Gene Expression Patterns in Peripheral Blood Correlate with the Extent of Coronary Artery Disease

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Abstract

Systemic and local inflammation plays a prominent role in the pathogenesis of atherosclerotic coronary artery disease, but the relationship of whole blood gene expression changes with coronary disease remains unclear. We have investigated whether gene expression patterns in peripheral blood correlate with the severity of coronary disease and whether these patterns correlate with the extent of atherosclerosis in the vascular wall.

Patients were selected according to their coronary artery disease index (CADI), a validated angiographical measure of the extent of coronary atherosclerosis that correlates with outcome. RNA was extracted from blood of 120 patients with at least a stenosis greater than 50% (CADI≥23) and from 121 controls without evidence of coronary stenosis (CADI=0).

160 individual genes were found to correlate with CADI (rho=0.2, P<0.003). Prominent differential expression was observed especially in genes involved in cell growth, apoptosis and inflammation. Using these 160 genes, a partial least squares multivariate regression model resulted in a highly predictive model (r² = 0.776, P<0.0001). The expression pattern of these 160 genes in aortic tissue also predicted the severity of atherosclerosis in human aortas, showing that peripheral blood gene expression associated with coronary atherosclerosis mirrors gene expression changes in atherosclerotic arteries.

In conclusion, the simultaneous expression pattern of 160 genes in whole blood correlates with the severity of coronary artery disease and mirrors expression changes in the atherosclerotic vascular wall.

Introduction

Coronary artery disease, a multifactorial chronic disease, is the leading cause of death in Western countries. Despite considerable advances in the prevention and treatment of coronary artery disease and its complications, morbidity and mortality remains high. In half of patients with coronary artery disease, the first manifestation is death [1]. Consequently, substantial efforts are being put into the development of new strategies for accurate noninvasive diagnosis of coronary artery disease and the identification of novel treatment targets [2].

Systemic and local inflammation has been shown to play a prominent pathologic role in atherosclerotic coronary artery disease [3]. Adhesion of leukocytes to activated endothelial cells and their migration into the arterial wall are thought to initiate, propagate, and destabilize coronary plaques. All types of blood constituents appear to play a role in plaque formation, although the majority of inflammatory lesions in atherosclerotic vascular tissue consist of foam cell macrophages and activated T-cells [4]. Several studies have found distinct gene expression patterns in atherosclerotic arteries [5–8]. While other pathways are likely also important, a consistent feature has been differential expression of inflammatory genes and genes involved in cell cycle control [9–12].

Microarray analysis of peripheral blood cells is a practical approach to study gene expression changes that may reflect not only genetic predisposition but also presence and activity of disease, environmental modifier effects, and treatment responses [13]. Total peripheral leukocyte count correlates with the severity of coronary atherosclerosis and is a strong predictor of cardiovascular outcome [14], but little is known about the role of phenotypic changes in circulating blood cells of patients with coronary atherosclerosis. In a recent micro-array analysis, 526 genes were found to be differentially expressed in isolated mononuclear cells from 41 patients [15]. Gene expression patterns of 50 of these genes together with 56 genes selected from the literature were subsequently shown to be associated with the presence of coronary artery disease in two independent cohorts. The aim of the present study was 1) to identify distinct genomic markers in peripheral whole blood that correlate with the severity of coronary artery disease using micro-array analysis and 2) to investigate to what extent gene expression patterns in peripheral blood mirror those in atherosclerotic arteries.

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Methods

Patient Selection and Characteristics

Patients and control subjects were recruited from individuals that had undergone catheterization in the Duke University Hospital Cardiac Catheterization Laboratory and participated in a proteomics study to discover candidate proteins that are differentially displayed in populations with and those without angiographic coronary artery disease [16]. After being approached and providing informed written consent, subjects had clinical and laboratory data collected. The investigation conforms to the principles outlined in the Declaration of Helsinki, and was approved by the Duke Institutional Review Board.

Patient selection, design and results from the main proteomics study have been reported previously [16]. Populations were initially defined in order to minimize differences in plasma proteins unrelated to the presence or absence of coronary artery disease. As a practical strategy, three different cohorts of subjects (cases and controls) were enrolled: 1) matched men (n = 106), who were matched for age and ethnic group, 2) unmatched men (n = 82), who did not fulfill the matching criteria and 3) unmatched women (n = 53). The severity of coronary artery disease was scored using the Duke Coronary Artery Disease Index (CAD-Index) [17,18]. The CAD-index is a prognostic assessment of the extent of coronary artery disease, accounting for the number and severity of lesions and diseased vessels and involvement of left anterior descending and left main disease.

Inclusion criteria for the coronary artery disease patient population (cases) were: age between 40 and 65 and coronary artery stenosis of >50% in at least one major coronary artery. Inclusion criteria for the control population (controls) were: age between 40 and 65 for matched men cohort only, no angiographically detectable coronary artery stenosis on cardiac catheterization within the last two years, normal left ventricular ejection fraction and normal regional wall motion. Exclusion criteria for controls were typical signs of angina, or any history or evidence of myocardial ischemia on stress testing, myocardial infarction or unstable angina, any history of peripheral arterial or cerebrovascular disease, or significant vascular stenosis on noninvasive imaging or angiography. Exclusion criteria also included myocardial infarction within one month (for cases), diabetes, uncontrolled hypertension (systolic blood pressure >180 mmHg or diastolic blood pressure >100 mmHg) or with end-organ damage, renal insufficiency (creatinine >2.0 mg/dL or BUN>40 mg/dL), active malignancy, significant valvular heart disease, NYHA Class III or IV heart failure, cigarette smoking >2 packs per day, total cholesterol >300 mg/dL or triglyceride >400 mg/dL, anemia (hemoglobin <12.5 g/dL for females or <13.5 g/dL for males), and hypotension (systolic blood pressure <90 mmHg and diastolic blood pressure <50 mmHg).

