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Aspirin Exposure Reveals Novel Genes Associated with Platelet Function and Cardiovascular Events

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Abstract

Objectives—To develop RNA profiles that could serve as novel biomarkers for the response to aspirin.

Background—Aspirin reduces death and myocardial infarction (MI) suggesting that aspirin interacts with biological pathways that may underlie these events.

Methods—We administered aspirin, followed by whole blood RNA microarray profiling, in a discovery cohort of healthy volunteers (HV1,n=50), and two validation cohorts of volunteers (HV2,n=53) or outpatient cardiology patients (OPC, n=25). Platelet function was assessed by platelet function score (PFS; HV1/HV2) or VerifyNow Aspirin (OPC). Bayesian sparse factor analysis identified sets of coexpressed transcripts, which were examined for association with PFS in HV1 and validated in HV2 and OPC. Proteomic analysis confirmed the association of validated

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Relationships with Industry:

- GSG (Consultant to United States Diagnostic Standards, Scientific Advisor to CardiDx, Pappas Ventures, and Universal Medicine, and Equity in CardioDx)
- TAM (Equity in Cellgenex)
- LKN (found online: https://www.dcri.org/about-us/conflict-of-interest/Newby-COI_2012-2013.pdf)
- The following authors (DV, GSG, JTC, JEL, RCB, TLO) have filed a provisional patent application regarding the Aspirin Response Signature

transcripts in platelet proteins. Validated gene sets were tested for association with death/MI in two patient cohorts (n=587, total) from RNA samples collected at cardiac catheterization.

Results—A set of 60 co-expressed genes named the “aspirin response signature” (ARS) was associated with PFS in HV1 ($r = -0.31$, $p = 0.03$), HV2 ($r = -0.34$, Bonferroni $p = 0.03$), and OPC ($p = 0.046$). Corresponding proteins for 17 ARS genes were identified in the platelet proteome, of which, six were associated with PFS. The ARS was associated with death/MI in both patient cohorts (odds ratio = 1.2, $p = 0.01$ and hazard ratio = 1.5, $p = 0.001$), independent of cardiovascular risk factors. Compared with traditional risk factors, reclassification (net reclassification index = 31 - 37%, $p \leq 0.0002$) was improved by including the ARS or one of its genes, *ITGA2B*.

Conclusions—RNA profiles of platelet-specific genes are novel biomarkers for identifying those do not response adequately to aspirin and who are at risk for death/MI.

Keywords

aspirin; platelets; genes; myocardial infarction; biomarkers

Introduction

Identification of novel biomarkers for individuals at risk for CAD mortality, primarily due to platelet-mediated cardiovascular events such as MI, is a priority for reducing the burden of cardiovascular disease. Although genome-wide surveys of genomic variation and gene expression can identify loci associated with CAD (1-3), few can serve as biomarkers for cardiovascular events(4).

Aspirin is prescribed for the prevention of cardiovascular events, suggesting that aspirin interacts with biological pathways that may underlie these events. Platelet function assays are a surrogate biomarker for the effects of aspirin and are associated with cardiovascular events.(5) However, platelet function testing is not widely available primarily due to technical complexity. In contrast, whole blood RNA profiling using PCR-based assays is currently a widely available diagnostic testing platform. (6,7) Therefore, we hypothesized that aspirin could be used as a probe in conjunction with whole blood RNA profiling to elucidate novel biomarkers for platelet function in response to aspirin and for cardiovascular outcomes.

Methods

Platelet Function Outcomes in Healthy Volunteers Cohorts at Duke University Medical Center (DUMC)

We previously described (8) discovery and validation healthy volunteer cohorts (HV1 and HV2, Supplemental Methods, Figure 1) and the platelet function score (PFS) - a composite metric of the following platelet function assays: PFA100 (collagen/epinephrine) closure time and the areas under the optical aggregometry curve induced by adenosine diphosphate (10, 5, 1uM), epinephrine (10, 1, 0,5 uM), and collagen (5, 2 mg/ml). We measured the PFS and mean platelet volume (MPV) in HV1 (n = 50) after 2 weeks of dosing with 325 mg/day non-

enteric coated, immediate release aspirin and HV2 (n = 53) after 4 weeks of dosing with 325 mg/day aspirin. In both cohorts whole blood RNA was collected into PAXgene® Blood RNA tubes (Becton, Dickinson, NJ, USA) before after aspirin exposure and stored at −80 C until microarray profiling. Platelet count was measured in platelet rich plasma in HV1.

Because three subjects in HV2 had participated in HV1, these were dropped from HV2, leaving 50 unique HV2 subjects. DUMC IRB approved the study protocols.

Platelet Function Outcomes in Patients At Risk For Cardiovascular Events At George Washington University (GWU)

We previously described (9) an outpatient cardiology cohort (OPC, Supplemental Methods, Figure 1) treated with 81mg/day aspirin assessed with the VerifyNow Aspirin device and whole blood RNA microarray analysis.

Clinical Outcomes in DUMC Patients

CATHGEN biorepository—The Catheterization Genetics (CATHGEN) biorepository has banked, whole blood RNA in PAXgene® tubes from DUMC patients from the time of cardiac catheterization, baseline medical history, and follow up for all-cause death and MI. (10,11) Two cohorts had available microarray data (Supplemental Methods, Figure 2):

Observational cohort: 224 sequential samples were selected for RNA analysis, of which, 191 had sufficient RNA for microarray analysis.

Case:control cohort: A nested case:control cohort of participants who had experienced death or MI (n = 250) after their index catheterization and age-, sex-, and race-matched controls (n = 250) who were free of death/MI > 2 years after cardiac catheterization was identified.(12) 447 had sufficient RNA for microarray analysis; 44 overlapped with the observational cohort and were dropped, leaving 403 subjects for analysis.

Follow-up for death/MI was ascertained in both cohorts in October 2011; the median follow-up was 3.8 years. Patients with incomplete follow-up were censored at the time of last contact. Patients who had a history of cardiac transplantation at the time of catheterization (n =5), died within seven days (n =1), or failed quality control (n =1) were excluded. The remaining datasets left 190 samples in the observational cohort (48 death/MI events) and 397 (202 death/MI events) in the case-control cohort.

RNA extraction, labeling, microarray hybridization, quality control, and normalization

See Supplementary Methods for full details. Two microarray platforms were utilized: Affymetrix U133A2 array (HV-1, pre-aspirin) and U133 plus 2.0 array (all others). The Robust Multichip Average (RMA) method was used for normalization.

