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METHODOLOGICAL ARTICLE

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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.

The work was carried out at Cornell University, Department of Population Medicine and Diagnostic Sciences, Ithaca, NY, USA.

KEYWORDS
dog, platelet, proteomics, releasate, secretion

Essentials

- Release of granule cargo is a critical platelet function.
- We define a core protein profile of the canine activated platelet secretome (CAPS).
- Species differences in platelet granule cargo proteins are identified.
- CAPS provides novel tools for using canine platelet defects as models of human disease.

1 | INTRODUCTION

The dog is an important comparative animal model of human hemostatic disease[1] and a translational large animal model for human medicine.[2] 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,[3] Glanzmann thrombasthenia,[4-6] storage pool diseases,[7] and immune-mediated thrombocytopenia[8] have been identified. Dogs also share with humans various neoplastic diseases, including spontaneous solid tumors and leukemias,[9,10] 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,[11-13] cancer,[14-16] uremia,[17-19] and diabetes mellitus.[20-22] 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.[23] To capitalize on those data, a solidly defined platelet secretion 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).[24] 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 scan speed have markedly improved the detection and identification of low-abundance proteins in digests of complex solutions,[25] enabling reliable identification of secretome cargo proteins. In an earlier study, Parsons and colleagues[26] 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.

2 | MATERIALS AND METHODS

2.1 | 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[24] and can also be found in detail in supplemental materials (Supplementary Method S1).

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

The in-solution protocol was modified from Parsons et al.[26] For protein extraction, 10× 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 (ProtiFi, Huntington, NY, USA) was according to vendor protocol and Zougman et al.[25,27] 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

TABLE 1 Dog demographics, platelet count, and quality control measures of final platelet suspensions

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, $\times 10^3/\mu L$	254 (±88)
Washed platelets:	
Platelets, $\times 10^3/\mu L$	524 (±283)
Red blood cells, $\times 10^6/\mu L$	0.0 (±0.0)
White blood cells, $\times 10^3/\mu L$	0.0 (±0.0)
CD18 positive cells, %	0.0025 (±0.002)
CD62P positive cells, %	3.5 (±1.2)

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.

addition of 3 µL of 12% aqueous phosphoric acid and 165 µL of S-Trap buffer (90% methanol in 0.1 mol/L of triethylammonium bicarbonate [TEAB], pH 8.5) before the protein mixtures were transferred onto the S-Trap micro column and centrifuged at 4000 *g* for 30 seconds. The sample tube was rinsed with 150 µL 90% methanol, 0.1 mol/L TEAB (pH 8.5), applied to the S-Trap micro column and centrifuged at 4000 *g* for 30 seconds. The S-Trap micro column was then washed with 150 µL 90% methanol, 0.1 mol/L TEAB (pH 8.5), centrifuged at 4000 *g* for 30 seconds, and the flow-through was discarded. Samples were placed into a new 2-mL tube for digestion, overlain with 25 µL of trypsin (40 ng/µL) in 50 mmol/L of TEAB (pH 8.5) and incubated at 37°C overnight. Peptides were then eluted off the S-Trap micro column stepwise and collected into a 2.0-mL Eppendorf tube, briefly: 40 µL of 50 mmol/L TEAB (pH 8.5) was added and followed by centrifugation at 4000 *g* for 30 seconds. Elution was repeated with 40 µL of 0.2% formic acid (FA), and 40 µL of 50% acetonitrile (ACN), 0.2% FA, respectively. All eluent was dried immediately in the Speed Vacuum and samples stored at −20°C.

2.3 | Nano–LC–tandem MS analysis

Lyophilized tryptic peptides were reconstituted in 20 µL of 0.5% FA for nano–LC–tandem MS analysis performed using an Orbitrap Fusion Tribrid (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).[25,28] 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).

2.4 | 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.[29] 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.