Blood Sampling and Gene Expression Analysis

The blood samples (2.5 mL) were collected in PAXgene™ Blood RNA tubes and total RNA was isolated using the standardized RNA Kit (PreAnalytiX, Qiagen) [19]. RNA isolation started with a centrifugation step to pellet nucleic acids in the PAXgene Blood RNA Tube. The pellet was then washed, and Proteinase K added to digest proteins. Alcohol was added to adjust binding conditions, and the sample was applied to a PAXgene RNA spin column. During a brief centrifugation, RNA selectively bound to the PAXgene silica-gel membrane and eluted using an optimized buffer. RNA was then quantified by absorbance at A260 nm and the purity was estimated by the ratio A260 nm/A280 nm. RNA integrity was confirmed by non-denaturing agarose gel electrophoresis. RNA was stored at −80°C until further analysis. The quality of 19 RNA samples was insufficient for microarray analysis due to degradation. The genome studies were conducted in the Novartis Genomics Factory, Basel, Switzerland.

Genome-wide transcript profiling was assessed using human HGU133A oligonucleotide expression probe arrays (Affymetrix, Santa Clara, CA, U.S.A.), comprising 22,483 probe sets. The experiments were done according to the recommendations of the manufacturer [20]. Data was normalized using MAS5 (Affymetrix); the data is publicly available at the Gene Expression Omnibus (GEO) repository (accession number GSE12298, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12298). As quality control, RT-PCR was performed on 8 selected genes in 2×20 subjects from the ‘matched men’ cohort.

Independent Evaluation of Predictive Gene Model in Human Aorta Tissue

To test whether the expression pattern in peripheral whole blood is representative for atherosclerosis in general, we have examined the capability of expression of genes derived from the peripheral blood cell study to predict the severity of atherosclerosis in human aortas. Gene expression data was generated using RNA extracted from a unique collection of freshly harvested human aortas with varying degrees of atherosclerosis (n = 67 donors). Donor identification, RNA extraction and micro-array methods (Affymetrix U95Av2) as well as gene expression signatures that differentiate between atherosclerotic disease states in human aortas have been reported previously [8]. As indicated in the original report, disease extent (normal, intermediate, severe) was scored by combining Sudan IV staining and raised lesion data. The “normal” or minimally diseased group showed no Sudan IV staining and contained no raised lesions, while the “intermediate” group showed more than 20% Sudan IV staining but contained no raised lesions. The “severe” group contained raised lesions covering more than 10% of the surface. We identified 20 normal, 25 intermediate and 22 severely diseased sections for this analysis.

Statistical Methods

Spearman rank correlation between CAD-index and gene expression was calculated (Partek Genomics Suite Version 6.3). An absolute correlation coefficient (rho) >0.2 was considered clinically relevant, corresponding to a p-value of 0.003 (n = 222). Among the 22,483 probe sets of the Affymetrix HGU133A chip, about 60 probe sets can be expected to have an absolute rho>0.2 by chance (false positives). Student’s t test, parametric correlation and rank correlation according to Spearman were performed with the statistical software package S-Plus Version 6.

Projections to Latent Structures (PLS) analysis including Orthogonal Signal Correction (OSC) (SIMCA-P Version 10.0) was used to identify gene sets that discriminate between increasing CAD-indices or the three classes (normal, intermediate and severe) of atherosclerosis in the aorta samples. To reduce gene selection bias, models were subsequently repeatedly built based on data from two cohorts to predict CAD in the third cohort. In addition, extensive cross-validation by leave-one-out technique and validation by response permutation was applied to 7 groups of approximately 32 subjects to reduce bias in creating a predictive gene set.

Results

Patient Demographics

Demographic data, medical history and medication of the study population are summarized in table 1. A history of hypertension
was significantly more common in the cases. Aspirin, statins, and blood pressure lowering agents were more frequently taken by the cases. All controls had no angiographically significant coronary artery disease (CAD-Index = 0). Within the cases, however, there was a wide distribution, with 81% of cases having a CAD-Index between 23 and 63. Although most cases (93%) had at least two-vessel disease or severe single-vessel disease, the distribution of cases is skewed towards the lower end of CAD-Index.

Clinical laboratory parameters were available for all subjects (table 1). Hematocrit and white blood cell counts were not significantly different. Total cholesterol and LDL-cholesterol levels were significantly lower in the coronary artery disease group, probably reflecting a higher use of statins.

**Prediction of Coronary Disease Using Risk Factors and Biochemical Markers**

Traditional risk factors, including body weight, smoking, and systolic and diastolic blood pressure did not correlate significantly with the extent of coronary disease in a rank correlation analysis. Total cholesterol (but not LDL-cholesterol) level was found to be inversely related with the CAD-index (rho = −0.41, P<0.0001), which may in part reflect the higher use of statins and better blood

