Identification of Aspirin Resistant Biomarkers Using Whole Blood Genome Profiling

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Abstract

IDENTIFICATION OF ASPIRIN RESISTANT BIOMARKERS USING WHOLE BLOOD GENOME PROFILING

Background: Anti-platelet therapy with aspirin has been proven to decrease atherothrombotic events and mortality in patients at high risk for cardiovascular disease. Despite this benefit, studies have shown that as many as 20% of patients taking aspirin will have recurrent ischemic vascular events. Many patients demonstrate inadequate anti-platelet response to aspirin on laboratory testing. We hypothesize that changes in gene expression in blood cells may shed light on the mechanisms of AR in adults at risk for ischemic events.

Objective: To identify genetic markers associated with aspirin resistance in a middle-aged population with known atherosclerosis or at elevated risk for coronary or peripheral vascular atherosclerosis.

Methods and Results: Blood samples were obtained from 131 patients with known cardiovascular diseases or at risk and divided into two groups (aspirin resistant and aspirin sensitive) according to their platelet function test results using VerifyNow® System. Whole blood total RNA was purified from 16 samples (8 resistant and 8 sensitive) with PAXgene Blood RNA kit for microarray assays. The RNA samples were reverse-transcripted and labeled with NuGen Ovation™ Amp V2 and FL-Ovation™ Biotin V2 Kits before being hybridized to the Affymetrix GeneChip® HG-U133 plus 2.0 arrays. The microarray data generated by the Affymetrix Command Console (.chp file) were exported to the GeneSpring 10.0.2 for further analysis. A total of 284 genes passed

the statistical ($p \leq 0.05$) and fold-change (≥ 1.8) threshold filtering. In order to gain functional insight into how the 284 genes might be related to aspirin resistance. We employed core analysis function of Ingenuity Pathway Analysis (IPA) to further explore statistically significant biological functions, molecular interaction networks and potential pathways associated with this gene list. Several pathways and gene markers, including THBS1 and PTGES were identified as possible explanations of the mechanisms of AR.

Conclusions: Whole blood gene expression profiling strategy may help solve the mystery of AR. Gene markers identified could be used as both diagnostic and therapeutic targets.

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Chapter I: Introduction

Aspirin (acetylsalicylate) is one of the most widely prescribed anti-platelet drugs worldwide [1] and its clinical effectiveness in the secondary prevention of cardiovascular events has been well established. According to a recent collaborative meta-analysis of 287 randomized trials of anti-platelet therapy which comprised more than 200 000 patients, aspirin reduces the risk of stroke, myocardial infarction or death by approximately 22% in patients with cardiovascular disease [2]. In patients with acute coronary syndrome as well as those undergoing percutaneous coronary intervention, anti-platelet therapy including aspirin has been shown to improve outcome and decrease mortality [3].

The target for aspirin is cyclo-oxygenase (COX) which is membrane-bound and exists in three isoforms, a constitutively expressed COX1, an inducible COX2 and recently alleged COX3 [4, 5]. COX1 is particularly abundant in platelets and largely responsible for generating TxA_2 which is a potent platelet agonist and vasoconsctrictor during thrombus formation. Platelets produce TxA2 in response to platelets activation. Platelet activation increases cytosolic calcium and protein kinase C, which induce phospholipase A2 (PLA2) translocation and activation. Membrane phospholipids are cleaved by PLA2,

followed by arachidonic acid (AA) release. AA is first converted to PGH_2 by COX then TxA_2 by thromboxane synthase. In fact, Fitzgerald et al. and Willerson et al. have independently shown that a higher formation of thromboxane was found in patients with unstable angina pectoris [6, 7], which is an indication of increased blood platelets reactivity.

Aspirin is a nonselective inhibitor of COX enzymes. But the affinity for COX-1 is 50 to 100 times that for COX-2 [8]. By acetylating COX-1 and COX-2 at position serine 529 and serine 516 in their respective active sites, Aspirin results in steric inhibition of AA binding thus irreversibly prevents its metabolism to TxA_2. Because platelets are anucleate, inhibition through COX-1 acetylation lasts for the lifespan of platelets (approximately 10 days) and new platelets must be generated to re-establish COX-1 activity. Thus, although aspirin has a short half-life (15-20 min), its pharmacodynamic half-life is 5 days.

However, aspirin is not equally effective for all the patients and up to 20% patients experienced recurrent cardiovascular events after PCI [9, 10]. These events raise the concept of "Aspirin Resistance" (AR). The phrase "Aspirin Resistance" is, however, used inaccurately to refer to the ineffectiveness of aspirin to protect patients from cardiovascular events. Rather than being resistant, patients who experience recurrent cardiovascular events despite aspirin therapy are more likely to exhibit variable response to anti-platelet drug [11].

Several different *in vitro* platelet function assays are used to measure aspirin responsiveness. Light transmission aggregometry (LTA): platelet aggregation could be assessed in platelet rich plasma (PRP) at 37^oC by LTA. PRP was obtained by

centrifugation of citrated whole blood for 10 min at 1000 rpm and adjusted to 250-450 × 10^9/L with platelet poor plasma if needed. The change in light transmittance was measured using a ChronoLog Aggregometer after the addition of AA to express the degree of aggregation, using platelet poor plasma as reference [12]. 1.6 mM (0.5 mg/mL) of AA is suggested as the appropriate concentration for this assay [13, 14]. Consequently, residual platelet aggregation ≥ 20% is used as the cut-off to identify aspirin resistant subjects despite daily aspirin therapy. Because this cut-off has been frequently used in the past and reported to be linked with higher incidence of adverse cardiac events [15-24]. Similarly, measurement of platelet aggregation through COX1-indepentdent pathways are also available with the use of COX1-independent agonists (ADP, epinephrine, and collagen) measured by LTA [16-18, 22-24]. Whole blood aggregometry: Whole blood aggregometry (WBA) measures electrical impedance (maximal amplitude) between two electrodes immersed in whole blood 5 min after addition of a platelet agonist (AA, 1.6 mM), using a ChronoLog Aggregometer [13, 25-27]. An impedance > 3Ω was chosen and used recently as representative of inadequate response to aspirin [28], although some investigators suggested a cut-off value of 0Ω [29, 30]. Platelet function analyzer (PFA-100®): PFA-100® (Dade Behring, IL, USA) is one of the most widely used point-of-care assays which measures the cessation of blood flow through a central aperture in a membrane coated with collagen and epinephrine [26, 27, 31]. Subjects whose closure time is in the normal range (<193s) despite aspirin treatment, as predetermined by the manufacture, are considered aspirin resistant. VerifyNow Aspirin®: The VerifyNow Aspirin® is another widely used point-of-care system (Accumetrics, CA, USA) which is based on turbidimetric optical detection of platelet aggregation in whole blood [25-27].

Whole blood was transferred into cartridges containing AA and lyophilized fibrinogen-coated beads. Consequently, the system converts light transmittance results into Aspirin Reaction Units which is dependent on degree of aggregation [12]. Subjects with Aspirin Reaction Units ≥ 550 are considered as aspirin resistant [32-34]. Urinary 11-dehydro-thromboxane B_2 measurement ($dTxB_2$): $dTxB_2$ is an inactive metabolite of thromboxane A_2 and detection of this metabolite in urine indicates systemic TxA_2 generation. $dTxB_2$ concentrations are measured using an enzyme immunoassay kit (11-dehydrothromboxane B2 EIA Kit, Cayman Chemical, MI, USA). Urinary $dTxB_2$ concentrations are normalized against urinary creatinine concentrations. Subjects with $dTxB_2$ levels ≥67.9 ng/mmol of creatinine are considered aspirin resistant [35].

However, according to the study done by Lordkipanidze et al [12], the prevalence of aspirin resistance varied widely (ranging from 2.8% to 59.5%) and showed poor agreement and correlation among different platelet function assays. Considering the fact that the various assays measured different aspects of platelet function, the apparent lack of agreement and correlation between assays is not very surprising. Interestingly, the reported high prevalence of aspirin nonresponsiveness mainly comes from studies that used COX1-independent platelet function assay, particularly the PFA-100® [11]. The major flaw of most platelet function assays is using arbitrary, clinically non-validated cut-off values to identify aspirin resistance. Although an association between laboratory methods determined aspirin resistance and worse clinical outcomes is observed in previous studies, the clinical relevance of different platelet function assays and their respective thresholds remains to be established [36-41].

Numerous studies have investigated the clinical effects of ex vivo determined aspirin responsiveness on cardiovascular outcomes. In a meta-analysis published in 2007, Snoep et.al addressed the question of whether laboratory aspirin resistance is related to a higher risk of cardiovascular recurrent events [42]. They included 15 full-text articles and 1 meeting abstract. The rate of laboratory aspirin resistance ranged from 5% to 65% (mean 27%). The pooled odds ratio of all cardiovascular events was 3.8 (95% CI 2.3-6.1) for laboratory aspirin resistance. However, the studies in this systematic review varied in several ways, including cardiovascular diseases the patients had experienced, aspirin dosage, duration of follow-up time, definition of outcome and the major one−platelet function test methods [42]. Nonetheless, despite these clinical diversities and differences in method, a consistent association was observed between the laboratory-determined extent of platelet inhibition by aspirin and clinical outcomes.