Real-Time PCR

See Supplementary Methods. Forty-five transcripts were selected for verification in the original RNA samples based on two criteria: 1) the strength of correlation of the probe set

with PFS and 2) the strength of membership between the probe set and the set of co-expressed genes of interest.

Platelet purification, Protein Sample Preparation, and Proteomics Analysis by LC-MS/MS

See Supplemental Methods.

Statistical Analysis

The raw and normalized microarray data are available in the Gene Expression Omnibus for the OPC cohort (GSE38511). The data for the HV1, HV2, and CATHGEN cohorts is available through the database of Genotypes and Phenotypes (phs000548.v1.p1 and phs000551.v1.p1). Unless stated otherwise, all tests were two-sided and were performed in R (2.10.0) or Matlab (R2010b); a p-value of < 0.05 was considered significant.

Discovery of coexpressed gene sets associated with PFS - Factor Modeling— The HV1, post-aspirin RMA normalized data were nonspecifically filtered (i.e., without regard to PFS) to remove probes with mean expression less than 2.0 (i.e., the gene was not expressed in whole blood) or with variance less than 0.25 (i.e., the gene was homogenously expressed), resulting in 2,929 probe sets for subsequent analysis. To discover “Factors” or sets of coexpressed genes representative of biological pathways, we used Bayesian factor regression modeling (BFRM, http://www.isds.duke.edu/research/software/west/bfrm/) (13,14) in an unsupervised fashion (i.e., without regard to PFS). Each of the probe sets used to estimate a particular Factor can be interpreted as a measurement of the activity of some (potentially unknown) biological pathway. Each sample can then be assigned a “Factor score”, which represents the aggregate expression of the transcripts within a Factor. The Factor scores can then be used for association with the phenotype of interest in subsequent analyses.

Factor projection, Gene membership within a Factor, Comparison of factor gene lists with selected gene sets, and Co-expression of transcripts represented by a Factor before and after aspirin exposure—See Supplemental Methods

Correlations between factor scores and platelet function—Pearson correlation was used to test for association between a Factor and PFS in HV1 and HV2. In the second validation cohort, OPC, we chose a one-sided t-test because we hypothesized a *lower* factor score in the aspirin-resistant vs. aspirin-sensitive groups.

Linear regression was used to assess the independent association of Factor scores and PFS after accounting for log-transformed MPV and/or platelet count.

Correction for multiple hypotheses testing—As HV1 was a hypothesis-generating pilot study we did not adjust p-values. In the first validation cohort, HV2, we adjusted p-values using Bonferonni correction. In the second validation cohort, we performed only one hypothesis test.

Analyses of RT-PCR data—The expression of each selected transcript relative to the three reference genes was expressed as Δ Cq (or "deltaCq", See Supplemental Methods) and correlated with the corresponding microarray probe set or platelet function score using Pearson tests of correlation.

Platelet proteomic dataset analysis—See Supplemental Methods.

Analyses of CATHGEN cohorts—Logistic or Cox proportional hazards regression models were created in the case:control or observational cohorts, respectively, to test for association between the Factor and death/MI. Each model tested the Factor alone as well as after controlling for baseline variables (Supplemental Data, Table 6) associated with the Factor of interest. The assumption of proportional hazards for each Cox model was met. Odds (or hazards) ratios, 95% confidence intervals, and p-values are reported.

To assess the independent association between the Factor and death/MI, logistic regression models were built on the combined CATHGEN cohorts by forcing Framingham risk factors (age, sex, smoking, diabetes, hypertension, hyperlipidemia), African-American [AA] race, cohort, platelet count, and the presence of CAD (defined as a CAD index(15) >32 or history of coronary artery bypass surgery/MI/percutaneous coronary intervention), into the model and adding the Factor score or individual probe set gene expression. To assess the incremental prognostic value of gene expression we compared the performance of competing models (risk factors ± Factor/probe set expression), using the areas under the receiver operating characteristics curve (ROC) (16), the net reclassification index (NRI, using risk categories of < 10%, 10-20%, or > 20%(17) or category-free NRI (18), and the integrated discrimination improvement [IDI] (17)).

Results

Discovery and validation of a set of co-expressed genes in whole blood that correlate with platelet function on aspirin

In the discovery cohort (HV1) we identified 20 Factors (numbered 1 - 20, Supplemental Data, Table 1) representing sets of highly correlated, co-expressed genes. To test the hypothesis that one or more of these gene sets were associated with PFS on aspirin, we correlated each set with PFS in HV1 and identified "Factor 14" (Figure 1A) and "Factor 3" ($r = 0.27$, p-value = 0.05). In the first validation cohort (HV2), we found a significant association between Factor 14 and PFS, with the same strength and direction as observed in HV1 (Figure 1B, Bonferroni adjusted p-value = 0.03), thus validating this association, however Factor 3 was not associated with PFS in HV2. We further validated Factor 14 with VerifyNow test results in the OPC cohort (Figure 2). Thus Factor 14, which we named the "aspirin response signature" (ARS), was validated in two independent cohorts as a set of co-expressed genes associated with platelet function on aspirin.

To verify the microarray-based expression of the ARS transcripts, we selected 45 of the 60 genes (see Methods for selection criteria) for verification in whole blood RNA from the HV2 cohort. Using RT-PCR, 42/45 transcripts significantly correlated with their microarray-based expression with 16/42 transcripts, including *ITGA2B, TREML1, MYL9,* and *MPL,*

strongly (r > 0.80) correlating with microarray based gene expression (Figure 3 and Supplemental Data, Table 2). For the majority of transcripts there was concordance between both the RT-PCR and microarray correlations with PFS (Supplemental Data, Table 3 and Figure 1). Therefore, RT-PCR assays validate the microarray-based expression associations with PFS for most ARS transcripts.

Aspirin response signature transcripts are primarily of platelet origin

We observed that the transcripts with the strongest correlation with PFS (Table 1) mapped to several well-known platelet transcripts: *ITGA2B, CLU, IGF2BP3, GP1BB*, and *SPARC*. Based on this observation we hypothesized that transcripts represented by the ARS were of platelet origin. To test this hypothesis, we examined the overlap and enrichment of the 60 genes represented by the ARS with pre-defined gene sets specific to various peripheral blood cell types. Up to 24 of the 60 ARS genes significantly overlapped with platelet- or megakaryocyte-specific genes, whereas none overlapped with non-platelet peripheral blood cell type genes (Supplemental Data, Tables 4 and 5). Further, in the CATHGEN cohorts, we found the strongest correlation between expression of the ARS and platelet count (r = 0.41, p < $2e^{-16}$) with no strong, positive correlations with any other peripheral blood cell type counts: white blood cells (r = −0.01, p = 0.87), lymphocytes (r = −0.25, p = 1.2e-05), neutrophils (r = 0.16, p = 0.01), or monocytes (r = 0.06, p = 0.27).

