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Platelet glycoproteins associated with aspirin-treatment upon platelet activation

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Abstract

Platelet glycoproteins are known to play central roles in hemostasis and vascular integrity and have pathologic roles in vascular occlusive diseases such as myocardial infarction and stroke. Characterizing glycoproteins within and secreted by platelets can provide insight into the mechanisms that underlie vascular pathologies and the therapeutic benefits or failure of anti-platelet agents. To study the impact of aspirin, which is commonly prescribed for primary and secondary cardiovascular prevention, on the platelet glycoproteome, we evaluated washed platelets from ten donors. The platelet glycoproteome, was studied using an iTRAQ in resting and stimulated states and with and without aspirin treatment. Using solid phase extraction of glycosite-containing peptides (SPEG), we were able to identify 799 unique N-linked glycosylation sites (glycosites) in platelets, representing the largest and the most comprehensive analysis to date. We were able to identify a number of glycoproteins impacted by aspirin treatment, which we validated using global proteomics analysis of platelets and their secreted proteins. In our analyses, metalloproteinase inhibitor 1 (TIMP1) was the single most significantly affected glycoprotein by aspirin treatment. ELISA assays confirmed proteomic results and validated our strategy. Functional analysis demonstrated that TIMP1 levels were highly correlated with platelet reactivity in vitro, with a correlation coefficient of -0.5 . The release of TIMP1 from platelets, which was previously unknown to be affected by aspirin treatment, may play important roles in hemostasis and/or vascular integrity. If validated, our findings may be useful for developing assays that assess platelet response to aspirin or other anti-platelet therapies.

Keywords

Cardiovascular disease; Glycomics; Glycoproteomics; Metalloproteinase inhibitor; Platelet; TIMP1

1 Introduction

Platelets are the main cellular effector of primary hemostasis. Platelets circulate in the blood in a quiescent state and are activated at sites of vascular injury by exposed subendothelial

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matrix components (e.g. collagen), shear stress, and soluble agonists (e.g. ADP, thrombin). Once activated, platelets adhere to subendothelial molecules and activated endothelial cells, aggregate to form a hemostatic plug, and release soluble mediators that cause feedback activation of hemostasis and promote inflammation and vascular repair. Platelets also participate in immune defense and surveillance through recruitment of professional phagocytes and T cells, modulation of cytokine production, and release of immune modulators, and antimicrobial substances. Pathological activation of platelets causes occlusion of blood flow. A thorough characterization of the molecular constituents of platelets is crucial to understand their normal physiologic functions in hemostasis and immunity as well as their pathologic roles in vascular occlusive diseases such as myocardial infarction and stroke.

Protein glycosylation is one of the most common PTMs, and many studies have shown that platelets contain the enzymes and sugar nucleotides required for glycosylation [1]. Glycoproteins are known to play an important role in platelet adhesion and aggregation. For example, a number of platelet adhesive receptors (GPIIb-IIIa, GPIb-IX-V) and their ligands (fibrinogen, VWF) are glycoproteins. Recently, it was shown that platelet biogenesis and function require glycosylation.[1] For these reasons, several recent studies have reported the characterization of glycoproteins, especially N-linked glycoproteins and their glycosites from platelets using glycopeptides enrichments methods like SPEs of glycopeptides, Lectin, electrostatic repulsion hydrophilic interaction chromatography, and strong cation exchange prefractionation [2–4]. Overall 250 N-glycosites have been previously reported from these studies [2–4]. Although glycoproteins are known to be important in platelet function, a characterization of platelet glycoproteins in response to platelet activation is lacking. Furthermore, no study has evaluated the impact of anti-platelet therapy on platelet glycoproteins. Therapeutic agents that inhibit platelet function, such as aspirin, are known to inhibit the secretion of numerous platelet proteins. However, it is unclear if this inhibitory effect affects all secreted molecules or only a subset, and the specific impact of aspirin on platelet glycoproteins has not been reported.