Because platelet activation and thrombus formation are influenced by many factors and various complex pathways, there may be several possibilities for aspirin resistance. According to the study done by Weber et al [43], aspirin resistance can be categorized into three mechanistic types: pharmacokinetic, pharmacodynamic, and thromboxane-independent activation pathways.

The platelets level is higher than normal in some situations when platelet turnover is high, such as during surgery, after trauma, or in patients having diabetes [44]. The resulting increased amount of active COX could synthesize more thromboxane despite aspirin treatment [45]. This temporary “resistance” was recently observed in a population of patients who had undergone coronary bypass [46]. In an observational study by Davi et al. [47], it was found that cardiovascular risk factors such as diabetes, hyper cholesterolemia

and hypertension were independently related to increased level of urinary 11dhTxB_2 excretion, which was an indicator of increased TxA_2 synthesis and represent the link between these risk factors and adverse cardiovascular events.

COX-2 is considered as a candidate for aspirin resistance and its existence and function in platelets is still controversial. Platelets don't have apparent COX-2 content, which is only constitutively expressed in a certain number of cells and is inducible by activating several signal transduction pathways. In a study by Rocca et al. [48], it was concluded that COX-2-derived PGE2 and TxA_2 may contribute to hemostatic responses by an unknown mechanism in clinical syndromes associated with high platelet turnover. Clinical evidence from recent studies of patients undergoing coronary artery bypass grafting (CABG) supports the existence of a possible COX-2 isoform in platelets and its role in aspirin resistance [45, 49, 50]. Furthermore, it was also hypothesized by some researchers that COX-2 in inflammatory cells and endothelial cells could contribute to an aspirin-insensitive thromboxane synthesis [51].

Drug interference is another mechanism that might cause aspirin resistance. One of the examples is the competitive interaction between aspirin and NSAIDs when they are concomitantly administered [52, 53], because the binding sites for both aspirin and NSAIDs are located within a narrow hydrophobic channel in the core of the COX enzyme. The antiplatelet activity of aspirin may also be influenced by its type of preparation. By measuring serum TxA_2 levels and AA-induced platelet aggregation, Cox *et al.* demonstrated that enteric-coated aspirin was less effective than equivalent doses of plain aspirin in inhibiting platelet function [54]. The authors attributed this observation to

the decreased bioavailability and poor absorption that an enteric-coated preparation has in the high-pH environment of the small intestine [11].

Noncompliance with aspirin therapy is associated with increased risk of adverse cardiovascular events [55] and may mislead researchers to identification of "fake" aspirin resistance [56, 57]. Actually, Cotter et al [56] have suggested that the correlation between laboratory aspirin resistance and recurrent cardiac events wouldn't exist anymore after exclusion of noncompliant patients. In a study of patients with cerebrovascular disease, aspirin was observed to be less effective for women and older individuals (age >63 years) compared to other subgroups [58], which raise the question for additional unknown mechanisms which could cause variable aspirin response in specific demographic groups of patients.

Natural variation in platelet reactivity is also largely influenced by genetic polymorphisms, which might either blunt platelets' response to agonists or sensitize platelets thus lowering the therapy effect.

COX-1 polymorphism−The human COX-1 gene contains 11 exons spanning a length of 24 kb on chromosome 9 and encodes more than 20 variants, although most of them are rarely found [59]. The role of single nucleotide polymorphisms (SNPs) of COX-1 in the mechanism of aspirin resistance is not fully understood yet. Some researchers suggested that no correlation can be established between the frequency of SNPs and the rate of recurrent cardiovascular events despite aspirin treatment [60-62]. Other researchers reported significant association between the COX-1 A842-G polymorphism and aspirin

response [63-65]. This genetic polymorphism might affect gene expression, enzyme activity, and interaction with pharmacological agents.

GP Ia/IIa and GP Ib alpha polymorphism− The GP Ib alpha subunit is highly polymorphic and considered to be the key one in the GP Ib-V-IX receptor [66]. The GP Ibα polymorphism contains four variants which differ in the number of a 39bp VNTR repeat they have (A,B,C and D ranging from 4 repeats to 1 repeat). All those polymorphisms are reported to play an essential role in cardiovascular diseases as potentially genetic risk factors [67-72]. According to the study by Macchi L et al. [73], the C807-T SNP found in GP Ia/IIa was shown to correlate with high GP Ia/IIa density on platelet membrane. Su et al. also found that the presence of GP Ia T allele may be a genetic marker of susceptibility to aspirin resistance [74].

GP IIb/IIIa polymorphism−This complex binds fibrinogen and von Willibrand factor and it is essential for platelet aggregation and hemostasis. Two allelic polymorphisms, the P1A1 and the P1A2 polymorphisms (C to T substitution at base 196 of the mRNA in exon 2) have been described. P1A2 was reported to be associated with increased platelet reactivity and decreased platelet inhibition with low-dose aspirin, which means platelet reactivity could be compensated in some alternative way despite aspirin treatment [73, 75].

Although many possibilities for aspirin resistance have been proposed from numerous studies worldwide, the exact mechanism underlying this phenomenon still remains unanswered. We hypothesize that changes in gene expression in blood cells contribute to aspirin resistance in adults at risk for ischemic events. Whole blood gene expression will

be profiled by microarray in patients with and without aspirin resistance, and differentially expressed genes will be identified by specialized microarray statistical methods. The resulting genes may have diagnostic value in screening for aspirin resistance in high-risk patients and may help identify ways to reverse or circumvent aspirin resistance.

Chapter II: Materials & Methods

AR Patients Identification

The aspirin resistance research project was reviewed and approved by George Washington University Institutional Review Board (IRB#070722). A total of 131 patients were recruited and identified by GW hospital Medical Faculty Associates Physicians in the course of routine medical exams, and by routine screenings for hyperlipidemia or hypertension. The patient inclusion criteria and exclusion criteria are listed in Table 1. The patients began aspirin therapy with supplied aspirin (non-enteric coated) at a dose of 81 mg/day for 7-10 days to be taken prior to breakfast. After post-aspirin treatment, the patients returned to the GW hospital for blood and urine collection. 2.5 ml of blood was drawn in Vacuette®2ml 9NC Coagulation Sodium Citrate 3.2% tubes (Greiner Bio-One, NC, USA) for platelet function test. 5 ml of blood was drawn in PAXgene Blood RNA tubes (BD, NJ, USA) for total RNA purification and genome-wide RNA profiling. 3 ml of blood was drawn in BD Vacutainer® K2 tubes for CBC w/difs. 2.5 ml of urine sample was collected in BD Vacutainer® urine C&S preservative plus plastic tubes for 11-dehydro-thromboxane B_2 measurement.

Platelet function test must be done in 2 hours after blood collection using VerifyNow® System and VerifyNow® Aspirin cartridge, both of which are manufactured by Accumetrics. This system is based on turbidimetric optical detection of platelet aggregation in whole blood. Whole blood was transferred into cartridges containing AA and lyophilized fibrinogen-coated beads. Consequently, the system converts light transmittance results into Aspirin Reaction Unit which is dependent on degree of aggregation. Patients with Aspirin Reaction Units ≥ 550 are considered as aspirin resistant.

Table 1. Inclusion and exclusion criteria for patients' recruitment.

Inclusion Criteria:
Patients who have already been prescribed aspirin by their physician, or who have already been recommended to take aspirin by their physician.
At risk patients are operationally defined as possessing a major risk factor for cardiac events.
All enrolled patients will be 40 to 80 year old males and females possessing any one of the following risk categories:
(1). known coronary artery disease or atherosclerosis, confirmed by carotid or peripheral arterial ultrasound, CT, or cardiac/peripheral catheterization and angiography.
(2). diabetes
(3). any of the following cardiovascular risk factors:
- male over age 40, female over age 50
- family history of coronary artery disease,
- smoking,
- hypertension,
- hyperlipidemia (as defined by Adult Treatment Panel III Guidelines: http://www.nhlbi.nih.gov/) or HDL <40mg/dL
- chronic renal insufficiency
Exclusion Criteria:
Known GI bleeding attributed to aspirin.
Active peptic ulcer disease.
History of peptic ulcer disease and currently not taking proton pump inhibitor.
Known aspirin allergy.
Current Warfarin or heparin treatment.
NSAIDs (except aspirin), clopidogrel, dipyridamole (Aggrenox) use within past 30 days.
Current steroid use.
Acute coronary syndrome within the past year.
TIA or stroke within the past year.
Fish oil-omega 3 supplements.
Any chronic systemic inflammatory condition (rheumatoid arthritis, SLE, HIV).

Whole Blood Total RNA Purification

The blood was drawn in PAXgene tubes and incubated for 2 hours at room temperature before moving to -20^{o}C or -80^{o}C freezer if RNA is not immediately isolated. Total RNA was purified from the whole blood sample using PAXgene Blood RNA kit (PreAnalytiX, A QIAGEN/BD Company) according to the manufacture's protocol. The final volume of purified total RNA was 80ul. 16 RNA samples (8 resistant and 8 sensitive) were further cleaned up for microarray analysis using RNeasy® Mini kit (QIAGEN Sciences, MD, USA), and the final volume was 50ul dissolved in RNase-free water. All the RNA samples were stored at -80^{o}C for further assays.

RNA Quantification and Quality Control

RNA concentration was quantitated by NanoDrop spectrophotometer ND-100 (Thermo Scientific) with purity expressed by the 260/280 ratio.

