cell inflammatory state. Under common pathological conditions including hypercholesterolemia, hypertension and diabetes, decreased NO availability may result in sustained activation of NFκB in response to shear stress, thereby promoting inflammatory processes in the endothelium (Grumbach et al., 2005; Balligand et al., 2009). Several other transcription factors such as KLF2 (Kruppel-like factor), Foxo-1 and Foxo-3a play a role in eNOS transcriptional activity in response to shear stress (SenBanerjee et al., 2004; Potente et al., 2005). Oscillatory shear stress (produced by turbulent blood flow) increases eNOS expression, but unlike laminar shear stress, via increased levels of ROS produced by NADPH oxidases (Cai et al., 2004). Importantly, production of ROS by the endothelia is quantitatively higher in response to oscillatory versus laminar shear stress (De Keulenaer et al., 1998). These observations explain the adverse effects of oscillatory shear on the phenotype of endothelial cells and possibly explain the pro-atherogenic features of endothelia exposed to disturbed turbulent flow (Balligand et al., 2009).

In addition to shear stress, transforming growth factor beta 1 (TGF-β1), a member of the TGF superfamily, whose members play key roles in the regulation of tissue growth and development, represents another significant mediator in eNOS transcription (Tai et al., 2004). TGF-β1 has been shown to increase eNOS human umbilical vein endothelial cell (HUVEC)

eNOS transcription in a concentration-dependent manner (Saura et al., 2002). Furthermore, atorvastatin treatment has been found to increase eNOS transcription via a TGF- $\beta$ 1 dependent pathway, leading to a reduction in atherosclerotic lesion size beyond its lipid lowering effects (Vecerova et al., 2012).

Two functionally relevant polymorphisms in the eNOS gene shown to influence eNOS transcriptional activity have been identified. The T(-786)C variant located in the gene promoter results in significantly reduced transcriptional activity, thus possibly leading to decreased eNOS-derived NO production (Nakayama et al., 1999). The T(-786)C variant has been linked to coronary artery spasm in the Japanese population and an increased risk of coronary artery disease (CAD) development in the Caucasian and Asian populations (Nakayama et al., 1999; Liu et al., 2013). Furthermore, eNOS transcriptional activity may also be regulated by microRNAs (miRs). A study has demonstrated that miRs based from 27-nt repeats located in intron 4 of the eNOS gene significantly suppress transcriptional activity, contrary to most so far identified miRs that act as inhibitors of translation or degrade mRNA (Zhang et al., 2005; Suarez et al., 2007). The levels of these 27-nt miRs vary according to the number of intronic repeats, which has been termed the 4 b/a polymorphism. Clinical genetic studies have yielded conflicting results in different populations with respect to this eNOS variant, some demonstrating an association with endothelial dysfunction (i.e., decreased NO production), an increased risk of arterial hypertension, coronary artery disease and ischemic stroke (Balligand et al., 2009; Li et al., 2010; Niu et al., 2011; Yao et al., 2013).

#### **1.4.2. Posttranscriptional regulation of eNOS**

Posttranscriptional regulation of eNOS mRNA stability is an important component of eNOS regulation and constitutes one of the mechanisms through which laminar shear stress affects eNOS activity (Weber et al., 2005). Under basal conditions, eNOS mRNA is very stable owing to multiple 3' untranslated region (3'-UTR) *cis*-mRNA elements that form stabilizing ribonucleoprotein (RNP) complexes (Ho et al., 2013). Upon cellular activation, the mRNA half-life can decrease dramatically, thus potentially lowering eNOS mRNA levels (Marsden et al., 1992). It has been shown that the 3'-UTR, encoded by exon 26, is critically important for eNOS mRNA steady state levels (Wang and Wang, 2000). A variety of mediators involved in atherogenesis, such as TNF- $\alpha$ , are known to decrease eNOS mRNA stability via modulation of RNP particle binding to 3'-UTR of eNOS (Tai et al., 2004). Translation elongation factor 1- $\alpha$ 1 (eEF1A1) and micro-RNAs (e.g., miR-155) are examples of the so far identified binding partners of the *cis*-acting sequences within eNOS mRNA

3'-UTR (Tai et al., 2004; Yan et al., 2008; Sun et al., 2012). The endothelial cell growth status also profoundly affects posttranscriptional eNOS regulation. A study has demonstrated a significant increase of eNOS mRNA levels in proliferating endothelial cells compared with confluent cells, not attributable to increased eNOS transcription (Searles et al., 1999). Another study by the same group showed that binding of monomeric actin (G-actin) and RNP targeted at a *cis*-element in the eNOS 3'-UTR was responsible for a reduction of eNOS mRNA stability in non-proliferating endothelial cells (Searles et al., 2004). As mentioned above, the role of miRs as regulators of eNOS gene expression has emerged. In one study, silencing of Dicer (a protein critical for microRNA maturation) led to an up-regulation of eNOS in endothelial cells (Suarez et al., 2007). This study identified two miRs (miR221 and miR222), that may be involved in the control of eNOS expression (Suarez et al., 2007). Significantly, miR-221/222 were not found to target the 3'-UTR of eNOS. A recent study has found that miR-222 exerts its inhibitory effects on eNOS expression through direct inhibition of the transcription factor ets-1 (Evangelista et al., 2013). In contrast, miR-155 inhibits eNOS gene expression predominantly through decreasing eNOS mRNA stability via binding to the 3'-UTR, thus serving as an important posttranscriptional modulator and a potential therapeutic target (Sun et al., 2012). Sparse data is available on the transcriptional regulation of miRs themselves, an area certainly warranting further research (Sun et al., 2012).

Nevertheless, changes in eNOS enzyme quantity only partially predict the overall enzyme function due to the abundant modulation of eNOS activity (Yang and Ming, 2006; Balligand et al., 2009; Fleming I, 2010). The chief posttranslational regulation mechanisms of eNOS will now be discussed.

### **1.4.3. Posttranslational regulation of eNOS**

The activity of eNOS is highly regulated by multiple mechanisms including eNOS lipidation, phosphorylation, O-glycosylation, S-nitrosylation, S-glutathionylation and direct protein-protein interactions. Furthermore, substrate and cofactor availability, as well as endogenous inhibitors of eNOS, all contribute to the “fine-tuning” of NO production.

#### **1) Regulation of eNOS by cellular trafficking**

Co-translationally, the eNOS protein undergoes irreversible myristoylation on a glycine (Gly2) residue and post-translationally a double reversible palmitoylation on specific cysteine (Cys15 and Cys26) residues. These modifications ensure the subcellular targeting of eNOS to the cytoplasmic aspect of the Golgi complex and to plasmalemma

caveolae (Belhassen et al., 1997; Feron et al., 1998; Sessa, 2004). The caveolae, i.e., small invaginations of the plasmalemmal membrane, enriched in cholesterol and sphingolipids, are determined by the membrane association and oligomerization of caveolins, a family of integral membrane proteins representing the principal components of caveolae. The caveolae serve as scaffolds for the assembly of multiprotein signaling complexes, including eNOS. The targeting of eNOS to caveolae is dynamically regulated, and upon specific stimuli, such as intracellular calcium increase, the enzyme can shuttle to intracellular compartments, i.e. the Golgi complex and cytoskeleton (Dedio et al., 2001). Shear stress and other agonists promote dissociation of eNOS from the inhibitory protein caveolin-1 (Cav-1), as will be discussed below, and promotes CaM binding to eNOS, thus facilitating its activation (Rizzo et al., 1998). A single-nucleotide polymorphism (SNP) in exon 7 of the eNOS gene, the G894T polymorphism, coding for a substitution of Glu by Asp at position 298 (Glu298Asp) in the oxygenase domain is associated with decreased NO production in endothelial cells under basal conditions and in response to shear stress (Joshi et al., 2007). Notably, the Glu298Asp variant is not located in the enzyme active site, but in the side chain region. This alteration seems to affect eNOS protein binding to Cav-1, thus resulting in its reduced association with the caveolar fraction. Importantly, purified eNOS enzymes containing the wild type and Asp298 sequence were found to have identical catalytic activity (Fairchild et al., 2001). Even though a full explanation of the mechanisms by which the Asp variant influences eNOS enzyme activity *in vivo* is still lacking (e.g., since the amino acid substitution is remote from the known caveolin binding or phosphorylation sites of eNOS and phosphorylation of the variant eNOS is unaffected), fairly robust data has been accumulated over the past years in favor of functional significance of this common variant (Balligand et al., 2009). Studies have demonstrated an association of the Asp allele with an enhanced systemic pressor response to phenylephrine (Philip et al., 1999), a lower basal flow with a preserved response to adenosine in coronary arteries (Naber et al., 2001), vasospastic angina pectoris (Yoshimura et al., 1998), essential hypertension resistant to conventional antihypertensive therapy (Jáchymová et al., 2001), and increased risk of carotid and coronary artery atherosclerosis (Lembo et al., 2001; Colombo et al., 2003; Balligand et al., 2009; Li et al. 2010).

## 2) Regulation of eNOS by protein-protein interactions

The eNOS enzymatic activity is profoundly affected by a multiprotein signaling complex that comprises eNOS and a conglomerate of adaptor and structural proteins, kinases, phosphatases and protein partners involved in cellular trafficking of eNOS (Yang and Ming,

2006; Balligand et al., 2009). One of the extensively studied protein interactions of eNOS in the endothelium is with Cav-1. Cav-1 interaction with eNOS results in steric inhibition of eNOS, which can be reversed by addition of CaM, supporting a reciprocal regulation of the enzyme by inhibitory Cav-1 versus activating calcium/CaM (Li et al., 1995; Feron et al., 1996). Cav-1 binding to the reductase domain of eNOS compromises its ability to bind CaM and to donate electrons to the heme subunit (Ghosh et al., 1998). Consistent with its inhibitory role, Cav-1 deficient mice demonstrate higher eNOS activity (Razani et al., 2001). However, it remains to be elucidated, whether the Cav-1/eNOS interaction plays an important role in atherosclerosis development in humans.

Another protein playing a crucial role in eNOS regulation is the heat shock protein 90 (Hsp90) (Balligand, 2002). Firstly, as a chaperone, Hsp90 is involved in the folding of NOS enzymes and determines the insertion of heme into the immature protein (Billecke et al., 2002). Additionally, Hsp90 acts in numerous signal transduction cascades. The binding of Hsp90 significantly increases eNOS activity. Hsp90 association with eNOS is enhanced upon stimulation with various stimuli, e.g., VEGF, histamine, shear stress, and estrogens (García-Cardena et al., 1998). The mechanism is mediated, in part, by the enhancement of CaM binding affinity to eNOS (Takahashi et al., 2003) and also by reversing the inhibitory action of Cav-1 on eNOS (Gratton et al., 2001). In addition, Hsp90 is essential for eNOS phosphorylation processes (Yang and Ming, 2006). Further eNOS protein partners include dynamin-2 and NOSIP. Dynamin-2 enhances eNOS enzymatic activity, whereas NOSIP relocates the enzyme to intracellular compartments, leading to a reduction of eNOS activity (Yang and Ming, 2006).

### 3) Regulation of eNOS by phosphorylation, glycosylation, nitrosylation and glutathionylation

Multiple signal transduction pathways converge to regulate eNOS via serin, threonin and tyrosin residue phosphorylation (Fleming, 2010). The responsible kinases include AMP-activated protein kinase (AMPK), protein kinase C (PKC), cAMP-dependent protein kinase (PKA), calmodulin-dependent protein kinase II (CaMKII), Proline-rich tyrosine kinase 2 (PYK2) and Akt kinase or protein kinase B (PKB). ENOS activation by shear stress, VEGF, estradiol and bradykinin occurs through Akt/PKB, PKA and CaMKII mediated phosphorylation of a specific serine residue (Ser1177). The proposed consequence of Ser1177 phosphorylation is the displacement of the C-terminal tail, thus freeing the FMN-binding domain and promoting electron transfer from the reductase to oxygenase domain (Balligand et al., 2009) Additional serine phosphorylation sites of eNOS were discovered (e.g., Ser114,

Ser615, Ser633), but their *in vivo* significance remains only partially elucidated (Fleming, 2010). Phosphorylation of a specific threonine residue (Thr 495) by AMPK and PKC poses an inhibitory effect on eNOS. This residue is constitutively phosphorylated in endothelial cells, representing a negative regulatory site yielding decreased enzyme activity through interference with the binding of CaM (Fleming et al., 2001). Tyrosine residue phosphorylation of eNOS has been demonstrated at positions 81 (Tyr81) and 657 (Tyr 657). Tyr81 phosphorylation occurs upon a variety of eNOS activating agonists (Fulton et al., 2008), but its functional impact remains to be determined, whereas Tyr657 phosphorylation, mediated by most likely by PYK2, inhibits eNOS activity (Fleming, 2010). The activity of eNOS in response to different agonists is determined by simultaneous alterations in Ser1177, Ser615, Thr495 and Tyr657 phosphorylation sites with resulting changes in enzyme behavior (Michell et al., 2001). It is plausible that such control mechanisms determine endothelial cell responsiveness to different agonists (Fleming, 2010). Additionally, dephosphorylation processes mediated by phosphatases acting selectively at certain phosphorylation sites (e.g., Ser1177 and Thr495) contribute to the regulation of eNOS activity (Greif et al., 2002).

There is growing evidence that *O*-linked glycosylation occurring under conditions of increased plasma glucose levels contributes to the regulation of eNOS. Hyperglycemia and glucosamine have been shown to inhibit eNOS activity through posttranslational glycosylation near the PKB/Akt phosphorylation site (Du et al., 2001). A further posttranslational modification of eNOS is *S*-nitrosylation. Membrane bound eNOS is constitutively *S*-nitrosylated and agonist stimulation promotes denitrosylation, paralleled with increased Ser-1177 phosphorylation, leading to enzyme activation (Erwin et al., 2006). Recently, *S*-glutathionylation has emerged as another posttranslational modification of eNOS (Chen et al., 2010), its role in eNOS uncoupling will be discussed further.

#### 4) Regulation of eNOS activity by modulation of substrate and cofactor availability

Availability of the enzyme substrate (L-arginine) and NO scavenging by free radicals determine the efficacy of NO production. Under physiological conditions, the intracellular L-arginine concentration by far exceeds the  $K_m$  for eNOS due to active L-arginine uptake via cationic amino acid transporters, including CAT-1 (Suschek et al., 2003), recycling of L-arginine from L-citrulline and recruitment of L-arginine from proteolysis (Hecker et al., 1990). However, under pathological conditions, L-arginine intracellular concentrations may decrease, hence eNOS might become substrate limited (Kamada et al., 2001). This may be due to the competition of eNOS for L-arginine as substrate with other enzymes, including

arginase and arginine decarboxylase, possibly causing relative L-arginine deficiency in the proximity of eNOS (Berkowitz et al., 2003; Hein et al., 2003, Förstermann and Sessa, 2012). The activity of arginase 1 was shown to be activated by *S*-nitrosylation. Increased nitrosylation of arginase 1 has been demonstrated in vessels of old rats coupled with up-regulation of the iNOS, as the source of nitrosylating species, providing a possible mechanism for the occurrence of endothelial dysfunction in aging vessels (Santhanam et al., 2007). As will be discussed further, under circumstances of L-arginine and/or BH<sub>4</sub> deficiency, eNOS transfers electrons to molecular oxygen, thereby producing superoxide (Balligand et al., 2009; Li and Förstermann, 2013).

The precise role of BH<sub>4</sub> in the formation of NO by eNOS still remains unclear, but it likely serves as an allosteric and/or redox cofactor (Landmesser et al., 2003). It is however clear that a normal endothelial NO production by eNOS is dependent on the optimal concentration of BH<sub>4</sub>, whereas a suboptimal concentration of BH<sub>4</sub> leads to eNOS uncoupling (Vasquez-Vivar et al., 2003). The crucial role of BH<sub>4</sub> availability for proper eNOS function was demonstrated in multiple animal and clinical studies. These studies have shown that administration of BH<sub>4</sub>, or GTP-cyclohydrolase I (CTPCH-1, the key enzyme for de novo BH<sub>4</sub> synthesis) overexpression in endothelial cells reverses endothelial dysfunction associated with diabetes, hypercholesterolemia and atherosclerosis (Tiefenbacher et al., 2000; Alp et al., 2004; Cai et al., 2005). Importantly, GTPCH-1 transcription increases in response to shear stress, parallel to increased eNOS transcription and activity, thereby optimizing the generation of NO by eNOS under circumstances of increased blood flow (Lam et al., 2006). Animal studies have demonstrated decreased BH<sub>4</sub> levels in atherosclerotic lesions (Vasquez-Vivar et al., 2002). In atherosclerosis, oxidation of BH<sub>4</sub> by superoxide and peroxynitrite to form dihydropterin (BH<sub>2</sub>) that is inactive for eNOS cofactor function, is paralleled with eNOS-dependent generation of superoxide and subsequent nitrosative stress secondary to peroxynitrite formation leading to a further decrease of BH<sub>4</sub> levels, thus forming a vicious cycle (Kuzkaya et al., 2003). Additionally, decreased synthesis or diminished BH<sub>4</sub> recycling have been suggested as mechanisms for the reduced BH<sub>4</sub> levels observed in atherosclerosis (Li and Förstermann, 2013).

##### 5) Endogenous eNOS inhibitors

eNOS activity can also be decreased by endogenous inhibitors such as asymmetric *N*G-methylated derivatives of L-arginine, e.g., asymmetric dimethylarginine (ADMA). ADMA is a naturally occurring amino acid originating from proteolysis of methylated

arginine residues. The methylation of arginine is catalyzed by the enzyme arginine methyltransferase (PRMT) type I (Vallance and Leiper, 2004). The intracellular ADMA concentration is controlled by dimethylarginine dimethylaminohydrolase (DDAH), which is the dominant degradation pathway of ADMA (Murray-Rust et al., 2001). ADMA levels have been shown to correlate with risk factors for atherosclerosis, the degree of endothelial dysfunction and severity of atherosclerosis (Vallance and Leiper, 2004; Boger and Ron, 2005; Yang and Ming, 2006). A high plasmatic concentration of ADMA has been associated with an elevated risk for acute coronary syndromes and predicts adverse cardiovascular events in patients undergoing percutaneous coronary intervention (PCI) (Valkonen et al., 2001; Lu et al., 2003). Transgenic overexpression of DDAH-1 was shown to increase NO production and reduce blood pressure in vivo (Dayoub et al., 2003). Conversely, deletion of DDAH was associated with endothelial dysfunction and arterial hypertension (Leiper et al., 2007). The precise mechanism responsible for the increased ADMA plasma concentrations observed in individuals with atherosclerosis, however, is not clear. In endothelial cells, type 1 PRMT is up-regulated by LDL leading to increased ADMA production (Boger et al., 2000). It has been shown that *S*-nitrosylation reduces DDAH activity, thereby leading to ADMA accumulation with subsequent eNOS inhibition (Leiper et al., 2002). Up-regulation of iNOS in atherosclerotic lesions has been demonstrated in multiple studies (Wilcox et al., 1997; Perotta et al., 2011). Such up-regulated iNOS leads to NO overproduction and increased peroxynitrite formation, thus causing DDAH inhibition. Consequently, ADMA levels increase, representing a possible mechanism of inhibition of constitutively expressed NOS during the course of atherosclerosis (Leiper et al., 2002). Furthermore, PRMT I and DDAH are redox-sensitive enzymes and oxidative stress has been shown to increase the activity of PRMT and reciprocally decrease the activity of DDAH, thereby leading to increased ADMA concentrations (Forstermann and Sessa, 2012). In addition to its inhibitory effects on eNOS activity, ADMA has also been shown to induce eNOS uncoupling (Antoniades et al., 2009)

#### 6) Regulation of NO bioavailability

The bioavailability of NO is determined not only by its production but also by its transport, storage and interaction with other molecules (Schulz et al., 2005). Reactions leading to NO inactivation mostly belong to the oxidative and nitrosative metabolism of NO (Kelm, 1999). In the presence of low superoxide levels, NO reacts with molecular oxygen to form nitrogen dioxide (NO<sub>2</sub>). NO<sub>2</sub> upon reaction with another NO molecule, forms the nitrosating species dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), which is subsequently hydrolyzed to nitrite. By contrast, in

the presence of high superoxide levels, NO reacts with  $O_2^-$  to form peroxynitrite ( $ONOO^-$ ), which can decompose to nitrate, or form  $NO_2$  and highly destructive hydroxyl radicals. However, low concentrations of peroxynitrite have been suggested to play a role in signal transduction processes (Mihm et al., 2003). Furthermore, low levels of peroxynitrite are readily detoxified by ROS scavengers (Brik et al., 2000). As mentioned previously, NO partly exerts its effects through S-nitrosylation of transcription factors, enzymes, membrane receptors and ion channels (Balligand et al., 2009). S-nitrosylation occurs by means of oxidative nitrosylation reactions with peroxynitrite, NO-thiol interactions and transnitrosation reactions (Bryan et al., 2004). The rate of S-nitrosylation is not enzymatically determined, hence the concentration of NO and the related species as well their subcellular localization are of profound significance. Accordingly, many S-nitrosylable proteins are topographically associated to eNOS, representing a key prerequisite for signaling specificity. On the other hand, denitrosylation is tightly regulated through the activity of S-nitrosoglutathione reductase, a specific denitrosating enzyme (Balligand et al., 2009).