Aspirin inhibits platelet activation by acetylating cyclooxygenase1 (COX1) at serine 530. COX1 acetylation blocks binding of arachonic acid and inhibits the enzymatic conversion of arachidonic acid to thromboxane A2 in platelets, thus reducing the ability of platelet to aggregate via feedback activation of thromboxane A2 receptors [5, 6]. Aspirin is recommended for primary and secondary prevention of cardiovascular disease events in high risk patients [7]. Recent studies have shown the effectiveness of aspirin treatment on platelets varies among individuals; however, the related impact of aspirin on platelet glycoproteome, proteome, and secretome among individuals are not very well understood. Aspirin is widely used to treat CV disease but its effect is modest and not all patients derive benefit. Aspirin is associated with diminished risk of some cancers but mechanism for this effect is not known.

The purpose of these studies was to: (1) more thoroughly characterize the platelet glycoproteome; and, (2) determine the impact of aspirin treatment on the glycoprotein composition of platelets in the presence and absence of agonist stimulation. An iTRAQ strategy was deployed to quantify the glycoproteome, proteome, and secretome in aspirin

and control-treated platelet samples. To further validate our quantitative proteomic approaches we utilized an ELISA assay to confirm fold changes among individual donors after aspirin treatment. Using these approaches, we have identified 427 glycoproteins from platelet lysates. We also identified several proteins not previously known to be affected by aspirin's inhibitory actions, and among these proteins the effects on metalloproteinase inhibitor 1 (TIMP1) were most prominent.

2 Materials and methods

2.1 Platelet preparation

Washed platelets were prepared from the blood of healthy volunteers (40% male, mean age 30.0 ± 3.5 years) for aggregation studies and analysis of platelet proteins/glycoproteins. Volunteers were eligible for study participation if they were free of chronic and acute medical illness and had not taken aspirin or nonsteroidal anti-inflammatory drugs in the past 10 days. The study was approved by the Johns Hopkins Institutional Review Board and all participants signed written informed consent. Briefly, blood was drawn into citrate anticoagulant (0.105 M final, Beckton-Dickinson Vacutainer, Franklin Lakes, New Jersey) and treated in the presence of aspirin (20 μ M final $\times$ 20 min, Sigma-Aldrich Corporation, Saint Louis, MO) or ethanol vehicle (0.03% final, Pharmco-Aaper Brookfield, CT) for 15 min. Platelets were isolated by differential centrifugation, treated with PGE1 (1 μ M final, Sigma-Aldrich) prior to centrifuging at $14\,000 \times g$, washed in acid-citrate-dextrose, then resuspended in Tyrode's buffer containing human fibrinogen (50 μ M final, AbD Serotec Raleigh, NC) to allow functional analyses.

2.2 Protein extraction

For protein digestion, the platelet pellet was first denatured in 1 mL of 8 M urea and 1M NH_4HCO_3 and sonicated thoroughly. The protein concentration was measured using a BCA protein assay kit (Thermo). The proteins were then reduced by incubating in 10 mM DTT at 55°C for 30 min and alkylated by addition of 20 mM iodoacetamide at room temperature for 30 min in the dark. Sample was diluted to make final concentration of urea 1 M. Trypsin 0.5 $\mu\text{g}/\mu\text{L}$ was added at a ratio of 1:50 (trypsin to Protein) and incubated at 37°C overnight. Peptides were purified with C18 desalting columns and dried using SpeedVac.

2.3 iTRAQ labeling of global peptides

Each iTRAQ (isobaric tags for relative and absolute quantitation) 4-plex reagent was dissolved in 70 μL of methanol. Tryptic peptide of each sample was added into 250 μL of iTRAQ dissolution buffer, then mixed with iTRAQ 4-plex reagent and incubated for 1 hour at room temperature. iTRAQ channels 114 was used for platelet at rest, and 115 was used for platelet with aspirin treatment and at rest, 116 was used for platelet after activation with collagen, 117 was used for platelet with aspirin treatment and activation with collagen. After iTRAQ labeling, the four channels were combined together. The reaction solution was cleaned up by SCX column. Then, peptides were dried and resuspended into 0.1% formic acid solution prior to MS analysis. Remaining 90% of the sample was used for glycopeptide analysis. In case of supernatant due to difference in protein concentration, only 114 and 115 channels were combined and a second set of sample was prepared by combining 116 and 117.