The quality control of purified total RNA was tested on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with Agilent RNA 6000 Nano Reagents and chips. In this analysis, RNA integrities were determined based on the ratio of intensity 18S and 28S to the background. RNA samples were selected using the RNA Integrity Number (RIN) cut-off >8.0.

Microarray Assays

An aliquot 10ul from each of the 16 RNA samples was diluted to 20ng/ul with molecular-grade water. Then we use 100ng (5ul) of purified RNA for reverse transcription. The resulting cDNA were amplified with Ovation™ RNA Amplification System V2 kit

(NuGEN Technologies Inc., CA, USA) according to the manufacture's protocol. The cDNA was further purified with DNA Clean & Concentrator™-25 kit (Zymo Research, CA, USA) and 3.75ug of cDNA (concentration measured by NanoDrop) from each sample was fragmented and labeled with FL-Ovation™ cDNA Biotin Module V2 kit (NuGEN Technologies Inc., CA, USA). We use 27ul out of 60ul of fragmented and labeled cDNA to make a hybridization cocktail with the Affymetrix GeneChip® Hybridization, Wash and Stain Kit. 80ul of hybridization cocktail was injected into each GeneChip® Human Genome U133 Plus 2.0 Array. The hybridization was carried out at 45^{o}C with 60rpm rotation in an Affymetrix Hybridization Oven 640 for 18 hours. Then, the arrays were stained using the Stain Module of Affymetrix's GeneChip® Hybridization, Wash and Stain Kit on an Affymetrix Fluidics Station 450, under control of Fluidics Control, a component of Affymetrix Genechip Commend Console (AGCC) system. The stained arrays were scanned on an Affymetrix GeneChip® Scanner 3000 7G, controlled by Scan Control Module of AGCC.

Microarray Data Analysis

The Microarray data was analyzed using GeneSpring GX 10.0.2 (Agilent Technologies, Santa Clara, CA, USA). After creating a new project, we chose Affymetrix Expression as experiment type and GCRMA as summarization algorithm, with baseline to median of all samples. All the 16 samples were divided into two groups: aspirin resistant (AR) group (treatment) and aspirin sensitive (AS) group (control). We used T-Test unpaired as the statistical analysis method, with p-value computation set as asymptotic and multiple testing correction set as no correction. By choosing a p-value cut-off ≤ 0.05, a list of 2551

genes was generated after the statistical analysis. The differentially expressed genes list was further filtered down to 284 genes by doing fold-change analysis with the cut-off ≥ 1.8. The differentially expressed 284-gene list was imported into Ingenuity Pathway Analysis software (Ingenuity Systems, Inc., Redwood City, CA, USA) to further explore associated statistically significant biological functions, molecular interaction networks and potential pathways.

Reverse Transcription Assays and Real-Time PCR

A total of 500ng RNA template was reverse transcribed to cDNA using iScript™ cDNA synthesis kit (BIO-RAD, CA, USA). The cDNA was diluted 5 times (20ul to 100ul) and 2ul was used for downstream real-time PCR analysis. Each real-time PCR reaction consisted of 2ul of cDNA, 1ul of each primer, 12.5ul of Power SYBR®Green PCR Master Mix (Applied Biosystems, Warrington, UK), and 8.5ul of molecular-grade water. Duplicates were performed for each sample. ABI Real Time PCR 7300 System was used for running the real-time PCR with the following condition: 50°C for 2min, 95°C for 10min, 40 cycles each contains 15sec at 95°C and 1min at 60°C, and a dissociation stage consisting of 15sec at 95°C, 1min at 60°C and another 15sce at 95°C. The 18S gene was used as an internal control for normalization purpose. The relative expression values were calculated using log2 transformation based on the average of duplicates against the values of 18S.

Chapter III: Results

Patients' Demographic and Clinical Data

By up-to-date, a total of 131 patients were involved in our study and their data (ID, ARU, age, gender and urinary creatinine level) are listed in table 2. All the patients are divided into 4 groups based on their ARU−AR group (9 patients, 6.87%), AS group 1 (23 patients, 17.56%), AS group 2 (40 patients, 30.53%) and AS group 3 (59 patients, 45.04%). The 16 patients whose whole blood total RNA was used for microarray analysis are labeled in green.

Table 2. Demographic and clinical data of all 131 patients involved in AR study.

Patient ID	**Groups**	**ARU**	**Age**	**Gender**	Creatinine, Urine (mg/dL)
AR Group (ARU ≥ 550):					
AR081	R	590	65	M	41
AR158	R	586	69	M	51
AR067	R	581	76	M	78
AR060	R	580	42	F	197
AR039	R	577	57	M	360
AR022	R	558	49	F	104
AR044	R	556	56	F	105
AR046	R	552	57	M	48
AR099	R	551	63	M	63

Patient ID	Groups	ARU	Age	Gender	Creatinine, Urine (mg/dL)
AS Group 1 (ARU 490-550):					
AR006	S	548	57	F	139
AR168	S	548	53	F	47
AR049	S	537	75	M	191
AR051	S	531	63	F	236
AR028	S	525	73	F	146
AR015	S	524	76	M	410
AR073	S	524	56	M	151
AR027	S	523	63	M	96
AR065	S	522	50	M	243
AR030	S	520	70	M	112
AR151	S	519	56	F	148
AR071	S	514	75	M	141
AR052	S	513	59	M	194
AR161	S	511	45	F	67
AR082	S	507	66	M	213
AR125	S	506	58	F	116
AR144	S	504	58	F	84
AR012	S	500	72	M	480
AR156	S	498	52	F	72
AR165	S	498	41	F	32
AR031	S	496	79	F	60
AR120	S	494	71	M	36
AR007	S	492	57	M	31
AS Group 2 (ARU 430-490):					
AR033	S	485	70	F	80
AR149	S	483	50	F	200
AR008	S	481	66	M	25
AR013	S	481	48	M	169
AR092	S	481	46	M	35
AR139	S	479	71	M	106
AR023	S	478	68	M	102
AR017	S	475	58	M	59
AR041	S	473	54	F	127
AR093	S	473	59	M	90
AR117	S	473	53	F	229
AR094	S	471			
AR002	S	467	63	M	203
AR167	S	466	40	F	165

Patient ID	**Groups**	**ARU**	**Age**	**Gender**	Creatinine, Urine (mg/dL)
AR075	S	465	62	F	137
AR050	S	464	65	M	81
AR057	S	463	46	F	54
AR085	S	462	68	M	34
AR066	S	461	60	F	41
AR109	S	461	69	M	570
AR054	S	460	58	F	174
AR059	S	459	63	M	166
AR122	S	458	50	F	20
AR155	S	455	53	F	19
AR045	S	454	44	M	151
AR029	S	448	55	F	122
AR062	S	448	69	F	229
AR121	S	445	66	F	114
AR018	S	442	71	F	71
AR095	S	442	65	M	82
AR098	S	442	61	M	136
AR010	S	441	59	M	37
AR114	S	438	75	M	151
AR173	S	437			
AR180	S	436			
AR162	S	432	40	M	75
AR014	S	431	59	M	212
AR001	S	430	57	F	63
AR132	S	430	73	M	144
AR150	S	430	52	F	78
AS Group 3 (ARU < 430):					
AR140	S	429	63	M	206
AR142	S	428	56	F	170
AR127	S	426	69	M	116
AR128	S	426	13	F	177
AR137	S	426	77	F	150
AR004	S	425	52	M	153
AR025	S	425	61	M	87
AR034	S	425	77	F	75
AR032	S	424	64	F	79
AR084	S	423	60	M	109
AR112	S	422	56	M	56
AR157	S	422	51	F	49

Patient ID	Groups	ARU	Age	Gender	Creatinine, Urine (mg/dL)
AR080	S	421	54	M	43
AR138	S	421	69	M	33
AR166	S	421	55	M	175
AR005	S	420	64	F	41
AR061	S	420	76	F	185
AR145	S	420	68	M	322
AR126	S	419	71	M	115
AR048	S	418	62	M	161
AR110	S	417	71	F	147
AR037	S	415	62	M	190
AR160	S	415	40	M	191
AR016	S	414	53	F	222
AR131	S	413	74	M	83
AR134	S	413	61	M	47
AR135	S	413	62	M	98
AR079	S	410	60		127
AR102	S	410	65	F	149
AR152	S	410	62	M	103
AR090	S	409	59	F	78
AR170	S	409			
AR141	S	408	54	M	83
AR154	S	408	60	F	360
AR164	S	407	46	M	164
AR021	S	406	47	M	333
AR086	S	406	62	F	198
AR106	S	406	62	M	58
AR107	S	406	75	M	137
AR108	S	406	76	M	233
AR133	S	406	51	M	252
AR147	S	406	77	M	85
AR153	S	406	65	F	
AR047	S	405	71	M	149
AR089	S	405	67	F	34
AR175	S	404			
AR097	S	403	64	M	248
AR136	S	403	54	M	179
AR104	S	402	80	M	75
AR103	S	401	67	M	139
AR105	S	400	53	M	533
AR163	S	400	55	F	444

Patient ID	Groups	ARU	Age	Gender	Creatinine, Urine (mg/dL)
AR172	S	400			
AR078	S	399	62	M	129
AR143	S	399	56	F	78
AR053	S	390	58	M	230
AR072	S	384	63	F	172
AR024	S	380	78	M	253
AR058	S	350	63	M	166

The Quality Control of 16 Purified Blood Total RNA Samples

The quality control of purified total RNA was tested on Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Reagents and chips. The RNA integrity profiles of all 16 samples are shown in Figure1. The major two peaks are referred to 18S and 28S rRNA. All 16 samples except for AR103 got RIN > 8.0, which indicated high quality and integrity of the samples.

