The canine activated platelet secretome (CAPS): A translational model of thrombin-evoked platelet activation response

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Abstract

Background: Domestic dogs represent a translational animal model to study naturally occurring human disease. Proteomics has emerged as a promising tool for characterizing human platelet pathophysiology; thus a detailed characterization of the core canine activated platelet secretome (CAPS) will enhance utilization of the canine model. The objectives of this study were development of a robust, high throughput, label-free approach for proteomic identification and quantification of the canine platelet (i) thrombin releasate proteins, and (ii) the protein subgroup that constitutes CAPS.

Methods: Platelets were isolated from 10 healthy dogs and stimulated with 50 nmol/L of γ-thrombin or saline. Proteins were in-solution trypsin-digested and analyzed by nano–liquid chromatography–tandem spectrometry. Core releasate proteins were defined as those present in 10 of 10 dogs, and CAPS defined as releasate proteins with a significantly higher abundance in stimulated versus saline controls (corrected P < .05).

Results: A total of 2865 proteins were identified; 1126 releasate proteins were present in all dogs, 650 were defined as CAPS. Among the differences from human platelets were a canine lack of platelet factor 4 and vascular endothelial growth factor C, and a 10- to 20-fold lower concentration of proteins such as haptoglobin, alpha-2 macroglobulin, von Willebrand factor, and amyloid-beta A4. Twenty-eight CAPS proteins, including cytokines, adhesion molecules, granule proteins, and calcium regulatory proteins have not previously been attributed to human platelets.

Conclusions: CAPS proteins represent a robust characterization of a large animal platelet secretome and a novel tool to model platelet physiology, pathophysiology, and to identify translational biomarkers of platelet-mediated disease.
INTRODUCTION

The dog is an important comparative animal model of human hemostatic disease and a translational large animal model for human medicine. In contrast to mouse models, companion dogs share the same environment as humans, have similar genetic diversity, and recapitulate human blood rheology and platelet pathophysiology. Well-characterized canine models of von Willebrand disease, Glanzmann thrombasthenia, storage pool diseases, and immune-mediated thrombocytopenia have been identified. Dogs also share with humans various neoplastic diseases, including spontaneous solid tumors and leukemias, where the role of platelets in pathogenesis is under active investigation.

Platelet proteomics in human patients has been used to identify changes in platelet proteins in association with conditions shared by humans and dogs, like neurodegenerative diseases, cancer, uremia, and diabetes mellitus. However, proteomic studies focused on canine platelet pathophysiology or discovery of novel disease biomarkers are lacking. A global platelet proteome of healthy dogs was reported by Trichler and colleagues in 2014. To capitalize on those data, a solidly defined platelet secretome profile of healthy dogs is also required for validation of dogs as relevant, translational animal models for disease-associated platelet pathophysiology and novel biomarker discovery.

Recently, we reported a shotgun proteomic characterization of the subset of activation-released secretion platelet proteins: the canine activated platelet secretome (CAPS). Two thousand platelet-associated proteins were identified, with 693 designated as CAPS proteins; however, the sample size was limited. In the present study, in-solution tryptic digests replaced the in-gel digests used in our preliminary study. Recent technical advances in nano–liquid chromatography (LC) and in mass spectrometry (MS) sensitivity and in-solution tryptic digest, and peptide purification enabled reliable identification of secretome cargo proteins. In an earlier study, Parsons and colleagues used this approach to identify a core platelet releasate in human thrombin-stimulated platelets from 32 healthy individuals. In the present study, a similar approach was utilized in 10 healthy dogs. The objective was to establish a high-throughput, affordable, label-free, shotgun proteomic protocol to reliably characterize canine platelet releasate proteins and the subset of actively secreted proteins, CAPS. The secretion status of CAPS proteins was defined on the basis of protein abundance in a thrombin-stimulated releasate relative to that of paired saline control samples.

MATERIALS AND METHODS

Blood sampling, platelet isolation, platelet activation, and releasate collection

Ten privately owned dogs weighing >5 kg were recruited (Table 1). The dogs were deemed healthy on the basis of normal physical examination, complete blood cell count (CBC), biochemistry profile, and urinalysis. Study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Cornell University (protocol no. 1994-0089) and undertaken with written informed client consent. Blood sampling, platelet isolation, platelet activation, and releasate collection were carried out as previously described and can also be found in detail in supplemental materials (Supplementary Method S1).