2.4 Glycopeptides capture

Dissolved C18 cleaned peptides in 5% ACN in 0.1% TFA. Add 1/10 of the final volume of 100 mM sodium periodate to samples, and incubate in dark at room temperature for 1 h with gentle shaking with hydrazide beads. Beads are then washed to remove any nonspecific binding. PNGaseF was used to detach peptides from glycan.

2.5 MS analysis

The sample was separated through a Dionex Ultimate 3000 RSLC nano system (Thermo Scientific) with a 75 $\mu\text{m} \times 50\text{ cm}$ C18 PepMap@RSLC separating column (Thermo Scientific) protected by a 5 mm guarding column (Thermo Scientific). Mobile phase flow rate was 0.35 $\mu\text{L}/\text{min}$ with 0.1% formic acid and 2% acetonitrile in water (A) and 0.1% formic acid 95% acetonitrile (B). The peptides were separated with gradient from 5–40% B in 104 min, MS analysis was performed using a Thermo Q Executive mass spectrometer (Thermo Scientific). AGC target for MS1 was set for 3×10^6 for MS1 in 60 ms maximum time. AGC target for MS/MS was 50 000 (at a resolution of 17 500, intensity threshold of 5×10^4 , High-energy collisional dissociation and maximum IT 100 ms) of the 20 most abundant ions. Charge state screening was enabled to reject unassigned, one, eight, and more than eight protonated ions. A dynamic exclusion time of 25 s was used to discriminate against previously selected ions.

2.6 Data analysis

Data generated using Q Exactive was searched using SEQUEST with proteome discoverer (PD) 1.3 (Thermo Scientific, Rockford, IL) against Human database refseq downloaded on August 13, 2014 having 71 861 proteins. Peptides were searched with trypsin protease, allowing only two missed cleavages. Search parameters used were 10 ppm precursor tolerance for precursor mass and 0.06 Da fragment ion tolerance, static modification of 4plex iTRAQ at N-terminus and Lysine, carbamidomethylation at cysteine, variable modifications of oxidation at methionine. Only for formerly glycopeptide analysis deamidation at asparagine variable modification was applied. Filters used for global data analysis included peptide rank 1, minimum of two peptides per protein, and 1% FDR threshold. Filters used for glycopeptide analysis was peptide rank 1, 1% FDR in proteome discoverer. Data were normalized for protein median.

2.7 Enzyme-linked immunosorbent assay

TIMP1 levels in platelet specimens were determined using an ELISA assay kit as per the manufactures protocol. The TIMP1 kit was purchased from R&D Systems. Briefly, 100 μL of platelet lysate was incubated in a 96-well microtiter plate precoated with TIMP1 capture antibody for 30 min at 4°C, followed by incubation with a HRP-conjugated mouse anti-human antibody for 30 min at 4°C. A substrate solution containing tetramethylbenzidine was then added and allowed to react for 20 min at room temperature. The wells were thoroughly washed between incubations. Absorbance readings were acquired at 450 nm using an absorbance reader (Biotek, Winooski, VT). Standard curves were generated from a four-parameter logistic curve fit using recombinant human TIMP1 (concentration range: 0-250 ng/mL). All specimens were assayed as duplicates. The mean of two values was obtained.

The total protein concentration was determined for all specimens using the BCA protein assay (Thermo Fisher Scientific Inc., Rockford, Illinois). The levels of TIMP1 were expressed as nanograms per milligram of total protein in platelet samples.