The complex, multilevel regulation of eNOS activity and NO bioavailability highlights the necessity to take into account parameters such as cellular compartment, cell type, intensity of stimulus (e.g., oscillating versus laminar shear stress), availability of substrate, cofactors, allosteric modulators, chaperones and inhibitors of eNOS as well as modulators of NO bioavailability, to be able to predict the overall NO output.

### **1.5. eNOS uncoupling, endothelial dysfunction and atherosclerosis**

Abundant experimental data implicates that dysfunctional eNOS enzymatic activity and increased oxidative stress play fundamental roles in atherosclerotic endothelial dysfunction. All major cardiovascular risk factors lead to a reduction in NO bioavailability, resulting from decreased NO production and augmented NO inactivation by ROS (Li and Förstermann, 2013). Under certain conditions, eNOS can become proatherogenic, i.e., uncoupling of eNOS, characterized by a diversion of electron flow from the enzyme substrate L-arginine to molecular oxygen resulting in the production of the superoxide radical instead of NO (Yang and Ming, 2006; Li and Förstermann, 2013). Accordingly, removal of endothelial cells in animal models of atherosclerosis prevented NO formation, but also reduced superoxide production (Ohara et al., 1997). As outlined above, several mechanisms are implicated in eNOS uncoupling, including  $BH_4$  deficiency, depletion of L-arginine and ADMA accumulation. S-glutathionylation has recently been identified as an additional mechanism involved in eNOS uncoupling (Förstermann and Sessa, 2012). Conditions of

increased oxidative stress promote S-glutathionylation of eNOS on two highly conserved cysteine residues (Cys689, Cys908) in the reductase domain of eNOS. This modification causes reversible eNOS uncoupling, leading to impaired endothelium dependent vasodilation (Chen et al., 2010). Importantly, LDL particles have also been shown to induce eNOS uncoupling (Pritchard et al., 1995). Currently, eNOS uncoupling is attributed a key role in atherogenesis. Apart from reduced NO production, it also potentiates pre-existing oxidative stress. ENOS uncoupling is considered to be a dynamic process, with uncoupled and coupled eNOS molecules simultaneously occurring in one cell, as has been shown in the hypercholesterolemic ApoE knockout mice (Ponnuswamy P et al., 2012). In this study, both superoxide and NO production by eNOS were detectable in endothelial cells. However, the deleterious effects of superoxide produced by uncoupled eNOS did not exceed the protective ones of eNOS-derived NO in this mouse model of atherosclerosis (Ponnuswamy P et al., 2012).

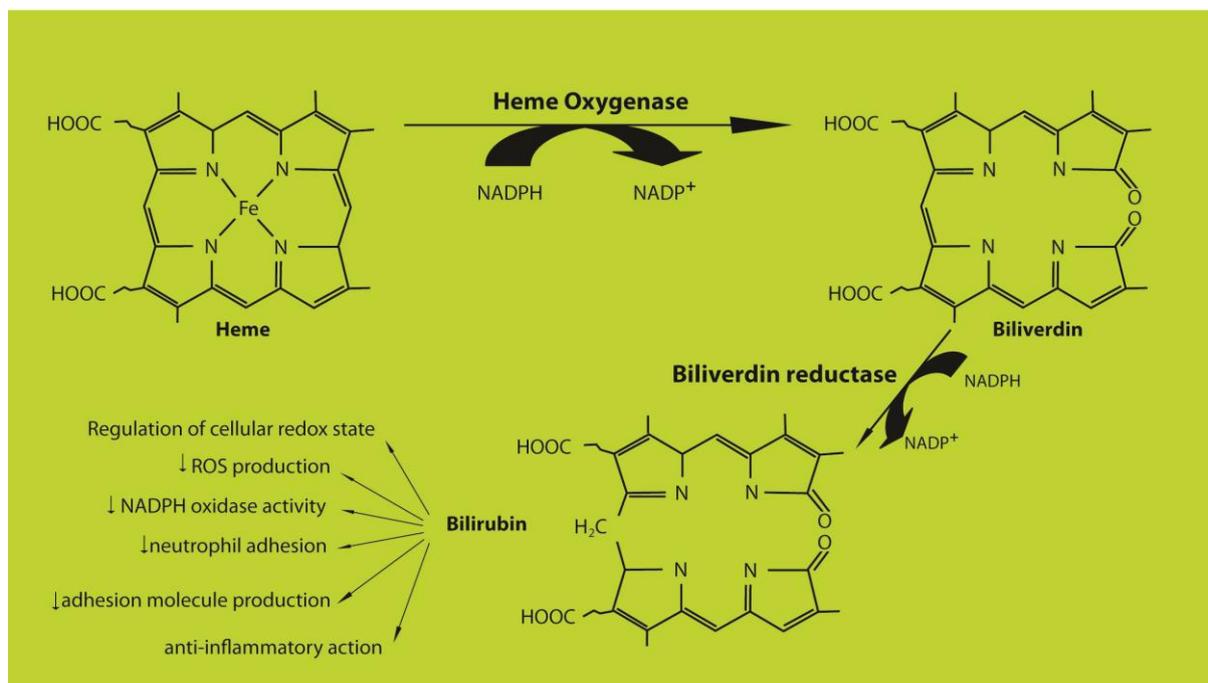
Multiple in vitro studies have demonstrated that a variety of atherogenic stimuli, such as ox-LDL, TNF $\alpha$  and thrombin suppress eNOS gene expression in cultured endothelial cells (Yang and Ming, 2006; Yan et al., 2008). However, most studies of eNOS gene expression in animal models of atherosclerosis demonstrate normal or even enhanced eNOS expression in atherosclerotic lesions (d'Uscio et al., 2001; Ming et al., 2004). Similarly, a study with human coronary atherectomy specimens demonstrated significantly higher eNOS expression in plaques of patients with acute coronary syndromes compared to plaques of stable angina patients (Rossi ML et al., 2005). However, other studies with human aortic and coronary artery specimens found a significant decrease in eNOS gene expression in endothelia overlying advanced atherosclerotic lesions, but not in those of early atherosclerotic samples (Wilcox et al., 1997; Fukuchi et al., 1999). This data suggests that endothelial dysfunction, at least at the early stages of atherosclerosis, is not attributable to a mere decrease in eNOS gene expression.

Numerous studies have shown that the eNOS gene knockout or eNOS inhibition promotes atherogenesis (Cayatte et al., 1994; Knowles et al., 2000) and eNOS gene transfer improves endothelial function and causes inhibition or regression of atherosclerotic lesions in animal models (Mujynya-Ludunge et al., 2005). Controversial results are reported in the ApoE $^{-/-}$  mice (an atherosclerosis-prone mouse model) that over-express the eNOS gene. One study found that these mice exhibited accelerated atherosclerotic lesion formation (Ozaki et al., 2002). However, other researchers have demonstrated a reduction of atherosclerotic lesions using the same experimental approach (van Haperen et al., 2002). Furthermore, it has

been demonstrated that eNOS, under certain circumstances, e.g., raised oxLDL levels, increased superoxide production, and this effect was significantly reduced by administration of L-NAME (Pritchard et al. 1995). These findings suggest a dual role of eNOS in atherosclerosis development. We can conclude that the reduction of NO bioavailability accompanying endothelial dysfunction and atherosclerosis is not the result of decreased eNOS protein levels, on the contrary, eNOS expression is generally enhanced in vascular disease. However, this compensatory mechanism is often futile, since the eNOS enzyme is subject to inhibition or becomes uncoupled (Forstermann and Sessa, 2012; Li and Forstermann, 2013). When summarized, current evidence suggests that atherosclerotic endothelial dysfunction is primarily caused by a dysregulation of eNOS enzymatic activity and NO inactivation through increased oxidative stress. ENOS uncoupling is perceived as an important mechanism contributing to endothelial dysfunction and increased oxidative stress in atherosclerosis. However, it remains a challenge to unravel the precise molecular mechanisms of eNOS deregulation in atherosclerosis.

### **1.6. Heme oxygenase**

The heme oxygenase (HO) protein family comprises of three isoforms, HO-1, 2, 3. These enzymes catalyze the first and rate-limiting step in the degradation of the protoporphyrin ring of heme, yielding equimolar quantities of biliverdin IXa, CO, and Fe<sup>2+</sup> (Tenhunen et al., 1968). Biliverdin is subsequently rapidly converted to bilirubin by biliverdin reductase, and Fe<sup>2+</sup>, a possible electron donor leading to ROS production, is sequestered into transferrin and then ferritin, thereby preventing its potential cytotoxic effects (Morita, 2005; Abraham and Kappas, 2008), **figure 4**.



**Figure 4. Oxidative metabolism of heme by HO-1 and biliverdin reductase and the versatile anti-inflammatory and antioxidative actions of bilirubin in biological systems**  
 CO-carbon monoxide, NADPH- reduced nicotinamide adenine dinucleotide phosphate, ROS-reactive oxygen species. (Adapted from Fig. 5 of Abraham and Kappas, 2008)

The HO-1, is a 32-kD inducible enzyme, that intriguingly utilizes heme as both a prosthetic group and a substrate (Ponka, 1999). The second isoform HO-2, a constitutively synthesized 36-kDa protein, is unresponsive to the inducers of HO-1. The third isoform, HO-3, also catalyzes heme degradation, but much less so when compared with HO-2. According to current findings, HO-3 is considered to be a pseudotranscript of HO-2 (Hayashi et al, 2004). HO-1 is induced by a variety of stimuli including heme, endotoxin, heavy metals, oxidants, hypoxia and hyperoxia, cytokines (IL-10, 13,18), growth factors (PDGF, VEGF), oxLDL and UV light (Ryter et al., 2006). Interestingly, NO is a potent inducer of HO-1 in many cells, including the endothelia (Marquis and Demble, 1998). A common feature of many of these inducers is the ability to generate ROS, suggesting a cytoprotective effect of HO-1 (Idriss et al. 2008). HO-1 is highly constitutively expressed in the spleen and other tissues of the reticuloendothelial system responsible for the degradation of aged erythrocytes (Abraham and Kappas, 2008). HO-1 is further highly expressed in the hematopoietic stem cells of the bone marrow, where it is anticipated to inhibit cellular differentiation by lowering the intracellular

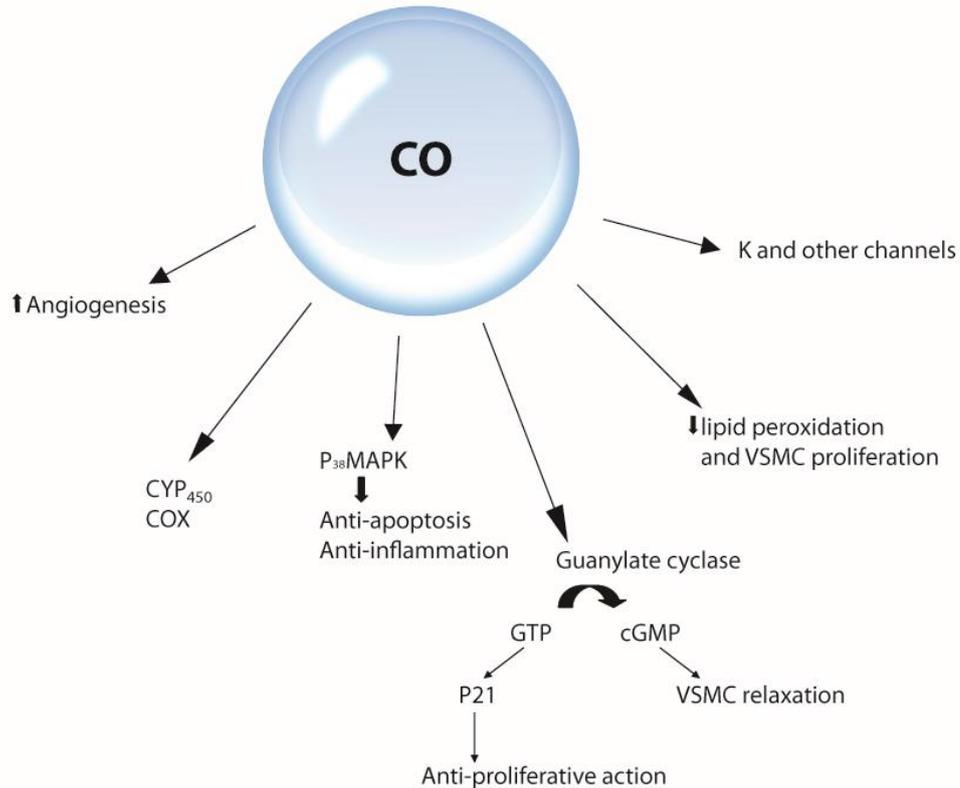
concentration of heme (Ryter et al., 2006). In addition, HO-1 expression is high in the liver parenchyma, owing to the uptake and degradation of plasma heme, hemoglobin, and methemalbumin. In other tissues, not directly involved in erythrocyte or hemoglobin metabolism, HO-1 typically occurs at undetectable or low levels under basal conditions but its expression is readily inducible by various chemical and physical stimuli, as mentioned above (Ryter et al., 2006). HO-2 is highly expressed in the testes, but the protein is abundantly expressed also in other tissues including the central nervous system, vasculature, liver, kidney, and gut (Ryter et al., 2006). Due to its dominant role in relation to vascular homeostasis and pathology, the molecular biology of HO-1 will further be discussed in detail.

The HO-1 was traditionally characterized as an endoplasmic reticulum (ER) associated protein. More recently, studies have indicated the targeting of HO-1 into other subcellular compartments including, but not limited to the nucleus, plasma membrane and mitochondria (Ryter et al., 2006). Kim et al. have described a functional association of HO-1 with the caveolae of the plasma membrane in endothelial cells (Kim et al., 2004). The possible functional subcellular compartmentalization of HO-1 enzymes uncovers an issue of organelle specific function of HO-1 metabolites, which is not yet fully characterized (Ryter et al., 2006). In the above mentioned study, HO-1 was detectable only in the ER under unstimulated conditions, whereas the exposure of endothelial cells to HO-1 inducers profoundly altered the subcellular distribution of HO-1. Moreover, variation in subcellular distribution was observed in an inducer-specific fashion. After stimulation, HO-1 was shown to co-localize with Cav-1. Interestingly, the down-regulation of Cav-1 resulted in a dramatic increase in HO-1 enzyme activity despite stable protein expression, indicating an inhibitory role of caveolin-1 on HO-1, similar to its inhibitory effect on eNOS (Feron et al., 1996; Kim et al., 2004). These observations suggest a possible functional role of HO-1 in caveolae and support the role of Cav-1 as an universal inhibitor of signaling mechanisms involving gaseous molecules produced in the caveolae (Kim et al., 2004). The regulation of HO-1 and its role in endothelial dysfunction and atherosclerosis development will be further discussed in extent.

### **1.6.1 Bilirubin and carbon monoxide - mechanisms of action in the vasculature**

Substantial evidence has been presented that biliverdin and bilirubin, natural antioxidants, represent a cellular defense strategy in response to oxidative stress, acting to prevent oxidant-mediated cell death (Kushida et al., 2002). High-normal serum levels of bilirubin are inversely related to atherogenesis (Hopkins et al., 1996). Atheroprotective actions of bilirubin include inhibition of LDL oxidation, scavenging of ROS and reactive

nitrogen species (RNS), attenuation of NADPH oxidase activity, resulting in decreased superoxide production and inhibition of PKC activity (Amit and Boneh, 1993; Neuzil and Stocker, 1994; Morita, 2005; Wang et al., 2007; Abraham and Kappas, 2008; Pae et al., 2010). In addition, bilirubin attenuates pro-inflammatory responses of the endothelium via inhibition of VCAM-1, MCP-1, and macrophage colony stimulating factor (M-CSF) (Kawamura et al., 2005). Furthermore, bilirubin provides protection against myocardial reperfusion injury through the suppression of lipid membrane oxidation (Clark et al., 2000), **figure 4**. CO, the second product of heme degradation, has a physiological role in the regulation of vascular tone similar to that of NO, in part acting through cGMP (Furchgott and Jothianandan, 1991). In addition, CO exerts cGMP independent actions on Ca<sup>2+</sup>-dependent potassium channels, leading to vasorelaxation through Ca<sup>2+</sup> desensitisation (Morita, 2005) and mediates a decrease in the cytochrome P450 (CYP<sub>450</sub>) dependent generation of vasoconstrictors (Wang, 1998b). Another cGMP independent mechanism of action is the activation of the mitogen-activated protein kinase (MAPK) pathway responsible for the protective effect of CO against lipopolysaccharide-induced pro-inflammatory cytokine production in macrophages and the antiproliferative effect on VSMC (Otterbein et al., 2000). Furthermore, CO has been shown to limit VSMC apoptosis (Liu et al., 2002). Finally, CO exerts a fibrinolytic effect through suppression of plasminogen activator inhibitor-1 (PAI-1) (Fujita et al., 2001), **figure 5**. Taken together, substantial data indicate that bilirubin and CO provide protection against the development of endothelial dysfunction and atherosclerosis.



**Figure 5. The role of CO in biological systems.**

cGMP-cyclic guanosine monophosphate; COX-cyclooxygenase; CYP 450- cytochrome P-450; GTP- guanosine triphosphate; NADPH- reduced nicotinamide dinucleotide phoshate; P21- cyclin-dependent kinase inhibitor 1 (CKI); p38MAPK-p38 mitogen activated protein kinase. (Adapted from Fig. 4 of Abraham and Kappas, 2008)

### 1.6.2. Regulation of HO-1 expression

HO-1 activity is principally regulated at the level of gene expression (Morita, 2005; Ryter et al., 2006; Stocker and Perrella, 2006). As discussed above, HO-1 expression is inducible in a rapid and temporary fashion by a broad range of chemical and physical stimuli.

Interestingly, only a few mediators that suppress HO-1 expression have been described (Pellacani et al., 1998). Following the discovery of the HO-1 transcriptional response, it has become evident that multiple modes of signal transduction are involved in the activation of HO-1 gene transcription, varying according to cell-type and inducer. The existing evidence indicates that most, if not all, HO-1 inducing stimuli act by modulating intracellular signal transduction pathways based on protein kinase cascades, which will be briefly reviewed (Ryter et al., 2006).

As mentioned previously, HO-1 stimulation is primarily controlled at the transcriptional level, which is governed by responsive elements localized in the promoter 5'-flanking region of the HO-1 gene (Morita, 2005). Compelling evidence shows that inducers of HO-1 activate different protein phosphorylation-dependent signaling cascades (e.g., MAPKs, tyrosine kinases, PI3K, protein kinases A, C, G) that ultimately converge to modulate an equally complex system of transcriptional regulators (Ryter et al., 2006; Stocker and Perella, 2006). Studies of the HO-1 promoter region have identified numerous response elements for transcription factors such as stress-responsive element (StRE), the antioxidant response element (ARE), as well as response elements for NF $\kappa$ B, activator proteins 1 and 2, and IL-6 (Alam and Den, 1992; Ryter et al., 2006). Likewise, multiple transcriptional factors including members of the Jun, Fos, CREB, ATF, Maf and the Cap'n'Collar basic leucine zipper protein (CNC-bZIP) families have been identified (Ryter et al., 2006). Among these, the nuclear factor-E2 related factor-2 (Nrf2), a member of the CNC-bZIP family, contains a potent transcription activation domain (Alam and Cook, 2003). Nrf2 activates a broad metabolic response to oxidative stress and Nrf-2 complexes have been associated with the HO-1 response to multiple agents (Itoh et al., 1999). Importantly, HO-1 is not inducible in Nrf2 null mice (Chan and Kan, 1999). OxLDL have also been shown to increase HO-1 expression via Nrf2 in macrophages (Ishii et al., 2004). However, other researchers have reported that Nrf2 deficient ApoE null mice experienced decreased atherosclerosis suggesting that Nrf2 expression may be pro-atherogenic despite its antioxidant actions (Araujo et al., 2012). The proposed mechanisms of Nrf2 pro-atherogenic actions are an increase in non-HDL cholesterol, promotion of foam cell formation due to an up-regulation of the macrophage CD36 scavenger receptor and an increased expression of IL-1 $\alpha$  in macrophages (Araujo et al., 2012). These contradictory effects of HO-1 and Nrf2 in atherogenesis underscore the complexity of pathways involved in the response to pro-oxidative and pro-atherogenic stimuli.