To confirm the platelet origin of the ARS genes, we analyzed purified platelet lysates by label-free proteomics in the HV2 cohort. We identified 17 proteins from the ARS gene set in the proteomics dataset, of which, six were associated with PFS including *ITGA2B*, *ITGB3*, and *MYL9* (Table 2), all in the same direction their corresponding transcripts. Therefore, from these data we conclude that a large number of ARS transcripts originate in platelets and are thus reporting on a coexpressed pathway of platelet transcripts and proteins associated with platelet function on aspirin.

Because mean platelet volume (MPV) is associated with platelet function (19) and the platelet origin of ARS transcripts, we assessed the extent to which the association between ARS and PFS was confounded by platelet volume or count. After controlling for MPV, the ARS remained significantly (adjusted regression coefficient for ARS = −0.5, standard error = 0.2, and p-value = 0.05 for HV1; and −0.87, 0.4, and p-value = 0.03 for HV2) associated with PFS. Further, in HV1, where platelet count and volume were both measured, the ARS remained significantly (−0.5 ± 0.2, p = 0.04) associated with PFS after their inclusion. Therefore, the association between ARS and platelet function is independent of other readily available platelet parameters such as count and MPV.

Prior to the administration of aspirin, the aspirin response signature is not associated with platelet function

Because pre-aspirin platelet function is a strong predictor of post-aspirin platelet function(8), we tested the hypothesis that the aggregate expression of the ARS genes was correlated with native, pre-aspirin PFS. In neither HV1 nor HV2 did we observe a correlation between the ARS and pre-aspirin PFS (Figure 3). Despite the absence of a correlation with PFS prior to aspirin, the ARS genes were similarly co-expressed before and after aspirin exposure

(Supplemental Data, Figure 2). Therefore, although the set of ARS genes are highly correlated with one another prior to aspirin exposure, their aggregate expression does not appear to contribute to native, pre-aspirin platelet function. Instead, the expression of the ARS genes specifically reflects platelet function *on aspirin*.

The aspirin response signature is an independent prognostic biomarker for cardiovascular events

Because of the association of the ARS with platelet function on aspirin and aspirin's role in preventing cardiovascular events, we tested the hypothesis that the ARS was associated with the risk of death/MI in two independent patient cohorts. In both case-control and observational cohorts, the ARS was significantly associated with death/MI in univariate analyses (odds ratio [OR] =1.2, 95% confidence interval [CI] =1.04-1.4, p =0.04 and hazard ratio [HR] =1.4, [CI] = 1.1-1.7, p =0.002, respectively). The majority of the individual transcripts represented by the ARS were also associated with death/MI in both cohorts. (Supplemental Data, Table 7)

To determine the extent to which the ARS or an individual probe set for *ITGA2B* was an independent prognostic biomarker for events, we combined the CATHGEN cohorts and found that the ARS (OR =1.3, CI = [1.1, 1.5], p =0.001) or the microarray-based expression of *ITGA2B* (probe set = 206494_s_at, OR = 1.5, CI = [1.2, 1.8], p = 0.0001) were independently associated with death/MI after adjustment for Framingham risk factors(20), race, platelet count, and presence of angiographic CAD.

To further assess the potential use of the ARS as a risk biomarker we tested the hypothesis that the ARS or *ITGA2B* probe set expression would improve measures of discrimination. Compared with a model using clinical risk factors alone, the inclusion of the ARS improved most measures of risk discrimination (Table 3, Figure 5A). Inclusion of *ITGA2B* probe set expression significantly improved all measures of discrimination (Table 3, Figure 5B). Thus, the ARS or the expression of an individual ARS transcript such as *ITGA2B* were independent prognostic biomarkers for risk of death/MI.

Discussion

We used aspirin as a probe to identify novel genes and biomarkers associated with platelet function and cardiovascular events. We hypothesized that administering aspirin while simultaneously assaying the blood transcriptome might identify sets of genes that are related to aspirin's cardioprotective effect. We identified a set of platelet-enriched, co-expressed genes and proteins, the "ARS", that was reproducibly associated with platelet function in response to aspirin. When tested as a prognostic biomarker, the ARS or an individual ARS transcript (e.g., *ITGA2B*), *independently* and *incrementally* predicted the risk of death/MI compared with traditional risk factors. Our data shows that 1) the genomic response to a pharmacologic "challenge" with aspirin can reveal genes that underlie platelet function on aspirin and mechanisms responsible for death/MI and 2) that whole blood RNA profiling may identify novel biomarkers that discriminate individuals at heightened risk for death/MI.

Transcripts associated with platelet function on aspirin are associated with cardiovascular events

We found neither association between the ARS and the presence of CAD, nor overlap between ARS genes those previously associated with CAD.(1,6) Instead, we found that the ARS was associated with death/MI after controlling for CAD and CAD risk markers. These findings highlight a unique and novel role that the biologic pathway represented by ARS genes have in the development of cardiovascular events, independent of CAD. We conclude that the biology of aspirin is complex and involves additional mechanisms beyond inhibiting platelet COX-1 and some of these mechanisms underlie risk for cardiovascular events.

A novel and translatable biomarker of platelet function in response to aspirin and the risk for cardiovascular events

Clinicians currently need a readily available biomarker for the response to aspirin. Despite the availability of platelet function assays, their widespread use is severely constrained by the need for specialized equipment and trained personnel. Point-of-care tests are available, but require testing to be completed within hours of phlebotomy; thus, they are out of reach for the vast majority of outpatients on aspirin. Further, most patients taking aspirin for chronic prevention are outpatients where results at the point-of-care are not required. Instead, testing in central laboratories, as is common for LDLc for statins, would be sufficient for determining aspirin response in the outpatient setting. Because of the coexpressed nature of the ARS genes, several individual transcripts (Table 1) correlated best with platelet function. We demonstrated that PCR for individual transcripts could be used in lieu of microarrays (Figure 3 and Supplemental Data, Table 2) for many ARS genes, thus demonstrating the feasibility of a blood-based diagnostic test.