3 Results

3.1 LC-MS/MS approach for glycoprotein identification

To investigate the effect of aspirin on the platelet glycoproteome, platelet samples from different donors ($n = 10$) were treated with and without aspirin. Collagen was used to activate platelets in vitro because it is a common physiologic agonist of platelets in vivo, and platelet aggregation in response to collagen in vitro is associated with incident and recurrent myocardial ischemic events in human clinical studies. Aggregation of washed platelets was recorded in the absence and presence of aspirin treatment. The platelet lysate from each individual in the four conditions described above was analyzed (Fig. 1). Tryptic peptides from platelet lysates were labeled with iTRAQ, and the combined iTRAQ-labeled peptides were subjected to global proteomics and N-linked glycoproteomics. The data was analyzed using Ref-Seq database and PD, subsequently filtered using 1% FDR and consensus sequence NXS/T motif. For glycoproteomics, at 1% FDR, 799 unique N-glycosites were identified from 1296 unique peptides (Supporting Information Table 1). The glycoproteins identified were subjected to GO annotation; Membrane Proteins were the most enriched category. Seventy percent of the identified glycoproteins were characterized as membrane, extracellular, or cell surface in origin.

At a threshold of p -value less than 0.05 from the analysis of technical replicates (1.2-fold), we identified 36 glycopeptides from 21 proteins that were differentially present in platelet lysates obtained from aspirin-treated versus control-treated platelets after activation with collagen (Table 1). Glycosites from proteins like Angiopoietin-1, CD226 antigen, glia-derived nexin, junctional adhesion molecule C, latent transforming growth factor beta 1, TIMP1, multimerin-1, thrombospondin-1, and vWF were identified to be differentially expressed between aspirin treatment and vehicle alone treatment at a p -value of less than 0.05. There were no significant glycoprotein changes observed on resting platelets in response to aspirin treatment (Table 1).

3.2 Global platelet proteomic profiles using iTRAQ quantitation on the same platelet samples

To further determine whether the observed glycoprotein changes were due to alterations in protein expression or glycosylation, the platelet samples were analyzed using quantitative global proteomics. Briefly, one microgram of iTRAQ-labeled platelet peptides from each donor was subjected to single proteomics analysis run. Across all ten donors 1532 proteins were identified (Supporting Information Table 2). The p -value for differential protein presence was determined using Student t -test with a minimum identification of the protein across five donors. Fifteen proteins were identified to be differentially present in platelet lysates obtained from aspirin-treated samples compared to controls after collagen stimulation (Table 2). The in vitro aggregation assay results were compared with global proteomics data and correlation coefficients calculated for the 15 proteins (Table 2). There

was a strong negative correlation between several proteins in the lysate fraction and the magnitude of platelet aggregation in vitro in response to collagen stimulation. The majority of these proteins are known to be secreted from platelets upon activation, which was confirmed by analyses of supernatant fractions (see supernatant experiments below).

The majority of the differentially expressed proteins (12 out of 15) were present in higher quantities in aspirin-treated platelet lysates, consistent with an expected reduction in platelet secretion after aspirin treatment. Proteins like Platelet factor 4, Platelet basic protein, Thrombospondin, SPARC precursor protein, and Serglycin are known to be secreted and differentially present after aspirin treatment [8]. In our study, three novel proteins were significantly affected by aspirin treatment: TIMP1, Vitamin D-binding protein, and Vitamin K-dependent protein S.

3.3 Platelet supernatant proteomic profiling using iTRAQ quantitation

We also examined the supernatants obtained after stimulating platelets with collagen from five of the subjects. Due to the differences in protein amounts collected before and after activation (protein concentration was much lower in supernatant than lysate fractions), samples were analyzed separately using LC-MS/MS. There was no difference in relative protein abundance between supernatant fractions from resting platelets with or without aspirin treatment. However, of the 830 proteins identified from supernatants (Supporting Information Table 3), 21 proteins were found to be differentially present between aspirin- and vehicle-treated samples after collagen stimulation (Table 3). The majority of proteins decreased upon aspirin treatment were consistent with lesser platelet secretion after aspirin treatment. Four glycoproteins multimerin, metalloproteinase inhibitor 1, thrombospondin, and latent-transforming growth factor beta-binding protein 1 showed significant differences in expression in both of the secretome and platelet lysates. This observation suggested that the glycoprotein changes observed from glycoproteomic analysis of platelet lysates could contribute to protein secretion.