### **1.6.3. HO-1 and atherosclerosis**

During the evolution of an atherosclerotic lesion, migration and proliferation of VSMC accompanied by accrual of extracellular matrix determine the progression into an advanced lesion. Wang and colleagues have demonstrated that expression of HO-1 is prominent in endothelial cells, macrophages, and foam cells in human and animal atherosclerotic lesions (Wang et al., 1998). Other researches have showed that HO-1 inducers reduce lesion size in atherosclerosis prone animals (Ishikawa et al., 2001). A study utilizing

adenovirus-mediated gene transfer of HO-1 has demonstrated that selective over-expression of HO-1 decreases lesion size in ApoE<sup>-/-</sup> mice (Juan et al., 2001). Another study with ApoE and HO-1 (ApoE<sup>-/-</sup>-HO-1<sup>-/-</sup>) deficient mice subjected to a western diet for 8 weeks found that these mice developed larger and more advanced lesions than ApoE<sup>-/-</sup> mice exposed to the same dietary conditions (Yet et al., 2003). Taken together, these findings provide strong evidence for a beneficial effect of HO-1 in experimental atherosclerosis.

Robust animal data justify interest in HO-1 in human atherosclerosis. The obvious implication is that increased HO-1 activity poses an antiatherogenic effect. In fact, the classical risk factors of atherosclerosis including hypertension, advanced glycation end products, cigarette smoke, oxidized lipids and various systemic inflammatory processes lead to increased HO-1 expression (Stocker and Perrella, 2006). Many researchers have therefore suggested that up-regulation of HO-1 may represent a protective mechanism counteracting endothelial dysfunction and atherosclerosis. Importantly, HO-1 deficiency has been described in humans, leading to accelerated atherosclerosis characterized by the presence of fatty streaks and fibrous plaques in early childhood, suggesting that HO-1 plays a crucial role in vasculoprotection against oxidative insults (Yachie et al., 1999). The concept of a causal relationship of HO-1 to cardiovascular diseases has been suggested by studies assessing polymorphisms in the 5'-flanking sequence of the HO-1 gene (Exner et al., 2004). Several polymorphisms have been identified in the HO-1 gene promoter. Undoubtedly, the most studied one so far is the (GT)<sub>n</sub> dinucleotide-length polymorphism. The number of glutathione thymidine (GT) dinucleotide repeats in the promoter of the HO-1 gene modulates the level of gene transcription (Exner et al., 2001; Morita, 2005). The long alleles, dichotomized at >25 or >27 GT repeats according to different authors (Exner et al., 2001; Kaneda et al., 2002), lead to decreased HO-1 inducibility, whereas the short alleles demonstrate adequate HO-1 expression upon stimulation. Genetic association studies have reported relevance of this polymorphism to disease states caused by increased oxidative stress such as abdominal aortic aneurysm, risk of restenosis after percutaneous intervention or increased susceptibility to the development of atherosclerosis in diabetic patients (Exner et al., 2001; Chen et al., 2002; Schillinger et al., 2002). In addition, a SNP labeled T(-413)A in the HO-1 promoter, was found to be associated with the incidence of CAD in a Japanese population (Ono et al., 2004). The authors showed that the AA genotype leads to substantially higher basal promoter activity, independent of the length of (GT)<sub>n</sub> repeats and that patients with this genotype had lower incidence of CAD (Ono et al., 2004). However, very little is known about the

interaction of transcription factors with these polymorphic regions (Stocker and Perrella, 2006).

Numerous studies have evaluated HO-1 expression in patients suffering from atherosclerosis (Wang et al., 1998). A study assessing HO-1 expression in monocytes and lymphocytes in patients with acute myocardial infarction (AMI), unstable and stable angina pectoris demonstrated significant differences of HO-1 expression, which was highest in patients with AMI and lowest in patients with stable angina pectoris (Chen et al., 2005). Another study in patients with angiographically defined CAD, HO-1 expression positively correlated with the disease burden (Li et al., 2006). Morsi et al. showed that HO-1 expression was only detectable in cells from advanced atherosclerotic lesions, in contrast to early lesions and healthy coronary artery specimens (Morsi et al., 2006). A plausible interpretation of these data is that higher HO-1 expression is a consequence of the atherosclerotic process, representing a defense mechanism. In conclusion, ample evidence from animal, as well as human studies indicates the key role of HO-1 in the pathogenesis atherosclerosis and imply HO-1 as a potential novel therapeutic target.

## **2. STUDY AIMS**

**The aim of our work was to examine the genetic determinants of endothelial dysfunction and atherosclerosis by assessing variations in the eNOS a HO-1 genes.**

**2.1.** We sought to identify whether genetic variations in the C-terminal part of eNOS, deemed crucial for proper enzyme function, are present in patients with coronary and/or peripheral artery disease.

**2.2.** We sought to elucidate the influence of the eNOS Glu298Asp polymorphism and of the HO-1 promoter (GT)<sub>n</sub> dinucleotide-length polymorphism on the extent and characteristics of coronary artery disease assessed comprehensively by means of coronary angiography, intravascular ultrasound and intravascular ultrasound derived virtual histology. Furthermore, we aimed to identify the impact of these gene polymorphisms on the evolution of coronary artery disease during the course of statin-based hypolipidaemic treatment in patients stable angina pectoris.

**2.3.** We aimed to determine the utility of the HO-1 promoter (GT)<sub>n</sub> dinucleotide-length polymorphism and other noninvasive markers as predictors of coronary artery disease severity and coronary artery plaque risk profile as assessed by coronary angiography, intravascular ultrasound and intravascular ultrasound derived virtual histology in patients with stable angina pectoris.

### **3. HYPOTHESES**

**We tested the following hypotheses:**

**3.1.** Genetic variants in the C-terminal part of the eNOS gene, shown to be crucial for proper enzyme function through influence on reduction equivalent transfer by the reductase domain, represent a novel genetic factor contributing to endothelial dysfunction and atherosclerosis development.

**3.2.** Will the eNOS Glu298Asp and the HO-1 promoter (GT)<sub>n</sub> dinucleotide-length polymorphisms, associated with increased risk of endothelial dysfunction and atherosclerosis according to multiple studies in different ethnic groups, impact the severity and characteristics of coronary artery disease assessed by coronary angiography, intravascular ultrasound and intravascular ultrasound derived virtual histology? Furthermore, do these polymorphisms influence the progression of coronary atherosclerosis during the course of statin-based hypolipidaemic treatment?

**3.3.** Does the HO-1 promoter (GT)<sub>n</sub> dinucleotide-length polymorphism, as part of a comprehensive spectrum of noninvasive tests, improve the prediction of coronary artery disease severity and coronary artery plaque risk profile?

## **4. MATERIALS AND METHODS**

#### 4.1. Study population

**Ethical Aspects.** All studies were approved by the institutional ethics committees of participating hospitals and all patients gave informed consent for the study procedures.

##### 4.1.1. Study population assessed for the influence of the eNOS Glu298Asp polymorphism and the HO-1 promoter (GT)<sub>n</sub> polymorphism on the extent and evolution of coronary artery atherosclerosis during statin treatment

Between November 2005 and April 2009, 70 patients with chronic stable angina were enrolled in the study. Randomization was performed after CAG in a 1:1 ratio into:

- 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, or newly assigned standard dose statin.

Basic clinical characteristics are summarized in table 1.

**Table 1. Baseline patient characteristics in both treatment groups**

data given as n(%) or mean ± standard deviation (Reproduced from Fig. 1 of Král et al., 2011)

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

#### 4.1.2 Study population assessed for the utility of the HO-1 promoter (GT)n polymorphism in the prediction of coronary artery disease severity and coronary artery plaque risk profile

Between November 2005 and April 2009, a total of 107 patients with stable angina were included in the study. 6 patients were excluded from analysis due to unsuitable VH-IVUS data. Genetic analysis was performed in 81 patients (80.2%). Patient characteristics are summarized in Table 2.

**Table 2. Patient demographics, type of therapy, lipid levels, pro-inflammatory markers, HO-1 promoter polymorphism, carotid ultrasound parameters, CAG, IVUS, and VH-IVUS parameters** (reproduced from Table 1 of Kovárník et al., 2013)

Age (years)	64.6 ± 9.8	HO-1 risk polymorphism	35 (43.2%)
Female	27 (26.5%)	IL-6	23.6 ± 29.2
Family history of CAD	50 (49.5%)	VCAM	731.9 ± 265.6
Arterial hypertension	85 (84.2%)	ICAM	328.9 ± 136.2
Diabetes mellitus	25 (24.8%)	TNF alpha	10.1 ± 7.8
Hyperlipidemia	73 (72.3%)	CD 40 ligand	9.1 ± 4.9
Smoking	62 (61.4%)	hsCRP	48 ± 5.8
Previous myocardial infarction	42 (41.6%)	IMT <sub>mass</sub> (mm)	2.6 ± 0.4
Beta-blockers	69 (68.3%)	IMT <sub>max</sub> (mm)	0.7 ± 0.1
ACEI	68 (67.3%)	Plaque in the carotid bulb	41 (41.8%)
Statins	62 (61.4%)	Angio score	7.8 ± 4.0
Total cholesterol (mmol/l)	4.79 ± 1.2	Percent atheroma volume (%)	47.0 ± 6.3
LDLc (mmol/l)	2.9 ± 1.1	Fibrous tissue (%)	56.4 ± 8.7
Triacylglycerides (mmol/l)	1.55 ± 1.1	Fibro-fatty tissue (%)	20.9 ± 9.7
HDLc (mmol/l)	1.25 ± 0.5	Dense calcification (%)	7.9 ± 6.0
Apolipoprotein A (mmol/l)	1.23 ± 0.2	Necrotic core (%)	13.8 ± 8.5
Apolipoprotein B 100 (mmol/l)	0.94 ± 0.3	VH-TCFA (patients)	56 (55.4%)
Framingham risk score (LDLc)	8.5 ± 3.2		
<i>ACEI = angiotensin-converting enzyme inhibitors; LDLc = low-density lipoprotein cholesterol; HDLc = high-density lipoprotein cholesterol. Data given as n (%) or mean ± standard deviation.</i>			

#### 4.1.3 Study population assessed for eNOS C-terminal variants

Between September 2008 to September 2009, 150 patients suffering from angiographically proven CAD and/or PAD were enrolled in the study. Basic clinical characteristics are summarized in Table 3.

**Table 3. Patient basic clinical characteristics**data given as n(%) or mean  $\pm$  standard deviation

Number of patients	150
Male sex	108 (72%)
Age	63,3 $\pm$ 9,3
CAD	118 (78,7%)
PAD	64 (42,7%)
Arterial hypertension	120 (80%)
Hyperlipidemia	110 (73,3%)
Diabetes mellitus	57 (38%)
Active smokers	68 (45,3%)

## 4.2. Molecular genetic methods

### 4.2.1 Genomic DNA isolation

Patient DNA was isolated from peripheral blood leukocytes using standard techniques.

### 4.2.2 Molecular genetic analysis of the eNOS and HO-1 genes

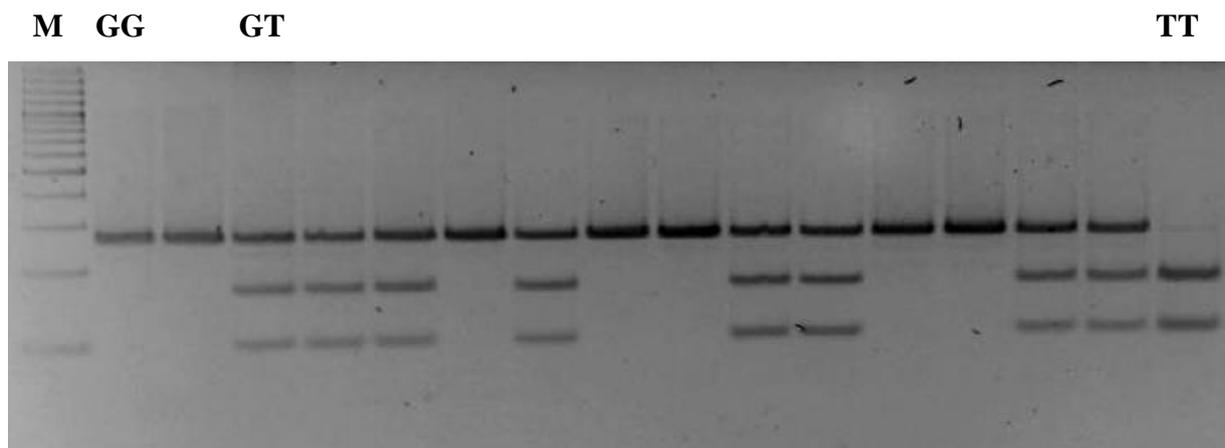
#### ENOS C-terminal variants

The polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify exon 25 and 26 of the eNOS gene. Amplification of all samples was performed on the MJ Research DYAD 220 Peltier Thermal Cycler (Conquer Scientific). The reaction was carried out in a final volume of 25  $\mu$ l containing 1  $\mu$ l of genomic DNA, 1  $\mu$ l of each primer (3,2 pmol) and 12,5  $\mu$ l of PP Master Mix. For exon 25 the following primers and PCR reaction parameters were used: forward primer ENOS25-sens, 5'-GGA GAC TTT CAC GTC CAG GG-3'; reverse primer ENOS25-anti, 5'-ACA GTG GAT CAG AAC CCG AC-3'; the mixture was incubated for 94°C 3 min, followed by twenty-nine cycles (each 30 s at 94°C, 30 s at 63°C, and 45 s at 72°C) and 72°C for 3 min. For exon 26, the following primers and PCR reaction parameters were used: forward primer ENOS26-sens, 5'-GGT TCC TGC TAA GGT CTC CG-3'; reverse primer ENOS26-anti, 5'-AAG AAA CAG GAA GCG GGT G-3'; the mixture was incubated for 94°C 3 min, followed by twenty-nine cycles (each 30 s at 94°C, 30 s at 59°C, and 45 s at 72°C) and 72°C for 3 min. The amplified exons 25 and 26 were separated by electrophoresis in 1.5% agarose gel. After staining with ethidium bromide, the amplified DNA was purified using the Qiagen Gel Extraction Kit (QIAGEN Ltd).

Subsequently, direct sequencing of exon 25 a 26 with forward and reverse primer in all samples was performed on the sequenator Prism ABI (Applied Biosystems). Sequence analysis of exons 25 a 26 with neighboring regions of introns 24, 25 and the 3'-UTR was performed with the Chromas Lite version 2.0 software (Technelysium Pty Ltd).

### **eNOS exon 7 Glu298Asp polymorphism**

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, **figure 6** (Gardemann et al., 2002; Colombo et al., 2003).



**Figure 6. Determination of the eNOS exon 7 polymorphism at codon 298**

Lane M-100-bp DNA ladder, GG lanes-homozygotes for Glu298 allele, GT lane-heterozygotes, TT lane-homozygote for Asp298 allele.

### **HO-1 promoter (GT)<sub>n</sub> polymorphism**

HO-1 gene promoter containing a poly (GT)<sub>n</sub> repeat was amplified by PCR with fluorescently labeled primers: sense primer (HMOX1\_S 5-AGAGCCTGCAGCTTCTCAGA-3) and antisense primer (HMOX1\_AS 5-ACAAAGTCTGGCCATAGG AC-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 (BIORAD, 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 labeled primers for Li-cor analysis and 6-FAM labeled 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 HO-1 gene by oxidative stress observed in promoters with less than 25 (GT)<sub>n</sub>, class S (short) alleles (protective HO-1), and lower induction in promoters with 25 or more (GT)<sub>n</sub>, class L (long) alleles (risk HO-1), as described previously (Exner et al., 2004; Morita, 2005). Homozygous class S and heterozygotes were grouped together and compared to homozygous class L carriers.

### **4.3. Invasive coronary artery assessment**

#### **Coronary angiography and IVUS**

Coronary angiography (CAG) was performed according to standard recommendations. Coronary artery atherosclerotic burden was assessed by means of our own angiographic scoring system (angio score). The score was calculated as a sum for all lesions with more than 20 % diameter stenosis found during CAG: 3 points for a stenosis > 50 % in the proximal third of an artery, 2 points for a stenosis < 50 % in the proximal third of an artery or a stenosis > 50 % distal to proximal third of artery and 1 point for a stenosis < 50 % distal to proximal third of an artery. After CAG and percutaneous coronary intervention (PCI), if 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  $\leq$  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-100) were used (Volcano Corp., Rancho Cordova, CA). After administration of 200  $\mu$ g of intracoronary nitroglycerin, the IVUS probe was introduced into the selected coronary artery beyond a distal fiducial point (well-defined side branch). Motorized pullback at 0.5 mm/s was performed through the rest of coronary artery. In the follow-up study, patients underwent repeated CAG and IVUS of the same coronary artery after 12 months.

### **IVUS and VH-IVUS analysis**

All measurements were performed in the catheterization laboratory of the General University Hospital of Charles University, Prague, Czech Republic and analyzed by a single operator. 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. 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 analyzed. Volumes were calculated using Simpson's rule and then normalized for pullback length. The primary IVUS endpoint for plaque magnitude assessment was the percent atheroma volume (PAV), which was calculated as  $(\Sigma(\text{EEMCSA} - \text{Lumen CSA}) / \Sigma \text{EEMCSA}) \times 100$ , where EEMCSA was the external elastic membrane cross-sectional area, and Lumen CSA was the luminal cross-sectional area. The change of PAV was computed as  $\text{PAV}_{\text{follow up}} - \text{PAV}_{\text{baseline}}$  for each patient. To avoid differences in pullback length, baseline and follow-up studies were normalized to the same overall length (the mean of the two studies). VH-IVUS uses spectral analysis of IVUS radiofrequency data to classify plaque components into four categories: fibrous, fibro-fatty, calcification, and necrotic core. VH-IVUS analyses are reported in relative amounts (percentages of plaque). The virtual histology-derived thin-cap fibroatheroma (VH-TCFA) was defined as a plaque with  $\text{PAV} > 40\%$  and consisting of more than 10 % of necrotic core in direct contact with the vessel lumen.

#### **4.4. Noninvasive predictors of coronary artery disease severity and risk profile**

##### **Framingham risk score (FRS)**

The FRS predicts the 10-year risk of coronary events according to a gender-specific model using age, diabetes mellitus (DM), total cholesterol or LDL (used in this study), systolic and diastolic blood pressure, and smoking (Mahmood et al., 2014). Risk score was calculated using  $\beta$ -coefficients to compute the linear function, as described previously (Wilson et al., 1998).

##### **Ultrasonographic examination of the carotid arteries**

Carotid intima-media thickness (IMT) measurement was performed using B-mode ultrasound with an 8 MHz linear probe on the outlying wall of the common carotid artery (ACC) and the internal carotid artery (ACI- on both sides) in longitudinal sections during end-diastole. For purposes of CAD prediction, we used IMTmass (sum of IMT in ACC and ACI for both sides) and IMTmax (maximum of all IMT measurements in every patient). Experienced ultrasonographers adjudicated carotid plaque presence in the carotid bulb if two of the following three criteria were met: (1) abnormal wall thickness (defined as IMT >1.5 mm); (2) abnormal shape (protrusion into the lumen, loss of alignment with adjacent arterial wall boundary); and (3) abnormal wall texture (brighter echoes than adjacent boundaries).

##### **Pro-inflammatory mediators and plasma lipids**

The following pro-inflammatory mediators were analyzed by means of the enzyme-linked immunosorbent assay (ELISA): VCAM-1, ICAM-1, TNF- $\alpha$ , CD 40 ligand, high-sensitivity C reactive protein (hsCRP), and IL-6. Furthermore, the complete plasma lipid examination was performed assessing total cholesterol (TC), LDL-C, HDL-C, triglycerides, Apo-A and Apo-B100.

#### **4.5. Statistical analysis**

Mean values  $\pm$  standard deviation or percentages were calculated for all variables. Differences between groups were compared using the  $\chi^2$  test. Statistical significance was calculated by Fischer's exact test for categorical variables and by Student's *t*-test for continuous variables. Data were analyzed using JMP 3.2 statistical software (SAS Institute, Cary, NC). A p-value of <0.05 was considered statistically significant. Multivariate statistical analysis was performed using IBM SPSS software version 17.0. Linear regression analysis was used for three dependent variables: angio score, PAV, and NC. Logistic regression was used for the dependent variable VH-TCFA. Optimal groups of predictors were chosen for achieving the best prediction of dependent variables.