Whole blood RNA testing is a well-established testing diagnostic testing platform. For cardiac allograft rejection and CAD diagnosis, whole blood microarray analyses were both transitioned to a PCR-based platform(6,7): AlloMap® and Corus® CAD, respectively. AlloMap® has been approved by the FDA and both are covered by major insurances. Therefore, there is a feasible path for blood-based RNA biomarkers to clinical adoption, FDA approval and insurance coverage.

Peripheral blood gene expression profiling reveals co-expressed transcripts of platelet origin associated with platelet function in response to aspirin

The genes underlying variable platelet function on aspirin have been difficult to identify(21) or explain a small portion of the observed variability(22). We hypothesized that whole blood RNA profiling, which *de facto* contains platelet transcripts, would yield biological pathways important for the response to aspirin. We demonstrated that the transcripts represented by the ARS are likely of platelet origin (Supplemental Data, Tables 4 and 5). When we analyzed platelet-enriched protein, we not only confirmed the well-known roles of *ITGA2B* and *ITGB3*, but also and ascribe new roles many other platelet genes: *MYL9, CLU, PPKAR2B, TREML1*, and *CTTN* with respect to platelet function on aspirin and cardiovascular events. Additionally, recent genome wide association studies identified a *PEAR1* polymorphism associated with platelet *PEAR1* levels and platelet function on aspirin.(22) We excluded the probe set (228618_at) mapping to *PEAR1* because its variance

(0.21) fell below our variance criteria (0.25, see Methods). However, in a *post hoc* analysis, *PEAR1* expression strongly correlated (r = 0.9) with ARS levels. Therefore, our approach identified previously known and novel platelet genes associated with platelet function in response to aspirin.

We observed an association between ARS and platelet function only after the administration of aspirin, suggesting that the latent effect of ARS genes on platelet function is unmasked in response to aspirin. Consistent with these findings, when we stratified the CATHGEN cohort by aspirin use, we observed that the association between the ARS and death/MI was higher in those using aspirin at the time of catheterization (OR = 1.4 vs. 1.1 in aspirin users vs. nonusers). We hypothesize that the molecular mechanisms represented by the ARS contributes minimally to native platelet function in the absence of aspirin. In contrast, when platelet COX-1, a protein not represented by the ARS, is suppressed by 325mg/day aspirin dosing(23), the effects of these platelet enriched genes is revealed such that the resulting level of platelet function is then determined by the ARS. Alternatively, aspirin exposure may alter the genomic and protein content of circulating platelets. The precise mechanism by which platelet function on aspirin is related to the expression of the ARS genes and proteins on aspirin is the subject of ongoing work.

Limitations

Several limitations deserve consideration. Neither platelet function nor mean platelet volume (MPV) were measured in CATHGEN. Therefore, we cannot know whether heightened ARS levels altered platelet function or volumes in addition to an increased risk of death/MI. To our knowledge, large cohorts with platelet function, banked RNA, longitudinal follow up, and a sufficient number of events are not available. Further, in our discovery and validation cohorts, the association of the ARS with PFS was independent of platelet count and MPV, suggesting the ARS provides an independent parameter of platelet function that underlies cardiovascular events. Second, although there was no association between the ARS and modifiable risk factors (e.g. diabetes, hyperlipidemia, or hypertension), because we did not assess the degree to which these risk factors were controlled we do not know if addressing these risk factors could modulate ARS levels. Finally, the comparison of the ARS gene set with that of platelets, megakaryocytes, and platelet proteomics analyses demonstrate that the top ARS genes correlative of platelet function on aspirin were of platelet origin. However, some ARS genes (e.g. *TTC7B* and *FSTL1*) are also expressed in non-platelet cell types, suggesting that mechanism(s) represented by ARS genes may involve more than just platelets.

Conclusion

In summary, we used aspirin as a probe in conjunction with RNA profiling and identified novel biomarkers that identify individuals at highest risk for death/MI independent of clinical risk factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

PFS	Platelet Function Score
ARS	Aspirin Response Signature
CAD	Coronary Artery Disease
MI	Myocardial Infarction
RT-PCR	Real-time Polymerase Chain Reaction
PCR	Polymerace Chain Reaction
RNA	Ribonucleic Acid
IRB	Institutional Review Board
DNA	Deoxyribonucleic Acid
LDLc	low density lipoprotein cholesterol

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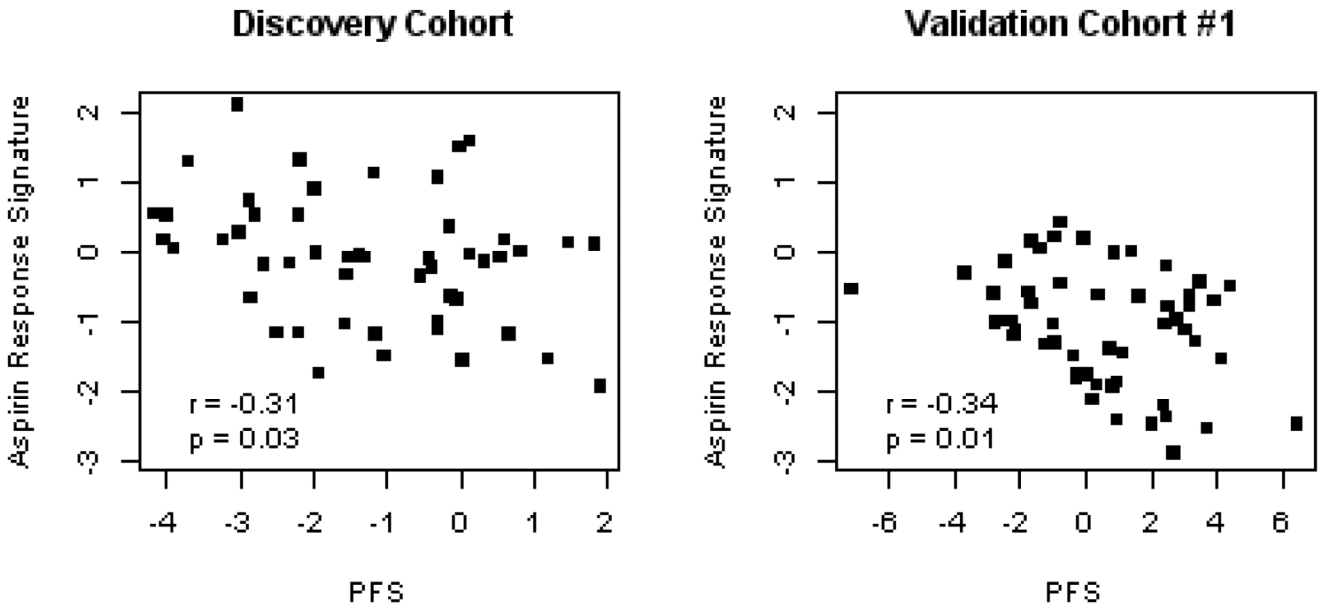

Figure 1. The aggregate expression of a set of coexpressed, whole blood genes correlates with platelet function in response to aspirin

Two independent cohorts of healthy volunteers were exposed to 325mg/day aspirin, followed by whole blood microarray profiling. Platelet function was assessed by the platelet function score (PFS(8)). The aggregate expression of a set of coexpressed genes (aspirin response signature [ARS], x-axis), is plotted against the PFS (y-axis) after aspirin exposure. Pearson correlation coefficients and p-values are reported.