Protein extraction, in-solution protein digest, and peptide purification

The in-solution protocol was modified from Parsons et al. For protein extraction, 10x RIPA Lysis Buffer (0.5 mol/L Tris-HCl, pH 7.4, 1.5 mol/L NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mmol/L EDTA; Sigma-Aldrich, St. Louis, MO, USA) was added to supernatants in a 1:9 ratio and extracted on ice for 15 minutes. Samples were immediately frozen and stored at −80°C. Preparation of the S-Trap micro spin column (ProtFi, Huntington, NY, USA) was according to vendor protocol and Zougman et al. All samples were thawed at 37°C, concentrated using Amicon 3 kDa cutoff ultrafiltration units (Merck Millipore, Billerica, MA, USA), dried in Speed Vacuum and stored overnight at −20°C. The samples were then reconstituted with 25 μL phosphate-buffered saline (pH 7.4), 1% sodium dodecyl sulfate w/v, 6 mol/L urea, 2 mol/L thiourea, and 10 mmol/L dithiothreitol (DTT) and incubated at 34°C for 1 hour. This was followed by alkylation with iodoacetamide (50 mmol/L final concentration) with incubation in the dark at room temperature (RT) for 45 minutes. The samples were quenched with DTT and incubated at RT for 15 minutes, followed by...
Lyophilized tryptic peptides were reconstituted in 20 μL of 0.5% FA for nano–LC–tandem MS analysis performed using an Orbitrap Fusion Trubrid (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer equipped with a nanospray Flex Ion Source, coupled with a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific). The tryptic peptides were eluted in a 120-minute gradient of 5%-38% ACN in 0.1% FA at 300 nL/min, followed by a 7-minute ramping to 90% ACN-0.1% FA and an 8-minute hold at 90% ACN-0.1% FA. The column was reequilibrated with 0.1% FA for 25 minutes before the next run. The Orbitrap Fusion was operated in positive ion mode with spray voltage set at 1.6 kV and source temperature at 275°C. External calibration for Fourier-transform (FT), ion trap, and quadrupole mass analyzers was performed. For data-dependent acquisition (DDA) analysis, the instrument was operated using FT mass analyzer in MS scan to select precursor ions followed by 3-second “Top Speed” data-dependent collision-induced dissociation ion trap tandem MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10 000 and normalized collision energy of 30%. MS survey scanned at a resolving power of 120 000 (full width at half maximum at m/z 200), for the mass range of m/z 375-1575. Dynamic exclusion parameters were set at 40 seconds of exclusion duration with ±10 ppm exclusion mass width. All data were acquired under Xcalibur 3.0 operation software (Thermo Fisher Scientific).

### Protein identification and quantification

The DDA raw files for CID tandem MS were subjected to database searches using Proteome Discoverer 2.2 (PD 2.2) software (Thermo Fisher Scientific) with the Sequest HT algorithm. For each dog, the two raw MS files of stimulated versus saline control samples, respectively, were combined for searching against a Canis lupus familiaris RefSeq database (CanFam3.1, downloaded from NCBI on January 12, 2018) containing 45 326 entries plus a common contaminant database of 246 entries. Two missed trypsin cleavage sites were allowed. Peptide precursor tolerance was set to 10 ppm, and fragment ion tolerance was set to 0.6 Da. Variable modification of methionine oxidation, deamidation of asparagine/glutamine and fixed modification of cysteine carbamidomethylation were set for the database search. High-confidence peptides defined by Sequest HT with a 1% false discovery rate by Percolator were considered for identification. Relative quantitation of identified proteins between thrombin-stimulated and saline control samples, was determined by the Label Free Quantitation workflow in PD 2.2. Precursor abundance intensity for each peptide identified was automatically determined, and unique peptides for each protein were summed and used to calculate the protein abundance (MS1 abundance) within the PD 2.2 software without normalization. Protein ratios were calculated on the basis of pairwise ratios for stimulated/saline control samples.

### Data analysis

All proteins were compiled in a large single spreadsheet using a custom python script. We retrieved the sequences of all protein accession numbers from Refseq [GCF_000002285.3_CanFam3.1] and aligned all against all with blastp (version 2.8.1+). Protein accessions with either the same protein name (protein description) or the same gene symbol and with global alignments of >98% sequence identity

### Table 1

| Breeds                     | Mixed breed (n = 6) | Labrador retriever (n = 2) | Beagle (n = 1) | Irish wolfhound (n = 1) |
|----------------------------|--------------------|---------------------------|---------------|-------------------------|
| Age, y                     | 5 (±3)             |                           |               |                         |
| Sex: F/FS/M/MN, n          | 2/5/0/3           |                           |               |                         |
| EDTA platelet count, x10^3/L | 254 (±88)         |                           |               |                         |

**Note:** All continuous variables are listed as mean ± standard deviation. CD18, leukocyte marker; CD62P, P-selectin; F, female; FS, female spayed; M, male; MN, male neutered.
were merged and mean MS1 abundance values were calculated. Of note, most of these merged protein accession numbers had the same gene symbol.

2.5.1 | Definition and analyses of core releasate proteins

Proteins present in 10 of 10 dogs were considered releasate proteins, and were compared to other platelet proteomes using homologous human Entrez Gene IDs (https://biodbnet-abcc.ncifcrf.gov). The abundance ranking of releasate proteins was compared to that of the human core releasate reported by Parsons and colleagues.