## **5. RESULTS**

**The PhD thesis is based on two papers published in impacted journals and one submitted manuscript:**

**Král A**, Kovárník T, Králík L, Skalická H, Horák J, Mintz GS, Uhrová J, Sonka M, Wahle A, Downe R, Aschermann M, Martásek P, Linhart A. Genetic variants in haem oxygenase-1 and endothelial nitric oxide synthase influence the extent and evolution of coronary artery atherosclerosis. *Folia Biol (Praha)*. 2011;57(5):182-90. (*IF* = 1,151)

Kovarnik T, **Kral A**, Skalicka H, Mintz GS, Kralik L, Chval M, Horak J, Skalicka L, Sonka M, Wahle A, Downe RW, Uhrova J, Benakova H, Cernohousova L, Martasek P, Belohlavek J, Aschermann M, Linhart A. The prediction of coronary artery disease based on non-invasive examinations and heme oxygenase 1 polymorphism versus virtual histology. *J Invasive Cardiol*. 2013;25(1):32-7. (*IF* = 1,569)

**Kral A**, Linhart A, Martasek P. Genetic variants in the C-terminus of the endothelial nitric oxide synthase in patients with coronary artery and peripheral artery disease. *Article in submission*.

## 5.1. Paper 1

Král A, Kovárník T, Králík L, Skalická H, Horák J, Mintz GS, Uhrová J, Sonka M, Wahle A, Downe R, Aschermann M, Martásek P, Linhart A:

**Genetic variants in haem oxygenase-1 and endothelial nitric oxide synthase influence the extent and evolution of coronary artery atherosclerosis.**

Folia Biol 57,182-90, 2011 (*IF* = 1,151)

## 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)

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**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.

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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 ( $\text{Fe}^{2+}$ ), 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  $\leq$  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-100) 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 fiducial point (well-defined side branch). Motorized pullback at 0.5 mm/s was performed through the rest of coronary artery. The proximal fiducial 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 fiducial 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 (Rodríguez-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  $(\sum(EEM_{CSA} - Lumen_{CSA}) / \sum EEM_{CSA}) \times 100$ , 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 (Rodríguez-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 le-

sions 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 *MboI* 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).

##### *HOI polymorphism*

After the DNA isolation (as described previously), the region of the *HOI* gene promoter containing a poly (GT)<sub>n</sub> 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 (BIORAD, 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)<sub>n</sub>, class S (short) alleles, and lower induction in promoters with 25 or more (GT)<sub>n</sub>, 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  $\chi^2$  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
<b>Male</b>	27 (84.4 %)	24 (63.2 %)	ns
<b>Age</b>	63.5 ± 9.0	65.5 ± 11.5	ns
<b>Arterial hypertension</b>	26 (81.3 %)	33 (86.8 %)	ns
<b>Diabetes mellitus</b>	9 (28.1 %)	9 (23.7 %)	ns
<b>Hyperlipoproteinaemia</b>	22 (68.8 %)	28 (73.7 %)	ns
<b>Active smokers</b>	22 (68.8 %)	23 (60.5 %)	ns
<b>History of MI</b>	16 (50.0 %)	12 (31.6 %)	ns
<b>Statin naive</b>	13 (40.6 %)	13 (34.2 %)	ns
<b>Betablockers</b>	21 (65.6 %)	26 (68.4 %)	ns
<b>ACE inhibitors</b>	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
<b>Fibrous</b>	54.4 ± 8.3	58.2 ± 9.2	0.08
<b>Fibro-fatty</b>	20.2 ± 9.7	22.3 ± 9.0	0.3
<b>Dense calcium</b>	9.9 ± 7.1	6.1 ± 4.6	0.009
<b>Necrotic core</b>	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 <i>ENOS</i>	Risk <i>ENOS</i>	Protective <i>HO1</i>	Risk <i>HO1</i>
<b>Fibrous</b>	56.0 ± 9.0	57.4 ± 8.1	57.3 ± 8.3	55.8 ± 8.9
	P = 0.54		P = 0.51	
<b>Fibro-fatty</b>	20.8 ± 8.9	22.0 ± 10.2	22.9 ± 10.4	18.9 ± 7.4
	P = 0.65		P = 0.12	
<b>Necrotic core</b>	14.2 ± 8.3	13.1 ± 8.8	12.1 ± 7.9	16.3 ± 9.1
	P = 0.61		P = 0.07	
<b>Dense calcium</b>	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).

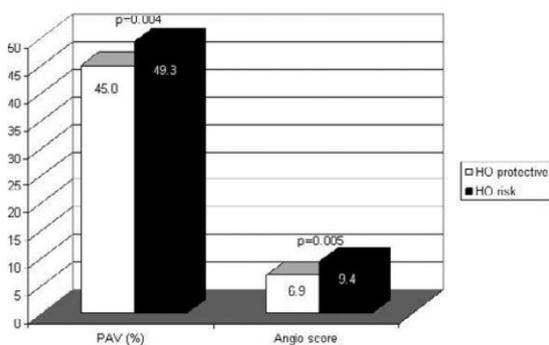


Fig. 1. Baseline percent atheroma volume and angio score according to the *HO1* polymorphism

#### 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:  $-0.33 \pm 2.70$  vs. standard group:  $1.28 \pm 4.20$ , 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*:  $0.77 \pm 4.10$  vs. risk *ENOS*:  $0.56 \pm 3.10$ , P = 0.8; PAV change in % protective *HO1*:  $0.81 \pm 4.0$  vs. risk *HO1*:  $0.21 \pm 4.0$ , P = 0.35).

## Discussion

### *HO1* 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 <i>ENOS</i>	Risk <i>ENOS</i>	Protective <i>HO1</i>	Risk <i>HO1</i>
<b>Fibrous</b>	-0.3 ± 6.3	-2.4 ± 7.6	-0.7 ± 8.2	-2.3 ± 4.8
	P = 0.22		P = 0.34	
<b>Fibro-fatty</b>	-1.0 ± 10.4	-5.1 ± 9.7	-5.7 ± 10.9	0.9 ± 7.4
	P = 0.09		P = 0.006	
<b>Necrotic core</b>	1.3 ± 7.0	3.9 ± 6.7	4.1 ± 7.4	0.4 ± 5.8
	P = 0.1		P = 0.03	
<b>Dense calcium</b>	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 TCFA 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 TCFA 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- $\alpha$  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 (Kausar 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-knock-out 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.

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## 5.2. Paper 2

Kovarnik T, Kral A, Skalicka H, Mintz GS, Kralik L, Chval M, Horak J, Skalicka L, Sonka M, Wahle A, Downe RW, Uhrova J, Benakova H, Cernohousova L, Martasek P, Belohlavek J, Aschermann M, Linhart A:

**The prediction of coronary artery disease based on non-invasive examinations and heme oxygenase 1 polymorphism versus virtual histology.**

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## The Prediction of Coronary Artery Disease Based on Non-Invasive Examinations and Heme Oxygenase 1 Polymorphism Versus Virtual Histology

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**ABSTRACT: Objective.** Prediction of coronary atherosclerosis in patients with stable angina based on non-invasive examinations. **Methods.** Pro-inflammatory markers, heme oxygenase-1 (HO-1) polymorphism, lipid levels, Framingham risk score (FRS), and carotid ultrasound were analyzed and compared to grayscale and virtual histology intravascular ultrasound (VH-IVUS). **Results.** A total of 101 patients were included, and genetic analysis was performed on 81 patients (80.2%). The HO-1 risk polymorphism was more frequent in patients post-myocardial infarction (61.3% vs 32%;  $P=0.097$ ), or with diabetes (68.4% vs 35.5%;  $P=0.011$ ) or a higher FRS (21.5 vs 15.7;  $P=0.014$ ). Plaques in patients with the HO-1 risk polymorphism contained less fibro-fatty tissue (17.1% vs 23.2%;  $P=0.005$ ) and more necrotic core (NC; 17.1% vs 12.7%;  $P=0.02$ ) and calcification (10.2% vs 5.7%;  $P=0.035$ ) compared to patients without the HO-1 risk polymorphism. Carotid intima media thickness ( $P=0.05$ ) and carotid bulb plaque ( $P=0.008$ ) predicted plaque burden. The level of Apo A inversely correlated with NC ( $P=0.047$ ;  $r = -0.27$ ) and was lower in patients with VH-thin-cap fibroatheroma (VH-TCFA; 1.19 mmol/L vs 1.3 mmol/L;  $P=0.04$ ). FRS correlated with NC ( $P=0.007$ ;  $r = 0.2$ ), with angiographic disease severity ( $P=0.032$ ;  $r = 0.21$ ) and was higher in patients with VH-TCFA (9.1 vs 7.8;  $P=0.03$ ). **Conclusion.** Carotid ultrasound and HO-1 polymorphism improve coronary atherosclerosis prediction.

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**Key words:** inflammation, plaque composition

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Acute coronary syndrome (ACS) is the first manifestation of coronary artery disease (CAD) in more than 50% of patients. Traditional risk stratification is limited. Many myocardial infarctions (MIs) occur in patients in the intermediate-risk group. Risk stratification can be improved by assessment of non-coronary atherosclerosis like carotid intima media thickness (IMT)<sup>1</sup> and markers of inflammation and oxidative stress.<sup>2</sup>

We recently published a study<sup>3</sup> of 107 patients, 70 of whom were genetically studied, that focused on the relationship between a genetic polymorphism for endothelial nitric oxide synthase and heme oxygenase-1 (HO-1) versus atherosclerosis development. The most interesting finding of this study was the correlation between the HO-1 polymorphisms and the extent of coronary atherosclerosis. The aim of the present study is to extend our initial results to examine how the analysis of genetic polymorphisms for HO-1 can improve prediction of the extent and risk profile of atherosclerosis based on traditional risk factors, pro-inflammatory markers, and carotid ultrasound.

HO is a microsomal enzyme that catalyzes heme degradation to iron, carbon monoxide (CO), and biliverdin, which is subsequently converted to bilirubin. CO and bilirubin are substances with vasodilatory, antioxidative, angiogenic, and anti-inflammatory properties. Iron is a potentially pro-oxidant agent, but is sequestered by ferritin.<sup>4</sup> The enzyme HO exists as isoenzymes HO-1 (inducible), HO-2 (constitutive), and HO-3 (probably only a pseudotranscript of HO-2).<sup>5,6</sup> The activity of the HO-1 gene is determined by the number of guanosine-thymidine (GT) dinucleotide repeats in the gene promoter. As the number of GT dinucleotide repeats increases, transcription of the gene, and thus its enzymatic activity, decreases.<sup>6</sup> Increased risk for CAD development was found in patients with an HO-1 gene polymorphism demonstrating a higher GT repetition.<sup>7</sup> Conversely, gene variants with a reduced number of GT repeats react to oxidative stress with increased transcriptional activity and thus act to protect against the development of atherosclerosis.<sup>8</sup>

The present study examined non-invasive predictors of the following coronary angiographic (CAG), intravascular ultrasound (IVUS), and virtual histology (VH-IVUS) parameters:

- (1) Angio score (angio) from CAG — a parameter of total atherosclerotic burden of coronary arteries.

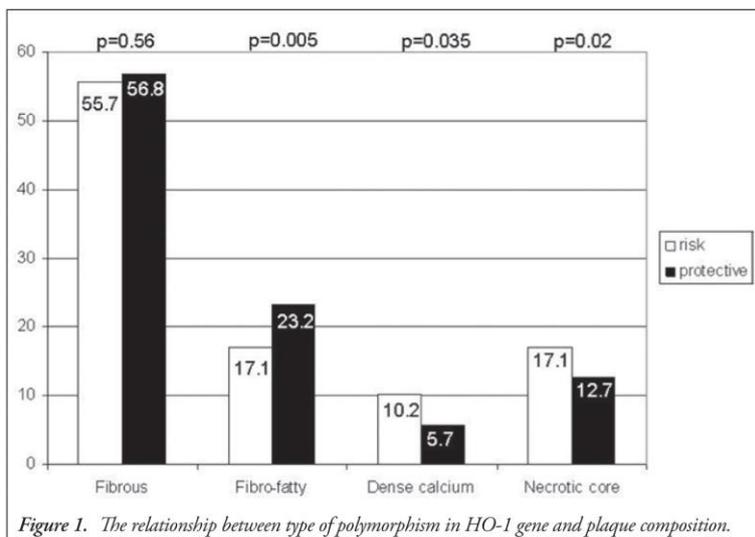


Figure 1. The relationship between type of polymorphism in HO-1 gene and plaque composition.

- (2) Percent atheroma volume (PAV) from IVUS — relative volume of selected non-culprit plaque.
- (3) Necrotic core (NC) from VH-IVUS — amount of necrotic core in selected non-culprit plaques.
- (4) Thin cap fibroatheroma (TCFA) from VH-IVUS — rupture-prone high-risk type of plaque<sup>9</sup> and a predictor of future cardiac events.<sup>10</sup>

**Methods**

**Study patients.** Because the objective of our study was to identify features of unstable plaques before the onset of ACS, only patients with stable angina pectoris (SAP) were included. All patients signed informed consent, and the study was approved by the local ethical committee.

**Framingham risk score (RS).** This risk score predicts a 10-year risk of coronary events according to a gender-specific model using age, diabetes mellitus (DM), total cholesterol or low density lipoprotein cholesterol (LDLc was used in this study), systolic and diastolic blood pressure, and smoking. Risk score was calculated using  $\beta$ -coefficients to compute the linear function as described by Wilson et al.<sup>11</sup>

**Ultrasonographic examination of the carotid arteries.** Carotid IMT measurement was performed using B-mode ultrasound with an 8 MHz linear probe on the outlying wall of the common carotid artery (ACC) and the internal carotid artery (ACI — on both sides) in longitudinal sections during end-diastole. For purposes of CAD prediction, we used  $IMT_{mass}$  (sum of IMT in ACC and ACI for both sides) and  $IMT_{max}$  (maximum of all IMT measurements in every patient). Experienced ultrasonographers adjudicated carotid plaque presence in the carotid bulb if two of the following three criteria were met: (1) abnormal wall thickness (defined as  $IMT > 1.5$  mm); (2) abnormal shape (protrusion into the lumen, loss of alignment with adjacent arterial wall boundary); and (3) abnormal wall texture (brighter echoes than adjacent boundaries).<sup>12</sup>

**Pro-inflammatory cytokines.** We analyzed the following pro-inflammatory markers: vascular cellular adhesive molecule (VCAM),

intercellular adhesive molecule (ICAM), tumor necrosis factor alpha (TNF alpha), CD 40 ligand, high-sensitivity C reactive protein (hsCRP), and interleukin 6 (IL-6). All markers were analyzed using the Enzyme Amplified Sensitivity Immunoassay (ELISA).

**HO-1 polymorphism.** Genomic DNA was isolated from peripheral blood leukocytes using standard procedures. The region of the HO-1 gene promoter containing a poly(GT)n repeat was amplified by polymerase chain reaction (PCR). We have divided alleles according to the number of GT repeats into two subclasses: promoters with less than 25 (GT)n — class S (short) alleles and promoters with 25 or more (GT)n — class L (long) alleles. Homozygous class S and heterozygous class S were grouped together (referred as protective type HO-1 polymorphism) and compared

to the homozygous class L carriers (referred as high-risk type of HO-1 polymorphism in the Results and Discussion sections). This group classification was used because both homozygous and heterozygous carriers of the class S allele show a reduced inflammatory response and thus are protective.<sup>8</sup> All patients signed informed content for genetic analysis.

**Catheterization and grayscale and VH-IVUS .** The following angiographic score was used to assess the atherosclerotic burden in the coronary arteries. We calculated the sum of the numeric indices (1, 2, or 3 points as stated below) associated with all lesions exhibiting more than 20% diameter stenosis:

- (1) 3 points: stenosis >50% in proximal third of a coronary artery.
- (2) 2 points: stenosis <50% in proximal third of a coronary artery or stenosis >50% in the mid or distal third of a coronary artery.
- (3) 1 point: stenosis <50% in the mid or distal third of a coronary artery.

After performing coronary angiography, the operator selected a target vessel for IVUS imaging. Only 1 native coronary artery with stenosis 20%-50% by angiography with no indication for either percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) was investigated in each patient. Plaque length >20 mm was suitable for study. Plaque length was defined as the length of a continuous arterial segment with plaque burden >20% based on IVUS assessment.

In case of similar findings in more than 1 coronary artery, the artery with the largest plaque burden was selected for the analysis.

The IVUS phased-array probe (Eagle Eye, 20 MHz, 2.9 Fr monorail), IVUS console, software, and motorized pullback device (research pullback, model R-100) were used for the studies (Volcano Corporation). After administration of 200  $\mu$ g of intracoronary nitroglycerin, the IVUS probe was introduced into the selected coronary artery at least 10 mm distal to the plaque. Motorized pullback at 0.5 mm/s was performed through the rest of the coronary artery all the way to the ostium. Plaque volume was expressed as a percent atheroma volume (PAV),

Table 1. Overall patient demographics, type of therapy, lipid levels, pro-inflammatory markers, type of gene polymorphisms, carotid ultrasound, CAG, IVUS, and VH-IVUS.

Age (years)	64.6 ± 9.8	HO-1 risk polymorphism	35 (43.2%)
Female	27 (26.5%)	IL-6	23.6 ± 29.2
Family history of CAD	50 (49.5%)	VCAM	731.9 ± 265.6
Arterial hypertension	85 (84.2%)	ICAM	328.9 ± 136.2
Diabetes mellitus	25 (24.8%)	TNF alpha	10.1 ± 7.8
Hyperlipidemia	73 (72.3%)	CD 40 ligand	9.1 ± 4.9
Smoking	62 (61.4%)	hsCRP	48 ± 5.8
Previous myocardial infarction	42 (41.6%)	IMT <sub>mass</sub> (mm)	2.6 ± 0.4
Beta-blockers	69 (68.3%)	IMT <sub>max</sub> (mm)	0.7 ± 0.1
ACEI	68 (67.3%)	Plaque in the carotid bulb	41 (41.8%)
Statins	62 (61.4%)	Angio score	7.8 ± 4.0
Total cholesterol (mmol/l)	4.79 ± 1.2	Percent atheroma volume (%)	47.0 ± 6.3
LDLc (mmol/l)	2.9 ± 1.1	Fibrous tissue (%)	56.4 ± 8.7
Triacylglycerides (mmol/l)	1.55 ± 1.1	Fibro-fatty tissue (%)	20.9 ± 9.7
HDLc (mmol/l)	1.25 ± 0.5	Dense calcification (%)	7.9 ± 6.0
Apolipoprotein A (mmol/l)	1.23 ± 0.2	Necrotic core (%)	13.8 ± 8.5
Apolipoprotein B 100 (mmol/l)	0.94 ± 0.3	VH-TCFA (patients)	56 (55.4%)
Framingham risk score (LDLc)	8.5 ± 3.2		

ACEI = angiotensin-converting enzyme inhibitors; LDLc = low-density lipoprotein cholesterol; HDLc = high-density lipoprotein cholesterol. Data given as n (%) or mean ± standard deviation.

which was calculated as  $\Sigma (EEM_{CSA} - \text{Lumen CSA})$  divided by  $\Sigma (EEM_{CSA}) \times 100$ , where  $EEM_{CSA}$  was the external elastic membrane cross-sectional area and Lumen CSA was the luminal cross-sectional area in the IVUS frames of the pullback sequence. Frames for IVUS analysis were taken from VH-IVUS mode, and all frames were analyzed.

Technical details of VH-IVUS as well as the analysis recommendations have been well-published.<sup>13</sup> VH-IVUS uses spectral analysis of IVUS radiofrequency data to classify plaques into four components: fibrotic tissue (F), fibrous-fatty tissue (FF), calcification (DC), and necrotic core (NC).<sup>14</sup> VH-IVUS analyses are reported in relative amounts (percentages of plaque). The definition of VH-derived TCFA (VH-TCFA<sup>15</sup>) was as follows: NC comprising more than 10% of the plaque in at least three consecutive cross-sections and direct contact of the NC with the vessel lumen.

**Statistical analysis.** Mean values ± standard deviation or percentages were calculated for all variables. Differences between the groups were compared by the  $\chi^2$  test. Statistical significance was calculated by Fischer's exact test for alternative variables. The statistical significance for continuous variables was determined by the Student's t-test. Data were analyzed using JMP 3.2 statistical software (SAS Institute). A P-value of <.05 was considered statistically significant. Multivariate statistical analysis was performed using IBM SPSS software version 17.0. Linear regression analysis was used for three dependent variables: angio, PAV, and NC. Logistic regression was used for the dependent variable VH-TCFA. Optimal groups of predictors were chosen for achieving the best prediction of dependent variables.