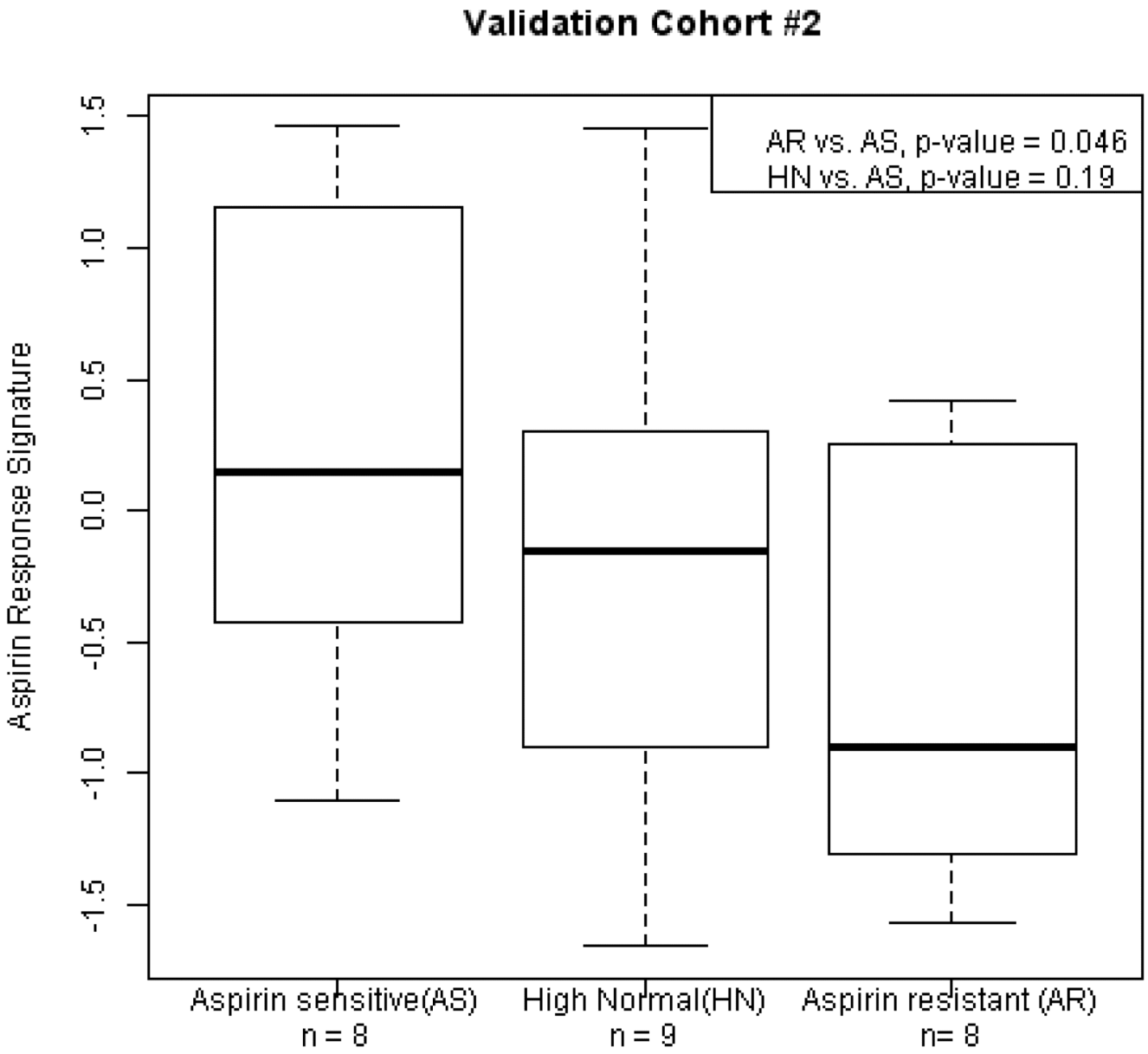

Figure 2. Aspirin response signature (ARS) is associated with platelet function in patients at risk for cardiovascular disease

Patients treated with 81mg/day aspirin were assessed with the VerifyNow Aspirin device.(9) Three categories of individuals were profiled by microarray based on their aspirin response units (ARU): Aspirin resistant (AR, ARU > 550); High normal (HN, 500 < ARU < 550); Aspirin sensitive (AS, ARU < 550). ARS values are for each group are plotted and compared using two-sample t-tests. P-values are one-sided.