2.5.2 | Definition and analyses of core CAPS proteins

The releasate protein abundances between thrombin-stimulated and vehicle control samples were compared using paired samples t tests. The resulting P values were corrected for multiple comparisons by adjusting the false discovery rate using the Benjamini-Hochberg procedure. Core CAPS proteins were subsequently defined as those with a significantly higher abundance in the stimulated sample, at a corrected significance level of 0.05. The core CAPS proteins were ranked from highest to lowest MS-1 abundance in the stimulated samples and characterized according to the MS-1 abundance ratio of paired stimulated and control samples, prediction of signal peptides with the SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/), and prediction of nonclassical protein secretion with the SecretomeP 2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/).

A protein-protein association network was built from the most confident protein-protein associations between 39 dog-specific proteins (including 28 CAPS proteins) and 100 other proteins based on different confidence channels (e.g., physical association from experimental data and functional associations from curated pathways, automatic text mining, and prediction methods) provided by the STRING database. The protein identifiers in Canis lupus familiaris were annotated using information of homologous genes, and a STRING confidence score of at least 0.4 was applied for finding protein association partners in the STRING database. Based on all these, 139 protein functional enrichments were performed by the Cytoscape StringApp. We considered the categories GO biological process, GO molecular function, GO cellular component, Reactome pathways, PFAM, SMART, InterPro protein domains, and Uniprot keywords, and used the entire genome as background. The nonredundant functional enriched terms with a false discovery rate (FDR) threshold of 5% and a STRING redundancy cutoff of 0.25 (maximal allowed Jaccard similarity index between members of two functional terms) were recorded.

3 | RESULTS

3.1 | Demographic summary and quality control

Table 1 summarizes dog demographics, platelet suspension cell purity, and status of baseline platelet activation before thrombin stimulation. Representative CD62P (P-selectin) expression of washed, unstimulated platelets is shown in Figure S2.

3.2 | Releasate proteome

A total of 2865 unique proteins, with an average sequence coverage of 13.6%, was identified in platelet-free supernatants from the combined thrombin-stimulated and saline control samples (Table S1). Of these, 1126 protein species were present in 10 of 10 dogs, with an average sequence coverage of 24.8% (Table S2). This canine platelet releasate demonstrated a 97% overlap of proteins when compared to reported human platelet proteomes, consistent with highly conserved structural, biochemical, and functional features shared between human and canine platelets.

Table S3 aligns the top 100 most abundant canine core releasate proteins with those of a similar study characterizing a core human platelet releasate from 32 donors. Among the high-abundance releasate proteins in dogs were those of known high concentration in humans including albumin, thrombospondin-1, platelet basic protein, fibrinogen, filamin-A, and talin-1. Platelet releasates also included extracellular matrix proteins and cytoskeletal molecules like actin. Beta and alpha forms of actin were present in the human platelet releasate proteome by Parsons et al., with alpha-actin as the dominant form. Beta-actin was the exclusive form in the dog. The majority of this beta-actin is likely derived from canine platelet microparticles as seen in our previous CAPS study, in which the secreted proteins were categorized as soluble or particulate fractions.

Table 2 compiles the top 100 releasate proteins differing in abundance rank between dog and human. The top 100 canine proteomes did not include 15 of the top 100 human platelet proteins. Some noteworthy differences were the absence of the major human proteins platelet factor 4 and platelet factor 4 variant, osteonectin, platelet glycoprotein V, thymosin β4, immunoglobulin class proteins (immunoglobulin kappa, gamma, alpha, and mu constants), apolipoprotein A-II, and alpha-1-acid glycoprotein 2. These findings were confirmed by the global canine platelet proteome, as only osteonectin, thymosin β4 and, apolipoprotein A-II were predicted to be present in their data set based on sequence homology to the same proteins in other species. Twelve other high-abundance human proteins were represented at 10- to 30-fold lower concentration in dogs, including alpha-1-antitrypsin, von Willebrand factor, alpha-1-acid glycoprotein 1, and apolipoprotein B-100.
### Table 2: Proteins in human and canine releasate proteomes that differ in rank abundance