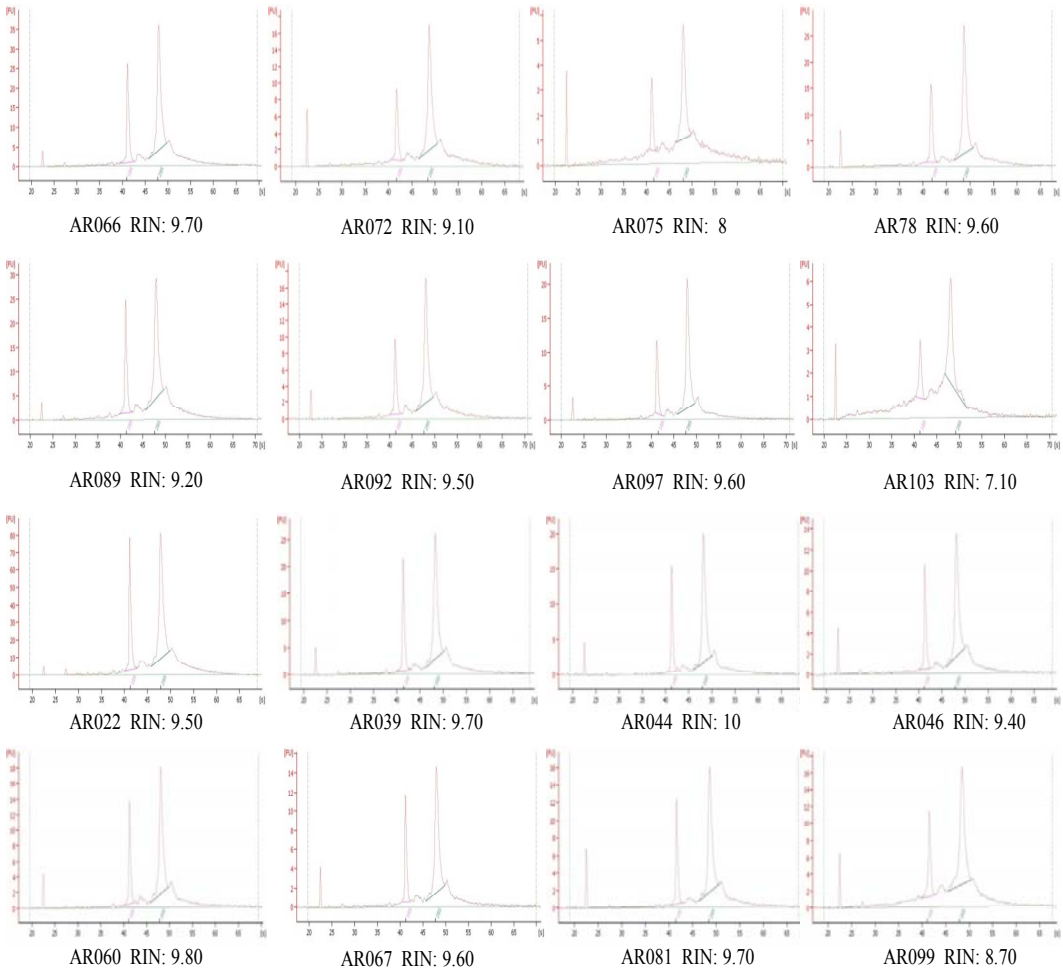
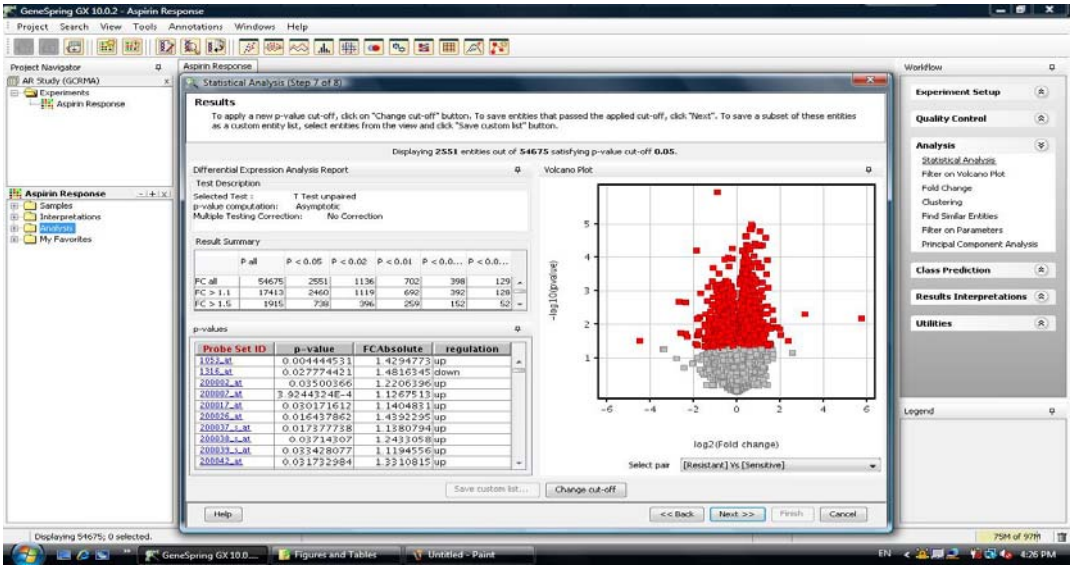


Figure 1. The RNA integrity profiles of all 16 samples. The major two peaks are referred to 18S and 28S rRNA. All 16 samples except for AR103 got RIN > 8.0, which indicated high quality and integrity of the samples.

Identification of Candidate Genes Associated With Aspirin Resistance

In order to identify genes differentially expressed between aspirin resistant group and aspirin sensitive group, we compared the expression profiles of 54675 probes. A total of 2551 genes remained after the statistical analysis by choosing a p-value cut-off ≤ 0.05 (Figure 2A). The differentially expressed gene list was further filtered down to 284 genes by doing fold-change analysis with the cut-off ≥ 1.8 (Figure 2B). 129 genes were up-regulated, whereas 155 were down-regulated in the AR group (Table 3).

A



B

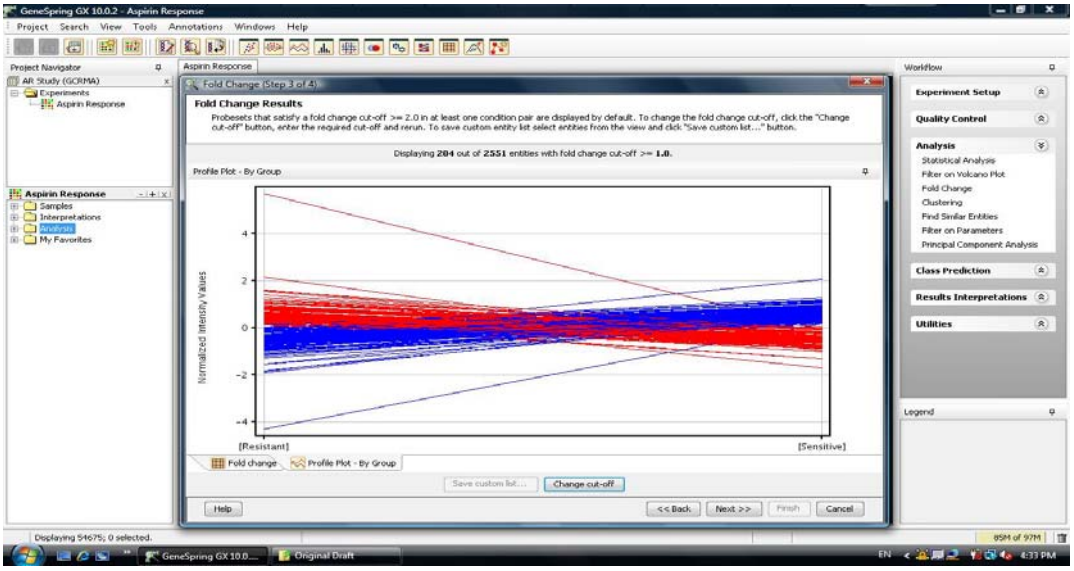


Figure 2. Microarray data analysis of all 16 samples using GeneSpring GX 10.0.2.

(A). Statistical analysis results. By choosing a p-value cut-off ≤ 0.05, a list of 2551 genes was generated after the statistical analysis. (B). Fold change analysis results. The

differentially expressed genes list was further filtered down to 284 genes by doing fold-change analysis with the cut-off ≥ 1.8.

Table 3. List of differentially expressed genes comparing AR group and control group. 284 genes remained after statistical analysis and fold-change analysis by GeneSpring GX 10.0.2. 129 genes were up-regulated whereas 155 genes were down-regulated in AR group.