**Table 1. Demographics and baseline characteristics.**

|                      | Matched Men |                      | Unmatched Men |                      | Unmatched Women |                      |
|----------------------|-------------|----------------------|---------------|----------------------|------------------|----------------------|
|                      | Controls    | Cases                | Controls      | Cases                | Controls         | Cases                |
|                      | n = 53      | n = 53               | n = 38        | n = 44               | n = 29           | n = 24               |
| Age at time of study (mean±SD) | 52±7        | 53±6                 | 51±6          | 58±7                 | <.001            | 52±7                 | 54±8                 | 0.27                  |
| Age at time of study (median, 25th–75th) | 52 (49–57)  | 52 (48–57)           | 50 (46–58)    | 55 (54–63)           | 56 (47–56)       | 54 (50–60)           | 0.15                  |
| Age at last catheterization (mean±SD)  | 51±6        | 51±7                 | 49±8          | 56±7                 | <.001            | 50±7                 | 53±7                 | 0.23                  |
| Age at last catheterization (median, 25th–75th) | 50 (47–55)  | 51 (47–56)           | 48 (44–57)    | 54 (52–62)           | 50 (45–54)       | 53 (49–57)           | 0.001                 |
| Ethnicity (Caucasian/African-American/Asian/Native Am) | 50/3/0/0/0 | 50/3/0/0/0           | 23/13/1/1/0   | 39/4/0/0/1           | 21/5/0/1/2       | 18/4/1/0/1           |                      |
| Smoking, n (%)       | 26 (49)     | 29 (55)              | 20 (53)       | 34 (77)              | <.001            | 9 (31)               | 13 (54)              | 0.02                  |
| Diabetes, n (%)      | 0 (0)       | 0 (0)                | 0 (0)         | 0 (0)                | 0 (0)            | 0 (0)                | 0 (0)                | 0 (0)                 |
| Hypertension, n (%)  | 16 (30)     | 27 (51)              | 15 (39)       | 27 (61)              | <.001            | 13 (45)              | 12 (50)              | 0.30                  |
| Myocardial infarction, n (%) | 0 (0) | 29 (55) | 2 (5) | 21 (48) | <.001 | 1 (3) | 10 (14) | <.001 |
| PCI, n (%)           | 0 (0)       | 15 (28)              | 0 (0)         | 13 (30)              | 0 (0)            | 0 (0)                | 4 (20)               | 0.001                 |
| CABG, n (%)          | 0 (0)       | 15 (28)              | 0 (0)         | 15 (34)              | 0 (0)            | 7 (29)               |                      |                      |
| Peripheral vascular disease, n (%) | 0 (0) | 2 (4) | 0 (0) | 5 (11) | 0 (0) | 3 (13) |                      |                      |
| Cerebrovascular disease, n (%) | 0 (0) | 1 (2) | 0 (0) | 4 (9) | 0 (0) | 3 (13) |                      |                      |
| Congestive heart failure, n (%) | 0 (0) | 6 (11) | 0 (0) | 4 (9) | 0 (0) | 2 (8) |                      |                      |
| Body Weight (kg) (mean±SD) | 97±19 | 94±16 | 101±19 | 94±30 | 0.27 | 89±27 | 84±24 | 0.43 |
| Systolic blood pressure (mmHg) (mean±SD) | 139±19 | 134±21 | 145±17 | 142±26 | 0.59 | 145±23 | 138±23 | 0.32 |
| Diastolic blood pressure (mmHg) (mean±SD) | 80±11 | 76±17 | 82±10 | 82±15 | 0.97 | 77±12 | 70±14 | 0.06 |
| LV ejection fraction (%) (mean±SD) | 64±7 | 56±11 | <.001 | 64±8 | 57±12 | 0.004 | 66±6 | 57±12 | 0.001 |
| Medication           |                      |                      |               |                      |                  |
| Aspirin, n (%)       | 18 (34)     | 47 (89)              | <.001         | 10 (26)              | 42 (95)          | <.001               | 6 (21)               | 21 (88)              | <.001 |
| ACE inhibitor, n (%) | 5 (9)       | 40 (75)              | <.001         | 7 (18)               | 26 (59)          | <.001               | 4 (14)               | 13 (54)              | <.001 |
| ARB, n (%)           | 3 (6)       | 0 (0)                | 0.07          | 2 (5)                | 5 (11)           | 0.03                | 0 (0)                | 5 (21)               |          |
| Beta blocker, n (%)  | 13 (25)     | 43 (81)              | <.001         | 6 (16)               | 37 (84)          | <.001               | 6 (21)               | 20 (83)              | <.001 |
| Calcium blocker, n (%) | 4 (8) | 10 (19) | <.001 | 5 (13) | 9 (20) | 0.07 | 3 (10) | 4 (17) | 0.19 |
| Statin, n (%)        | 10 (19)     | 38 (72)              | <.001         | 4 (11)               | 29 (66)          | <.001               | 5 (17)               | 15 (63)              | <.001 |
| Fibrate, n (%)       | 2 (4)       | 4 (8)                | 0.15          | 0 (0)                | 8 (18)           | 0 (0)               | 1 (4)                |                      |          |
| Clinical Laboratory parameters |                      |                      |               |                      |                  |
| Total Cholesterol (mg/dL) (mean±SD) | 196±29 | 167±32 | <0.01 | 195±39 | 177±39 | 0.06 | 204±39 | 181±52 | 0.12 |
| Triglycerides (mg/dL) (mean±SD) | 183±120 | 142±71 | 0.05 | 155±96 | 181±142 | 0.40 | 146±67 | 161±76 | 0.50 |
| LDL Cholesterol (mg/dL) (mean±SD) | 117±22 | 100±31 | <0.01 | 117±35 | 102±40 | 0.10 | 119±31 | 110±40 | 0.10 |
| HDL Cholesterol (mg/dL) (mean±SD) | 44±11 | 39±9 | 0.03 | 47±12 | 42±9 | 0.09 | 56±21 | 49±16 | 0.24 |
| HbA1c (%) (mean±SD) | 5.4±0.4 | 5.5±0.5 | 0.30 | 5.8±1.1 | 5.7±0.8 | 0.92 | 5.6±0.6 | 5.6±0.6 | 0.81 |
| Creatinine (mg/dL) (mean±SD) | 1.0±0.1 | 1.1±0.1 | 0.11 | 1.1±0.1 | 1.1±0.2 | 0.89 | 0.8±0.2 | 1.0±0.5 | 0.12 |
| Hematocrit (%) (mean±SD) | 44±5 | 43±3 | 0.15 | 43±2 | 43±3 | 0.89 | 41±3 | 40±2 | 0.03 |
| White blood cell count (10^3/L) (mean±SD) | 5.6±1.3 | 6.2±2.1 | 0.06 | 5.9±1.7 | 6.4±1.9 | 0.27 | 6.7±2.3 | 6.9±2.1 | 0.51 |

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lipid control in cases. In addition, other parameters were found to positively (potassium, blood urea nitrogen, phosphorus and osmolality) or negatively (calcium and HDL-cholesterol) correlate with CAD-index (\(\rho > 0.2\)). Of note, important clinical markers such as LDL-C (\(\rho = 0.02\)), CRP (\(\rho = -0.12\)) and homocysteine (\(\rho = 0.02\)) exhibited a poor correlation with CAD-index, which could result from treatment of affected individuals. In a multivariate correlation analysis, the combination of risk factors and biochemical markers only poorly predicted the extent of coronary artery disease (\(r^2 = 0.229\)).

**Gene Expression**

Gene expression data from 222 out of 241 subjects were available for this analysis (110/121 cases and 112/120 controls); RNA from the remaining 19 subjects did not pass quality control due to degradation.

In a univariate analysis, 160 genes were found to correlate with CAD-Index with an absolute rank correlation coefficient (\(\rho\)) >0.2 (\(P<0.003\)). All probesets correlating with CAD-Index are listed in table 2. Most of these genes are known to be involved in hematopoietic cell differentiation, cell growth or growth arrest, apoptosis, cell adhesion, matrix modulation and inflammatory and immune response, processes known to modulate atherosclerosis.