| Gene symbol | Protein identification                                              | Rank | Human | Canine |
|-------------|---------------------------------------------------------------------|------|-------|--------|
| ACTB        | Actin, cytoplasmic 1                                               | 89   | 3     |
| YWHAE       | 14-3-3 protein epsilon isoform X2                                  | 193  | 40    |
| ACTA1       | Actin, alpha skeletal muscle                                        | 31   | 1030  |
| ACTA2, ACTC1| Actin, aortic smooth muscle, actin, alpha cardiac muscle 1         | Absent| 21    |
| ACTG1       | Actin, cytoplasmic 2                                               | 5    |       |
| ADA         | Adenosine deaminase isoform X2                                     | Absent| 73    |
| ORM1        | Alpha-1-acid glycoprotein 1                                         | 41   | 565   |
| SERPINA1    | Alpha-1-antitrypsin                                                | 14   | 190   |
| A2M         | Alpha-2-macroglobulin                                              | 20   | 432   |
| APP         | Amyloid beta A4 protein                                            | 33   | 1094  |
| APOA2       | Apolipoprotein A-II                                                | 78   | Absent|
| APOB        | Apolipoprotein B-100                                               | 48   | 1943  |
| CCL14       | C-C motif chemokine 14                                             | Absent| 10    |
| CKB         | Creatine kinase B-type                                             | Absent| 58    |
| HP          | Haptoglobin                                                        | 12   | 274   |
| IGHG2       | Ig gamma-2 chain C region                                          | 36   | Absent|
| IGHG3       | Ig gamma-3 chain C region                                          | 46   | Absent|
| IGHG4       | Ig gamma-4 chain C region                                          | 49   | Absent|
| IGKC        | Ig kappa chain C region                                            | 27   | Absent|
| IGLC2       | Ig lambda-2 chain C regions                                        | 44   | Absent|
| IGHM        | Ig mu chain C region                                               | 58   | Absent|
| ITIH2       | Inter-alpha-trypsin inhibitor heavy chain H2                      | 86   | 870   |
| ITIH4       | Inter-alpha-trypsin inhibitor heavy chain H4 isoform X1(803); isoform X3(905) | 84 | 802:905 |
| KRT10       | Keratin, type I cytoskeletal 10                                    | Absent| 23    |
| KRT9        | Keratin, type I cytoskeletal 9                                     | Absent| 94    |
| KRT1        | Keratin, type II cytoskeletal                                      | Absent| 32    |
| KRT77       | Keratin, type II cytoskeletal 1b                                   | Absent| 83    |
| KRT6A       | Keratin, type II cytoskeletal 6A isoform X2                        | Absent| 89    |
| LTA4H       | Leukotriene A-4 hydrolase                                          | Absent| 51    |
| CTS4        | Lysosomal protective protein                                        | 95   | 1088  |
| HTIMP2      | Metalloproteinase inhibitor 2                                      | Absent| 87    |
| BIN1        | Myc box-dependent-interacting protein 1                            | Absent| 62    |
| SERPINE1    | Neuroserpin isoform X1                                             | 671  | 52    |
| PF4         | Platelet factor 4                                                  | 7    | Absent|
| PF4V1       | Platelet factor 4 variant                                          | 4    | Absent|
| GP5         | Platelet glycoprotein V                                            | 16   | Absent|
| PSAP        | Proactivator polypeptide                                           | 90   | 1331  |
| PRSS57      | Serine protease 57                                                 | Absent| 98    |
| SPARC       | SPARC (Osteonectin)                                                | 10   | Absent|
| TMSB4X      | Thymosin beta 4                                                    | 26   | Absent|
| TUBB4B      | Tubulin beta-4B chain                                              | 220  | 20    |
| N/A         | Uncharacterized protein                                            | 6    | Absent|
| VWF         | von Willebrand factor                                              | 25   | 321   |

**Note:** The proteins are sorted in alphabetical order.

1 Parson et al.26

2 Proteins previously identified in human platelets based on proteomic or experimental evidence.
3.3 | Canine activated platelet secretome

Of the 1126 releasate proteins from 10 of 10 dogs, 650 were classified as CAPS proteins (Table S4). The repertoire of CAPS consists of bioactive molecules with transport, procoagulant, anticoagulant, proteolytic, antiproteolytic, proangiogenic or anti-angiogenic activities, cytokines, immune-related proteins, and growth factors. The two methods had 386 CAPS core proteins in common, while 303 proteins were solely identified by the in-gel method and 264 proteins solely by the in-solution method (Figure 1 and Table S5).

The rank order of the 100 most abundant CAPS proteins is presented in Table 3. Some major contributors to canine platelet secretory cargo are C-C motif chemokine 14, trem-like transcript 1, glia-derived nexin, and metalloproteinase inhibitors 1 and 2. Of these, only metalloproteinase inhibitor 1 is also in the human top 100. In addition to the noteworthy absence of platelet factor 4, von Willebrand factor was not one of the 100 most abundant CAPS. Vascular endothelial growth factor c was not detected; however, CAPS included other growth factors like extracellular matrix protein 1, transforming growth factor beta-1, platelet-derived growth factor subunit beta, angiopoietin-1, and latent-transforming growth factor beta-binding protein 1.

Independent tests for secretion-specific features of the 650 CAPS proteins were performed by the Secretome P and SignalP databases. This analysis resulted in annotation of 113 proteins (17.4%) whose secretion was predicted to be triggered either by nonclassical or classical (signal peptide) secretory pathways, respectively (Table S6).

3.4 | Characterization of canine-specific proteins

We identified 28 CAPS proteins and an additional 11 releasate proteins not previously reported in human platelets (Table 4). Of these, the highest abundant proteins included C-C motif chemokine 14, neuroserpin, serine protease 57, interleukin-8, peptidyl-prolyl cis-trans isomerase C, carcinoembryonic antigen–related cell adhesion molecule 21 (CEACAM21), protein-arginine deiminase type 3, myristoylated alanine-rich C-kinase substrate, and 45-kDa calcium-binding protein. Proteins detected in lower abundance included microfibril-associated glycoprotein 4, nib-like protein 1, cathepsin Z, neuronal cell adhesion molecule, neuropilin-1, angiotensin-converting enzyme, cathepsin L1, and interleukin-34.