2.5 | 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

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,[26] and were compared to other platelet proteomes using homologous human Entrez Gene IDs (https://biodbnet-abcc.ncifcrf.gov).[26,30-35] The abundance ranking of releasate proteins was compared to that of the human core releasate reported by Parsons and colleagues.[26]

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.[36] 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 (eg, physical association from experimental data and functional associations from curated pathways, automatic text mining, and prediction methods) provided by the STRING database.[37] 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.[38] 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,[26,30-35] 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.[26] 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,[26] 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,[39,40] as seen in our previous CAPS study, in which the secreted proteins were categorized as soluble or particulate fractions.[24]

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,[23] 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	Absent
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
IGHA1	Ig alpha-1 chain C region	37	Absent
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
CTSA	Lysosomal protective protein	95	1088
HTIMP2	Metalloproteinase inhibitor 2	Absent[‡]	87
BIN1	Myc box-dependent-interacting protein 1	Absent[‡]	62
SERPINI1	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.

[†]Parsons et al.[26]

[‡]Proteins previously identified in human platelets based on proteomic or experimental evidence.

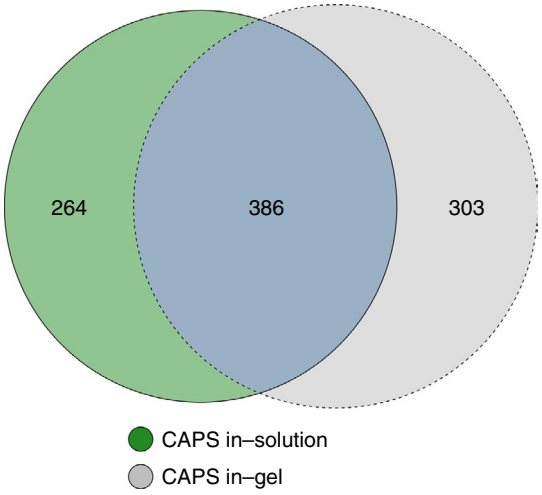

FIGURE 1 Venn diagram comparing the number of CAPS proteins identified by the in-gel and in-solution methods. The methods coidentified 386 core CAPS proteins, whereas 303 proteins were solely identified by the in-gel approach and 264 solely by the in-solution approach. Of note, if several RefSeq identifications of the in-gel CAPS proteins mapped to the same protein of the in-solution CAPS core, only one in-gel protein was chosen as coidentified. This led to the exclusion of four proteins from the in-gel CAPS core (XP_013974803, XP_005628024, XP_022266309, and XP_005636600) for the present comparison and Venn diagram. CAPS, canine activated platelet secretome

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, niban-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	FERMT3	Fermitin 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 Ib 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

(Continues)

TABLE 3 (Continued)

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	TGFB1	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	CLIC1	Chloride intracellular channel protein 1
63	PNP	Purine nucleoside phosphorylase
64	TIMP2	Metalloproteinase inhibitor 2 precursor
65	CORO1B	Coronin-1B
66	CSRP1	Cysteine and glycine-rich protein 1
67	CAP1	Adenylyl cyclase-associated protein 1; X1; X2
68	HPSE	Heparanase
69	SELP	P-selectin isoform X1
70	PRSS57	Serine protease 57
71	PYGB	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	LOC607368	Immunoglobulin lambda-1 light chain isoform X22; 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	TWF2	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/06	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	TIMP3	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	NT5C3	Cytosolic 5′-nucleotidase 3A isoform X1
100	CNN2	Calponin-2

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

dogs, low granule release of von Willebrand factor, and high release of proteins like C-C motif chemokine 14, trem-like transcript 1, interleukin-8, glia-derived nexin, and metalloproteinase inhibitors 1 and 2. The core CAPS defined in the present study identified additional secreted proteins such as CEACAM21, ezrin, several cathepsins, and cystatin C. Together, these interspecies secretome differences may reflect variations in protein processing and trafficking to platelet granules, or species differences in protease-activated receptor-mediated granule release.

The CAPS findings are relevant for understanding canine disease states and use of the canine model in translational studies. For example, the absence of platelet factor 4 in the CAPS may explain the absence of heparin-induced thrombocytopenia in dogs. In humans, heparin-induced thrombocytopenia results from formation of neoantigens consisting of complexes of platelet factor 4 and heparin bound to the platelet surface.[41] Also, several proteins within the family of immunoreceptor tyrosine-based inhibition motif–containing receptors warrant further investigation. Trem-like transcript 1, for example, is solely found in megakaryocytes and platelets[42] and is reported as a more sensitive marker of platelet activation than P-selectin.[43] Thrombin activation leads to release of soluble trem-like transcript 1, which has anti-inflammatory properties[44] and has been detected as increased in patients with sepsis.[45]