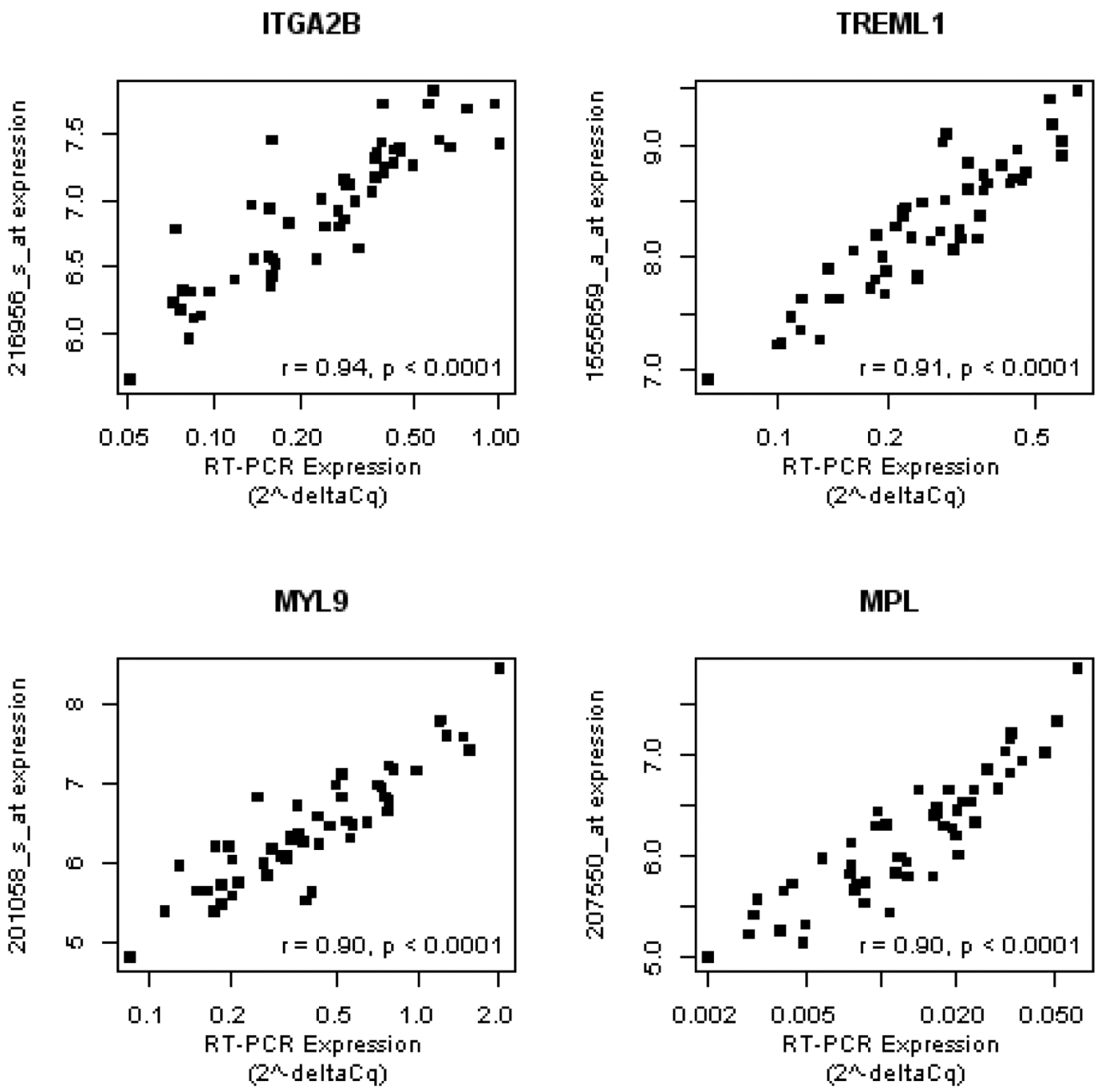

Figure 3. PCR-based assays verify the microarray-based gene expression values for aspirin response signature genes

Real-time PCR assays were designed to verify selected transcripts represented by the aspirin response signature (ARS) in the HV2 cohort. The deltaCq for each assay was correlated with the RMA normalized, probe set expression for the corresponding ARS gene using Pearson correlation (see Supplementary Data, Table 2). For the four genes with the highest PCR vs. microarray-based correlation (*ITGA2B*, *MYL9*, *TREML1*, and *MPL)*, we plot the relative quantity ($2^{-\text{deltaCq}}$, x-axis, log-scale) vs. the corresponding probe set expression (y-axis), correlation coefficient, and p-value.

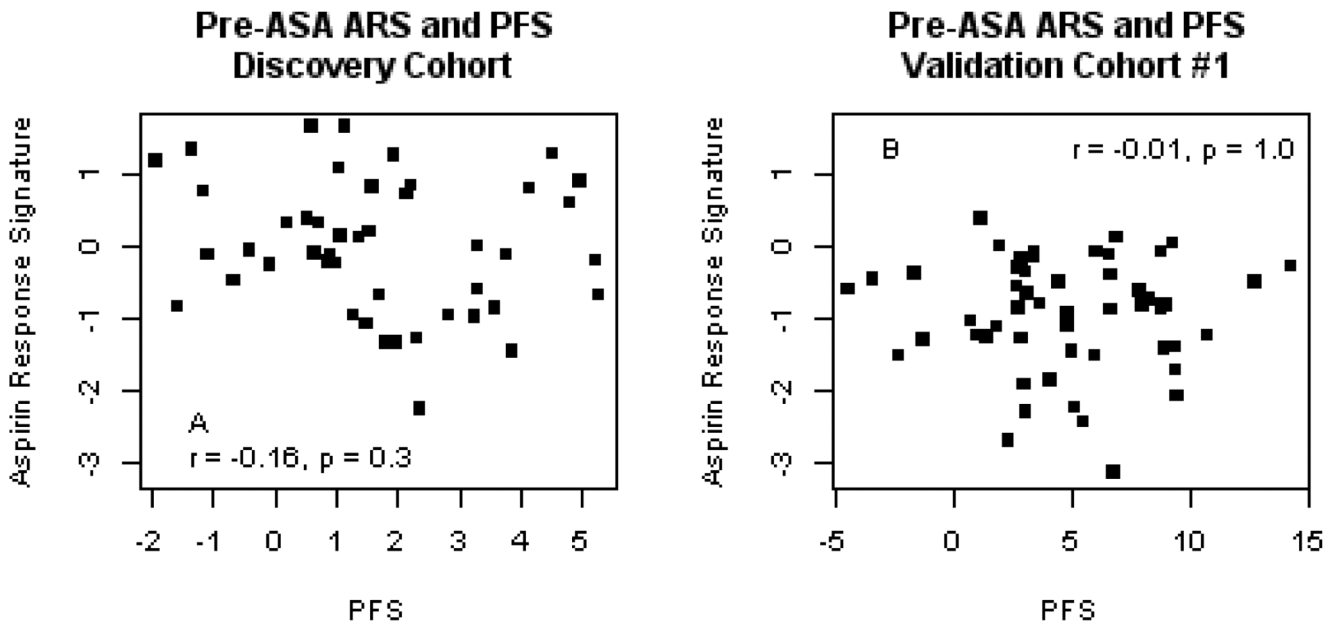

Figure 4. A set of coexpressed peripheral blood genes does not correlate with native, pre-aspirin platelet function

The aggregate expression of coexpressed genes, is plotted against the platelet function before the administration of aspirin in the discovery cohort (HV1, A, n = 45) and validation cohort (HV2, B, n = 50) healthy volunteers. Pearson correlation coefficients and p-values are reported. ARS = aspirin response signature; PFS = platelet function score.