3.4 Validation of TIMP1 changes by ELISA

To further validate glycoproteomic data from ITRAQ experiments, we chose to evaluate TIMP1, which had not been reported to be affected by aspirin treatment previously. Relative abundance of TIMP1 was 50–100% higher in platelet lysates obtained from aspirin-treated samples versus vehicle after activation with collagen (Fig. 2). The variation in abundance of TIMP1 was due to differential donor response to aspirin. Similar results for TIMP1 were observed in proteomic and glycoproteomic analysis (Fig. 2). Conversely, supernatants from these samples showed 50% lower levels of TIMP1 after aspirin-treatment that was consistent with a decrease in protein secretion after aspirin treatment.

4 Discussion

The purpose of this study was to characterize the platelet glycoproteome and determine the impact of aspirin on platelet glycoproteins in greater detail. We used MS to identify changes in the proteins and glycoproteins presenting in platelet lysates and supernatants in the presence and absence of collagen stimulation and aspirin treatment. Quantitative proteomics

was performed using iTRAQ labeling followed by MS. We were able to identify 799 glycosites, which more than triples the number reported from previous studies [2–4]. Ontology analyses suggested that most of the identified glycoproteins were membrane, extracellular, or cell surface in origin, consistent with the important roles of platelets and protein glycosylation in cell-cell adhesion. Analysis of the whole platelet proteome allowed identification of 1532, which also provides greater resolution than previously reported [8].

We identified 36 glycopeptides from 21 proteins that were differentially present in platelet lysates obtained from aspirin-treated versus control-treated platelets after collagen activation. Using an FDR of 1%, fewer than 2% of data deviated from the threshold of 1.2 suggesting very stringent thresholds in our analyses. Because aspirin inhibits platelet activation and secretion, we expected to observe greater relative abundance of glycoproteins in lysates from aspirin- compared to vehicle-treated samples, which is what we observed. We also expected to observe a similar phenomenon when examining the whole proteome by iTRAQ instead of the glycoproteome, which again was consistent with observations. Concordantly, we expected to observe lesser abundance of proteins differentially present in supernatants. Several of the glycoproteins and proteins that were differentially present in lysates from aspirin versus Vehicle-treated samples are known to be present in platelet granules including, fibrinogen, VWF, multimerin, thrombospondin, platelet factor 4, platelet basic protein, and SPARC protein. The impact of aspirin on these proteins was consistent with those previously reported by Coppinger et al. [8] However, several of the identified glycoproteins are not known to be contained in granules, to be secreted, or both, such as TIMP1, CD226, junctional adhesion molecule c, and Angiopoietin-1.

When comparing glycoproteomics to global proteomics data, proteins like thrombospondin 1, metalloproteinase inhibitor 1, fibrinogen gamma chain, and alpha 1 antichymotrypsin were the only glycoproteins that showed significant differences in expression at both protein and glycoprotein levels after aspirin treatment upon collagen activation. Proteins like latent-transforming growth factor beta-binding protein 1, multimerin 1, and junctional adhesion molecule showed significant change in glycoprotein analysis; however, the same changes were not observed in global proteomics analysis. This could be a result of changes in glycosylation of these proteins due to aspirin treatment or changes in these proteins might be below our significance threshold. Proteins like kallistatin, angiopoietin 1, and afamin were not identified in global proteomics analysis. This phenomenon can be explained by the fact that glycosite analysis is a subproteome analysis as a result decreasing the complexity compared to global proteomics analysis. Glycosite analysis results in enrichment of a subset of peptides leading to identification of the low abundant proteins, which would not have been detected in global analysis.