For assigning functional terms to the 39 canine-specific proteins, the 100 most confidently associated proteins were identified using the STRING database. From these 139 proteins, we identified 44 nonredundant enriched functional terms (FDR < 0.05; Table S7). The association network is shown in Figure 2. The proteins fall within two functional clusters: (i) immune response and signaling and (ii) nucleotide metabolism. The most connected canine-specific proteins are interleukin-8; guanine nucleotide-binding protein subunit beta-5; CEACAM21; regulator complex protein LAMTOR3; and C-C motif chemokine 14 with 65, 61, 28, 27, and 19 protein associations, respectively, which makes them prime candidates for follow-up studies.

4 | DISCUSSION

We established an in-solution platelet proteomics method which facilitated the identification of 1126 core releasate proteins, and 650 significantly secreted core thrombin-stimulated CAPS proteins across 10 healthy dogs. This allowed us to solidify and build on our preliminary list of CAPS proteins, using a less expensive and less labor-intensive method. Advantages of the in-solution versus in-gel method included increased quality in the detection of CAPS proteins with fewer one-peptide identifications (3.2% vs 7.2%) and superior identification of functionally significant endosome-derived granule cargo proteins. Both methods identified 386 common proteins, and the cores were particularly reproducible for highly secreted proteins, with 72% of the top 25 highly secreted proteins in common. Moreover, 88% of these proteins were ranked within 0-5 positions, indicating very similar ranking profiles. Differences in CAPS definitions accounts for most discrepancies between the two CAPS cores. Of the 264 core in-solution CAPS proteins not identified with the in-gel method, 68.9% were secreted with a ratio < 2 and hence were excluded from the in-gel CAPS core, where inclusion required a ratio > 2 in at least 2 of 3 dogs. Additionally, the in-gel approach included the secretome particulate fraction, increasing sensitivity for detecting microparticle-associated proteins compared to the in-solution approach.

Several noteworthy species-specific CAPS findings reproduced between the studies,24 include the absence of platelet factor 4 in...
### TABLE 3  The 100 most abundant proteins in the canine platelet thrombin secretome (CAPS)