Genes up-regulated in AR group:

UniGene ID	Gene Symbol	Description	Fold Change
Hs.712540	HLA-DRB4	major histocompatibility complex, class II, DR beta 4	54.136
Hs.211751	BEGAIN	brain-enriched guanylate kinase-associated homolog (rat)	8.760
Hs.647090	TMEM176B	transmembrane protein 176B	5.105
Hs.313	SPP1	secreted phosphoprotein 1	4.052
Hs.272398	ETV7	ets variant gene 7 (TEL2 oncogene)	3.971
Hs.146688	PTGES	prostaglandin E synthase	3.623
Hs.502314	LOC400713	zinc finger-like	3.195
Hs.654720	BIN3	bridging integrator 3	3.141
Hs.490789	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	2.952
Hs.72026	PRSS21	protease, serine, 21 (testisin)	2.919
Hs.527193	RPS23	ribosomal protein S23	2.917
Hs.493639	C9orf72	chromosome 9 open reading frame 72	2.702
Hs.532231	COPG2	coatomer protein complex, subunit gamma 2	2.650
Hs.470126	KYNU	kynureninase (L-kynurenine hydrolase)	2.646
Hs.351475	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	2.640
Hs.710500	HAVCR2	hepatitis A virus cellular receptor 2	2.630
Hs.560087	CLECL1	C-type lectin-like 1	2.618
Hs.42151	HNMT	histamine N-methyltransferase	2.551
Hs.487562	LOC100132288	similar to hypothetical protein MGC27019 /// similar to tektin 4	2.504
Hs.647110	LOC285972	hypothetical protein LOC285972	2.374
Hs.212885	CNRIP1	cannabinoid receptor interacting protein 1	2.352
Hs.78619	GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	2.331
Hs.596210		CDNA clone IMAGE:4824710	2.321
Hs.631558	TNNT1	troponin T type 1 (skeletal, slow)	2.320
Hs.329266	HIP1	huntingtin interacting protein 1	2.315
Hs.501114	RNF165	ring finger protein 165	2.274
Hs.411925	TMEM38B	transmembrane protein 38B	2.265
Hs.329266	HIP1	Huntingtin interacting protein 1	2.262
Hs.351811	CLEC4D	C-type lectin domain family 4, member D	2.257
Hs.339918	CLLU1	chronic lymphocytic leukemia up-regulated 1	2.210
Hs.441550	ABHD12	abhydrolase domain containing 12	2.200
Hs.466165	PGLS	6-phosphogluconolactonase	2.191

UniGene ID	Gene Symbol	Description	Fold Change
Hs.476358	CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	2.189
Hs.516629	ICA1L	islet cell autoantigen 1,69kDa-like	2.187
Hs.495912	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	2.184
Hs.567359	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	2.173
Hs.633978	FLJ35390	hypothetical LOC255031	2.169
Hs.130838	LOC285835	hypothetical protein LOC285835	2.151
Hs.504301	TMEM45B	transmembrane protein 45B	2.149
Hs.6909	MOXD1	monooxygenase, DBH-like 1	2.145
Hs.591602	SSFA2	sperm specific antigen 2	2.138
Hs.518834	B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	2.111
Hs.42151	HNMT	histamine N-methyltransferase	2.102
Hs.127675	CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	2.101
Hs.404741	NFE2L3	nuclear factor (erythroid-derived 2)-like 3	2.097
Hs.658686		CDNA clone IMAGE:5268630	2.092
Hs.350875	ZNF570	zinc finger protein 570	2.084
Hs.417948	TCN2	transcobalamin II; macrocytic anemia	2.077
Hs.645458	MOBKL3	MOB1, Mps One Binder kinase activator-like 3 (yeast)	2.076
Hs.293077	CHPT1	Choline phosphotransferase 1	2.074
Hs.525462	C1orf41	chromosome 1 open reading frame 41	2.061
Hs.339918	CLLU1	chronic lymphocytic leukemia up-regulated 1	2.058
Hs.522699	COX7B	cytochrome c oxidase subunit VIIb	2.057
Hs.143929	CLEC7A	C-type lectin domain family 7, member A	2.054
Hs.444024	SNX19	sorting nexin 19	2.042
Hs.253576	CCDC147	coiled-coil domain containing 147	2.042
Hs.143929	CLEC7A	C-type lectin domain family 7, member A	2.042
Hs.709491	LOC645513	Hypothetical LOC645513	2.014
Hs.374067	UBE3B	ubiquitin protein ligase E3B	2.013
Hs.444229	ARHGAP24	Rho GTPase activating protein 24	2.010
Hs.660143	APOBEC3F	apolipoprotein B mRNA editing enzyme	2.007
Hs.567359	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	2.006
Hs.647450	PROCR	protein C receptor, endothelial (EPCR)	2.005
Hs.525549	BTBD7	BTB (POZ) domain containing 7	2.003
Hs.584788	PDE7A	phosphodiesterase 7A	2.001
Hs.272225	TRPM6	transient receptor potential cation channel, subfamily M, member 6	2.001
Hs.468840	PLEK	pleckstrin	1.992
Hs.127675	CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	1.990
Hs.444229	ARHGAP24	Rho GTPase activating protein 24	1.988
Hs.22587	SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein	1.988
Hs.645492	MCART1	mitochondrial carrier triple repeat 1	1.987
Hs.70769	C6orf162	chromosome 6 open reading frame 162	1.978
Hs.440553	ZNF473	zinc finger protein 473	1.977
Hs.655657	EXOC6	exocyst complex component 6	1.973
Hs.351475	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	1.970
Hs.170568	TATDN1	TatD DNase domain containing 1	1.954
Hs.436564	PREP	Prolyl endopeptidase	1.953
Hs.333823	MRPL13	mitochondrial ribosomal protein L13	1.952
Hs.592347	RNMT	RNA (guanine-7-) methyltransferase	1.949
Hs.511991	NPHP3	nephronophthisis 3 (adolescent)	1.927
Hs.12056	ASGR1	asialoglycoprotein receptor 1	1.925
Hs.88663	CENPQ	centromere protein Q	1.922
Hs.699402	LOC202181	hypothetical protein LOC202181	1.921

UniGene ID	Gene Symbol	Description	Fold Change
Hs.193491	TUBB6	tubulin, beta 6	1.919
Hs.477547	TMCC1	transmembrane and coiled-coil domain family 1	1.917
Hs.655623	PIN4	protein (peptidylprolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	1.915
Hs.200250	CREM	cAMP responsive element modulator	1.907
Hs.436445	OSGIN2	oxidative stress induced growth inhibitor family member 2	1.906
Hs.502	TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	1.904
Hs.438993	BCAT1	branched chain aminotransferase 1, cytosolic	1.903
Hs.522356	ORM1 /// ORM2	orosomucoid 1 /// orosomucoid 2	1.895
Hs.478729	TMEM44	transmembrane protein 44	1.888
Hs.654449	PGBD3	piggyBac transposable element derived 3	1.879
Hs.126248	COL9A3	collagen, type IX, alpha 3	1.862
Hs.131700	LOC100129461	Hypothetical protein LOC100129461	1.854
Hs.592283	STAG3	stromal antigen 3	1.853
Hs.288658	ZNF35	zinc finger protein 35	1.850
Hs.269988	ROD1	ROD1 regulator of differentiation 1 (S. pombe)	1.845
Hs.72325	HINT3	histidine triad nucleotide binding protein 3	1.844
Hs.633978	FLJ35390	hypothetical LOC255031	1.833
Hs.660728	ZNF418	zinc finger protein 418	1.833
Hs.660628		Partial mRNA; ID YG39-1A	1.819
Hs.477325	EAF2	ELL associated factor 2	1.819
Hs.655165	C7orf44	chromosome 7 open reading frame 44	1.816
Hs.532019	MRPL1	mitochondrial ribosomal protein L1	1.811
Hs.441601	FLJ42957	FLJ42957 protein	1.809
Hs.492716	WDR67	WD repeat domain 67	1.808
Hs.508266	COMMD6	COMM domain containing 6	1.807
Hs.712570	ZNF193	zinc finger protein 193	1.805

Genes down-regulated in AR group:

Hs.387679	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	22.157
Hs.232165	CD177	CD177 molecule	6.378
Hs.161839	MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	5.105
Hs.591391	DEFA4	defensin, alpha 4, corticostatin	5.089
Hs.434181	TREML4	triggering receptor expressed on myeloid cells-like 4	4.840
Hs.307835	PGM5	phosphoglucomutase 5	3.787
	LOC643224	similar to tubulin, beta 8	3.647
Hs.529517	LTF	lactotransferrin	3.577
Hs.710902	LOC100129014	hypothetical protein LOC100129014	3.569
Hs.619315	LTBP1	latent transforming growth factor beta binding protein 1	3.500
Hs.654595	VIL1	villin 1	3.496
Hs.204238	LCN2	lipocalin 2	3.376
Hs.41	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	3.324
Hs.419815	EGF	epidermal growth factor (beta-urogastrone)	3.311
Hs.124128	KIAA2022	KIAA2022	3.178
Hs.443789	C6orf60	chromosome 6 open reading frame 60	2.824
Hs.82906	MPL	myeloproliferative leukemia virus oncogene	2.806
Hs.656425	PEX6	peroxisomal biogenesis factor 6	2.774
Hs.159430	FNDC3B	fibronectin type III domain containing 3B	2.757
Hs.89714	CXCL5	chemokine (C-X-C motif) ligand 5	2.728
Hs.504370	LOC283174	Hypothetical LOC283174	2.683
Hs.226390	RRM2	ribonucleotide reductase M2 polypeptide	2.566
Hs.411312	ITGA2B	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	2.504
Hs.369703	SLC35D3	solute carrier family 35, member D3	2.501