Using log-transformed data with signal intensities >80, only 19 probesets were found to be significantly differentially expressed in a multiway ANOVA (smoking, age, gender, cohort, race, CAD (i.e. case vs. control) and CAD-index as fixed factors or random effects, respectively) (table 2). However, when only taking the 20 controls with the least predicted CAD versus the 20 cases with the most predicted disease into account, a formal comparison yielded 90 out of the 160 probesets with statistically significant differential expression (\(p<0.05\), no adjustment for multiple comparisons) (table 2). rt-PCR confirmed the Affymetrix results for 7 of the 8 genes tested in 20 cases and 20 controls (FKBP8, ITPK1, MARCH2, PNPLA2, TUBA3, UBXD1, FTL); the remaining gene (PINK1) did not show a significantly different expression on rt-PCR.

**Correlation of Gene Expression Profile with Coronary Disease**

All 160 genes with \(\rho > 0.2\) were included in the PLS analysis, with CAD-Index as the only response variable. Polynomial regression analysis of the resulting t1-scores versus CAD-Index resulted in the prediction model including 95% confidence range of the regression and the 95% prediction interval with \(r^2 = 0.764\) (\(p<0.001\)) (figure 1). Predictive accuracy was found to be excellent in the overall population (RMSEE (root mean square error of estimation) = 0.323), but improved with increasing threshold of CAD (RMSEE = 0.249 for controls vs cases with CAD>40; RMSEE = 0.204 for controls vs cases with CAD>60 and RMSEE = 0.172 for controls vs cases with CAD>70).

In order to test for robustness of the model, the PLS analysis was performed separately for each of the three cohorts, with the model repeatedly constructed using two cohorts (training sample) and tested in the third cohort (test sample). While the controls remain quite stable in the range of -2 standard deviations, the t1-scores of the cases were located mainly in the +2 standard deviation range and increase with increasing CAD-Index (figure 2). This relationship is clearly present in each cohort. Cross-validation of the model was also performed by dividing the data into 7 groups of on average 32 subjects and then developing a number of parallel models from reduced data with one of the groups deleted. The omitted group was then used as a test data set, and the differences between actual and predicted CAD-Indices were subsequently calculated for these data points. The reduced models validation demonstrated a \(Q^2\) cum of 0.776, indicating an excellent predictive ability.

A Variable Importance in the Projection (VIP) of each gene for the separate PLS analyses of the three cohorts compared to the PLS analysis including all subjects was calculated. The VIP of the first 24 genes shows only little variation between the three cohorts suggesting a rather high stability of the prediction model (figure 3). A set of eight genes appears to have the highest impact on the model (FTL, FKBP8, TUBA3, PNPLA2, UBXD1, MARCH2, ITPK1, PINK1, in order of contribution; listed in bold in table 2). A PLS analysis only involving these eight highest ranking genes in the VIP analysis showed that the expressions profiles of these eight genes are also able to predict the CAD-Index (\(r^2 = 0.752\)). Adding traditional risk factors and biochemical markers do not significantly improve this model (\(r^2 = 0.782\)).

**Test of Predictability in Human Aorta Tissue Samples**

Since the genes whose expression contributes to prediction of CAD were studied within circulating leukocytes, we sought to define whether they actually reflect a molecular process that is ongoing within atherosclerotic arteries or not. Furthermore, as a test of reproducibility of the contribution of these 160 genes to predicting atherosclerotic disorders, we have investigated whether the in situ expression pattern of our 160 genes derived from peripheral blood could also adequately predict the severity of aorta atherosclerotic lesions. To achieve this goal, we have used gene expression data extracted from a large set of human aortas obtained from heart donors (\(n = 67\)), an independent human model of atherosclerosis. Excluding genes that are not present on the microarray used in the aorta expression study, the expression pattern of the remaining genes accurately separated the aorta samples according to the severity of atherosclerosis (figure 4). These results indicate that gene expression changes in peripheral blood are correlated with the extent of coronary atherosclerosis.

**Discussion**

In this large-scale expression analysis of peripheral whole blood cells, we have found 160 genes whose expression correlates with the severity of angiographically documented coronary artery atherosclerosis. Taking into account that the CAD-Index is a semi-quantitative estimate of the extent of coronary atherosclerotic disease, which implies variation across subjects even with the same degree of disease, the prediction based on expression pattern of these genes is robust. Our findings are also robust as assessed by internal validation and consistency across three distinct subgroups. Importantly, the in situ expression pattern of the 160 genes derived from the peripheral blood analysis was also predictive of the severity of atherosclerosis in human aorta tissue. This provides validation of the association of this set of genes with atherosclerosis and support for the concept that peripheral blood gene expression reflects pathophysiology in the vascular wall. Taken together, the molecular signature in peripheral blood for varying degrees of coronary artery disease is remarkably consistent with that seen in the atherosclerotic arterial wall, providing valuable new information of the pathways and their genes that are involved in the atherosclerotic process.

Peripheral blood is easily accessible and routinely used for diagnostic laboratory analysis and thus is a good resource for additional tests that might define extent of coronary artery disease. Several inflammatory markers, including high sensitivity C-
### Table 2. List of 160-gene model predictive of the extent of coronary artery disease.