| Rank | Gene | Protein identification |
|------|------|------------------------|
| 1    | THBS1| Thrombospondin-1       |
| 2    | ALB  | Serum albumin precursor, serum albumin isoform X1 |
| 3    | FGB  | Fibrinogen beta chain  |
| 4    | FGA  | Fibrinogen alpha chain |
| 5    | FGG  | Fibrinogen gamma chain isoform X1 |
| 6    | FLNA | Filamin-A              |
| 7    | TLN1 | Talin-1 isoform X2     |
| 8    | PPBP | Platelet basic protein precursor |
| 9    | CCL14| C-C motif chemokine 14 |
| 10   | LOC480784, LOC609402| Hemoglobin subunit beta-like |
| 11   | FN1  | Fibronectin isoform X7 |
| 12   | TIMP1| Metalloproteinase inhibitor 1 isoform X1 |
| 13   | VCL  | Vinculin               |
| 14   | TUBA4A | Tubulin alpha-4A chain isoform X1 |
| 15   | TUBB4B| Tubulin beta-4B chain isoform X2 |
| 16   | PFN1 | Profilin-1             |
| 17   | PLEK | Pleckstrin             |
| 18   | LDHA | l-lactate dehydrogenase A chain; X1 |
| 19   | NID1 | Nidogen-1              |
| 20   | GSN  | Gelsolin               |
| 21   | TREML1| Trem-like transcript 1 protein isoform X1 |
| 22   | TUBB1| Tubulin beta-1 chain isoform X1 |
| 23   | FERM3T| Fermin family homolog 3 |
| 24   | F2   | Prothrombin            |
| 25   | CFL1 | Cofilin-1              |
| 26   | ALDOA| Fructose-bisphosphate aldolase A |
| 27   | HSPA8| Heat shock cognate 71 kDa protein |
| 28   | SERPINE1| Plasminogen activator inhibitor 1 precursor |
| 29   | YWHAZ| 14-3–3 protein zeta/delta |
| 30   | TF   | Serotransferrin        |
| 31   | LOC1008, 55558(40) | Hemoglobin subunit alpha-like |
| 32   | TAGLN2| Transgelin-2           |
| 33   | ILK  | Integrin-linked protein kinase |
| 34   | TUBA1A, TUBA3C, LOC610636, LOC100856405, TUBA1C| Tubulin alpha-1A chain, tubulin alpha-1B chain isoform X1, tubulin alpha-1C chain |
| 35   | PARVB| Beta-parvin isoform X1 |
| 36   | LTA4H| Leukotriene A-4 hydrolase |
| 37   | SERPINI1| Neuroserpin isoform X1 |
| 38   | GP1BA| Platelet glycoprotein lb alpha chain precursor |
| 39   | RAP1B| ras-related protein Rap-1b |
| 40   | RSU1 | ras suppressor protein 1 isoform X1 |
| 41   | MSN  | Moesin isoform X1      |
| 42   | CKB  | Creatine kinase B-type |
| 43   | SERPINE2| Glia-derived nexin  |
| 44   | PROS1| Vitamin K-dependent protein S |
| Rank | Gene | Protein identification |
|------|------|------------------------|
| 45   | BIN1 | myc box-dependent-interacting protein 1 isoform X16 |
| 46   | APOA1| Apolipoprotein A-I |
| 47   | EHD3 | EH domain-containing protein 3 |
| 48   | BIN2 | Bridging integrator 2 isoform X1 |
| 49   | TGFβ1| Transforming growth factor beta-1 precursor |
| 50   | SRGN | Serglycin |
| 51   | ITGB3| Integrin beta-3 isoform X1 |
| 52   | CAPN1| Calpain-1 catalytic subunit |
| 53   | PGK1 | Phosphoglycerate kinase 1 |
| 54   | TKT | Transketolase |
| 55   | ADA  | Adenosine deaminase isoform X2 |
| 56   | PKM  | Pyruvate kinase PKM isoform X1 |
| 57   | MMRN1| Multimerin-1 isoform X1 |
| 58   | PDLIM1| PDZ and LIM domain protein 1 |
| 59   | GDI1 | rab GDP dissociation inhibitor alpha |
| 60   | PLG  | Plasminogen precursor |
| 61   | LIMS1| LIM and senescent cell antigen-like-containing domain protein 1, X2; X5 |
| 62   | CIC1 | Chloride intracellular channel protein 1 |
| 63   | PNP  | Purine nucleoside phosphorylase |
| 64   | TIMP2| Metalloprotease inhibitor 2 precursor |
| 65   | CORO1B| Coronin-1B |
| 66   | CSRP1| Cysteine and glycine-rich protein 1 |
| 67   | CAP1 | Adenyl cyclase-associated protein 1; X1; X2 |
| 68   | HPSE | Heparanase |
| 69   | SELP | P-selectin isoform X1 |
| 70   | PRSS57| Serine protease 57 |
| 71   | PYPB| Glycogen phosphorylase, brain form |
| 72   | LTBP1| Latent-transforming growth factor beta-binding protein 1 isoform X1; X7 |
| 73   | PRDX1| Peroxiredoxin-1 isoform X1 |
| 74   | C4A  | Complement C4-A |
| 75   | GPI  | Glucose-6-phosphate isomerase |
| 76   | LO67368| Immunoglobulin lambda-1 light chain isoform X2; X16; X44; X34; X32; X40; X2; X3; X8 |
| 77   | VASP | Vasodilator-stimulated phosphoprotein; X1 |
| 78   | AHSG | Alpha-2-HS-glycoprotein |
| 79   | ECM1 | Extracellular matrix protein 1 |
| 80   | TF2  | Twinfilin-2 |
| 81   | EMILIN1| EMILIN-1, partial |
| 82   | RAB11B| ras-related protein Rab-11B |
| 83   | ACTR3B| Actin-related protein 3 |
| 84   | VCP  | Transitional endoplasmic reticulum ATPase isoform X1 |
| 85   | GSTP1| Glutathione S-transferase pi 1 |
| 86   | ABRACL| Costars family protein ABRACL |
| 87   | CALU | Calumenin isoform X1 |
| 88   | ANXA5| Annexin A5 isoform X1 |

(Continues)
TABLE 3 (Continued)

| Rank | Gene   | Protein identification                      |
|------|--------|--------------------------------------------|
| 89   | Sep/O6 | Septin-6 isoform X1                        |
| 90   | PLA2G7 | Platelet-activating factor acetylhydrolase; X1 |
| 91   | MDH1   | Malate dehydrogenase, cytoplasmic           |
| 92   | IL8; CXCL8 | Interleukin-8 precursor                     |
| 93   | YWHAB  | 14-3–3 protein beta/alpha                  |
| 94   | TIP3   | Metalloproteinase inhibitor 3 precursor     |
| 95   | PPIB   | Peptidyl-prolyl cis-trans isomerase B       |
| 96   | CAPZA1 | F-actin-capping protein subunit alpha-1     |
| 97   | PGD    | 6-phosphogluconate dehydrogenase, decarboxylating |
| 98   | ARHGAP1| Rho GTPase-activating protein 1 isoform X1  |
| 99   | NTSC3  | Cytosolic 5′-nucleotidase 3A isoform X1     |
| 100  | CNN2   | Calponin-2                                 |

Note: The proteins are ranked from highest to lowest abundance.