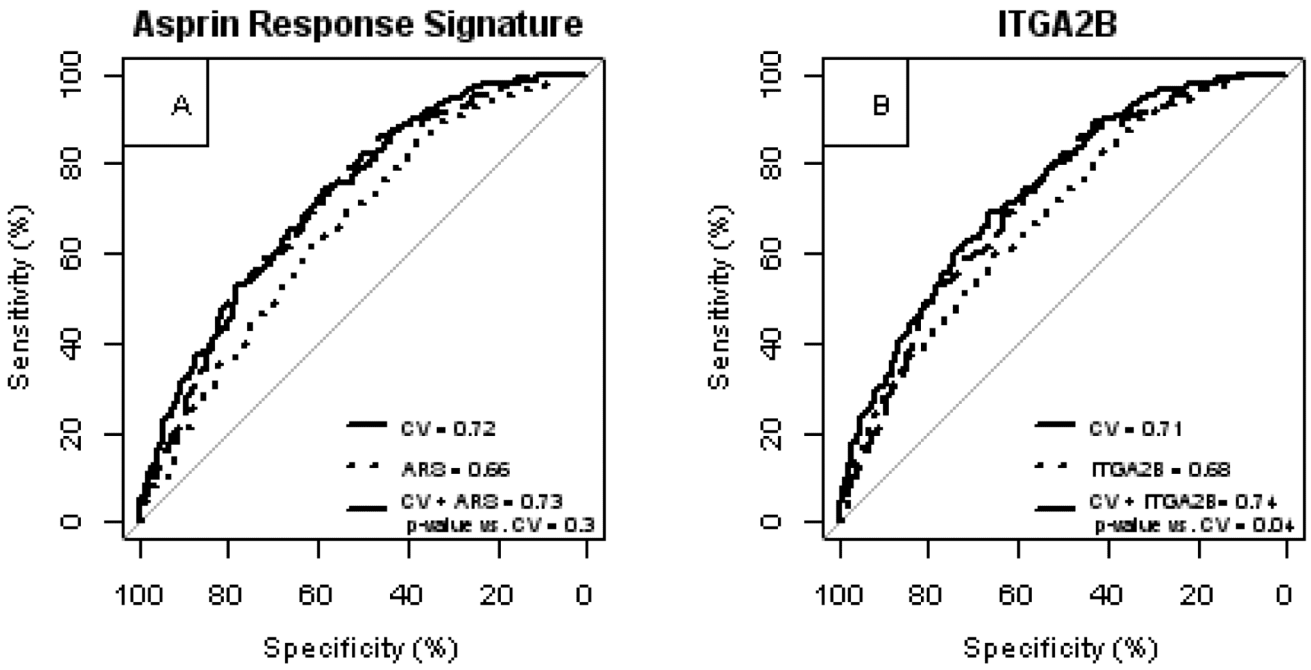

Figure 5. Peripheral blood gene expression adds additional prognostic information for death or myocardial infarction

Patients in the case:control and observational cohorts were combined and analyzed with respect to death/myocardial infarction (MI) outcomes. The receiver operating characteristics curves were plotted for predictive models containing cardiovascular risk factors, platelet count, presence of coronary artery disease, cohort (collectively, CV) and gene expression, or both were compared. ARS = aspirin response signature. The probe set, 216956_s_at represents *ITGA2B* gene expression.

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Table 1

Genes represented by the ARS and their correlation with platelet function with aspirin*

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS beta coefficient *	Combined P-value
Factor 14	n/a	n/a	−0.76088	0.0017
Individual Factor 14 transcripts				
208782_at	*FSTL1*	follistatin-like 1	−1.6579	0.0003
201059_at	*CTTN*	cortactin	−1.2817	0.0015
201906_s_at	*CTDSPL*	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	−1.3795	0.0025
1555659_a_at	*TREML1*	triggering receptor expressed on myeloid cells-like 1	−1.0767	0.0034
212667_at	*SPARC*	secreted protein, acidic, cysteine-rich (osteonectin)	−1.214	0.0048
216956_s_at	*ITGA2B*	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−1.0689	0.0048
230942_at	*CMTM5*	CKLF-like MARVEL transmembrane domain containing 5	−1.1641	0.0061
57588_at	*SLC24A3*	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	−1.3053	0.0063
207550_at	*MPL*	myeloproliferative leukemia virus oncogene	−0.931	0.0066
219090_at	*SLC24A3*	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	−1.1123	0.0080
208791_at	*CLU*	clusterin	−0.9584	0.0085
206494_s_at	*ITGA2B*	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−0.7279	0.0087
227189_at	*CPNE5*	copine V	−1.2062	0.0088
220496_at	*CLEC1B*	C-type lectin domain family 1, member B	−1.2077	0.0090
206493_at	*ITGA2B*	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−0.8966	0.0094
206049_at	*SELP*	selectin P (granule membrane protein 140kDa, antigen CD62)	−1.1642	0.0104
203819_s_at	*IGF2BP3*	insulin-like growth factor 2 mRNA binding protein 3	−1.2947	0.0123
225354_s_at	*SH3BGRL2*	SH3 domain binding glutamic acid-rich protein like	−1.0895	0.0146

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS beta coefficient *	Combined P-value
		2		
207808_s_at	*PROS1*	protein S (alpha)	−1.1049	0.0174
207206_s_at	*ALOX12*	arachidonate 12-lipoxygenase	−0.8756	0.0207
212813_at	*JAM3*	junctional adhesion molecule 3	−1.0454	0.0215
1560262_at	*LRRC32*	leucine rich repeat containing 32	−0.9376	0.0226
204628_s_at	*ITGB3*	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	−0.968	0.0242
214146_s_at	*PPBP*	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	−0.713	0.0243
211026_s_at	*MGLL*	monoglyceride lipase	−1.0027	0.0249
208792_s_at	*CLU*	clusterin	−0.8122	0.0266
201108_s_at	*THBS1*	thrombospondin 1	−0.9169	0.0276
201058_s_at	*MYL9*	myosin, light chain 9, regulatory	−0.5909	0.0287
206390_x_at	*PF4*	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	−0.9017	0.0296
206655_s_at	*GP1BB*	glycoprotein Ib (platelet), beta polypeptide	−0.8934	0.0317
209651_at	*TGFB1I1*	transforming growth factor beta 1 induced transcript 1	−0.7618	0.0326
207414_s_at	*PCSK6*	proprotein convertase subtilisin/kexin type 6	−0.8566	0.0351
200665_s_at	*SPARC*	secreted protein, acidic, cysteine-rich (osteonectin)	−0.8261	0.0410
212077_at	*CALD1*	caldesmon 1	−0.5688	0.0505
203817_at	*GUCY1B3*	guanylate cyclase 1, soluble, beta 3	−0.8511	0.0546
227088_at	*PDE5A*	phosphodiesterase 5A, cGMP-specific	−0.918	0.0571
226152_at	*TTC7B*	tetratricopeptide repeat domain 7B	−0.7986	0.0594
206167_s_at	*ARHGAP6*	Rho GTPase activating protein 6	−0.8437	0.0677
37966_at	*PARVB*	parvin, beta	−0.7708	0.0717
208601_s_at	*TUBB1*	tubulin, beta 1	−0.5959	0.0736
204115_at	*GNG11*	guanine nucleotide binding protein (G protein), gamma 11	−0.5622	0.1229
241133_at	*PRSS1*	protease, serine, 1 (trypsin 1)	−0.4814	0.1243