| Symbol | U133A ID | 95Av2 ID | Name                                                                 | Pathway                     | Rho  |
|--------|----------|----------|----------------------------------------------------------------------|------------------------------|------|
| AIF1   | 207823_s_at | 37011_at 33641_g_at 3640_at 37764_at | allograft inflammatory factor 1 | Angiogenesis                  | 0.21 |
| MMP19 | 204575_s_at | 3640_at 37764_at | matrix metalloproteinase 19 | Angiogenesis                  | 0.22 |
| EPM   | 207346_at |          | epimorphin | Response to metal ion | Extracellular matrix modulation | 0.22 |
| MMP24 | 78047_s_at |          | Matrix metalloproteinase 24 (membrane-inserted) | Angiogenesis                  | 0.20 |
| CADD   | 209833_at | 822_s_at 1211_s_at | CASP2 and RIPK1 domain containing adaptor with death domain | Apoptosis                     | 0.25 |
| WDR13  | 222138_s_at | 727_at | WD repeat domain 13 | Apoptosis                     | 0.24 |
| PDE4D  | 210837_s_at | 38526_at | phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila) | Apoptosis                     | 0.22 |
| AK2    | 212174_at | 40789_at 40788_at | adenylate kinase 2 | Apoptosis                     | 0.28 |
| FOLH1  | 217487_x_at | 1740_g_at 1739_at 1655_s_at | folate hydrolase (prostate-specific membrane antigen) 1 | Apoptosis                     | 0.22 |
| TGM5   | 207911_s_at | 33001_s_at | transglutaminase 5 | Apoptosis                     | 0.24 |
| PSAP1  | 220403_s_at |          | p53-regulated apoptosis-inducing protein 1 | Apoptosis                     | 0.25 |
| NALP1  | 211822_s_at |          | NACHT, leucine rich repeat and PYD (pyrin domain) containing 1 | Apoptosis                     | 0.26 |
| LRGAL5 | 203236_s_at | 766_at 38091_at | galectin 9 | Cell adhesion                | 0.25 |
| ICAM1  | 202637_s_at | 32640_at | intercellular adhesion molecule 1 (ICD54), human rhinovirus receptor | Cell adhesion                | 0.21 |
| PCDHG3 | 205717_s_at | 657_at 35609_at 1691_at 1690_at 1169_at | protocadherin gamma subfamily C, 3 | Cell adhesion                | 0.20 |
| GLPLD1 | 206265_s_at | 934_at 1293_s_at | glycosylphosphatidylinositol specific phospholipase D1 | Cell adhesion                | 0.21 |
| CDH11  | 207173_x_at | 36976_at 2087_s_at | cadherin 11, type 2, OB-cadherin (osteoblast) | Cell adhesion                | 0.24 |
| DSC3   | 206032_at | 32417_at | desmocollin 3 | Cell adhesion                | 0.20 |
| LAMB3  | 209270_at | 36929_at | laminin, beta 3 | Cell adhesion                | 0.25 |
| PKP4   | 214874_at | 33475_at | plakophilin 4 | Cell adhesion                | 0.22 |
| FN1    | 214702_at |          | Fibronectin 1 | Cell adhesion                | 0.21 |
| Ile45  | 48659_at |          | IGFBP-2-Binding Protein, Ile45 (FLJ12438) | Cell adhesion                | 0.22 |
| PINK1  | 209018_s_at | 35361_at | PTEN induced putative kinase 1 | Cell growth & growth arrest | 0.22 |
| FKBPH8 | 40850_at | 40850_at | FK506 binding protein 8, 38kDa | Cell growth & growth arrest | 0.25 |
| UBXD1  | 220757_s_at |          | UBX domain-containing protein 1 | Cell growth & growth arrest | 0.21 |
| RXRA   | 202426_s_at | 405_at 32800_at | retinoid X receptor, alpha | Cell growth & growth arrest | 0.24 |
| RIS1   | 213338_at | 35692_at | Ras-induced senescence 1 | Cell growth & growth arrest | 0.28 |
| NFYC   | 202215_s_at | 40466_at | nuclear transcription factor Y, gamma | Cell growth & growth arrest | 0.30 |
| CLN3   | 209275_s_at | 497_at | ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease) | Cell growth & growth arrest | 0.27 |
| RARA   | 211605_s_at | 1337_s_at | retinoic acid receptor, alpha | Apoptosis                     | 0.26 |
| HCFC1  | 202473_x_at | 37910_at | host cell factor C1 (VP16-accessory protein) | Cell growth & growth arrest | 0.23 |
| PSG3   | 203399_x_at | 40857_f_at | pregnancy specific beta-1-glycoprotein 3 | Cell growth & growth arrest | 0.22 |
| Symbol | U133A ID  | 95Av2 ID  | Name                                                                 | Pathway                                      | Rho |
|--------|-----------|-----------|----------------------------------------------------------------------|----------------------------------------------|-----|
| STA2   | 204226_at | 38341_at  | staufen, RNA binding protein, homolog 2 (Drosophila)                 | Cell growth & growth arrest                  | 0.26|
| STAU2  | 208427_s_at | 36411_at  | ELAV (embryonic lethal, abnormal vision, Drosophila)-like            | Cell growth & growth arrest                  | 0.25|
| TP53I1 | 214667_s_at | 36136_at  | tumor protein p53 inducible protein 11                               | Cell growth & growth arrest                  | 0.31|
| NPR3   | 219789_at | 34519_at  | natriuretic peptide receptor C/guanylate cyclase C                  | Cell growth & growth arrest                  | 0.21|
|        |           |           |                                                                    | Cell growth & growth arrest                  |     |
| PTP4A1 | 200730_s_at | 843_at    | protein tyrosine phosphatase type IVA, member 1                      | Cell growth & growth arrest                  | 0.27|
| STC2   | 203439_s_at | 32043_at  | stanniocalcin 2                                                      | Cell growth & growth arrest                  | 0.24|
| SEMA3C | 203788_s_at | 377_g_at  | sema domain, immunoglobulin domain (lg), short basic domain, secreted, (semahorin) 3C | Cell growth & growth arrest                  | 0.28|
| CCNA1  | 205899_at | 1914_at   | cyclin A1                                                            | Cell growth & growth arrest                  | 0.20|
| PTPRH  | 206084_at | 1658_g_at | protein tyrosine phosphatase, receptor type, R                       | Cell growth & growth arrest                  | 0.23|
| LHX2   | 206140_at | 40528_at  | LIM homeobox 2                                                       | Cell growth & growth arrest                  | 0.21|
|        |           |           |                                                                    | Cell growth & growth arrest                  |     |
| CPSF4  | 206688_s_at | 35743_at  | cleavage and polyadenylation specific factor 4, 30kDa                | Cell growth & growth arrest                  | 0.22|
|        |           |           |                                                                    | Inflammatory response                        |     |
| i-4   | 207377_at | 31735_at  | type 1 protein phosphatase inhibitor                                 | Cell growth & growth arrest                  | 0.21|
| MEIS2  | 207480_s_at | 41388_at  | Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse) | Cell growth & growth arrest                  | 0.20|
| NF2    | 211092_s_at | 38007_at  | neurofibrin 2 (bilateral acoustic neuroma)                           | Cell growth & growth arrest                  | 0.23|
|        |           |           |                                                                    | Cytoskeleton                                 |     |
| BRRN1  | 212949_at | 41639_at  | barren homolog (Drosophila)                                          | Cell growth & growth arrest                  | 0.32|
| CDC42  | 214230_at | 960_g_at  | cell division cycle 42 (GTP binding protein, 25kDa)                 | Cell growth & growth arrest                  | 0.23|
| ZMYND10 | 216663_s_at | 32993_s_at | zinc finger, MYND domain containing 10                               | Cell growth & growth arrest                  | 0.25|
| CRO4   | 222301_at | 40468_s_at | transcripational activator of the c-fos promoter                    | Cell growth & growth arrest                  | 0.22|
| PPP2R5B| 635_s_at  | 635_s_at  | protein phosphatase 2, regulatory subunit B (BS6), beta isoform      | Cell growth & growth arrest                  | 0.20|
|        |           |           |                                                                    | Immune response                              |     |
| TRIM45 | 219923_at | 41639_at  | tripartite motif-containing protein 45                               | Cell growth & growth arrest                  | 0.23|
| TDRKH  | 221053_s_at | 959_at    | tudor and KH domain containing                                       | Cell growth & growth arrest                  | 0.26|
| PB1    | 221212_x_at | polybromo 1 |                                                                     | Cell growth & growth arrest                  | 0.25|
| NEIL1  | 219396_s_at | 39736_at  | nea endonuclease VIII-like                                          | Cell growth & growth arrest                  | 0.31|
|        |           |           |                                                                    | Cytoskeleton                                 |     |
| PMS2L5 | 2179_at   | 179_at    | Postmeiotic segregation increased 2-like 5                           | Cell growth & growth arrest                  | 0.24|
| BLOC1S | 202592_at | 635_s_at  | biogenesis of lysosome-related organelles complex-1, subunit 1       | Cell growth & growth arrest                  | 0.23|
| BRF2   | 218955_at | 40462_s_at | subunit of RNA polymerase III transcription initiation factor, BRF1-like | Cell growth & growth arrest                  | 0.23|
| ASNA1  | 202024_at | 38188_s_at | arsenical pump-driving ATPase                                        | Cell growth & growth arrest                  | 0.21|