UniGene ID	Gene Symbol	Description	Fold Change
Hs.10414	KLHDC8A	kelch domain containing 8A	2.476
Hs.130692	C12orf39	chromosome 12 open reading frame 39	2.437
Hs.356270	LOC647081 /// SDHD	succinate dehydrogenase complex, subunit D, integral membrane protein	2.432
Hs.499659	GARNL4	GTPase activating Rap/RanGAP domain-like 4	2.422
Hs.703408	TSHZ2	teashirt zinc finger homeobox 2	2.359
Hs.283091	RETN	resistin	2.358
Hs.533566	H19	H19, imprinted maternally expressed transcript	2.357
Hs.467662	ITGB1BP1	integrin beta 1 binding protein 1	2.355
Hs.371980	LOC650392	Hypothetical protein LOC650392	2.353
Hs.703408	TSHZ2	Teashirt zinc finger homeobox 2	2.350
Hs.201555	ABHD7	abhydrolase domain containing 7	2.339
Hs.218040	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	2.335
Hs.271771	SNCA	synuclein, alpha (non A4 component of amyloid precursor)	2.296
Hs.709211	FSTL1	follistatin-like 1	2.292
Hs.642748	TTC16	tetratricopeptide repeat domain 16	2.287
Hs.36761	HRASLS	HRAS-like suppressor	2.261
Hs.656425	PEX6	peroxisomal biogenesis factor 6	2.261
Hs.21239	RPH3A	rabphilin 3A homolog (mouse)	2.211
Hs.191911	NFIA	nuclear factor I/A	2.206
Hs.567260	DSC1	desmocollin 1	2.198
Hs.463421	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	2.193
Hs.84665	MYOT	myotilin	2.177
Hs.164226	THBS1	thrombospondin 1	2.174
Hs.271605	TSHZ2	teashirt zinc finger homeobox 2	2.168
Hs.436975	CLOCK	clock homolog (mouse)	2.152
Hs.567828	DPY19L4	dpy-19-like 4 (C. elegans)	2.151
Hs.534307	CCND3	Cyclin D3	2.125
Hs.712554	CD6	CD6 molecule	2.124
Hs.199743	ME3	malic enzyme 3, NADP(+)-dependent, mitochondrial	2.120
Hs.414880	USF1	upstream transcription factor 1	2.112
Hs.469199	RNF103	ring finger protein 103	2.095
Hs.298987	LOC100130097	Hypothetical protein LOC100130097	2.095
Hs.567524	C10orf132	chromosome 10 open reading frame 132	2.083
Hs.473927	PDE9A	phosphodiesterase 9A	2.073
Hs.654825	DOCK9	Dedicator of cytokinesis 9	2.069
Hs.512856	SEC14L5	SEC14-like 5 (S. cerevisiae)	2.045
Hs.72885	AZU1	azurocidin 1 (cationic antimicrobial protein 37)	2.040
Hs.213642	SLC35D1	solute carrier family 35 (UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter), member D1	2.040
Hs.99863	ELA2	elastase 2, neutrophil	2.037
Hs.484918	CMAH	CMP-N-acetylneuraminate monooxygenase pseudogene	2.035
Hs.63788	PCCB	Propionyl Coenzyme A carboxylase, beta polypeptide	2.032
Hs.163244	LRRN1	leucine rich repeat neuronal 1	2.030
Hs.709829	LOC728052	similar to hCG2031213	2.019
Hs.444957	ATP8A2	ATPase, aminophospholipid transporter-like, class I, type 8A, member 2	2.002
Hs.239370	GPSM1	G-protein signaling modulator 1 (AGS3-like, C. elegans)	2.002
Hs.2006	GSTM3	glutathione S-transferase M3 (brain)	2.000
Hs.591088	IL11RA	interleukin 11 receptor, alpha	1.996
Hs.494261	PSAT1	phosphoserine aminotransferase 1	1.993
Hs.659681	BCOR	BCL6 co-repressor	1.983
Hs.89839	EPHA1	EPH receptor A1	1.960
Hs.269591	EXPH5	exophilin 5	1.949

UniGene ID	Gene Symbol	Description	Fold Change
Hs.6638	MYEF2	myelin expression factor 2	1.949
Hs.411312	ITGA2B	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	1.945
Hs.607775		Transcribed locus	1.942
Hs.708032	TM4SF1	transmembrane 4 L six family member 1	1.940
Hs.676982	CXorf50	chromosome X open reading frame 50 /// hCG1731871 /// hypothetical protein LOC100132401	1.922
Hs.153322	PLCL1	phospholipase C-like 1	1.921
Hs.1565	NEDD4	neural precursor cell expressed, developmentally down-regulated 4	1.916
Hs.442530	TBXA2R	thromboxane A2 receptor	1.904
Hs.511454	PLXNA4	plexin A4	1.903
Hs.665654	ZMYM2	zinc finger, MYM-type 2	1.901
Hs.591378	ZNF57	zinc finger protein 57	1.894
Hs.14248	STON2	stonin 2	1.879
Hs.212774	UBA6	ubiquitin-like modifier activating enzyme 6	1.876
Hs.239	FOXM1	forkhead box M1	1.871
Hs.58685	CD5	CD5 molecule	1.871
Hs.619315	LTBP1	latent transforming growth factor beta binding protein 1	1.869
	TncRNA	trophoblast-derived noncoding RNA	1.860
Hs.656856	ZNF662	zinc finger protein 662	1.858
Hs.654448	DEFA1 /// DEFA3	defensin, alpha 1 /// defensin, alpha 3, neutrophil-specific	1.856
Hs.536663	ITGB5	Integrin, beta 5	1.846
Hs.564945	LOC653786	otoancorin pseudogene	1.844
Hs.655297		Neuroblastoma cDNA, clone:Nbla00136, full insert sequence	1.844
Hs.473648	GART	Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase	1.835
Hs.709609	TTLL5	tubulin tyrosine ligase-like family, member 5	1.835
Hs.332708	FBLN5	fibulin 5	1.834
Hs.501622	TMEM16J	transmembrane protein 16J	1.825
Hs.466539	CLIP3	CAP-GLY domain containing linker protein 3	1.821
Hs.259412	C1orf63	chromosome 1 open reading frame 63	1.819
Hs.646418		Transcribed locus, moderately similar to NP_001026424.1 heterogeneous nuclear ribonucleoprotein A3	1.816
Hs.706739	AGER	advanced glycosylation end product-specific receptor	1.815
Hs.154329	TMEM16E	transmembrane protein 16E	1.815
Hs.371001	EIF3B	eukaryotic translation initiation factor 3, subunit B	1.808

Characterization of the 284 Genes Using Ingenuity Pathways Analysis (IPA)

In order to gain functional insight into how the 284 genes might be related to aspirin resistance. We employed core analysis function of IPA to further explore statistically significant biological functions, molecular interaction networks and potential pathways associated with our 284-gene list. Of the returned result, we found that hematological

system development and function was ranked as #1 in the Top Networks section and cardiovascular disease was ranked as #3 in the Top Bio functions Diseases and Disorders section, both of which were statistically significant. The cardiovascular disease function group contains 36 molecules which are listed in Table 4. These molecules are classified into nine subgroups which are ischemia, angina pectoris, cardiovascular disorder, infarction, stroke, retraction, thromboembolism, atrial fibrillation and diastolic dysfunction. We noticed that there are two genes- HLA-DRB4 and HLA-DQA1, in the 284 gene list but not in this 36 gene list. Although these two genes are not reported by any study to be related to blood coagulation, considering their highest fold-change value, we still included them with the other 36 genes in Table 4 as our candidate genes. The hematological system development and function network is shown in Figure 3. Molecules in green and red are up-regulated and down-regulated respectively. After comparing the molecules between cardiovascular disease function group and hematological system development and function network, checking their microarray data and gene ontology annotations (molecular function, biological process and cellular component), we eventually identified two genes−THBS1 and PTGES as our candidate biomarkers. Another network was established for these two genes showing the direct and indirect interplays between them and their interacting partners (Figure 4).

Table 4. 36 out of 284 genes were identified as cardiovascular disease-related after core analysis using IPA. We manually added another two genes−HLA-DQA1 and HLA-DRB4 to the list because of their high fold-change value. In this list, 17 genes were up-regulated whereas 21 genes were down-regulated in AR group.