## Results

**Patient population.** Between November 2005 and April 2009, a total of 107 patients with SAP were included in the study. Data from 6 patients were unsuitable for VH-IVUS. Genetic analysis was performed in 81 patients (80.2%). Patient demographics are summarized in Table 1. The following arteries were analyzed: left anterior descending artery in 64 patients (63.4%), right coronary artery in 32 patients (31.7%), and left circumflex artery in 4 patients (4%).

**Angio score.** The highest angio scores were found in patients with the following: history of myocardial infarction (MI) (9.5 ± 3.9 vs 6.5 ± 3.6;  $P=.0001$ ), risk type of HO-1 polymorphism (9.1 ± 4.2 vs 6.9 ± 3.1;  $P=.008$ ), patients with LDLc <2.6

mmol/L on therapy (8.8 ± 3.8 vs 6.9 ± 4.0;  $P=.018$ ), statin therapy (8.8 ± 3.8 vs 6.9 ± 4.0;  $P=.018$ ), and past history of hyperlipidemia (8.3 ± 4.2 vs 6.4 ± 3.0;  $P=.03$ ). The FRS significantly correlated with the angio score ( $P=.032$ ;  $r = 0.21$ ). Predictors of the angio score from multivariate analysis are summarized in Table 2.

**Prediction of PAV.** Higher levels of PAV were found in patients with a risk type of HO-1 polymorphism (48.6 ± 6.6% vs 45.8 ± 5.8%;  $P=.04$ ) and in patients with plaque in the carotid bulb (48.6 ± 5.7% vs 44.7 ± 6.8%;  $P=.008$ ). PAV correlated with  $IMT_{mass}$  ( $P=.05$ ;  $r = 0.22$ ). Predictors of PAV from multivariate analyses are summarized in Table 3.

**Prediction of NC.** The percentage of NC measured using VH-IVUS analysis correlated with FRS ( $P=.007$ ;  $r = 0.2$ ), PAV ( $P=.04$ ;  $r = 0.2$ ) and inversely with the level of apolipoprotein A (Apo A;  $P=.047$ ;  $r = -0.27$ ). Higher content of NC was found in patients with risk type of HO-1 polymorphism (17.1 ± 8.9% vs 12.7 ± 7.8%;  $P=.02$ ) and with HDLc less than 1.6 mmol/L (15.1 ± 8.5% vs 10.4 ± 7.4%;  $P=.02$ ). Besides the NC percentage, the PAV also correlated with DC ( $P=.02$ ;  $r = 0.23$ ) and inversely with FF ( $P=.05$ ;  $r = -0.19$ ). Predictors of NC from multivariate analyses are summarized in Table 4.

**Prediction of VH-TCFA.** Patients with VH-TCFA lesion phenotype more often had an LDLc <2.6 mmol/L on treatment (58.9% vs 34.9%;  $P=.018$ ), lower level of Apo A (1.19 ± 0.16 mmol/L vs 1.3 ± 0.26 mmol/L;  $P=.04$ ), higher FRS (9.1 ± 3.1 vs 7.8 ± 3.1;  $P=.03$ ), and higher angio score (8.9 ± 4.2 vs 6.4 ± 3.2;  $P=.001$ ) compared to patients without VH-TCFA. A trend was observed for a more frequent occurrence of VH-TCFA lesion phenotype in patients with the risk type of HO-1 polymorphism

Table 2. Predictors of angio score from multivariate analysis. The model contained the following predictors: LDL <2.6, FRS, MI history (overall model, R=0.458; P<.001).

Parameters Included in Model	Unstandardized Coefficients - B	Significance	95% CI for B
(Constant)	4.119	.000	
LDLc <2.6	1.335	.119	-0.348–3.019
FRS	0.115	.005	0.036–0.193
MI history	2.813	.001	1.180–4.447

FRS = Framingham risk score; MI = myocardial infarction.

Table 3. Predictors of PAV from multivariate analysis. The model contained the following predictors: VCAM, hsCRP, IMT<sub>mass</sub>, carotid plaque, MI history (R=0.491; P=.004).

Parameters Included in Model	Unstandardized Coefficients - B	Significance	95% CI for B
(Constant)	31.970	.000	22.098–41.842
VCAM	0.004	.104	-0.001–0.01
hsCRP	-0.214	.081	-0.454–0.027
IMT <sub>mass</sub>	3.947	.026	0.481–7.413
Carotid plaque	3.540	.015	0.711–6.369
MI history	-0.837	.566	-3.732–2.059

VCAM = vascular cellular adhesive molecule; hsCRP = high-sensitivity C reactive protein; IMT = intima media thickness; MI = myocardial infarction.

Table 4. Predictors of NC from multivariate analysis. The model contained the following predictors: HO-1 risk polymorphism, carotid plaque, and Apo A (R=0.540; P=.002).

Parameters Included in Model	Unstandardized Coefficients - B	Significance	95% CI for B
(Constant)	23.741	.001	10.330–37.152
HO-1 risk	4.788	.031	0.463–9.113
Carotid plaque	3.772	.067	-0.278–7.821
Apo A	-11.039	.033	-21.157–0.922
MI history	-0.837	.566	-3.732–2.059

HO-1 risk = risk type of polymorphism for heme-oxygenase 1; Apo A = apolipoprotein A; MI = myocardial infarction.

(51.1% vs 30.3%; P=.06). However, the important role of the HO-1 polymorphism in the prediction of VH-TCFA was confirmed by multivariate analyses. The predictors of VH-TCFA from the multivariate analysis are summarized in Table 5.

The polymorphism of HO-1 gene. The risk type of polymorphism in the HO-1 gene was found more frequently in patients with a history of MI (61.3% vs 32%; P=.0097) and in patients with diabetes (68.4% vs 35.5%; P=.011). In addition, patients with the high-risk type of polymorphism in the HO-1 gene had a higher FRS (21.5 ± 12.5 vs 15.7 ± 8.0; P=.014), higher angio score (9.1 ± 4.2 vs 6.9 ± 3.1; P=.008), and also a higher PAV (results are mentioned above) than patients with the protective

type of polymorphism in the HO-1 gene. The relationship between the specific type of HO-1 polymorphism and the plaque composition is shown in Figure 1.

## Discussion

The main findings of our study are as follows:

- (1) High-risk type polymorphism of HO-1 gene was found more frequently in high-risk patients (MI in past, DM, higher FRS).
- (2) High-risk type polymorphism of HO-1 correlated with extent of atherosclerosis (angio score and PAV) as well as plaque risk profile (larger NC and more frequent VH-TCFA).
- (3) Carotid ultrasound and Apo A level can improve the prediction of coronary atherosclerosis PAV (carotid ultrasound) and plaque risk profile (Apo A).
- (4) Complex risk assessment is necessary for prediction of high-risk patients and the extent of coronary atherosclerosis.

**Polymorphism in HO-1 gene and high-risk patients.** We found a higher occurrence of a high-risk polymorphism in HO-1 gene in patients with a past history of MI, in patients with DM, and in patients with higher FRS. The common factor for cardiovascular disease and DM is an increased activity of reactive oxygen species (ROS).<sup>15</sup> Low activity of HO-1 increases levels of intracellular ROS that is associated with insulin resistance in adipocytes.<sup>16</sup> The HO-1 system has been shown to suppress insulin resistance<sup>17</sup> and enhance insulin sensitivity. Hemin, an inducer of the HO system, is effective against streptozocin-induced diabetes.<sup>18</sup> Oda et al<sup>19</sup> have shown a negative association between bilirubin level and glycosylated hemoglobin (HbA1c) in healthy Japanese men and women.

In animal studies, the absence of HO-1 renders animals more susceptible to myocardial ischemia/reperfusion damage,<sup>20</sup> while induction of HO-1 can act protectively against cardiac ischemia/reperfusion *in vivo*.<sup>21</sup> Induction of HO-1 increases adult cardiomyocyte tolerance to ischemia after *in vivo* transplantation.<sup>22</sup> Furthermore, CO has been shown to inhibit platelet aggregation.<sup>23</sup> These findings are consistent with the higher occurrence of MI in patients with the high-risk type of HO-1 polymorphism.

The activity of HO-1 plays an important protective role not only in development of DM and MI, but also in the development of arterial hypertension. The HO-1 system serves as a negative control mechanism to the pressor activity of angiotensin II<sup>21</sup> while CO regulates blood pressure cooperatively with NO.<sup>24</sup> The higher frequency of DM and arterial hypertension are consistent with the higher FRS in patients with the risk polymorphism in the HO-1 gene.

**Polymorphism in HO-1 gene and prediction of coronary atherosclerosis.** We found higher angio scores, greater PAV, more NC together with DC, and lower FF tissue percentages in patients with the risk type of HO-1 polymorphism. Greater PAV and necrotic core are known risk features of unstable plaques. On the other hand, the role of calcifications inside

Table 5. Predictors of VH-TCFA from multivariate analysis. The model contained the following predictors: HO-1 risk polymorphism, plaque in carotid bulb, Apo A, angio and FRS, correct prediction rate of 80.9%.

Parameters Included in Model	Regression Coefficient B	Significance	Exp(B)	95% CI for Exp(B)
(Constant)	2.525	.433	12.485	
HO-1 risk	1.760	.034	5.815	1.144–29.567
Carotid plaque	1.599	.044	4.947	1.046–23.392
Apo A	-3.148	.138	0.043	0.001–2.739
Angio	0.184	.146	1.202	0.938–1.539
FRS	-0.159	.266	0.853	0.644–0.644

*HO-1 risk = risk type of polymorphism for heme-oxygenase 1; Apo A = apolipoprotein A; angio = angio score; FRS = Framingham risk score.*

plaque remains unclear. Calcified plaques are thought to be stable; however, microcalcifications increase plaque vulnerability, ie, they increase risk of plaque rupture for higher plaque stiffness.<sup>25</sup> Thus, carriers of the high-risk type of HO-1 polymorphism developed more pronounced coronary atherosclerosis with a higher-risk profile. Li et al<sup>26</sup> found higher expression of HO-1 in patients with CAD and levels of HO-1 protein were highest in those with a greater disease burden. It seems that HO-1 expression is a consequence of the disease process and so may be a defense mechanism.<sup>27</sup> The mechanism of anti-atherosclerotic effects of HO-1 is the reduction of ROS, reduction of inflammatory mediators, and a reversion of decreased activity of endothelial NO synthase caused by oxidized LDLc and TNF alpha.<sup>28</sup> It was found that CO has an anti-apoptotic effect<sup>29</sup> and induction of HO-1 prevents cell death.<sup>30</sup> These pathways are probably responsible for transforming the plaque composition from primarily fibrous and fibro-fatty to containing increasingly more necrotic tissue with calcifications.

**Carotid ultrasound and Apo A level.** We did not find a robust correlation between carotid IMT and CAD; rather, we found only borderline correlation between IMT<sub>mass</sub> and plaque volume. A similar finding was published in the study done by Kranjec.<sup>31</sup> It is known that carotid IMT is an independent but relatively modest predictive factor for CAD.<sup>32</sup> Moreover, Wald et al<sup>33</sup> published a large meta-analysis of 18 studies including 44,861 patients that found a detection rate lower than 65% for CAD based on carotid IMT or carotid plaque.

In contrast, patients with plaque present in the carotid bulb had higher PAV. The presence of carotid plaques seems to be a better predictor for coronary atherosclerosis<sup>34</sup> and cardiac event than elevated IMT.<sup>35-37</sup> Nicholls et al described in more than 4000 patients the relationship between increasing burden of coronary atherosclerosis, as determined by IVUS, and subsequent clinical outcomes.<sup>38</sup>

Apo A inversely correlated with amount of NC (better than HDLc). Furthermore, Apo A and the high-risk type of HO-1 polymorphisms were identified as independent predictors of NC using multivariate analysis. We found correlations between VH-TCFA and levels of ApoA. Van Craeyld et al<sup>39</sup> found in an experimental mouse model that increased HDLc following ApoA-1 gene transfer elevates collagen content in atherosclerotic lesions.

These results confirm the role of Apo A in the process of plaque stabilization. The necessity to implement Apo A assessment among classical risk factors was reiterated in the study by Walladius et al.<sup>40</sup>

Complex risk assessment is necessary for prediction of high-risk plaque profile. FRS, age, and angio score correlated with occurrence of VH-TCFA. Rodriguez-Granillo et al<sup>41</sup> did not find a significant correlation between the occurrence of VH-TCFA and separate atherosclerotic risk factors; more complex risk assessment using the FRS was a predictor for VH-TCFA in the study done by Marso,<sup>42</sup> and higher FRS was associated with a higher occurrence of VH-TCFA in the PREDICT study.<sup>43</sup> FRS also correlated

with NC content in plaques and with angio score. These data underline the necessity of more complex assessment of atherosclerotic risk factors such as FRS for plaque risk profile and extent of coronary atherosclerosis.

The more frequent occurrence of VH-TCFA and higher angio score in patients with LDLc <2.6 mmol/L was problematic. However, these patients were in the well-treated high-risk group where we found more diabetics (68% vs 41.3%;  $P=.02$ ), more smokers (55.7% vs 35.9%;  $P=.05$ ), and more patients with a prior MI (69.1% vs 32.8%;  $P=.0003$ ). It is not surprising that lipid levels in well-treated patients with DM or a history of an MI were lower compared to patients with “only” hyperlipidemia. However, lipid-lowering therapy successfully decreased the level of LDLc, but unfortunately failed to change plaque composition in terms of reducing NC as it was demonstrated in the IBIS-2<sup>44</sup> and HEAVEN<sup>45</sup> trials.

The presence of VH-TCFA in 56.4% of patients with stable angina agrees with a study done by Hong et al,<sup>46</sup> which analyzed all three coronary arteries with VH-IVUS and found 1.7 VH-TCFAs per patient with stable angina.

## Conclusions

The main finding of this study is the correlation between the HO-1 risk type polymorphism and high-risk plaque features (higher plaque volume, larger NC). These types of plaque together with more frequent DM can probably explain the higher number of MIs in patients with risk HO-1 polymorphism. Based on our results, we can recommend implementation of genetic polymorphism for HO-1 screening in addition to the traditional risk assessment of CAD. HO-1 GT repeat genotyping and the subsequent distribution of patients based on the presence of high-risk HO-1 polymorphisms (generally, the presence of more than 25 GT repeats in the HO-1 gene promoter) and protective HO-1 polymorphisms (generally, the presence of less than 25 GT repeats in the HO-1 gene promoter, as described in the Methods section) is currently relatively easy to perform. Analysis of these polymorphisms may improve the prediction accuracy of the plaque risk profile, especially the prediction of necrotic core and VH TCFA. We expect that inducers of HO-1 or CO-donors may represent a new treatment approach for patients with CAD.

Additional non-invasive parameters were shown to further improve CAD prediction. The presence of plaque in carotid bulb correlated with plaque volume and was a better predictor than carotid IMT. Low level of Apo A was a predictor for high-risk plaque features, such as a large NC and the finding of a VH-TCFA.

The presented study indicates that genetic risk factors are likely to play an important diagnostic and eventually treatment role in the comprehensive management of cardiovascular disease.

**Study limitations.** A limitation of the present study is the relatively small number of patients enrolled, especially considering the assessment of possible correlation between the atherosclerotic risk factors and plaque composition. Additionally, only 80.2% of patients underwent genetic analysis; however, the study sample size is sufficient to generate statistically significant results and thus allow initial conclusions to be drawn. Finally, the study allowed detailed VH-IVUS assessment of only one plaque in a single coronary artery per subject.

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### 5.3. Paper 3

Kral A, Linhart A, Martasek P:

**Genetic variants in the C-terminus of the endothelial nitric oxide synthase in patients with coronary artery and peripheral artery disease.**

*Article in submission*

# **Genetic variants in the C-terminus of the endothelial nitric oxide synthase in patients with coronary artery disease and peripheral artery disease**

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## Abstract

Nitric oxide (NO), produced by the endothelial nitric oxide synthase (eNOS), is essential for endothelial and vascular homeostasis. The C-terminal part of eNOS is crucial for adequate enzyme function: therefore, genetic variants affecting this part of the protein would be expected to have profound effects on its function. The aim of the study was to search for genetic variations in the C-terminal part of eNOS, encoded by exons 25 and 26 in patients with symptomatic atherosclerosis. 150 patients with symptomatic atherosclerosis involving either the coronary or lower limb arteries were enrolled in the study. Two novel variants in exons 25, the G3617C polymorphism (synonymous variant) and exon 26, the G3911A polymorphism in the noncoding mRNA (the 3' untranslated region-UTR) and a novel variant in intron 24 were discovered in 3 different individuals. Significantly, the 3'UTR has been shown to be critically important for eNOS mRNA steady state levels. Thus, any variant in this region might alter the binding affinity of regulatory *trans*-acting elements, and thereby influence the posttranscriptional regulation of eNOS. In conclusion, our study did not demonstrate significant genetic variation in the C-terminal part of eNOS in individuals suffering from symptomatic atherosclerosis. Nevertheless, a novel variant in the noncoding mRNA of eNOS was discovered in one patient, a finding that warrants further investigation of its possible impact on eNOS mRNA stability.

**Abbreviations:** ADMA - asymmetric dimethylarginine, AMI - acute myocardial infarction, Asp - aspartic acid, bp - base pairs, BH<sub>4</sub> - tetrahydrobiopterine, CAD - coronary artery disease, eNOS - endothelial nitric oxide synthase, FAD - flavin adenine dinucleotide, FMN - flavin mononucleotide, Glu - glutamic acid, iNOS - inducible nitric oxide synthase, NADPH - reduced nicotinamide adenine dinucleotide phosphate, nNOS - neuronal nitric oxide synthase, NO - nitric oxide, PAD - peripheral artery disease, PCR - polymerase chain reaction, 3'-UTR- 3' - untranslated region

## Introduction

Atherosclerosis with its various clinical manifestations represents the most frequent cause of mortality in developed countries. To date, many risk factors of atherosclerosis have been identified, but the genetic determinants of the development, extent and severity of atherosclerosis is still poorly characterized. Nitric oxide (NO) represents the principal regulator of vascular tone (Vallance et al., 2000) whose further effects include prevention of leukocyte adhesion (Lefer et al., 1997), thrombocyte-endothelial interaction (Radomski et al.,

1987), scavenging of superoxide radicals (Loscalzo et al., 1995) and inhibition of vascular smooth muscle cell proliferation (Garg et al., 1989) Therefore, it has been suggested that a reduction in basal NO production may predispose to hypertension, vasospasm, thrombosis, and atherosclerosis development (Cosentino et al., 1998; Cook et al., 1997; Oemar et al., 1998; Rudic et al., 1999).

NO is produced by the nitric oxide synthase (NOS) enzymes. The NOS enzyme family consists of three isoforms (endothelial-eNOS, neuronal-nNOS and inducible, iNOS) which share a similar structure, a N-terminal heme (oxygenase) domain connected by a calmodulin-binding amino acid sequence to a reductase domain. The reductase domain, accepts electrons from NADPH and transports them via two flavins, FAD and FMN, to the iron molecule of the heme (Raman et al., 1999). Tetrahydrobiopterin (BH<sub>4</sub>), is an essential enzyme cofactor, and in its absence, the eNOS and nNOS isoforms transfer electrons to molecular oxygen, leading to production of the deleterious superoxide radical (Vasquez-Vivar et al. 1998). The essential role of the C-terminal parts of the reductase domain of NOS for the rate of reduction equivalent transfer to the heme part of the protein and thus proper enzyme function has been described previously (Roman et al., 2000 a, b; Jáchymová et al., 2005). These C-termini slow electron transfer between the two flavins, enabling tight electron coupling, thereby suppressing superoxide production (Raman et al., 1999). In endothelial dysfunction, electron coupling is altered, giving rise to an increased amount of free radicals (Schulz et al., 2008; Yang et al., 2009; Li and Forstermann, 2013).