| SRT5   | 221010_s_at | 40462_s_at | transcriptional activator of the c-fos promoter                    | Cell growth & growth arrest                  | 0.22|
|        |           |           |                                                                    | Cell growth & growth arrest                  |     |
| HIST1H4G | 208551_at | 38188_s_at | histone 1, H4g                                                       | Cell growth & growth arrest                  | 0.27|
| SLC5   | 211767_at | 38188_s_at | subunit of RNA polymerase III transcription initiation factor, BRF1-like | Cell growth & growth arrest                  | 0.27|
| MAN2A2 | 202032_s_at | 41766_at  | mannosidase, alpha, class 2A, member 2                               | Cell-cell interaction                        | 0.28|
| GJB1   | 215243_s_at | 41076_at  | gap junction protein, beta 3, 31kDa (connexin 31)                    | Cell-cell interaction                        | 0.24|
| PLXNA2 | 207290_at | 40395_at  | plexin A2                                                            | Cell-cell interaction                        | 0.21|
| ADH1B  | 209613_s_at | 35730_at  | alcohol dehydrogenase IB (class I), beta polypeptide                | Cell-cell interaction                        | 0.22|
|        |           |           |                                                                    | Immune response                              |     |
| FGA    | 205650_s_at | 38825_at  | fibrinogen, A alpha polypeptide                                       | Coagulation                                  | 0.26|
|        |           |           |                                                                    | Cell adhesion                                |     |
| Symbol | U133A ID | 95Av2 ID | Name | Pathway | Rho |
|--------|----------|----------|------|---------|-----|
| SERPINB8 | 206034_at | 36312_at | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 8 | Inflammatory response | 0.23 |
| TUBA3 | 212639_x_at | 32272_at | tubulin alpha 3 | Cytoskeleton | 0.28 |
| ADD1 | 214726_x_at | 32146_s_at | adducin 1 (alpha) | Cytoskeleton | 0.21 |
| ARHGAP4 | 204425_at | 39649_at | Rho GTPase activating protein 4 | Cytoskeleton | 0.23 |
| LGL1 | 206123_at | 804_s_at | lethal giant larvae homolog 1 (Drosophila) | Cytoskeleton | 0.22 |
| SERPINB8 | 206034_at | 36312_at | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 8 | Inflammatory response | 0.23 |
| TUBA3 | 212639_x_at | 32272_at | tubulin alpha 3 | Cytoskeleton | 0.28 |
| ADD1 | 214726_x_at | 32146_s_at | adducin 1 (alpha) | Cytoskeleton | 0.21 |
| ARHGAP4 | 204425_at | 39649_at | Rho GTPase activating protein 4 | Cytoskeleton | 0.23 |
| LGL1 | 206123_at | 804_s_at | lethal giant larvae homolog 1 (Drosophila) | Cytoskeleton | 0.22 |
| KRT6A | 209125_at | 39016_r_at | keratin 6A | Cytoskeleton | 0.28 |
| SMXP | 219772_s_at | | small muscle protein, X-linked | Cytoskeleton | 0.20 |
| STXBP2 | 209367_at | 38259_at | syntaxin binding protein 2 | Cytoskeleton - Exocytosis | 0.25 |
| PLUR | 211924_s_at | 189_s_at | plasminogen activator, urokinase receptor | Extracellular matrix modulation | 0.20 |
| COL13A1 | 211809_s_at | | collagen, type XIII, alpha 1 | Extracellular matrix modulation | 0.25 |
| ABCG2 | 214033_at | | Up-regulated gene 7 | Extracellular matrix modulation | 0.26 |
| ITPK1 | 210740_s_at | 35715_at | inositol 1,3,4-triphosphate 5/6 kinase | Hematopoietic cell differentiation | 0.30 |
| FTL | 212788_x_at | 35083_at | ferritin, light polypeptide | Hematopoietic cell differentiation | 0.24 |
| FANCC | 205187_at | 39517_s_at | Fanconi anemia, complementation group C | Hematopoietic cell differentiation | 0.23 |
| SMAD5 | 205187_at | 1952_s_at | | Hematopoietic cell differentiation | 0.22 |
| NOTCH2 | 202445_s_at | 38083_at | Notch homolog 2 (Drosophila) | Hematopoietic cell differentiation | 0.20 |
| GATA1 | 210446_at | 36787_at | GATA binding protein 1 (globin transcription factor 1) | Hematopoietic cell differentiation | 0.25 |
| LSR | 217212_s_at | 938_at | interleukin 9 receptor | Hematopoietic cell differentiation | 0.20 |
| KRAS | 219657_s_at | | | Hematopoietic cell differentiation | 0.24 |
| NFX1 | 202585_s_at | 34667_at | nuclear transcription factor, X-box binding 1 | Hematopoietic cell differentiation | 0.26 |
| EPHB2 | 211165_x_at | 902_s_at | EPH receptor B2 | Hematopoietic cell differentiation | 0.23 |
| PRKAR1B | 212559_at | 1091_at | protein kinase, cAMP-dependent, regulatory, type I, beta | Hematopoietic cell differentiation | 0.25 |
| PAFH18 | 200815_s_at | 32569_at | platelet-activating factor acetylhydrolase, isoform lb, alpha subunit 45KDa | Hematopoietic cell differentiation | 0.27 |
| NFX1 | 202585_s_at | 34667_at | nuclear transcription factor, X-box binding 1 | Hematopoietic cell differentiation | 0.26 |
| Symbol | U133A ID | 95Av2 ID | Name | Pathway | Rho |
|--------|---------|---------|------|---------|-----|
| KCNMB1 * | 209948_at | 38298_at | potassium large conductance calcium-activated channel, subfamily M, beta member 1 | Ion channel | 0.22 |
| CHRNA5 | 206533_at | 36397_at | cholinergic receptor, nicotinic, alpha polypeptide 5 | Ion channel | 0.21 |
| SAH | 210377_at | 33279_s_at, 33278_at | SA hypertension-associated homolog (rat) | Lipid metabolism | 0.30 |
| HCLS1 | 207833_s_at | 37764_at | holocarboxylase synthetase (biotin-(propionyl-Coenzyme A-carboxylase (ATP-hydrolysing)) ligase | Metabolic homeostasis | 0.21 |
| MAOA | 204388_s_at | 41772_at, 41771_g_at, 41770_at | monoamine oxidase A | Neurotransmission | 0.28 |
| GABRA6 * | 207182_at | 34025_at | gamma-aminobutyric acid (GABA) A receptor, alpha 6 | Neurotransmission | 0.23 |
| CEPBA | 204039_at | 32550_r_at | CCAAT/enhancer binding protein (C/EBP), alpha | Progenitor cell differentiation | 0.35 |
| SOX4 * | 201416_at | 33131_at | SRY (sex determining region Y)-box 4 | Cell growth & growth arrest | 0.20 |
| ZNF305 | 206507_at | 37083_s_at, 37082_at | zinc finger protein 305 | Progenitor cell differentiation | 0.21 |
| ZNF1A2 * | 220567_at | 41771_g_at | zinc finger protein, subfamily 1A, 2 | Progenitor cell differentiation | 0.23 |
| ZNF3 * | 219605_at | 41770_at | zinc finger protein 3 (A8–51) | Progenitor cell differentiation | 0.22 |
| CAPN5 * | 205166_at | 38504_at | calpain 5 | Response to injury | 0.25 |
| MTF1 | 205323_s_at | 38945_at | metal-regulatory transcription factor 1 | Response to metal ion | 0.25 |
| CA12 | 203963_at | 36454_at | carbonic anhydrase XII | Response to metal ion | 0.26 |
| NEDD4L | 212445_s_at | 39356_at | neural precursor cell expressed, developmentally down-regulated 4-like | Response to metal ion | 0.22 |
| CABIN1 * | 202624_s_at | 37652_at | calcineurin binding protein 1 | T/B cell proliferation | 0.21 |
| SH3BP2 | 209370_s_at | 1303_at | SH3-domain binding protein 2 | T/B cell proliferation | 0.23 |
| TNFRSF5 | 35150_at | 35150_at, 35149_at | tumor necrosis factor receptor superfamily, member 5 | T/B cell proliferation | 0.22 |
| HDAC5 * | 202455_at | 38295_at | histone deacetylase 5 | T/B cell proliferation | 0.28 |
| PIP | 206509_at | 41094_at, 325_s_at | prolactin-induced protein | Inflammatory response | 0.23 |
| GAD2 * | 216651_s_at | 32280_at, 32279_at | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | T/B cell proliferation | 0.21 |
| PNPLA2 * | 39854_r_at | 39854_r_at | patatin-like phospholipase domain containing 2 | Triglyceride homeostasis | 0.24 |
| MGLL | 211026_s_at | 35792_at | monoglyceride lipase | Triglyceride homeostasis | 0.27 |
| MJD | 216657_at | 36819_at | Machado-Joseph disease (spinocerebellar ataxia 3, olivopontocerebellar ataxia 3, autosomal dominant, ataxin 3) | Ubiquitination | 0.2 |
| FBXO31 | 219784_at | 37652_at | F-box only protein 31 | Ubiquitation | 0.26 |
| GGA3 | 218115_s_at | 37959_at | golgi associated, gamma adaptin ear containing, ARF binding protein 3 | Ubiquitilation | 0.26 |
| N4BP1 | 48612_at | 37891_at | Nedd4 binding protein 1 | Ubiquitilation | 0.21 |
| BC002942 | 31837_at | 31837_at | hypothetical protein BC002942 | Ubiquitilation | 0.28 |
| MGC21416 | 212340_at | 37891_at | hypothetical protein MGC21416 | Ubiquitilation | 0.27 |
| DHRF2p586F1822 | 37891_at | 37891_at | DHRF2p586F1822 | Ubiquitilation | 0.28 |
| CMT1A | 215999_at | 31781_at | CMT1A duplicated region transcript 1 | Ubiquitilation | 0.23 |
| KIAA0241 | 212475_at | 39761_at | KIAA0241 protein | Ubiquitilation | 0.28 |
reactive protein (CRP) are associated with cardiovascular risk, independently from traditional risk factors [21]. Nevertheless, there is debate as to the additional prognostic value of these tests beyond traditional risk factors [22]. Other non-invasive analyses, such as coronary multislice CT can identify the extent of coronary artery disease, but such tests require specialized equipment and involvement of intravenous contrast and radiation. A simple blood test that predicts the extent of coronary artery disease could involve use of intravenous contrast and radiation. A simple blood test that predicts the extent of coronary artery disease could involve use of intravenous contrast and radiation. A simple blood test that predicts the extent of coronary artery disease could involve use of intravenous contrast and radiation. A simple blood test that predicts the extent of coronary artery disease could involve use of intravenous contrast and radiation. 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peripheral cells of CAD patients might in at least in part reflect a general pro-inflammatory state that leads to degenerative changes [49–52].