Genes up-regualted in AR group

UniGene ID	Gene Symbol	Description	Fold Change
Hs.712540	HLA-DRB4	major histocompatibility complex, class II, DR beta 4	54.136
Hs.313	SPP1	secreted phosphoprotein 1	4.052
Hs.146688	PTGES	prostaglandin E synthase	3.623
Hs.490789	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	2.952
Hs.493639	C9orf72	chromosome 9 open reading frame 72	2.702
Hs.42151	HNMT	histamine N-methyltransferase	2.551
Hs.329266	HIP1	huntingtin interacting protein 1	2.315
Hs.476358	CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	2.189
Hs.495912	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	2.184
Hs.6909	MOXD1	monooxygenase, DBH-like 1	2.145
Hs.374067	UBE3B	ubiquitin protein ligase E3B	2.013
Hs.647450	PROCR	protein C receptor, endothelial (EPCR)	2.005
Hs.584788	PDE7A	phosphodiesterase 7A	2.001
Hs.592347	RNMT	RNA (guanine-7-) methyltransferase	1.949
Hs.200250	CREM	cAMP responsive element modulator	1.907
Hs.438993	BCAT1	branched chain aminotransferase 1, cytosolic	1.903
Hs.478729	TMEM44	transmembrane protein 44	1.888

Genes down-regualted in AR group

Hs.387679	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	22.157
Hs.619315	LTBP1	latent transforming growth factor beta binding protein 1	3.500
Hs.204238	LCN2	lipocalin 2	3.376
Hs.226390	RRM2	ribonucleotide reductase M2 polypeptide	2.566
Hs.411312	ITGA2B	integrin, alpha 2b	2.504
Hs.218040	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	2.335
Hs.271771	SNCA	synuclein, alpha (non A4 component of amyloid precursor)	2.296
Hs.709211	FSTL1	follistatin-like 1	2.292
Hs.191911	NFIA	nuclear factor I/A	2.206
Hs.164226	THBS1	thrombospondin 1	2.174
Hs.436975	CLOCK	clock homolog (mouse)	2.152
Hs.414880	USF1	upstream transcription factor 1	2.112
Hs.654825	DOCK9	Dedicator of cytokinesis 9	2.069
Hs.213642	SLC35D1	solute carrier family 35, member D1	2.040
Hs.99863	ELA2	elastase 2, neutrophil	2.037
Hs.444957	ATP8A2	ATPase, aminophospholipid transporter-like, class I, type 8A, member 2	2.002
Hs.442530	TBXA2R	thromboxane A2 receptor	1.904
Hs.511454	PLXNA4	plexin A4	1.903
Hs.665654	ZMYM2	zinc finger, MYM-type 2	1.901
Hs.212774	UBA6	ubiquitin-like modifier activating enzyme 6	1.876
Hs.706739	AGER	advanced glycosylation end product-specific receptor	1.815

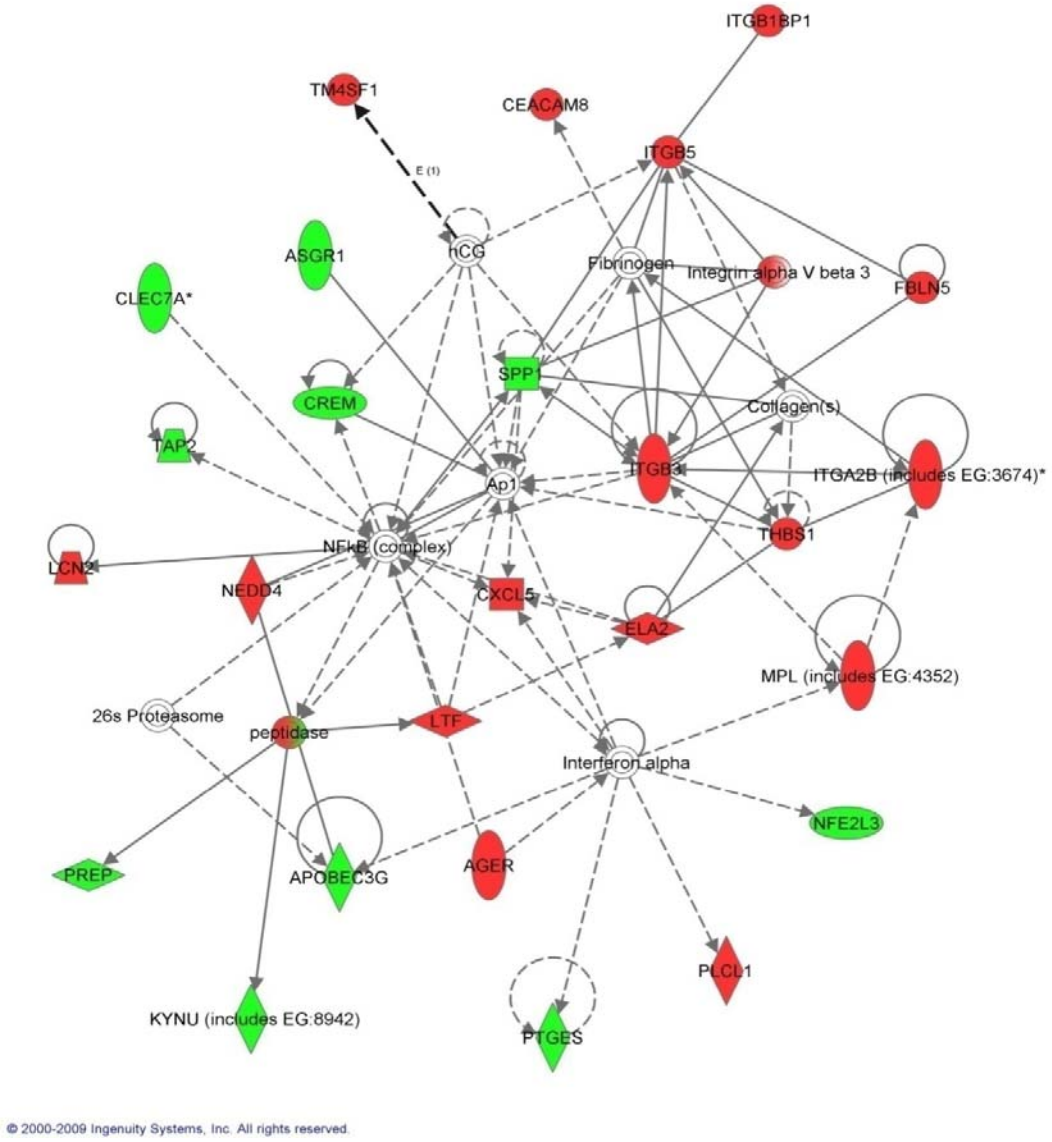


Figure 3. The hematological system development and function network generated by IPA. This network represents the most significant molecular interactions associated with the 284 genes identified by microarray analysis. Several genes in this list are closely related to blood coagulation process, such as ITGA2B, ITGB3, THBS1, PTGES, AGER and DMD. Molecules in green and red are up-regulated and down-regulated respectively.

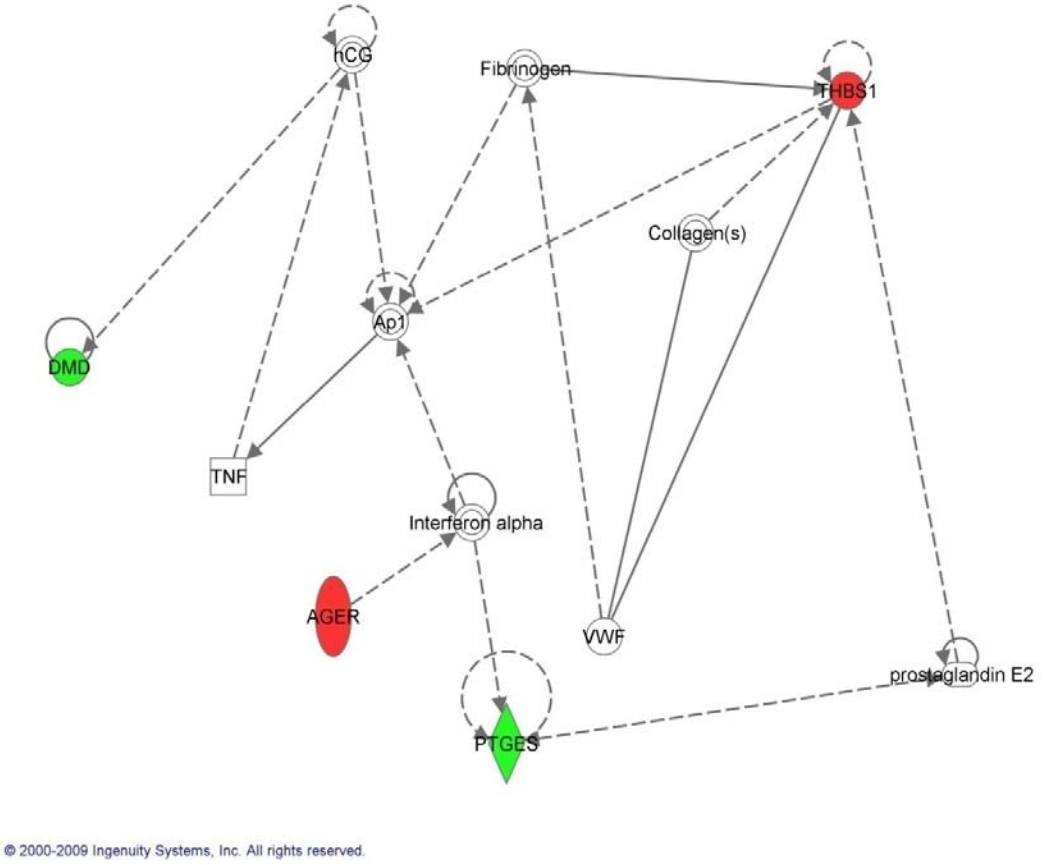


Figure 4. Customized network showing the direct and indirect interplays between our candidate biomarkers (THBS1 and PTGES) and their interacting partners.

Confirmation of Candidate Biomarkers' Expression Level:

So far, we got the real-time PCR data for 4 genes which are HLA-DRB4, HLA-DQA1, THBS1 and PTGES (Figure 5). The real-time PCR data showed consistent result with microarray analysis data in terms of the gene expression level.