We identified TIMP1 as a glycoprotein that was differentially present in aspirin treated compared to vehicle-treated platelet lysates after collagen activation. These results were confirmed in whole proteome analyses, analysis of platelet supernatants, and by ELISA of platelet lysates. TIMP1 functions as an irreversible inhibitor of matrix metalloproteinase 1 (MMP1). MMPs are peptidases that are involved in degradation of extracellular matrix. TIMP1 also promotes cell proliferation. Depending on the environment, TIMP1 stimulates metastasis and formation or reduces tumor angiogenesis. TIMP1, TIMP2, and TIMP4 are

known to be present in platelets and are rapidly released upon activation [9]. The cellular location of platelet TIMPs is controversial. Villeneuve et al. reported that TIMPs are not stored in platelet alpha granules; instead, they are localized in submembranous structures distinct from alpha-granules [9]. Thrombospondin, a well-recognized alpha-granule protein, was the only other protein that was observed to show significant differences across glycoproteome, global proteome, and secretome analyses. However, unlike thrombospondin, TIMP1 is not known to be present in platelet granules and the impact of aspirin on its secretion has not previously been described. The observation that aspirin reduces platelet release of TIMP1 suggests that aspirin's effects in vivo may be complex. One might speculate, for example, that the antiaggregatory effects of aspirin might be mitigated by diminished inhibition of metalloproteinase activity and worsened vascular integrity.

In our glycoproteomic analysis TIMP1 was shown to have two N-glycosites at position 53 and 101. It has been previously documented that glycan structure specifically fucose in the outer arm on the TIMP1 can impact its activity of inhibiting MMP [10]. Another role of TIMP1 is to function as a growth factor via binding to CD63 and ITGB1 and activating cellular signaling cascades. Serum concentrations of TIMP1 are directly correlated to platelet count, consistent with release from platelets during blood clotting, and plasma TIMP1 levels have been reported to be a risk factor for CVD [11].

We found several other glycoproteins to be differentially presented in aspirin-treated compared to vehicle-treated samples after collagen activation; however, these proteins were not verified by orthogonal approaches. CD226 is an adhesion molecule known to be present on platelets and endothelial cells and that allows for heterotypic cell adhesion [12]; however, its physiologic significance and the impact of aspirin on its expression is not characterized. Angiopoietin 1 is known to be presented in platelets and secreted upon activation; however, its subcellular location has not been defined nor has the effect of aspirin on its secretions [13]. Angiopoietin is known to play a role in maintaining vascular integrity and has been implicated in tumor progression and angiogenesis [14]. Another protein differentially expressed was junctional adhesion molecule C, which is known to be on the surface of platelets but its secretion has not previously been defined [15]. A soluble form of junctional adhesion molecule C is known to be a mediator of angiogenesis [16]. These findings require additional verification, and their implication for the biology of platelet function and response to aspirin treatment are unknown.

5 Concluding remarks

In summary, this study provides the most complete characterization of the platelet glycoproteome to date, and is the first study to characterize the impact of aspirin on platelet glycoproteins. As expected, aspirin reduced the ability of platelets to secrete granular glycoproteins such as thrombospondin 1. In addition and unexpectedly, aspirin also modifies the ability of platelets to secrete TIMP1, and potentially several other proteins not known to be secreted or contained within platelet granules, but possessing adhesive and angiogenic functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

COX1	cyclooxygenase1
PD	proteome discoverer
SPE	solid phase extraction
TIMP1	metallopeptidase inhibitor 1

Significance of the study

Aspirin is widely used to treat cardiovascular disease. The effectiveness of aspirin treatment on platelets varies among individuals. In this study, we evaluated the impact of in vitro aspirin exposure on the platelet glycoproteome, proteome, and secretome in healthy volunteers. Seven hundred ninety-nine unique N-linked glycosylation sites (glycosites) were identified, representing the largest, and the most comprehensive analysis of the platelet glycoproteome to date. As expected, aspirin reduced the ability of platelets to secrete granular glycoproteins such as thrombospondin 1 and platelet factor 4. In addition, several proteins were identified that were not previously reported to be secreted by platelets or affected by aspirin, including CD226, junctional adhesion molecule C, angiopoietin-1, and metalloproteinase inhibitor 1 (TIMP1). Our studies demonstrate an association between platelet glycoprotein secretion and platelet function response to aspirin exposure, and the potential for platelet glycoprotein analyses to elucidate the molecular mechanisms that underlie differential response to aspirin treatment.