The presence of CEACAM21, a member of the carcinoembryonic antigen gene family, is a new CAPS finding. The protein is absent from the global platelet human proteome and murine secretomes, but was consistently found in high abundance (ranked 132) and actively secreted (average thrombin/saline ratio, 5.0) in all the dogs. CEACAM21 is one of the most connected proteins in our protein-protein association analysis and is associated with many enriched functional terms related to immune response. In addition to its yet unknown role in hemostasis, CEACAM21 appears to be involved in immune activation and remodeling of the extracellular matrix. While CEACAM21 function remains poorly defined, it has been identified as a highly expressed candidate gene for tumor progression and tumor growth in aggressive prostate cancer and is significantly overexpressed in immune active tumor tissue of high-grade serous ovarian cancer. Indeed, several CAPS proteins warrant future comparative investigations in cancer biology. Ezrin previously has been found necessary for development of metastasis in a mouse model of osteosarcoma, consistent with the association of high tumor ezrin expression and early metastasis in canine osteosarcoma and poor survival in human pediatric osteosarcoma. Cathepsins and metalloproteinase inhibitors are implicated in the regulation of tumor progression, and cystatin C has been associated with cancer metastasis. The present study establishes canine platelets as a relevant source for discovery of important biological mediators.

The CAPS proteins were also analyzed for secretion-specific annotations. We found that 82.6% of core CAPS proteins (537/650 proteins) were not annotated in the nonclassical or classical human secretory pathway, that is, signal peptide-mediated protein transfer from the endoplasmic reticulum to the Golgi complex and finally to the plasma membrane for release. As described for human platelet releasate proteomes, CAPS proteins represent granule contents released by exocytosis, plasma membrane-derived microparticle proteins, exosomes, and membrane-bound proteins shed from the platelet surface. However, many nonsecretion predicted proteins, like prothrombin and lysosomal enzymes, alpha-<i>v</i>-<i>i</i>-<i>d</i>-iduronidase, and the canine-specific protein cathepsin Z, are clearly secreted proteins; thus, categorization based on secretory pathway is at present not broadly applicable for platelet releasate proteins.

Differences in protease activated receptor (PAR) expression, structure, and signaling may explain some species differences in the thrombin-evoked platelet secretion profile. Selective cargo release and potential species differences in protein granule compartments cannot be excluded. Here and in the global canine platelet proteome, PAR4 was the only PAR family member detected. To date, PAR expression, presence of homo- or heterodimerization, and glycoprotein-Ib-IX/PAR4 cooperative

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| Gene        | Protein identification                                      | Rank<sup>a</sup> | Releasate | CAPS |
|-------------|-------------------------------------------------------------|-------------------|-----------|------|
| CCL14       | C-C motif chemokine 14<sup>b</sup>                          | 10                | 9         |      |
| SERPIN1     | Neuroserpin isoform X1<sup>b</sup>                         | 52                | 37        |      |
| PRSS57      | Serine protease 57<sup>b</sup>                              | 98                | 70        |      |
| IL8; CXCL8  | Interleukin-8 precursor<sup>b</sup>                        | 129               | 92        |      |
| PPIIC       | Peptidyl-prolyl cis-trans isomerase C isoform X1<sup>c</sup> | 183               | 133       |      |
| CEACAM21    | Carcinoembryonic antigen-related cell adhesion molecule 21<sup>b</sup> | 192               | 137       |      |
| PADI3       | Protein-arginine deiminase type-3                           | 224               | 161       |      |
| MARCKS      | Myristoylated alanine-Rich C-kinase substrate               | 296               | 213       |      |
| SDF4        | 45-kDa calcium-binding protein<sup>b</sup>                  | 309               | 222       |      |
| MFAP4       | Microfibril-associated glycoprotein 4<sup>b</sup>           | 366               | 263       |      |
| PSTPIP1     | Proline-serine-threonine phosphatase-interacting protein 1<sup>b</sup> | 383               | 274       |      |
| FAM129B     | Nibin-like protein 1                                         | 428               | 306       |      |
| CTSZ        | Cathepsin Z<sup>b</sup>                                     | 430               | 308       |      |
| NRCAM       | Neuronal cell adhesion molecule isoform X1<sup>b</sup>      | 442               | 308       |      |
| EFHD1       | EF-hand domain-containing protein D1                        | 455               | 316       |      |
| NRP1        | Neurilpin-1 isoform X2<sup>b</sup>                         | 456               | 327       |      |
| ACE         | angiotensin-converting enzyme<sup>b</sup>                   | 506               | 354       |      |
| CTS1        | Cathepsin L1<sup>b</sup>                                    | 540               | 380       |      |
| NT5C3B      | 7-Methylguanosine phosphate-specific 5′-nucleotidase isoform X1<sup>b</sup> | 541               | 381       |      |
| IL34        | Interleukin-34 isoform X3<sup>b</sup>                       | 728               | 498       |      |
| GBP1        | Guanylate-binding protein 1<sup>b</sup>                     | 740               | NS        |      |
| GSTA4       | Glutathione S-transferase A4 isoform X1<sup>c</sup>         | 765               | 518       |      |
| GBA         | Glucosylceramidase<sup>b</sup>                              | 776               | 526       |      |
| RGCC        | Regulator of cell cycle RGCC                                | 854               | NS        |      |
| SELENBP1    | Selenium-binding protein 1<sup>c</sup>                      | 865               | NS        |      |
| FABP3       | Fatty acid-binding protein, heart<sup>c</sup>                | 889               | NS        |      |
| DIRAS2      | GTP-binding protein Di-Ras2<sup>b</sup>                     | 922               | NS        |      |
| GN5         | Guanine nucleotide-binding protein subunit-beta-5<sup>b</sup> | 926               | NS        |      |
| DDHD2       | Phospholipase DDHD2 isoform X1<sup>c</sup>                  | 934               | 596       |      |
| NTSC2       | Cytosolic purine 5′-nucleotidase isoform X1<sup>b</sup>      | 949               | NS        |      |
| LAMTOR3     | Regulator complex protein LAMTOR3<sup>b</sup>                | 977               | 608       |      |
| SEMA4B      | Semaphorin-4B<sup>b</sup>                                   | 1000              | 618       |      |
| IDUA        | Alpha-L-iduronidase isoform X1<sup>b</sup>                  | 1009              | 622       |      |
| MATK        | Megakaryocyte-associated tyrosine-protein kinase isoform X1<sup>b</sup> | 1044              | NS        |      |
| PGPEP1      | Pyroglutamyl-peptidase 1<sup>c</sup>                        | 1046              | 636       |      |
| DHDH        | Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase<sup>b</sup>  | 1094              | NS        |      |
| FGF1        | Fibroblast growth factor 1 isoform X2<sup>b</sup>           | 1096              | 648       |      |
| NPEPL1      | Probable aminopeptidase NPEPL1<sup>c</sup>                  | 1102              | NS        |      |
| PGM3        | Phosphoacetylglucosamine mutase isoform X1<sup>c</sup>       | 1103              | NS        |      |