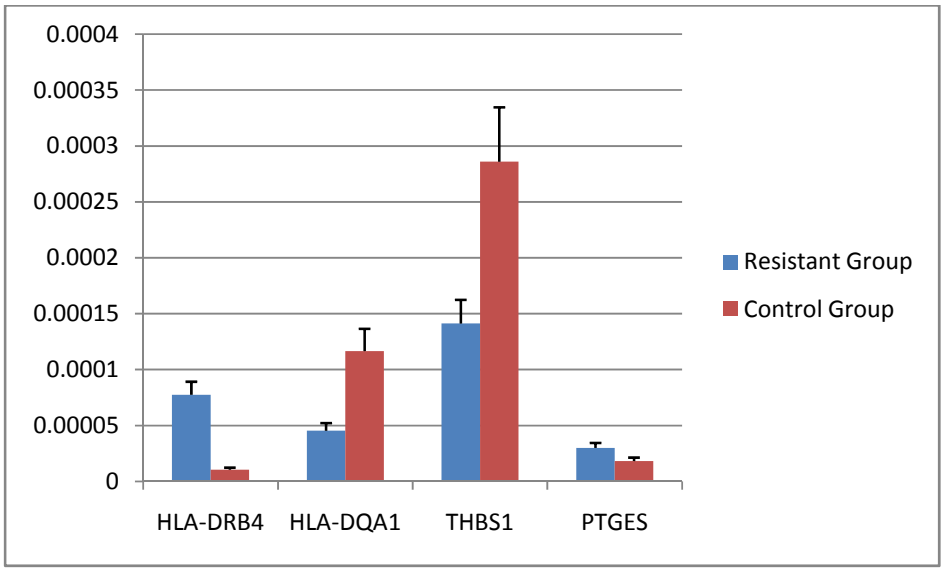


Figure 5. Confirmation of Candidate Biomarkers' Expression Level. Gene expression levels were compared between AR Resistant Group (in blue) and Control Group (in red) for HLA-DRB4, HLA-DQA1, THBS1 and PTGES. The experiment was repeated twice with duplicates.

Chapter IV: Discussion

As introduced in the background, a lot of research has been done trying to explain the factors and mechanisms contributing to aspirin resistance, most of which are based on studying genetic polymorphism of COX-1 and other platelet receptors. Considering the fact that the mechanism behind aspirin resistance phenomenon might be very complicated and influenced by multiple factors, we came up with the idea of using microarray technology to profile the whole blood gene expression. Preliminary studies have documented quantitative mRNA differences in disease states ranging from asthma to mental stress, when compared with control populations. We have to note that the major limitation of our method is the fact that blood-derived mRNA mainly comes from lymphocytes which are not necessarily involved in blood coagulation process. But a growing literature also indicates that changes in peripheral lymphocyte-derived gene expression may, to some extent, mirror changes in the circulation and other organ systems. We must mention that there used to be 3 major technical difficulties in obtaining high quality RNA from whole blood. First, blood cells gene expression could be changed ex vivo due to stresses induced by separation method. Second, high level of RNase and enzyme inhibitors in the blood could degrade RNA rapidly or interfere with

amplifications. Finally, high levels of globin message contained in red blood cells could overwhelm other signals on the array. These 3 problems have recently been solved by using PAXgene Blood RNA tubes and PAXgene Blood RNA kit. The PAXgene Blood RNA tube contains a fixative which stops RNA transcription and degradation immediately after blood drawing, thereby preventing ex vivo changes in expression. The fixative could also stable the blood sample for up to 3 days at room temperature by neutralizing RNase activity. Finally, the reagents in PAXgene Blood RNA kit contain special peptide-nucleic acid (PNA) blocking primers that prevent reverse transcription of the globin messages.

AR patients were identified by running the platelet function test 7−10 days after aspirin therapy started. We feel that if this test could be run both before and after patients took aspirin, it might provide us with more insights into how aspirin works well in the patient's body and how "aspirin resistant" the patient is. Whether the microarray analysis should be performed in the same way also remains to be discussed. In addition, more follow-up clinical data should be collected and analyzed to see if the AR patients identified by us would also develop any cardiovascular event despite aspirin therapy, which indicates an agreement or disagreement between lab-determined aspirin resistance and clinically determined aspirin resistance.

When we looked at the 284-gene list into details, we found that it is still short of some molecules whose functions are closely associated with blood clot formation, especially those which were reported to be detected in human blood mRNA by other studies. The number of returned genes partially depends on the threshold we set for the fold-change analysis. A cut-off ≥ 1.8 was chosen because we wanted to end up with a list around 200

genes. In future study, the list would be expanded by decreasing the stringency to include more potential candidate biomarkers. We also want to mention that some genes on the list, such as TBXA2R, ITGA2B and ITGB3, are excluded from being candidate biomarkers not because they are functionally unrelated to blood coagulation. Instead, their microarray data regarding gene expression regulation can't support and explain aspirin resistance. Two candidate biomarkers were finalized eventually, which are THBS1 and PTGES.

Von Willebrand factor (vWF) plays an important role in the initial platelets tethering to the subendothelium at high shear after vascular wall injury. VWF also functions as a carrier for coagulation factor VIII, not only prolonging its lifespan in the circulation but also increasing its concentration at sites of vascular injury [76]. VWF performs its function in a size-dependent form, since a selective deficiency of large multimers of vWF is associated with a bleeding diathesis, type IIA von Willebrand disease [77]. THBS1 could reduce vWF multimer size by cleaving the linking disulfide bonds which hold the subunits together [78]. THBS1 is a trimeric protein which exists in both plasma (produced and released by endothelial cells) and platelets α-granules (produced by megakaryocytes). John E. Pimanda et al. collected platelet lysates from both $THBS1^{+/+}$ and $THBS1^{-/-}$ mice. They found that the average vWF multimer size was partially reduced in $THBS1^{+/+}$ but not in $THBS1^{-/-}$ platelets [79]. They also observed smaller vWF multimers in thrombin-activated $THBS1^{+/+}$ platelet releasate compared to $THBS1^{-/-}$ platelet releasate, which supports a role for THBS1 in controlling the multimer size of the very large vWF multimers during platelet activation. In the same study, platelets function tests are also performed using platelet aggregometer and PFA-100®. Both methods

showed a more pronounced platelets aggregation in THBS1$^{-/-}$ mice than that in THBS1$^{+/+}$ mice. Therefore, down-regulated expression of THBS1, as what is observed in our analysis, could lead to insufficient processing of large vWF multimers and more rapid and pronounced thrombus formation. Actually, the thrombotic microangiopathies such as throbotic thrombocytopaenic purpura and the haemolytic uraemic syndrome are observed in a population who have a higher than normal average vWF multimer size [80]. The persistence of unprocessed ultralarge VWF multimers in the circulation is also thought to precipitate platelet clumping in arterioles and capillaries, resulting in tissue ischemia [81]. However, we must note that both plasma vWF and platelet vWF are involved in blood coagulation and their individual contribution is still controversial. It was observed that people with normal plasma and low platelet vWF have either normal or mildly prolonged bleeding times [82], which may indicate a minor role for platelet vWF. In addition, plasma vWF and platelet vWF are separately processed by THBS1 in endothelial cells and α-granule respectively. The THBS1 mRNA expression level in endothelial cells needs to be further investigated.

Prostaglandin E synthase (PTGES) is an enzyme which converts cyclooxygenase (COX)-derived prostaglandin H_2 to PGE_2. PTGES exists in two distinct forms: constitutively expressed cytosolic form (cPTGES) and inducible membrane-associated form (mPTGES). These two PTGESs differentiate in structure, enzyme properties and their functional coupling mechanism-cPTGES is mainly coupled with the constitutive COX-1, whereas mPTGES is preferentially linked with the inducible COX-2 [83]. Lars Kaestner et al. [84] have suggested a role of PGE_2 in activating calcium channels on erythrocytes membrane. The resulting influx of calcium activates another potassium channel and the lipid-

scramblase on erythrocytes membrane, which could further induce cell shrinkage and plasma membrane remodeling. This might be an indication of potential participation of erythrocytes in blood coagulation, because cell shrinkage and plasma membrane remodeling might be linked with an augmented aggregation of erythrocytes and the formation of solid blood clot. Sabrina Gross et al. found that PGE_2, which is produced by aterial wall in response to inflammation, could bind to EP3 receptors on platelets membrane and decrease intracellular cAMP concentration by inhibiting adenylate cyclase [85]. This would lead to increased platelets sensitivity to agonists thus facilitating thrombosis formation. In the same study, another indirect mechanism in which PGE_2 could exacerbate atherothrombosis was also suggested. They found that PGE_2 could stimulate macrophages to produce metalloproteases which subsequently degrade fibrous cap and weaken plaque. Despite validating a role of vascular-wall produced PGE_2 in facilitating thrombosis formation, Sabrina Gross et al. can't demonstrate an equal role of platelet-produced PGE_2 [85]. There are also opposite opinions saying that PGE_2 stimulates cAMP formation in platelets, which leads to inhibition of platelet aggregation [86]. Several studies have suggested an increased production of PGE_2 resulting from the up-regulation of PTGES [83, 87, 88]. However, based on the fact that PTGES existing in different blood cells may varied in their function under certain biological process like thrombosis formation [85, 89], we can't attribute any cardiovascular pathophysiological condition to the up-regulation of PTGES before validating their source in our samples. Another concern is that because PTGES exists in two different isoforms and mPTGES is more involved in various human diseases [90-93], a better understanding of the

association between increased PTGES expression level in AR patients and aspirin resistance awaits further identification of the PTGES in our samples.

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