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[46] and the human[26] and murine secretomes,[47] 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[48] and is significantly overexpressed in immune active tumor tissue of high-grade serous ovarian cancer.[49] Indeed, several CAPS proteins warrant future comparative investigations in cancer biology.[14-16] 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.[50] Cathepsins[51] and metalloproteinase inhibitors[52] are implicated in the regulation of tumor progression, and cystatin C has been associated with cancer metastasis.[53] 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,[26,54] 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-L-iduronidase, and the canine-specific protein cathepsin Z, are clearly secreted proteins[54]; 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.[55] Here and in the global canine platelet proteome,[23] PAR4 was the only PAR family member detected. To date, PAR expression, presence of homo- or heterodimerization, PAR4/P2Y_{12} association, and glycoprotein-Ib-IX/PAR4 cooperative

TABLE 4 Novel platelet proteins in the canine thrombin releasate and/or canine platelet secretome (CAPS) relative to human and murine platelets

Gene	Protein identification	Rank[a]	
		Releasate	CAPS
CCL14	C-C motif chemokine 14[b]	10	9
SERPINI1	Neuroserpin isoform X1[b]	52	37
PRSS57	Serine protease 57[b]	98	70
IL8; CXCL8	Interleukin-8 precursor[b]	129	92
PPIC	Peptidyl-prolyl cis-trans isomerase C isoform X1[c]	183	133
CEACAM21	Carcinoembryonic antigen-related cell adhesion molecule 21[b]	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[b]	309	222
MFAP4	Microfibril-associated glycoprotein 4[b]	366	263
PSTPIP1	Proline-serine-threonine phosphatase-interacting protein 1[b]	383	274
FAM129B	Niban-like protein 1	428	306
CTSZ	Cathepsin Z[b]	430	308
NRCAM	Neuronal cell adhesion molecule isoform X1[b]	442	308
EFHD1	EF-hand domain-containing protein D1	455	316
NRP1	Neuropilin-1 isoform X2[b]	456	327
ACE	angiotensin-converting enzyme[b]	506	354
CTSL	Cathepsin L1[b]	540	380
NT5C3B	7-Methylguanosine phosphate-specific 5′-nucleotidase isoform X1[b]	541	381
IL34	Interleukin-34 isoform X3[b]	728	498
GBP1	Guanylate-binding protein 1[b]	740	NS
GSTA4	Glutathione S-transferase A4 isoform X1[c]	765	518
GBA	Glucosylceramidase[b]	776	526
RGCC	Regulator of cell cycle RGCC	854	NS
SELENBP1	Selenium-binding protein 1[c]	865	NS
FABP3	Fatty acid-binding protein, heart[c]	889	NS
DIRAS2	GTP-binding protein Di-Ras2[b]	922	NS
GNB5	Guanine nucleotide-binding protein subunit beta-5[b]	926	NS
DDHD2	Phospholipase DDHD2 isoform X1[c]	934	596
NT5C2	Cytosolic purine 5′-nucleotidase isoform X1[b]	949	NS
LAMTOR3	Regulator complex protein LAMTOR3[b]	977	608
SEMA4B	Semaphorin-4B[b]	1000	618
IDUA	Alpha-L-iduronidase isoform X1[b]	1009	622
MATK	Megakaryocyte-associated tyrosine-protein kinase isoform X1[b]	1044	NS
PGPEP1	Pyroglutamyl-peptidase 1[c]	1046	636
DHDH	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase[b]	1094	NS
FGF1	Fibroblast growth factor 1 isoform X2[b]	1096	648
NPEPL1	Probable aminopeptidase NPEPL1[c]	1102	NS
PGM3	Phosphoacetylglucosamine mutase isoform X1[c]	1103	NS

Note: The proteins are sorted according to abundance rank.

NS, not secreted.

[a]MS 1 abundance rank of in total 1226 canine protein species in the thrombin releasate and in total 650 CAPS secretome.

[b]Directly annotated as at least one enriched functional pathway or term.

[c]Directly connected to a protein annotated as at least one enriched functional pathway or term, ie indirectly annotated. For detailed information, see Table S7.