Figure 1. Workflow of the platelet analysis with and without aspirin treatment at rest and after stimulation. Proteins and glycopeptides were analyzed using iTRAQ quantitative method.

Figure 2. Ratio of TIMP1 with and without aspirin treatment in donors from proteomics, glycoproteomics, and ELISA analysis of platelets or supernatant.

N-glycopeptides differentially present in platelet lysates comparing aspirin- and vehicle-treated samples after activation with collagen

Table 1

Accessions	Peptide	Protein name	# PSMs	Activated 117/116	Activated <i>p</i> -value
578808854	DIENFNSIQK	Afamin	5	1.249	0.019
4505529	SVQEIQATFFYFTPNKTEDTIFLR	Alpha-1-acid glycoprotein 2	25	1.194	0.033
50659080	TLNQSSDELQLSMGNAMFVK	Alpha-1-antichymotrypsin	5	1.262	0.015
315434240	ATTNNSVLQK	Angiotensin-1	15	1.302	0.000
153266841	VYKPSAGNNSLYR	Beta-2-glycoprotein 1	4	1.549	0.005
530414082	NFTSKFPR	CD226 antigen	8	1.654	0.012
115298678	TVLTPATNHMGNVTFIPANR	Complement C3	1	1.227	0.001
356582273	FSDGLENSSTQFEVK	Complement C4-A isoform 2 preproprotein	7	1.201	0.015
148664205	NLSLRLEGLQEK	Endothelial cell-selective adhesion molecule	7	1.769	0.026
70906437	VDKDLQSLLEDILHQVENKTSEVK	Fibrinogen gamma chain isoform gamma-A	103	2.059	0.012
70906437	DLQSLLEDILHQVENKTSEVK	Fibrinogen gamma chain isoform gamma-A	17	1.953	0.028
70906437	VDKDLQSLLEDILHQVENK	Fibrinogen gamma chain isoform gamma-A	280	1.338	0.001
70906437	DLQSLLEDILHQVENK	Fibrinogen gamma chain isoform gamma-A	52	1.309	0.001
211904152	NKDIVTVANAVFVKNAEIEVPFVTR	Glia-derived nexin	6	1.770	0.003
328927055	TSLKIWNVTR	Junctional adhesion molecule C	6	1.446	0.034
21361302	SQILEGLGFNLTELSQSDVHR	Kallistatin	8	1.342	0.002
21361302	FLNDTMAVYEAK	Kallistatin	8	1.217	0.004
530367593	KEYYNLNDASLCDNVLPNVTK	Latent-transforming growth factor beta-binding protein 1	12	1.307	0.001
530367593	ECYYNLNDASLCDNVLPNVTK	Latent-transforming growth factor beta-binding protein 1	1	1.243	0.001
4507509	SHNRSEEFLLAGK	Metalloproteinase inhibitor 1	9	2.219	0.000
4507509	FVGTPEVNVQTTLYQR	Metalloproteinase inhibitor 1	57	1.540	0.000
4507509	AKFVGTPENVNQTTLYQR	Metalloproteinase inhibitor 1	15	1.327	0.023
45269141	FNPGAESVVLNSNTLK	Multimerin-1	54	1.640	0.000
45269141	HPFTGDNCTIK	Multimerin-1	20	1.359	0.005
45269141	LQNLTLPTNASIK	Multimerin-1	34	1.346	0.000
73858570	VLSNNSDANLELINTWVAK	Plasma protease C1 inhibitor	9	1.204	0.003
40317626	VSCPIMPSCNATVPDGECCPR	Thrombospondin-1	47	1.531	0.001
40317626	KVSCPIMPSCNATVPDGECCPR	Thrombospondin-1	47	1.490	0.014