We intentionally did not separate peripheral blood cells or leukocyte subtypes. There is currently little pathophysiological evidence that the study of leukocyte subgroups would add to our predictive model and the isolation process could, in itself, affect the gene expression pattern. Using whole blood cells not only allows aggregate RNA expression analysis per patient without the need to pool rare subtypes, but is also more practical from a clinical perspective. Leukocyte levels in all groups were very similar, although it cannot be excluded that the percentage of specific subtypes differ between groups, and hence that different numbers of subtypes are responsible for the observed effect. Peripheral whole blood might also include differential expression signatures from reticulocytes, platelets or rare hematopoietic progenitors.

In a recent paper, Wingrove et al reported 526 differentially expressed genes (>1.3-fold expression) from a genome-wide microarray analysis of peripheral blood mononuclear cells of 27 cases with angiographically documented CAD and 14 controls [15]. The authors found that 14 genes, out of a set of 106 genes including the 50 most significant genes from the microarray analysis and 56 genes selected from the literature, were associated with the presence of CAD and the severity of CAD in two independent cohorts. The overlap between our study and the Wingrove study at the individual gene level appears to be very limited. This might be in part due to the considerably different design of our study. Not only did we prefer a correlation-based approach, the Wingrove study also used a much smaller subset of patients for unbiased microarray-based gene discovery, and added 56 literature-based genes for the subsequent analysis in their two cohorts. As a result of our correlation analysis, we also did not exclude genes with differential expression below 1.3-fold; since atherosclerosis is a chronic disease, small changes in gene expression might accrue over time and result in a clinically relevant phenotype. Moreover, in contrast with our study, a substantial proportion of microarray samples in the Wingrove analysis were taken from patients presenting with an acute coronary syndrome, which might have significantly influenced expression levels. Another reason for the discrepancies between the two studies might be the different types of microarray used and different types of cells studied. In our study, we analyzed RNA from whole blood in all patients, in contrast with isolated mononuclear cells used in the discovery phase of the Wingrove study. An Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, Ca; USA) comparing the 366 genes with p < 0.05 (from the 526 probesets) and our 160 genes with rho > 0.2 shows that similar biological functions were hit, despite the different microarrays and different matrices used (data not shown). In any case, the discrepancies between both studies suggest that these results need to be validated in larger and more diverse populations.