The eNOS has the longest C-terminus of the three isoforms-42 amino acids, compared to 21 amino acids in the iNOS and 33 amino acids in the nNOS (Figure 1). NO synthesis rate is inversely related to the length of the C-terminal extension in the particular isoform, hence, is highest in iNOS and lowest in eNOS (Raman et al., 1999). Therefore, polymorphisms or mutations in the C-terminal part of eNOS could profoundly affect enzyme function through a change in the rate of electron flow and thus play a role in the pathogenesis of endothelial dysfunction and atherosclerosis. The eNOS gene, located on chromosome 7q35-36, is composed of 26 exons (Marsden et al., 1993). Several common genetic variants in the eNOS gene have been identified, including the T-786C variant located in the eNOS promoter that has been shown to suppress the eNOS gene transcription and the 4 b/a polymorphism representing variability in the number of 27-nt repeats in intron 4 (Wang et al., 1996; Tsukada et al., 1998; Shimasaki et al., 1998; Nakayama et al., 1999). These intronic repeats give rise to micro RNAs (miRs) that act as inhibitors of transcription, contradictory to most miRs that act as inhibitors of translation or degrade mRNA (Zhang et al., 2005). The most extensively

studied in relation to endothelial dysfunction and atherosclerosis is the 894G/T or Glu298Asp polymorphism in exon7. The variant protein demonstrates decreased basal association to caveolae (small invaginations of the plasmalemmal membrane serving as scaffolds for the assembly of multiprotein signaling complexes) as well as reduced dissociation from the inhibitory protein caveolin-1 in response to eNOS agonists (Joshi et al., 2007). Studies have shown that individuals homozygous for the Asp298 allele demonstrate decreased NO production in response to agonist stimuli (Joshi et al., 2007), presumably accounting for the predisposition to the development of endothelial dysfunction (Naber et al. 2001) and atherosclerosis (Hingorani et al., 1999; Colombo et al., 2002; Li et al., 2010).

Surprisingly, little attention has been focused on the search for genetic variants in the reductase domain of the eNOS which is essential for proper enzyme function, as mentioned above (Jáchymová, et al., 2005). We hypothesized that variants in the C-terminal part of the reductase domain of eNOS could represent an unrecognized genetic factor contributing to eNOS uncoupling, endothelial dysfunction and atherosclerosis development. Furthermore, we postulated that variants in the noncoding mRNA-the 3' untranslated region (3'-UTR) encoded by exon 26, demonstrated to be crucial for eNOS mRNA stability (Ho et al., 2013) may prove to be an additional mechanism leading to altered eNOS activity in individuals with endothelial dysfunction and atherosclerosis.

In the present study, we searched for genetic variants in exons 25 and 26 of the eNOS gene, encoding the C-terminus and the 3'-UTR in patients with coronary artery disease (CAD) and peripheral artery disease (PAD).

## **Materials and Methods**

### Patient population

A total of 150 patients admitted to the Second Department of Medicine, Department of Cardiovascular Medicine of the General University Hospital in Prague from September 2008 to September 2009 were enrolled in the study if they suffered from angiographically proven CAD and/or PAD. The study was approved by the institutional ethics committee and all patients gave informed consent to the genetic analysis.

### Genetic analysis

The DNA was extracted from peripheral blood leukocytes using standard techniques. The polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify exon 25 and 26 of the eNOS gene. Amplification of all samples was performed on

the MJ Research DYAD 220 Peltier Thermal Cycler (Conquer Scientific). The reaction was carried out in a final volume of 25 µl containing 1 µl of genomic DNA, 1 µl of each primer (3,2 pmol) and 12,5 µl of PP Master Mix. For exon 25 the following primers and PCR reaction parameters were used: forward primer ENOS25-sens, 5'-GGA GAC TTT CAC GTC CAG GG-3'; reverse primer ENOS25-anti, 5'-ACA GTG GAT CAG AAC CCG AC-3'; the mixture was incubated for 94°C 3 min, followed by twenty-nine cycles (each 30 s at 94°C, 30 s at 63°C, and 45 s at 72°C) and 72°C for 3 min. For exon 26, the following primers and PCR reaction parameters were used: forward primer ENOS26-sens, 5'-GGT TCC TGC TAA GGT CTC CG-3'; reverse primer ENOS26-anti, 5'-AAG AAA CAG GAA GCG GGT G-3'; the mixture was incubated for 94°C 3 min, followed by twenty-nine cycles (each 30 s at 94°C, 30 s at 59°C, and 45 s at 72°C) and 72°C for 3 min.

The amplified exons 25 and 26 were separated by electrophoresis in 1.5% agarose gel. After staining with ethidium bromide, the amplified DNA was purified using the Qiagen Gel Extraction Kit (QIAGEN Ltd). Subsequently, direct sequencing of exon 25 a 26 with forward and reverse primer in all samples was performed on the sequenator Prism ABI (Applied Biosystems). Sequence analysis of exons 25 a 26 with neighboring regions of introns 24, 25 and the 3'-UTR was performed with the Chromas Lite version 2.0 software (Technelysium Pty Ltd).

## Results

Basic clinical characteristics of the patient cohort are summarized in Table 1. Sequencing of exon 25 revealed a novel G3617C variant in one patient. This variant leads to a substitution of cytosine (C) for guanosine (G), representing a synonymous mutation since both triplets-CGG a CGC encode the amino acid arginine (R) (Figure 2). Furthermore, a novel G→A polymorphism in intron 24, located 12 bp before the start of exon 25 was identified in one other patient. Sequencing of exon 26 revealed a novel G3911A variant leading to a substitution of adenosine (A) for guanosine (G), in the noncoding mRNA 6 bp from the stop codon (Figure 3).

## Discussion

The C-terminal part of eNOS is responsible for a substantial deceleration of intraprotein electron transfer, thereby facilitating tight electron coupling and minimal electron leak from intraprotein transport resulting in suppressed generation of free radical species (Roman et al., 2000a). An important mechanism leading to endothelial dysfunction and

atherosclerosis is eNOS uncoupling, i.e. altered electron coupling by the enzyme, characterized by a diversion of electron flow from L-arginine to molecular oxygen resulting in the production of the superoxide radical instead of NO (Schulz et al., 2008; Yang et al., 2009; Li and Forstermann, 2013). Several mechanisms are involved in the development of eNOS uncoupling including depletion of L-arginine, accumulation of asymmetric dimethylarginine (ADMA), BH<sub>4</sub> deficiency and eNOS S-glutathionylation, which occurs under conditions of increased oxidative stress (Förstermann and Sessa, 2012). However, genetic factors contributing to the development of eNOS uncoupling and dysregulation in endothelial dysfunction and atherosclerosis have remained elusive.

Given its significance for proper enzyme function, genetic variants in the C-terminal part of eNOS could alter electron transfer with a resulting disequilibrium between superoxide and NO production. Nevertheless, to our best knowledge, no functionally significant genetic variations in the C-terminal part of eNOS have been reported to date, maybe in part, due to a lack of focus on this area. We postulated that genetic variations in the C-terminal part of eNOS might represent a genetic basis for endothelial dysfunction, one of the initial steps in atherogenesis (Davignon et al., 2004). We tested this hypothesis on a cohort of patients with symptomatic atherosclerosis involving either the coronary or lower limb arteries. Genotyping of exons 25 and 26 which constitute the C-terminal and neighboring region of the reductase domain of eNOS was performed with the use of direct sequencing, a time-consuming, but in return, the most precise method applied in the search for novel genomic variations.

The genomic analysis of exons 25 and 26 of the eNOS gene, however, demonstrated minimal genetic variation in our patient population. No amino acid-altering variants in exons 25 and 26 were detected, nevertheless, a novel variant in the 3'-UTR of exon 26- the G3911A variant was described. The 3'-UTR, containing multiple, evolutionarily conserved pyrimidine-rich sequence elements, is critically important for eNOS mRNA steady state levels (Wang et al., 2000). Under basal conditions, eNOS mRNA is highly stable due to multiple 3'-UTR *cis*-mRNA elements that form stabilizing ribonucleoprotein (RNP) complexes (Wang and Wang, 2000; Ho et al., 2013). A variety of mediators involved in atherogenesis, such as tumor necrosis factor alpha (TNF- $\alpha$ ), oxidized low density lipoproteins (ox-LDL) and thrombin have been shown to decrease eNOS mRNA stability and thus dramatically decrease eNOS mRNA levels via modulation of *trans*-acting element binding to 3'-UTR regions of eNOS (Eto et al., 2001; Searles, 2006). Translation elongation factor 1- $\alpha$ 1 (eEF1A1), heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), sONE (NOS3AS), small interfering RNAs (siRNAs) and micro-RNAs (e.g. miR-155) are examples of the presently identified

binding partners of the *cis*-acting sequences within eNOS mRNA 3'-UTR (Robb et al., 2004; Yan G et al. 2008; Sun et al., 2012; Ho et al., 2013).

Given the significance of the region for eNOS mRNA stability and hence eNOS protein levels, any genetic variant in the eNOS 3'-UTR could dramatically alter the binding affinity of *trans*-acting elements, and thereby modulate posttranscriptional regulation of eNOS (Wang et al., 2000). Importantly, no sequence variants in the human eNOS 3'-UTR have been reported to date. Therefore, our finding of a novel genetic variant in the 3'-UTR of the eNOS certainly warrants further investigation of its possible impact on eNOS posttranscriptional regulation.

In conclusion, our study demonstrated minimal variation in exons 25 and 26 of the eNOS gene encoding the C-terminal region of the protein in individuals suffering from symptomatic atherosclerosis. The fact that no amino acid-altering variants in the C-terminus of eNOS were found may reflect its pivotal significance for proper eNOS function, when any changes in sequence would be too deleterious. The potential functional significance of the novel variant in the 3'-UTR of human eNOS will be the subject of future research.

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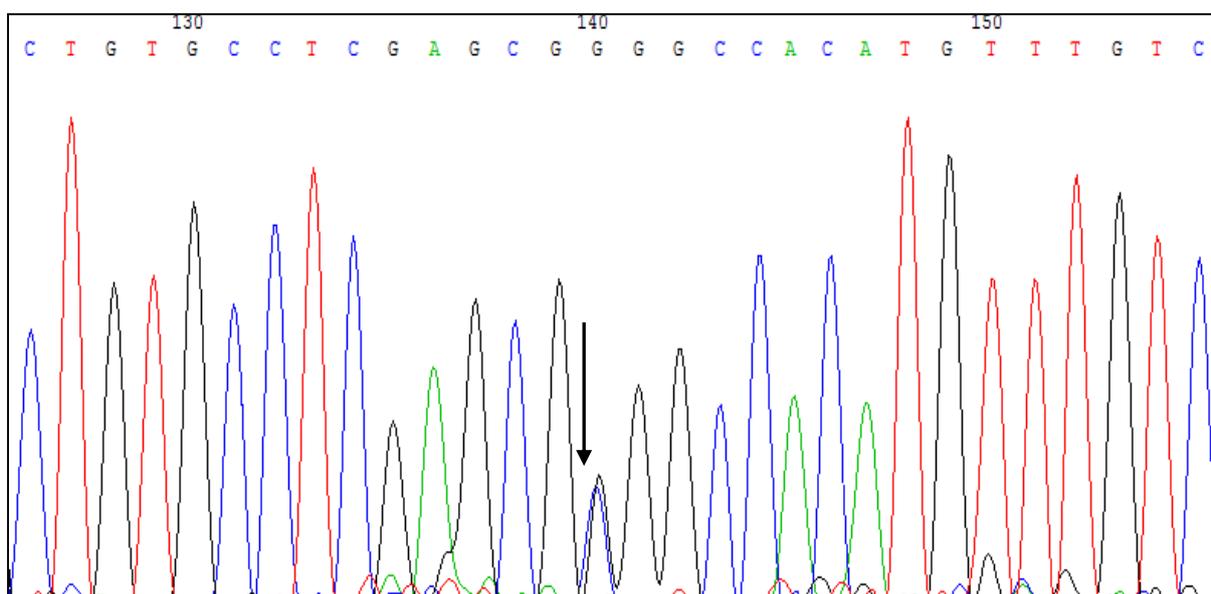
**Table 1.** Basic clinical characteristics of the patient population data given as n(%) or mean  $\pm$  standard deviation

Number of patients	150
Male sex	108 (72%)
Age	63,3 $\pm$ 9,28
CAD	118(78,7%)
PAD	64(42,7%)
Hypertension	120(80%)
Hyperlipidemia	110(73,3%)
Diabetes mellitus	57(38%)
Active smokers	68 (45,3%)

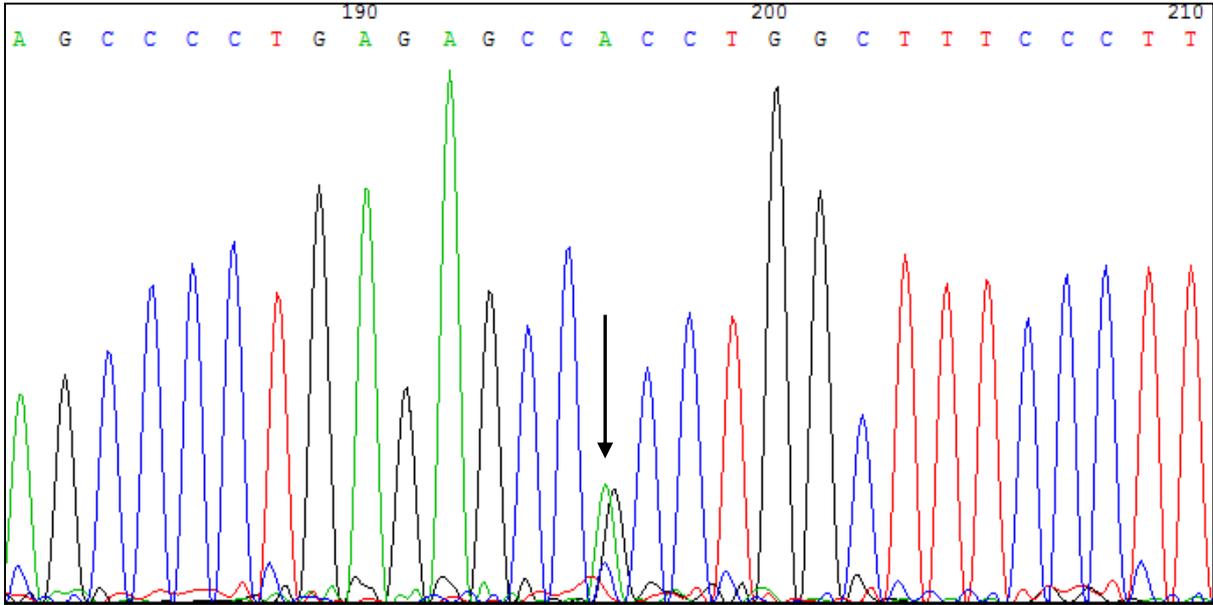
**Figure 1.** C-terminal parts of three mammalian NOS isoforms. (Adapted from Fig. 2 of Jáchymová et al., 2005)



**Figure 2.** Novel synonymous G3617C variant localized in exon25 of the eNOS gene.



**Figure 3.** Novel G3911A variant localized in the noncoding region (3'-UTR) of exon 26 of the eNOS gene



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## **6. RESULTS SUMMARY AND DISCUSSION**

## 6.1. Paper 1, Folia Biologica 57, 182-190, 2011

eNOS derived NO is the pivotal mediator of vascular homeostasis and alterations in eNOS activity and/or increased NO degradation are considered key mechanisms in endothelial dysfunction and atherosclerosis, disease states characterized by increased oxidative stress. The HO-1 is perceived as one of the chief cellular defense mechanisms against oxidative stress. However, the contribution of the eNOS and HO-1 gene variations to the development of endothelial dysfunction and atherosclerosis remains only partially elucidated.

We studied the influence of the eNOS exon 7 Glu298Asp polymorphism and the HO-1 promoter (GT)<sub>n</sub> polymorphism on the extent and characteristics of coronary artery atherosclerotic involvement assessed by means of CAG, IVUS and IVUS-VH. In addition, we assessed the influence of these polymorphisms on the changes in coronary atherosclerotic involvement during the course of hypolipidaemic statin-based therapy.

### *Baseline plaque characteristics*

We did not observe significant differences in baseline plaque composition with respect to the eNOS a HO-1 polymorphisms in the study population. Importantly, we found a higher prevalence of TCFA in HO-1 risk variant carriers (70.0 % vs. 46.2 %,  $p = 0.047$ ).

### *Coronary artery atherosclerotic burden and plaque magnitude*

The total coronary atherosclerotic burden, as expressed by the angio score, was significantly greater in HO-1 risk variant carriers (HO-1 risk  $9.4 \pm 4.0$  vs. protective  $6.9 \pm 3.2$ ,  $p = 0.005$ ), but did not differ with respect to the eNOS genotype (eNOS risk  $8.1 \pm 3.9$  vs. eNOS protective  $7.8 \pm 3.6$ ,  $p = 0.69$ ). Similarly, the PAV was significantly higher in carriers of HO-1 risk variant (HO-1 risk  $49.3 \% \pm 6.6$  vs. protective  $45.0 \% \pm 5.5$ ,  $p = 0.004$ ), but did not differ with respect to the eNOS genotype (eNOS risk  $46.7 \% \pm 6.8$  vs. protective  $46.7 \% \pm 5.9$ ,  $p = 0.98$ ).

### *Changes of plaque composition*

A significant interaction between eNOS and HO-1 genotypes and changes in plaque composition was observed, the results are summarized in table 4.

**Table 4. Changes of plaque composition according to eNOS and HO-1 genotypes**

(Reproduced from Tab. 4 of Král et al., 2011)

Plaque tissue change in %	Protective ENOS	Risk ENOS	Protective HO-1	Risk HO-1
<b>Fibrous</b>	-0.3 ± 6.3	-2.4 ± 7.6	- 0.7 ± 8.2	- 2.3 ± 4.8
	p = 0.22		p = 0.34	
<b>Fibro-fatty</b>	-1.0 ± 10.4	-5.1 ± 9.7	- 5.7 ± 10.9	0.9 ± 7.4
	p = 0.09		<b>p = 0.006</b>	
<b>Necrotic core</b>	1.3 ± 7.0	3.9 ± 6.7	4.1 ± 7.4	0.4 ± 5.8
	p = 0.1		<b>p = 0.03</b>	
<b>Dense calcium</b>	0.5 ± 6.0	3.7 ± 5.3	2,8 ± 6,8	1,3 ± 3,9
	<b>p = 0.02</b>		p = 0.29	

## Discussion

The key finding of this study is that patients with the HO-1 risk promoter polymorphism had greater coronary artery atherosclerotic burden (as expressed as a higher angio score) and more prominent atherosclerotic plaques in coronary arteries (as expressed as higher PAV). Another significant finding was the higher prevalence of the VH-TCFA type of plaques in these patients. Importantly, plaques with higher proportions of necrotic tissue, such as the TCFA plaques are related to the development of unstable plaques. These plaques are prone to rupture, representing a frequent mechanism of acute coronary syndromes (Virmani et al., 2006; Stone et al., 2011).

The formation and progression of the plaque necrotic core is caused by apoptosis of foam cells coupled with impaired phagocytosis of the dead cells (Tabas et al., 2009). Concurrently, the structural integrity of the TCFAs is directly linked to the survival of VSMC of the thin-fibrous cap, which prevents exposure of plaque content to the haemostatic system (Larsen et al., 2010). In animal studies, HO-1 induction was associated with a significant increase in plaque cap thickness and a reduction in the necrotic core size and plaque lipid accumulation, contributing to plaque stabilization (Cheng et al., 2009). In contrast, HO-1

knockout led to severely reduced numbers of VSMC due to increased apoptosis (Yet et al., 2003). These findings indicate that HO-1, through CO and its further signaling pathways, inhibits apoptosis in VSMC, macrophages and foam cells, thus attenuating key processes that contribute to plaque destabilization (Larsen et al., 2010). In addition, CO concurrently inhibits VSMC migration and proliferation (Morita et al., 1997; Rodriguez et al., 2010), additional crucial processes of atherosclerosis development (Abraham and Kappas, 2008). Furthermore, CO exerts a potent anti-inflammatory effect on plaque macrophages and endothelia through multiple pathways including decreased TNF- $\alpha$  and IL-6 production, inhibition of adhesion molecule expression, e.g., E-selectin and VCAM-1, and increased IL-10 production (Yet et al., 2003, Larsen et al., 2010). Importantly, HO-1 over-expression in endothelia significantly attenuates inflammatory mediator production and reverses impaired eNOS expression induced by ox-LDL and TNF- $\alpha$ , thus alleviating endothelial dysfunction (Kawamura et al., 2005). Through these pleiotropic effects, HO-1 seems to confer protection against the progression of the atherosclerotic process as well as destabilization of advanced plaques.