Accessions	Peptide	Protein name	# PSMs	Activated 117/116	Activated <i>p</i> -value
40317626	VVNSTTGPGHEHLR	Thrombospondin-1	389	1.227	0.000
530382394	LSQNSVNLSPSSHANNLSVVTYSKCT	Uncharacterized protein C6orf106 isoform X1	14	1.361	0.008
88853069	FEDGVLDPDYPRNISDGFDPDNDVDAALALPAHSYSGR	Vitronectin	37	1.437	0.006
88853069	NISDGFDPDNDVDAALALPAHSYSGR	Vitronectin	58	1.213	0.000
89191868	ASPPSSSCNISSGEMQK	von Willebrand factor preproprotein	16	1.438	0.001
89191868	IDGSGNFQVLLSDRYFNK	von Willebrand factor preproprotein	9	1.349	0.005
89191868	GQVYLQCGTPCNLTCT	von Willebrand factor preproprotein	20	1.335	0.004

Table 2
Correlation of proteins differentially present in platelets after aspirin treatment with collagen-induced platelet aggregation

Accession	Description	PSMs	117.116	p value	Correlation	Supernatant
530376993	Platelet factor 4 isoform X1	280	1.661	0.000	-0.618	Y
4505735	Platelet factor 4 variant precursor	191	1.617	0.000	-0.590	Y
4505981	Platelet basic protein preproprotein	476	1.609	0.000	-0.521	Y
4507171	SPARC precursor	173	1.416	0.000	-0.622	Y
45935371	Serglycin precursor	39	1.310	0.000	-0.071	Y
40317626	Thrombospondin-1 precursor	1624	1.262	0.000	-0.531	Y
192447438	Vitamin K-dependent protein S preproprotein	73	1.258	0.000	-0.721	Y
70906437	Fibrinogen gamma chain isoform gamma-A	760	1.253	0.000	-0.663	Y
578809023	Vitamin D-binding protein isoform X1	35	1.248	0.004	-0.024	Y
70906435	Fibrinogen beta chain isoform 1 preproprotein	1034	1.235	0.000	-0.668	Y
4502027	Serum albumin preproprotein	1541	1.223	0.002	-0.519	Y
4507509	Metalloproteinase inhibitor 1 precursor	38	1.220	0.000	-0.507	Y
48762934	Collagen alpha-2(I) chain precursor	16	0.766	0.015	-0.225	Y
4504345	Hemoglobin subunit alpha	813	0.727	0.011	0.129	Y
4504351	Hemoglobin subunit delta	373	0.712	0.012	0.144	Y
4504349	Hemoglobin subunit beta	807	0.697	0.011	0.109	Y

Table 3
Global proteins differentially present in supernatants of platelets comparing aspirin- to vehicle-treated samples after collagen activation

Accession	Description	PSMs	117/116	p value
32189392	Peroxiredoxin-2	6	1.32	0.04
195972866	Keratin, type I cytoskeletal 10	34	1.30	0.01
578809106	PREDICTED: immunoglobulin J chain isoform X1	5	1.25	0.03
47132620	Keratin, type II cytoskeletal 2 epidermal	51	1.25	0.04
119395750	Keratin, type II cytoskeletal 1	64	1.21	0.02
45269141	Multimerin-1	308	0.83	0.01
424036738	Trem-like transcript 1 protein isoform b	23	0.83	0.03
40317626	Thrombospondin-1	2861	0.83	0.01
4502551	Calumenin isoform a	40	0.82	0.00
4503123	Connective tissue growth factor	17	0.81	0.04
530367593	Latent-transforming growth factor beta-binding protein 1 isoform X4	329	0.81	0.01
261337165	Latent-transforming growth factor beta-binding protein 1 isoform LTBP-1L	340	0.81	0.01
4507171	SPARC	217	0.80	0.01
4505981	Platelet basic protein preproprotein	1232	0.79	0.02
189163485	Lysosomal protective protein isoform b	22	0.78	0.03
63025222	Transforming growth factor beta-1	50	0.78	0.01
4507509	Metalloproteinase inhibitor 1	64	0.77	0.02
4506427	Retinoic acid receptor responder protein 2	20	0.77	0.00
22538814	C-C motif chemokine 5 isoform 1	33	0.76	0.02
530376993	Platelet factor 4 isoform X1	473	0.72	0.02
4505735	Platelet factor 4 variant	346	0.70	0.01