Of the 160 genes we found to be correlated with the extent of CAD, only 19 were significantly differentially expressed between all cases and controls, while gene expression was significantly different for 90 genes when comparing 20 patients with the least predicted CAD-index to 20 patients with the highest predicted CAD-index. Most of our cases only have mild to moderate disease, with only a minority having extensive disease. Thus, in part as a result of our proteomics-driven patient selection, there is likely to be a very gradual transition from controls to cases, with the distribution of cases being skewed towards the lower end of CAD-index. We therefore assumed that the difference between controls and cases was not likely to be very large, hence our preference for a correlation-based analysis. Furthermore, since the average age of the controls was 52 years, it is highly likely that some degree of coronary atherosclerosis is present in these subjects. Interestingly,
patients with normal angiograms but with microvascular dysfunction may also demonstrate peripheral monocyte activation, although not to the extent seen in patients with angiographically documented coronary artery disease [53]. Our findings that the present model also accurately predicts the severity of coronary artery disease in female patients, in whom advanced coronary artery disease is less likely at the age of 50, is reassuring. It is notable that CRP and LDL did not predict disease in our population. However, while these are excellent markers for future cardiovascular events [54], their ability to predict the severity of angiographically documented CAD is known to be low [55–58]. We even observed an inverse correlation between LDL-cholesterol levels and CAD-index. This might be at least in part due to differences in treatments, especially in statin use. Statins might indeed blunt gene expression differences in vascular cells and circulating monocytes to certain extent, which might have influenced our findings [59,60].

In conclusion, the combined predictive value of differentially expressed genes in peripheral blood correlates with the extent of coronary atherosclerosis. Importantly, the expression pattern of the same genes is also correlated with the extent of disease in atherosclerotic aortas. While these findings need prospective validation in further populations, our findings also suggest that gene expression profiles might represent a novel and promising non-invasive test to assess the presence and extent of coronary artery disease. Although the extent of angiographic disease is a strong predictor of clinical outcome, further studies in larger and unselected populations will also be needed to examine the

Figure 2. Partial least squares plot per cohort. Results of the partial least squares regression analysis with 160 genes applied separately to each of the three cohorts (A) “Matched Men” (B) “Unmatched Men” and (C) “Unmatched Women”. Models were each time constructed in two cohorts and then tested in the third cohort. Individual patients are ordered by their CAD-index. Labels represent the individual CAD-index. Controls (full line) have all CAD-index 0, and the CAD-index of cases (dotted line) increases from 23 up to 100. While the controls remain quite stable in the range of -2 standard deviations, the t1-scores increase with increasing CAD-index (t1 indicates the t1 score vector result from the PLS analysis).

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**Figure 3. VIP.** Variable Importance in the Projection (VIP) for the separate PLS analyses of the three different cohorts compared to the PLS analysis including all subjects. Displayed are the 24 probesets with the highest VIP. The curve shows a steep decrease for the first 8 genes (listed in table 2); the contribution of further genes is comparable as suggested by almost linear curves.

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**Figure 4. Partial least squares discriminant analysis in atherosclerotic aortas.** Result of the partial least squares discriminant analysis (t1/t2 score plot) including all aorta samples; n = 67. Dots represent normal aortas, squares represent intermediate atherosclerosis and diamonds indicate severe aorta atherosclerosis. Using expression data in aorta samples, the PLS analysis using the 160 peripheral blood genes adequately separates normal aortas from intermediate and severe atherosclerotic aortas (the ellipse indicates Hotelling’s T2 95% confidence region; t1 and t2 indicate the t1 and t2 score vector results from the PLS-DA analysis).

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potential role of gene expression patterns in predicting outcome and to address potential confounding factors.

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Author Contributions
Conceived and designed the experiments: PRS MDG DV WEK GSG PJGC CBG. Performed the experiments: MDG DV MHSJ GSG PJGC CBG. Analyzed the data: PRS PG DV WEK CLN CBG. Contributed reagents/materials/analysis tools: MDG DV MHSJ. Wrote the paper: PRS PJGC CBG.
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