Significant differences in HO-1 expression have been detected in patients with different forms of CAD and HO-1 expression has been shown to correlate with increasing coronary lesion complexity and stenosis grade (Chen et al., 2005; Song et al., 2009). Similarly, Cheng et al. have demonstrated in a study of carotid endarterectomy specimens that HO-1 expression was specifically up-regulated in vulnerable plaques characterized by increased lipid and macrophage accumulation and low collagen and VSMC content and that HO-1 expression correlated with levels of pro-inflammatory and plaque destabilizing cytokines and chemokines (Cheng et al., 2009). In addition, other researchers have shown that products of the HO-1 enzymatic reaction enhance NO bioavailability by modulating eNOS expression and activity, preventing NO inactivation and by compensating for NO deprivation (Pae et al., 2010). In light of the current evidence, enhanced HO-1 expression in atherosclerotic lesions most likely represents a compensatory atheroprotective and anti-inflammatory response, acting to restore vascular function, in part through enhancement of NO bioavailability, and prevent plaque instability. However, under certain conditions, the levels of HO-1 protein and HO-1 activity may be discordant, presumably due to posttranslational modifications. Therefore, further research is required to clarify why highly up-regulated HO-1 may be overwhelmed and thus fail to halt the progression of atherosclerotic lesions (Araujo et al., 2012).

HO-1 activity is principally regulated at the level of gene transcription. Thus, any mechanism affecting the transcriptional activity of the HO-1 gene can profoundly alter HO-1

enzymatic activity. Undoubtedly, the most extensively studied genetic variant affecting HO-1 expression is the HO-1 (GT)<sub>n</sub> polymorphism located in the gene promoter. As described previously, the number of GT dinucleotide repeats in the gene promoter modulates the level of gene transcription (Yamada et al., 2000). The long alleles (L) demonstrate decreased HO-1 transcriptional activity upon induction, compared with the short alleles (S) (Taha et al., 2010). Multiple studies have reported the relevance of this polymorphism to the risk of coronary artery disease (Chen et al., 2002; Kaneda et al., 2002; Schillinger et al., 2002; Araujo et al., 2012). According to our findings, the HO-1 (GT)<sub>n</sub> promoter polymorphism plays an important role in coronary artery atherosclerosis development, progression and possibly in plaque type formation as well. The blunted induction of HO-1 gene transcription in the 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 advanced coronary atherosclerosis, such as we have observed in our population. Moreover, our results, as well as other current findings, 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 promoting VSMC survival in the plaque fibrous cap (Yet et al., 2003; Cheng et al., 2009; Larsen et al., 2010).

The observed influence of the (GT)<sub>n</sub> HO-1 promoter variant on changes in plaque composition is somewhat puzzling. We observed an unfavorable type of plaque transformation in patients with the protective HO-1 variant, characterized by a decrease of fibro-fatty tissue and an increase of necrotic core proportion during statin treatment therapy. Paradoxically, the HO-1 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 favorable plaque composition changes observed in patients with the HO-1 risk promoter variants might possibly be caused by statin therapy. Statins have been shown to induce HO-1 expression through impeding the prenylation of small G proteins such as Ras. Additionally, the p38MAPK and the PI3K-Akt kinase pathways are involved in statin mediated HO-1 induction (Lee et al., 2004; Chen et al., 2006; Ali et al., 2009). We speculate that impaired inducibility of HO-1 expression upon pro-atherogenic stimuli in the HO-1 L/L variant carriers may represent one of the principal mechanisms contributing to the atherosclerotic plaque formation and progression in these individuals. Thus, ameliorated HO-1 expression induced by statins may have lead to the favorable plaque composition changes observed in risk HO-1 carriers in our study. Importantly, almost 40 % of patients were statin naive prior to trial enrolment, and all statins utilized in our trial have been

demonstrated to induce HO-1 expression (Chen et al., 2006). We further hypothesize that the atherosclerotic process in carriers of the protective HO-1 promoter variant may be driven primarily by different mechanisms not as susceptible to statin therapy, thereby possibly explaining the unfavorable plaque composition changes observed in these patients.

Conversely, the eNOS Glu298Asp polymorphism had no impact on the severity of coronary artery atherosclerotic involvement quantified by angio score, or baseline plaque magnitude and composition in our cohort. Multiple studies have evaluated the relationship of the Glu298Asp eNOS polymorphism to the risk of CAD with conflicting results (Colombo et al., 2003; Jaramillo et al., 2010; Li et al., 2010), but to our best knowledge, no study to date has evaluated the impact of this eNOS variant on coronary artery atherosclerotic involvement assessed by IVUS and IVUS-VH.

Substantial evidence demonstrating that alterations in NO synthesis promote atherosclerosis in experimental animals has been presented. ApoE-deficient mice treated with L-NAME experienced significant progression of aortic atherosclerosis (Kauser et al., 2000). Likewise, long-term inhibition of eNOS by L-NAME administration to rats resulted in induction of coronary inflammation and atherosclerosis (Tomita et al., 1998). Another study has shown that endothelial over-expression of arginase II induced endothelial dysfunction and enhanced atherosclerosis in mice (Vaisman et al., 2012). The importance of eNOS in vasculoprotection was further demonstrated in a study utilizing the ApoE/eNOS double-knockout mice model. In this study, eNOS deficient mice developed accelerated atherosclerosis including coronary artery disease and aortic aneurysm (Kuhlen cordt et al., 2001b). Additional studies have confirmed that eNOS gene knockout promotes atherogenesis (Knowles et al., 2000), and eNOS gene transfer or eNOS over-expression improves endothelial function along with regression of atherosclerotic lesions in experimental animals (Mujynya-Ludunge et al., 2005; Jiang et al., 2012). On the other hand, most studies in atherosclerotic animal models have shown unchanged or even augmented eNOS expression in atherosclerotic lesions despite the presence of endothelial dysfunction (d'Uscio et al., 2001; Ming et al., 2004). A study of human coronary artery specimens found a significant decrease in eNOS gene expression only in endothelial cells overlying advanced atherosclerotic lesions, as opposed to early atherosclerotic lesion samples (Fukuchi et al., 1999). Thus, current evidence implies that endothelial dysfunction and atherosclerosis are determined primarily by impaired NO bioavailability caused by deregulated eNOS enzymatic activity, eNOS uncoupling and NO inactivation through increased oxidative stress, rather than eNOS gene down-regulation.

Extensive research has focused on the possible genetic determinants of altered eNOS derived NO production. Multiple eNOS gene polymorphisms with variable clinical impact have been identified (Wang and Wang, 2000; Cook, 2006). The following polymorphisms influence eNOS transcriptional activity: the T(-786)C variant in the eNOS promoter has been shown to affect gene transcription by a significant influence on eNOS promoter activity; the 4 b/a polymorphism in intron 4 determines the number of 27-nt miRs that act as suppressors of eNOS transcriptional activity (Balligand et al., 2009). Differently, the eNOS 894 G/T (Glu298Asp) polymorphism does not affect gene transcription, but instead influences eNOS posttranslational regulation. The Asp variant is associated with decreased NO production by endothelia in response to shear stress (Joshi et al., 2007). The proposed mechanisms leading to an altered NO production by the variant enzyme are decreased basal association to caveolae and reduced dissociation from caveolin-1 in response to shear stress (Joshi et al., 2007). Another work by the same group showed that an amino acid substitution at position 298 of the eNOS protein leads to a significant reduction in amino acid distances, thereby possibly affecting protein geometry and thus eNOS protein–protein interactions (Joshi and Bauer, 2008). These alterations seem to affect eNOS protein function and possibly explain the enhanced disease risk associated with the Asp allele (Joshi et al., 2011). Numerous studies in support of the functional significance of this eNOS variant have been presented (Yoshimura et al., 1998; Philip et al., 1999; Naber et al., 2001; Gardemann et al., 2002; Colombo et al., 2003; Li et al., 2010).

Although we did not find a relationship between the presence of the eNOS risk variants and the magnitude of coronary atherosclerosis and baseline plaque composition, we observed an association of this eNOS polymorphism with atherosclerotic plaque composition changes during statin treatment. In patients with the eNOS protective variant (Glu/Glu), we found favorable plaque composition changes characterized by a significantly lower increase in plaque calcified tissue as well as a statistically nonsignificant trend toward smaller progression of the necrotic core during statin therapy, 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 Glu298Asp polymorphism on plaque composition changes during statin treatment. It has been demonstrated that oxLDL decrease eNOS expression via activation of the Rho-Rho kinase pathway (Blum et al., 2009). Statins lead to enhanced eNOS expression through multiple mechanisms including a modest reduction of oxLDL levels and a concurrent inhibition of the Rho-Rho kinase pathway. Furthermore, statins increase eNOS activity via activation of the Akt kinase pathway and inhibition of Cav-1 expression in

endothelial cells (Kureishi et al., 2000; Blum et al., 2009; Balakumar et al., 2012). We speculate that the effects of statins may be more pronounced in the wild-type eNOS enzyme carriers, and therefore, the greater up-regulation of eNOS activity could contribute to the favorable changes in plaque composition observed in these individuals. However, the extent to which our observations were affected by statin treatment remains to be elucidated by future investigations.

## 6.2. Paper 2, Journal of Invasive Cardiology 25, 32-37, 2013

The prediction of CAD and its risk profile is one of the key objectives in preventive cardiology. Abundant clinical data implicate an important role of the HO-1 promoter (GT)<sub>n</sub> polymorphism in relation to atherosclerosis. We studied the utility of the HO-1 promoter (GT)<sub>n</sub> polymorphism and other noninvasive markers in the prediction of coronary artery disease severity and coronary artery plaque risk profile.

In this study, we evaluated a spectrum of non-invasive predictors of the following parameters:

- 1) Angio score derived from CAG – expressing total coronary atherosclerotic burden
- 2) Percent atheroma volume (PAV) derived from IVUS – relative volume of a selected atherosclerotic plaque
- 3) Necrotic core (NC) derived from VH-IVUS – relative amount of necrotic core of a selected atherosclerotic plaque
- 4) Thin cap fibroatheroma (TCFA) derived from VH-IVUS - a high risk rupture-prone type of plaque, predictive of future coronary events.

The spectrum of noninvasive examinations assessed in this study included the HO-1 (GT)<sub>n</sub> promoter polymorphism, pro-inflammatory cytokine and plasma lipid levels and parameters derived from an ultrasonographic examination of carotid arteries.

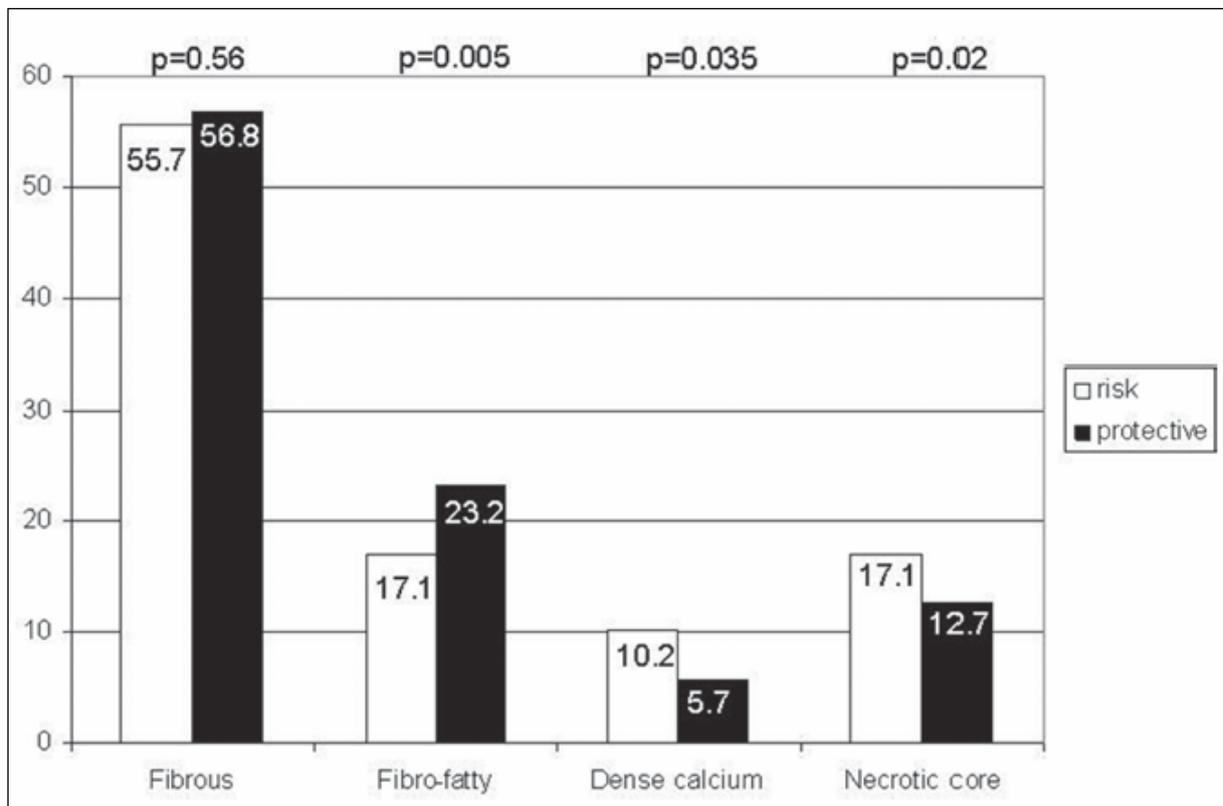
### *HO-1 polymorphism and prediction of coronary atherosclerosis*

Similarly to our previous study (Král et al., 2011), the risk HO-1 polymorphism was found to influence the extent of coronary atherosclerosis expressed as a higher angio score ( $9.1 \pm 4.2$  vs.  $6.9 \pm 3.1$ ;  $p = 0.008$ ). Furthermore, risk HO-1 variant carriers showed more prominent plaques, indicated as higher PAV ( $48.6 \pm 6.6\%$  vs.  $45.8 \pm 5.8\%$ ;  $p = 0.04$ ).

In addition, plaques in HO-1 risk variant carriers were characterized by higher proportions of necrotic core ( $17.1 \pm 8.9\%$  vs.  $12.7 \pm 7.8\%$ ;  $p = 0.02$ ). The proportion of dense calcium and fibro-fatty components of coronary plaques also differed significantly with respect to the HO-1 variant, **figure 7**. A statistically non-significant trend was observed for a more frequent occurrence of the VH-TCFA lesion phenotype in risk HO-1 variant carriers ( $51.1\%$  vs.  $30.3\%$ ,  $p = 0.06$ ). However, the significance of the HO-1 risk polymorphism in the prediction of VH-TCFA was confirmed by multivariate analysis, **table 5**.

**Figure 7. Relationship between HO-1 promoter polymorphism and plaque composition**

(Reproduced from Fig. 1 of Kovárník et al. 2013)



*Other noninvasive parameters in the prediction of coronary atherosclerosis*

In addition to the HO-1 risk variant, LDL <2.6 mmol/L on hypolipidaemic therapy correlated with the extent of coronary atherosclerosis, i.e., angio score ( $8.8 \pm 3.8$  vs.  $6.9 \pm 4.0$ ;  $p = 0.018$ ). Furthermore the FRS showed a significant, albeit modest correlation with the angio score ( $p = 0.032$ ;  $r = 0.21$ ), a finding confirmed by multivariate analysis (data not shown). In addition to the HO-1 risk variant, PAV was also higher in patients with plaques in the carotid bulb ( $48.6 \pm 5.7\%$  vs.  $44.7 \pm 6.8\%$ ;  $p = 0.008$ ). Furthermore, ApoA was a significant predictor of NC in the multivariate analysis (data not shown). Patients with VH-TCFA lesions had lower levels of ApoA ( $1.19 \pm 0.16$  mmol/L vs.  $1.3 \pm 0.26$  mmol/L;  $p = 0.04$ ), and higher FRS ( $9.1 \pm 3.1$  vs.  $7.8 \pm 3.1$ ;  $p = 0.03$ ). In addition to the HO-1 risk polymorphism, only carotid plaque was an independent predictor of VH-TCFA according to multivariate analysis, **table 5**.

**Table 5. Predictors of VH-TCFA from multivariate analysis**

Logistic regression analysis, correct prediction rate 80,9%. (Reproduced from Tab. 5 of Kovárník et al., 2013)

Parameters Included in Model	Regression Coefficient B	Significance	Exp(B)	95% CI for Exp(B)
(Constant)	2.525	.433	12.485	
HO-1 risk	1.760	.034	5.815	1.144–29.567
Carotid plaque	1.599	.044	4.947	1.046–23.392
Apo A	-3.148	.138	0.043	0.001–2.739
Angio	0.184	.146	1.202	0.938–1.539
FRS	-0.159	.266	0.853	0.644–0.644

*HO-1 risk = risk type of polymorphism for heme-oxygenase 1; Apo A = apolipoprotein A; angio = angio score; FRS = Framingham risk score.*

## Discussion

The principal finding of this study is the correlation between the risk HO-1 promoter variant and total coronary atherosclerotic burden, as well as high-risk coronary plaque features such as higher PAV and larger necrotic core. Additionally, the risk HO-1 promoter variant predicted the occurrence of the VH-TCFA type of plaque. Our results are of profound significance, given the risk attributable to these plaque features (Stone et al., 2011). Additional noninvasive tests found to predict coronary artery disease severity and risk profile were the FRS, presence of carotid bulb plaque and decreased ApoA levels.

HO-1 plays a key role in maintaining vascular redox homeostasis and in the prevention against vascular injury. Accumulating evidence shows that HO-1 expression in the vasculature exerts protective effects against atherosclerosis due to its antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative, and immunomodulatory properties (Abraham and Kappas, 2008; Araujo et al., 2012). As mentioned previously, HO-1 is highly expressed in all key cell types found in human atherosclerotic lesions. Additionally, abundant data from animal models shows that modulation of HO-1 expression significantly alters atherogenesis (Ishikawa et al., 2001, Juan et al., 2001; Yet et al., 2003; Cheng et al., 2009). Enhanced HO-1 expression appears to be protective against the development of both early and advanced atherosclerotic plaques. Its antioxidant and anti-inflammatory properties may impede the development of early plaques (Ishikawa et al., 2001; Orozco et al., 2007), while its anti-

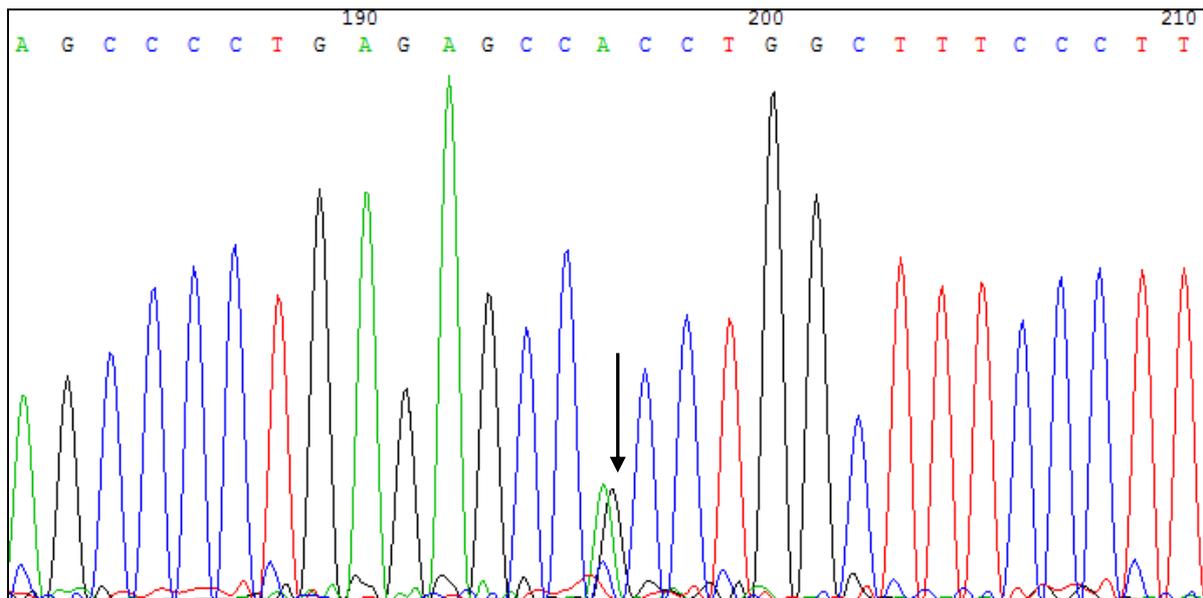
apoptotic activities may represent an important mechanism against lesion progression into an advanced plaque and reduce plaque vulnerability for rupture (Cheng et al., 2009; Li et al., 2011). The impaired HO-1 expression in risk HO-1 promoter variant carriers (Yamada et al., 2000; Taha et al., 2010), leading to increased oxidative stress, vascular inflammation and enhanced apoptosis of foam cells and VSMCs (Araujo et al., 2012), is likely responsible for the greater magnitude and higher risk profile of coronary atherosclerosis we have observed in our patients. Based on our findings, we suggest to incorporate the assessment of the HO-1 (GT)<sub>n</sub> promoter polymorphism into the spectrum of noninvasive predictors of coronary artery disease severity and risk profile.

