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=0.0097), or with diabetes (66.4% vs 35.5%; P=0.011) or a higher FRS (21.5 vs 15.7; P=0.04). 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) 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; 1.9 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

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) and markers of inflammation and oxidative stress. We recently published a study3 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 sequestrated by ferritin. The enzyme HO exists as isoenzymes HO-1 (inducible), HO-2 (constitutive), and HO-3 (probably only a pseudotranscript of HO-2). 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. Increased risk for CAD development was found in patients with an HO-1 gene polymorphism demonstrating a higher GT repetition. 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.

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.
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 (FRS). 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.\textsuperscript{11}

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$\text{\textit{mass}}$ (sum of IMT in ACC and ACI for both sides) and IMT$\text{\textit{max}}$ (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).\textsuperscript{12}

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) and compared 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 µg 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),

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The relationship between type of polymorphism in HO-1 gene and plaque composition.}
\end{figure}
groups were compared by the χ² test. Statistical significance was calculated for all variables. Differences between the groups were compared 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.

which was calculated as Σ (EEM CSA – Lumen CSA) x 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. 13 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). 14 VH-IVUS analyses are reported in relative amounts (percentages of plaque). The definition of VH-derived TCFA (VH-TCFA) 13 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 χ² 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=.047; r = - 0.27). Higher content of NC was found in patients with risk type of HO-1 polymorphism (13.2 ± 8.9% vs 17.1 ± 8.9%; P=.04; r = - 0.2) and inversely with the level of apolipoprotein 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.
Non-Invasive Prediction of Coronary Atherosclerosis

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

<table>
<thead>
<tr>
<th>Parameters Included in Model</th>
<th>Unstandardized Coefficients - B</th>
<th>Significance</th>
<th>95% CI for B</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Constant)</td>
<td>4.119</td>
<td>.000</td>
<td></td>
</tr>
<tr>
<td>LDLc &lt;2.6</td>
<td>1.335</td>
<td>.119</td>
<td>-0.348–3.019</td>
</tr>
<tr>
<td>FRS</td>
<td>0.115</td>
<td>.005</td>
<td>0.036–0.193</td>
</tr>
<tr>
<td>MI history</td>
<td>2.813</td>
<td>.001</td>
<td>1.180–4.447</td>
</tr>
</tbody>
</table>

\( FRS = \text{Framingham risk score}; \ MI = \text{myocardial infarction}. \)

Table 3. Predictors of PAV from multivariate analysis. The model contained the following predictors: VCAM, hsCRP, IMT_mass, carotid plaque, MI history (\( R=0.491; \) \( P=.004 \)).

<table>
<thead>
<tr>
<th>Parameters Included in Model</th>
<th>Unstandardized Coefficients - B</th>
<th>Significance</th>
<th>95% CI for B</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Constant)</td>
<td>31.970</td>
<td>.000</td>
<td>22.098–41.842</td>
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<tr>
<td>VCAM</td>
<td>0.004</td>
<td>.104</td>
<td>-0.001–0.01</td>
</tr>
<tr>
<td>hsCRP</td>
<td>-0.214</td>
<td>.081</td>
<td>-0.454–0.027</td>
</tr>
<tr>
<td>IMT_mass</td>
<td>3.947</td>
<td>.026</td>
<td>0.481–7.413</td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>3.540</td>
<td>.015</td>
<td>0.711–6.369</td>
</tr>
<tr>
<td>MI history</td>
<td>-0.837</td>
<td>.566</td>
<td>-5.732–2.059</td>
</tr>
</tbody>
</table>

\( \text{VCAM} = \text{vascular cellular adhesive molecule}; \ \text{hsCRP} = \text{high-sensitivity C reactive protein}; \ \text{IMT} = \text{intima media thickness}; \ MI = \text{myocardial infarction}. \)

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

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

\( \text{HO-1 risk} = \text{risk type of polymorphism for heme-oxgenase 1}; \ \text{Apo A} = \text{apoliprotein A}; \ MI = \text{myocardial infarction}. \)

(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). Low activity of HO-1 increases levels of intracellular ROS that is associated with insulin resistance in adipocytes. The HO-1 system has been shown to suppress insulin resistance and enhance insulin sensitivity. Hemin, an inducer of the HO system, is effective against streptozocin-induced diabetes. Oda et al 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, while induction of HO-1 can act protectively against cardiac ischemia/reperfusion in vivo. Induction of HO-1 increases adult cardiomyocyte tolerance to ischemia after in vivo transplantation. Furthermore, CO has been shown to inhibit platelet aggregation. 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 while CO regulates blood pressure cooperatively with NO. The higher frequency of DM and arterial hypertension are consistent with the higher occurrence of MI 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-TCFAs 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%.

<table>
<thead>
<tr>
<th>Parameters Included in Model</th>
<th>Regression Coefficient B</th>
<th>Significance</th>
<th>Exp(B)</th>
<th>95% CI for Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Constant)</td>
<td>2.252</td>
<td>.433</td>
<td>12.485</td>
<td></td>
</tr>
<tr>
<td>HO-1 risk</td>
<td>1.760</td>
<td>.034</td>
<td>5.815</td>
<td>1.144–29.567</td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>1.599</td>
<td>.044</td>
<td>4.947</td>
<td>1.046–23.392</td>
</tr>
<tr>
<td>Apo A</td>
<td>-3.148</td>
<td>.138</td>
<td>0.043</td>
<td>0.001–2.739</td>
</tr>
<tr>
<td>Angio</td>
<td>0.184</td>
<td>.146</td>
<td>1.202</td>
<td>0.938–1.539</td>
</tr>
<tr>
<td>FRS</td>
<td>-0.159</td>
<td>.266</td>
<td>0.853</td>
<td>0.644–0.644</td>
</tr>
</tbody>
</table>

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

The presence of VH-TCFAs in 56.4% of patients with stable angina agrees with a study done by Hong et al.,

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 TCFAs. 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-IUS assessment of only one plaque in a single coronary artery per subject.

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**References**