Note: The proteins are sorted according to abundance rank.
NS, not secreted.
<sup>a</sup>MS 1 abundance rank of in total 1226 canine protein species in the thrombin releasate and in total 650 CAPS secretome.
<sup>b</sup>Directly annotated as at least one enriched functional pathway or term.
<sup>c</sup>Directly connected to a protein annotated as at least one enriched functional pathway or term, ie indirectly annotated. For detailed information, see Table S7.
The identified CAPS proteins should not be regarded as the definitive final set of reference CAPS proteins. The core CAPS proteins were restricted to those present in 10 of 10 dogs and were statistically defined by comparison with a saline-treated control sample. Many more proteins were present in 9 of 10 or 8 of 10 dogs. Haptoglobin was secreted in high abundance, yet identified in only 8 of 10 dogs. Proteins were identified according to individual isoforms. Isoform variations might be biologically irrelevant, but using an isoform-specific approach and applying a 10 of 10 dog criterion might have resulted in exclusion of CAPS proteins. This is exemplified by integrin alpha-IIb, which was identified without an isoform annotation in 8 of 10 dogs and with the annotation of isoforms 1 and 2 in
the remaining two dogs. Another challenge was the identification of proteins identical by name but not accession number, and with a global sequence identity alignment of <98%. This was the case for the human platelet secretion protein complement C3, which in four dogs was identified differently than in the remaining six dogs. Another potential limitation was that platelet suspensions were not adjusted to a standard platelet concentration. However, the variation in platelet number did not have a demonstrable effect on either the rate or maximal extent of platelet aggregation following agonist stimulation. We compensated for difference in platelet count by standardizing the concentration of releasate protein used for MS analysis. In addition, the saline control samples were processed identically to the activated samples such that proteins present in the saline controls likely reflected plasma proteins not removed by washing (contaminants), or proteins released nonspecifically during sample preparation, handling, or secondary to stir-related platelet activation. The number of proteins identified in the canine releasate was 4-fold greater than a similar in-solution human platelet releasate proteome. This likely reflects differences in methodology, including differences in platelet activation and initial centrifugation, replacement of acetone protein precipitation with filter-aided sample concentration, and S-Trap processing before tandem MS in this study. Methodology differences, including in mass spectrometers, software programs, or search algorithms between studies, might account for differences in protein identifications. Nonstandard peptide readouts including semitryptic searches were not performed, but this is consistent with previous human studies.

In conclusion, the methodologies described for platelet activation, isolation of releasate proteins, and proteomic analyses are readily adaptable to study platelet activation response across species and in response to other soluble agonists. The healthy dog CAPS proteome demonstrates species differences in granule cargo relevant for future comparative studies of thrombus formation, tumor metastasis, and wound healing. Same-species comparisons of CAPS from healthy and diseased dogs will also provide insights into platelets’ role in mediating inflammatory and neoplastic disease.

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AUTHOR CONTRIBUTIONS
SEC provided the concept, designed the study, conceived experiments, analyzed and interpreted data, and wrote the manuscript. JLC, RG, and MBB designed the study, analyzed and interpreted data, and wrote the manuscript. SES analyzed data and contributed to the manuscript. ATK provided the concept and study design and contributed to data interpretation and writing the manuscript. PBS and PBM provided protocols and contributed to data interpretation and writing the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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