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS beta coefficient *	Combined P-value
203680_at	*PRKAR2B*	protein kinase, cAMP-dependent, regulatory, type II, beta	−0.5049	0.1365
205442_at	*MFAP3L*	microfibrillar-associated protein 3-like	−0.4724	0.1385
212151_at	*PBX1*	pre-B-cell leukemia transcription factor 1	−0.6059	0.1729
212573_at	*ENDOD1*	endonuclease domain containing 1	−0.7276	0.1735
230690_at	*TUBB1*	tubulin, beta 1	−0.578	0.1864
230645_at	*FRMD3*	FERM domain containing 3	−0.6391	0.2102
225974_at	*TMEM64*	transmembrane protein 64	0.38321	0.2227
1553842_at	*BEND2*	chromosome × open reading frame 20	−0.5657	0.2258
228708_at	*RAB27B*	RAB27B, member RAS oncogene family	−0.4836	0.2512
227180_at	*ELOVL7*	ELOVL family member 7, elongation of long chain fatty acids (yeast)	−0.3943	0.2823
212148_at	*PBX1*	pre-B-cell leukemia transcription factor 1	−0.3139	0.2970
203414_at	*MMD*	monocyte to macrophage differentiation-associated	−0.4287	0.3236
1552773_at	*CLEC4D*	C-type lectin domain family 4, member D	0.37545	0.3543
222717_at	*SDPR*	serum deprivation response (phosphatidylserine binding protein)	−0.3009	0.3830
224823_at	*MYLK*	myosin, light chain kinase	−0.2911	0.4644
214974_x_at	*CXCL5*	chemokine (C-X-C motif) ligand 5	−0.1621	0.5011
229778_at	*C12ORF39*	chromosome 12 open reading frame 39	−0.2032	0.5020
235331_x_at	*PCGF5*	polycomb group ring finger 5	0.22781	0.5470
212651_at	*RHOBTB1*	Rho-related BTB domain containing 1	−0.2395	0.5755
206110_at	*HIST1H3H*	histone cluster 1, H3h	−0.2021	0.5827
215779_s_at	*HIST1H2BG*	histone cluster 1, H2bg	−0.2623	0.5896
207815_at	*PF4V1*	platelet factor 4 variant 1	−0.0927	0.6139
226188_at	*LGALSL*	lectin, galactoside-binding-like–	0.23728	0.6142
221556_at	*CDC14B*	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	−0.2127	0.6530
207156_at	*HIST1H2AG*	histone cluster 1, H2ag	−0.1534	0.6882
210387_at	*HIST1H2BG*	histone cluster 1, H2bg	−0.1494	0.6906

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS beta coefficient *	Combined P-value
225166_at	*ARHGAP18*	Rho GTPase activating protein 18	0.15541	0.7353
206272_at	*RAB4A*	RAB4A, member RAS oncogene family	0.09962	0.7967
210986_s_at	*TPM1*	tropomyosin 1 (alpha)	0.08749	0.8404
227451_s_at	*C6ORF79*	chromosome 6 open reading frame 79	−0.0134	0.9791

* = the beta coefficient for the expression of either the aggregate expression of the ARS or each probe set represented by the ARS using the combined HV1 and HV2 datasets from a regression model containing gene expression and cohort (HV1 vs. HV2) with corresponding p-value; PFS = platelet function score.

Table 2

Aspirin response signature proteins identified in platelet protein and their correlations with PFS on aspirin

Protein Name	Correlation with PFS	p-value
TBB1	−0.32	0.02
GP1BB	−0.29	0.03
ITA2B	−0.29	0.03
ITB3	−0.28	0.04
MYL9	−0.27	0.05
RB27B	−0.26	0.06
LEGL	−0.24	0.08
TSP1	−0.24	0.08
CALD1	−0.22	0.11
SRC8	−0.21	0.12
SH3L2	−0.20	0.16
CXCL7	−0.20	0.15
SDPR	−0.18	0.19
PLF4	−0.18	0.20
SPRC	−0.14	0.31
PDE5A	−0.09	0.49
CLUS	0.06	0.67

Table 3
Measures of discrimination with and without inclusion of gene expression profiles

Measure	Traditional Risk Factors	Traditional Risk Factors + ARS	Traditional Risk Factors + 216956_s_at** (*ITGA2B*)
Area under ROC curve 95% confidence interval [CI] p-value*	0.72 [0.68-0.76] n/a	0.73 [0.69-0.77] 0.3	0.74 [0.70-0.78] 0.04
Net reclassification index (<10%, 10-20%, > 20%) CI p-value	-	0.06 [0.02 – 0.10] 0.005	0.12 [0.07 - 0.17] < 1e-05
Net reclassification index (category-free) CI p-value	-	0.31 [0.15 – 0.47] 2e-04	0.37 [0.21 – 0.54] 8.7e-06
Integrated discrimination improvement CI p-value	-	0.01 [0.002 – 0.02] 0.006	0.03 [0.02 – 0.05] 2e-05

* all p-values are for comparisons with 'Traditional Risk Factors' model which includes: age, sex,African-American race, smoking, diabetes, hypertension, hyperlipidemia, cohort, and the presence of coronary artery disease;

** The 216956_s_at probe set represents *ITGA2B* gene expression on the Affymetrix microarray; ARS = aspirin response signature; ROC = receiver operating characteristic