### 6.3. Paper 3, manuscript in submission

ENOS uncoupling is one of the principal phenomenon underlying endothelial dysfunction and atherosclerosis. The C-terminal part of eNOS is crucial for proper electron coupling, and hence enzyme function. The aim of this study was to search for genetic variants in the C-terminus of the reductase domain of eNOS encoded by exons 25 and 26 in patients with angiographically documented CAD and PAD. In addition, the noncoding mRNA (3'-UTR) of exon 26, shown to have a significant role in eNOS posttranscriptional regulation, was evaluated for sequence variations.

Allelic variations were assessed by means of direct sequencing. A novel synonymous (G3617C) variant in exon 25 was found in one patient. Furthermore, a novel variant (G3911A) leading to a substitution of adenosine (A) for guanosine (G) in the noncoding mRNA 6 bp from the stop codon of exon 26 was discovered in one other patient, figure 8.

**Figure 8.** Novel G3911A variant localized in the noncoding region (3'-UTR) of exon 26 of the eNOS gene. Reproduced from Fig. 3 of *Král et al, manuscript submitted*



### Discussion

The C-terminal part of the reductase domain of eNOS is crucial for proper enzyme function. It slows the rate of reduction equivalent transfer to the catalytic domain of the protein (Roman et al, 2000 a, b; Jáchymová et al., 2005), and thus enables tight electron coupling with suppression of superoxide production (Raman et al., 1999). Therefore, we hypothesized that polymorphisms or mutations in the C-terminal part of eNOS could profoundly affect enzyme

function through a change in the rate of electron flow and coupling, and thus play a role in the pathogenesis of endothelial dysfunction and atherosclerosis. The genomic analysis of exons 25 and 26 of the eNOS gene, however, demonstrated minimal genetic variation in our patient population. No amino acid-altering variants in exons 25 and 26 were detected, nevertheless, a novel variant in the noncoding region-3'-UTR of exon 26 was found. As outlined previously, posttranscriptional regulation of eNOS mRNA stability is an important component of the immensely complex system of eNOS regulation. Under unstimulated conditions, eNOS mRNA is highly stable due to multiple 3'-UTR *cis*-mRNA elements that form stabilizing RNP complexes (Wang and Wang, 2000; Ho et al., 2013). A range of mediators involved in atherogenesis, such as tumor TNF- $\alpha$ , have been shown to decrease eNOS mRNA stability via modulation of RNP binding to the 3'-UTR of eNOS (Tai et al., 2004). Given the significance of the 3'-UTR in eNOS posttranscriptional regulation, it is plausible that genetic variants in the 3'-UTR could alter the binding affinity of *trans*-acting elements, and thereby influence eNOS mRNA stability (Wang and Wang, 2000). Thus, our finding of a novel genetic variant in the 3'-UTR of the human eNOS gene warrants further investigation of its possible impact on eNOS posttranscriptional regulation.

## **7. CONCLUSIONS AND FUTURE PERSPECTIVES**

Endothelial dysfunction is one of the key steps in atherosclerosis initiation and progression, but to date, the genetic factors contributing to endothelial dysfunction and atherosclerosis development are poorly characterized. The eNOS and HO-1 are enzymes crucial for vascular homeostasis, thus, alterations in their regulation and function are involved in atherogenesis (Ignarro and Napoli, 2004; Abraham and Kappas, 2008; Forstermann and Sessa, 2012; Araujo et al., 2012). Principally, endothelial dysfunction and atherosclerosis are characterized by decreased NO bioavailability (Yang and Ming, 2006; Li and Forstermann, 2013). ENOS uncoupling, a phenomenon with multifactorial genesis, is one of the chief mechanisms leading to decreased NO bioavailability in the vasculature (Li and Forstermann, 2013). According to current evidence, HO-1 up-regulation during the course of the atherosclerotic disease represents one of the key mechanisms acting to restore NO bioavailability and vascular homeostasis (Abraham and Kappas, 2008; Araujo et al., 2012).

Numerous genetic association studies have evaluated the relationship of several common eNOS polymorphisms to the risk of endothelial dysfunction and atherosclerosis development, in part, with conflicting results (Li et al., 2010; Liu et al., 2013; Yao et al., 2013). The eNOS G894T (Glu298Asp) polymorphism has been linked to increased risk of atherosclerosis in multiple studies (Gardemann et al., 2002; Colombo et al., 2003; Li et al., 2010). Notably, the decreased eNOS Asp variant activity is not related to decreased cellular eNOS levels, consistent with the notion that intimate spatial and temporal regulation is required for proper eNOS function in endothelial cells (Joshi et al., 2007). Similarly, the HO-1 (GT)<sub>n</sub> promoter polymorphism, shown to profoundly influence HO-1 expression, and hence enzymatic activity, has been linked to increased risk of atherosclerosis in many studies (Abraham and Kappas, 2008; Araujo et al., 2012). However, very limited data are available on the impact of these eNOS and HO-1 polymorphisms on the extent and characteristics/risk profile of coronary artery atherosclerotic disease (Colombo et al., 2002; Liang et al., 2013). Moreover, reports assessing the influence of eNOS and HO-1 gene variants on the progression of coronary artery atherosclerosis are completely lacking. Furthermore, little attention has been devoted to the search for variations in exons 25 and 26, encoding the C-terminal part of the eNOS reductase domain, shown to be crucial for proper enzyme function (Roman et al., 2000 a, b; Jáchymová et al., 2005) and the 3'-UTR, demonstrated to have a key role in eNOS mRNA stability (Wang and Wang, 2000; Ho et al., 2013).

We thus sought to evaluate the genetic determinants of endothelial dysfunction and atherosclerosis by assessing the impact of common eNOS and HO-1 polymorphisms on coronary artery disease severity and risk profile assessed comprehensively by means of CAG,

IVUS and IVUS-VH, and their evolution during hypolipidaemic treatment. In addition, we searched for sequence variations in exons 25 and 26 of the eNOS gene, encoding the C-terminus of eNOS and the 3'-UTR.

We did not observe an association of the eNOS G894T polymorphism with the extent and risk profile of coronary atherosclerosis characterized in detail by a broad spectrum of parameters derived from CAG and IVUS. Nevertheless, we observed an association of this eNOS variant with plaque composition changes during hypolipidaemic statin-based therapy. Statins have been shown to increase eNOS activity through several mechanisms. These actions include an up-regulation of eNOS expression by counteracting the inhibitory effects of ox-LDLs and via a TGF- $\beta$ 1 dependent pathway (Blum et al., 2009; Vecerova et al., 2012). In addition, statins increase eNOS mRNA stability and enzyme activity through inhibition of the Rho-Rho kinase pathway (Blum et al., 2009). Further beneficial actions of statins on posttranslational eNOS regulation are exerted through the activation of the PI3K-Akt pathway leading to increased binding affinity of eNOS for calmodulin, while concurrently reducing the levels of the inhibitory Cav-1 (Kureishi et al., 2000; Blum et al., 2009; Balakumar et al., 2012). We hypothesize that effects of statins on posttranslational eNOS regulation, namely eNOS protein caveolar association and other protein-protein interactions, may be more pronounced in the wild-type eNOS carriers, thus possibly leading to the restoration of adequate eNOS activity contributing to the favorable plaque composition changes we have observed in these individuals. Further investigations on this relationship are certainly warranted.

Our study demonstrated no amino-acid altering variants in the C-terminal part of eNOS in patients with atherosclerosis, a finding that may reflect the pivotal significance of the C-terminus for proper eNOS function, when any changes in sequence would be too deleterious. Given the significance of the 3'-UTR for the regulation of eNOS mRNA stability, our finding of a novel genetic variant in the 3'-UTR of the human eNOS gene warrants further investigation of its possible impact on eNOS posttranscriptional regulation.

According to our findings, the risk HO-1 promoter polymorphism (L/L) correlates with greater coronary artery atherosclerotic burden and high-risk coronary plaque features, such as more prominent plaques, larger necrotic core proportion and a higher prevalence of the TCFA type of plaque. Other researchers have demonstrated that the protective HO-1 promoter genotypes exhibit higher HO-1 expression under conditions of high oxidative stress, such as endothelial dysfunction and atherosclerosis (Morita 2005; Chen et al. 2012). Therefore, the observed greater disease burden and higher risk profile of CAD in HO-1 risk

variant carriers is attributable to insufficient HO-1 activity resulting in persistent oxidative stress and inflammation in the vascular wall. On the other hand, the protective HO-1 variant did not prevent negative plaque composition changes during statin therapy in our patients, suggesting that adequate HO-1 activity may be overwhelmed in atherosclerosis and thus fail to impede the progression of atherosclerotic lesions. We conclude that impaired induction of HO-1 expression in HO-1 risk promoter variant carriers is responsible for the development of more advanced coronary atherosclerosis with higher risk plaques we have observed in our patients.

To the best of our knowledge, no study to date has evaluated the relationship of eNOS and HO-1 genetic variants to coronary atherosclerosis characterized in such a thorough manner and larger-scale studies are warranted to confirm and possibly further extend our findings. Our results significantly contribute to the recognition of the genetic background of endothelial dysfunction and atherosclerosis and the elucidation of possible causes of variable vascular responses to hypolipidaemic therapy. In light of our results, HO-1 promoter polymorphism assessment in individuals with suspected coronary artery disease seems desirable, given the unequivocally higher risk of the L/L variant carriers characterized by greater disease burden and high-risk coronary artery plaque features. Thus, we suggest to incorporate the assessment of the HO-1 (GT)<sub>n</sub> promoter polymorphism into the spectrum of noninvasive tests utilized for coronary artery disease severity and risk profile prediction. Larger-scale studies focusing specifically on eNOS Glu298Asp polymorphism interaction with coronary artery plaque characteristics and composition changes during statin treatment are essential, given our interesting preliminary findings.

Nevertheless, atherosclerotic endothelial dysfunction is certainly only in part attributable to variations in the eNOS gene, as an immense number of interactions and regulatory mechanisms converge to fine-tune eNOS function, ensuring tailored NO production. Furthermore, functional DNA variants may be modified by numerous environmental factors, such as cigarette smoking (Wang et al., 1996). Exogenous NO supply, used to date (e.g., nitrates), in no way resembles the prompt regulation of eNOS activity in response to specific stimuli in distinct cell types and subcellular compartments. The very complex molecular regulation of eNOS appears essential for coordinate signaling of an effector, whose actions are critically dose and localization dependent. Thus, the desired strategy would be one that restores endogenous eNOS-derived NO production in situ (Balligand et al., 2009). Some currently used drugs improve endothelial function through multiple beneficial actions on eNOS, including increased enzyme abundance and activity.

These include drugs affecting the renin–angiotensin–aldosterone system (RAAS), statins, calcium channel blockers, thiazolidinediones, and certain beta blockers (e.g., nebivolol, carvedilol) (Forstermann and Sessa, 2012; Seals et al., 2014).

Further advances in the understanding of the molecular biology of eNOS uncoupling and decreased NO bioavailability will help to identify potential novel therapeutic approaches aimed at specific signaling pathways (Kietadisorn et al., 2012). Future pharmacological strategies may aim to restore proper enzyme activation, e.g., through modulation of Cav-1 activity, or restore eNOS catalytic activity by replenishing substrate and cofactor supply, inducing proper protein conformation, phosphorylation state, and interaction with Hsp90. Alternatively, upstream signaling elements such as VEGF receptor 2 (VEGFR2) and specific PI3K/Akt isoforms, may represent potential therapeutic targets (Balligand et al., 2009). A further promising strategy striving to preserve or restore proper eNOS function despite an unfavorable local redox environment aims at targeting specific redox-sensitive cysteine thiols in the reductase domain involved in S-glutathionylation-induced eNOS uncoupling (Chen et al., 2010; Maron and Michel, 2012). In addition, eNOS transcription enhancers (AVE9488 and AVE3085) show promising preclinical data, leading to enhanced eNOS activity and vasoprotective actions in experimental animals (Kietadisorn et al., 2012).

Currently, up-regulation of HO-1 is perceived as a protective mechanism counteracting endothelial dysfunction and atherosclerosis in humans. However, further investigations of patients with HO-1 deficiency and patients with insufficient expression of HO-1 in response to risk factors of atherosclerosis, such as HO-1 L/L genotype carriers are required. Measurement of HO-1 protein levels and HO-1 activity in the plasma and at the cellular level in individuals with different HO-1 genotypes will be necessary, as oxidative stress may elicit different degrees of HO-1 response in various cell types (Morita, 2005). Correlation of the HO-1 genotype with tissue and cellular HO-1 activity in response to oxidative stress is certainly an area warranting future research.

Given the role currently attributed of HO-1 in atheroprotection and restoration of NO bioavailability, HO-1 induction or the delivery of the end products of heme degradation represent promising therapeutic approaches. Specific pharmacological interventions under development include induction of HO-1 expression with targeted gene delivery or drugs such as heme arginate, administration of CO by inhalation or use of CO releasing molecules (CORM), and administration of biliverdin/bilirubin or inhibitors of bilirubin conjugation, resulting in increased unconjugated bilirubin levels (Morita, 2005; Abraham and Kappas, 2008; Araujo et al., 2012). Current observations indicate that the protective effects of HO-1

may differ according to the protein level and the specific site of expression. However, contemporary HO-1 inducers and HO-1 gene delivery techniques do not produce an organ-specific overexpression of HO-1. Thus, the key for future therapeutic applications of HO-1 modulation lies in proper targeting of enhanced HO-1 expression at a specific site, e.g., the vascular wall, to enable maximal cytoprotection at the site of the atherosclerotic lesion with minimal adverse effects (Morita, 2005).

## **8. SUMMARY OF PUBLICATION ACTIVITY**

## 8.1. Papers related to PhD thesis

**Kral, A.**, T. Kovarnik, L. Kralik, H. Skalicka, J. Horak, G.S. Mintz, J. Uhrova, M. Sonka, A. Wahle, R. Downe, M. Aschermann, P. Martasek, and A. Linhart. 2011. Genetic variants in haem oxygenase-1 and endothelial nitric oxide synthase influence the extent and evolution of coronary artery atherosclerosis. *Folia Biologica*. 57:182-190. (IF = 1,15)

Kovarnik, T., **A. Kral**, H. Skalicka, G.S. Mintz, L. Kralik, M. Chval, J. Horak, L. Skalicka, M. Sonka, A. Wahle, R.W. Downe, J. Uhrova, H. Benakova, L. Cernohousova, P. Martasek, J. Belohlavek, M. Aschermann, and A. Linhart. 2013a. The prediction of coronary artery disease based on non-invasive examinations and heme oxygenase 1 polymorphism versus virtual histology. *The Journal of Invasive Cardiology*. 25:32-37. (IF = 1,56)

**Kral A**, Linhart A, Martasek P. Genetic variants in the C-terminus of the endothelial nitric oxide synthase in patients with coronary artery and peripheral artery disease. (Manuscript in submission)

## 8.2. Original publications unrelated to the thesis

Kralova, J., Z. Kejik, T. Briza, P. Pouckova, **A. Kral**, P. Martasek, and V. Kral. 2010. Porphyrin-cyclodextrin conjugates as a nanosystem for versatile drug delivery and multimodal cancer therapy. *Journal of Medicinal Chemistry*. 53:128-138. (IF = 5,24; cited 46x)

Kejik, Z., T. Briza, J. Kralova, P. Pouckova, **A. Kral**, P. Martasek, and V. Kral. 2011. Coordination conjugates of therapeutic proteins with drug carriers: a new approach for versatile advanced drug delivery. *Bioorganic & Medicinal Chemistry Letters*. 21:5514-5520. (IF = 2,55; cited 6x)

Kovarnik, T., G.S. Mintz, H. Skalicka, **A. Kral**, J. Horak, R. Skulec, J. Uhrova, P. Martasek, R.W. Downe, A. Wahle, M. Sonka, V. Mrazek, M. Aschermann, and A. Linhart. 2012.

Virtual histology evaluation of atherosclerosis regression during atorvastatin and ezetimibe administration: HEAVEN study. *Circulation Journal*. 76:176-183.

(IF = 3,76; cited 19x)

Widimsky, P., F. Rohac, J. Stasek, P. Kala, R. Rokyta, B. Kuzmanov, M. Jakl, M. Poloczek, J. Kanovsky, I. Bernat, O. Hlinomaz, J. Belohlavek, **A. Kral**, V. Mrazek, V. Grigorov, S. Djambazov, R. Petr, J. Knot, D. Bilkova, M. Fischerova, K. Vondrak, M. Maly, and A. Lorencova. 2012. Primary angioplasty in acute myocardial infarction with right bundle branch block: should new onset right bundle branch block be added to future guidelines as an indication for reperfusion therapy? *European Heart Journal*. 33:86-95. (IF = 10,47; cited 14x)

Jachymova, M., A. Muravska, T. Palecek, P. Kuchynka, H. Rehakova, S. Magage, **A. Kral**, T. Zima, K. Horky, and A. Linhart. 2012. Genetic variation screening of TNNT2 gene in a cohort of patients with hypertrophic and dilated cardiomyopathy. *Physiological Research*. 61:169-175. (IF = 1,56; cited 1x)

Kovarnik, T., **A. Kral**, H. Skalicka, L. Skalicka, O. Dostal, L. Kralik, P. Martasek, M. Aschermann, J. Horak, A. Linhart, A. Wahle, and M. Sonka. 2013. Prediction of coronary vessel involvement on the basis of atherosclerosis risk factor analysis. *Bratislavské Lekárske Listy*. 114:413-417. (IF = 0,34)

### 8.3. Citations of papers related to PhD thesis (WoS)

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

Kral, A; Kovarnik, T; Kralik, L; Skalicka, H; Horak, J; Mintz, GS; Uhrova, J; Sonka, M; Wahle, A; Downe, R; Aschermann, M; Martasek, P; Linhart, A.

**FOLIA BIOLOGICA Volume: 57 Issue: 5 Pages: 182-190 Published: 2011**

*Times Cited (5):*

1. Association Between Heme Oxygenase 1 Gene Promoter Polymorphisms and Susceptibility to Coronary Artery Disease: A HuGE Review and Meta-Analysis

By: Qiao, Huaiyu; Sai, Xiaoyong; Gai, Luyue; et al.

AMERICAN JOURNAL OF EPIDEMIOLOGY Volume: 179 Issue: 9 Pages: 1039-1048

Published: MAY 1 2014

2. Stress Perception and (GT)<sub>n</sub> Repeat Polymorphism in Haem Oxygenase 1 Promoter Are Both Risk Factors in Development of Eating Disorders

By: Slachtova, L.; Kaminska, D.; Chval, M.; et al.

FOLIA BIOLOGICA Volume: 59 Issue: 6 Pages: 233-239 Published: 2013

3. Review of the Cost Effectiveness of Pharmacogenetic-Guided Treatment of Hypercholesterolaemia

By: Sorich, Michael J.; Wiese, Michael D.; O'Shea, Rebekah L.; et al.

PHARMACOECONOMICS Volume: 31 Issue: 5 Pages: 377-391 Published: 2013

4. The Prediction of Coronary Artery Disease Based on Non-Invasive Examinations and Heme Oxygenase 1 Polymorphism Versus Virtual Histology

By: Kovarnik, Tomas; Kral, Ales; Skalicka, Hana; et al.

JOURNAL OF INVASIVE CARDIOLOGY Volume: 25 Issue: 1 Pages: 32-37 Published:

JAN 2013

5. Microsatellite Polymorphism in Haem Oxygenase 1 Gene Promoter in Multiple Sclerosis

By: Zbornikova, P.; Kralik, L.; Lelkova, P.; et al.

FOLIA BIOLOGICA Volume: 58 Issue: 2 Pages: 69-74 Published: 2012

**The Prediction of Coronary Artery Disease Based on Non-Invasive Examinations and Heme Oxygenase 1 Polymorphism Versus Virtual Histology**

Kovarnik, T; Kral, A; Skalicka, H; Mintz, GS; Kralik, L; Chval, M; Horak, J; Skalicka, L; Sonka, M; Wahle, A; Downe, RW; Uhrova, J; Benakova, H; Cernohousova, L; Martasek, P; Belohlavek, J; Aschermann, M; Linhart, A

**JOURNAL OF INVASIVE CARDIOLOGY Volume: 25 Issue: 1 Pages: 32-37**

**Published: JAN 2013**

***Times Cited (1):***

Association Between Heme Oxygenase 1 Gene Promoter Polymorphisms and Susceptibility to Coronary Artery Disease: A HuGE Review and Meta-Analysis

By: Qiao, Huaiyu; Sai, Xiaoyong; Gai, Luyue; et al.

AMERICAN JOURNAL OF EPIDEMIOLOGY Volume: 179 Issue: 9 Pages: 1039-1048

Published: MAY 1 2014

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