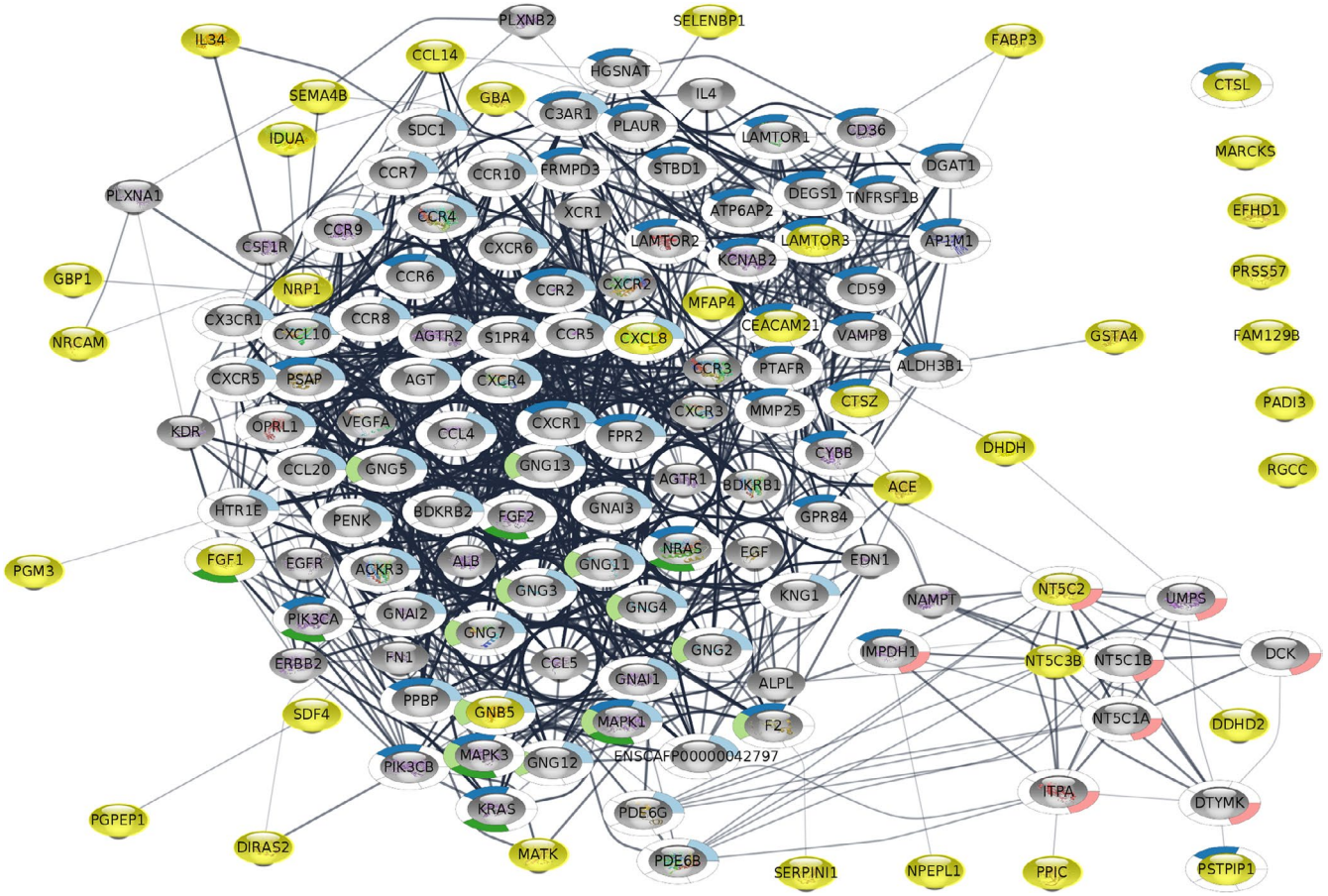

FIGURE 2 STRING network and functional enrichment of 39 canine-specific proteins. The network shows their most confident protein-protein associations annotated in the STRING database with 100 other proteins. The canine-specific proteins are marked in yellow. The ring around the genes illustrates the five most enriched Reactome pathways (see Methods and Table S7). Pathways ranked in order of significance: "G alpha (i) signaling events" (light blue), "innate immune system" (dark blue), "thrombin signaling through proteinase activated receptors (PARs)" (light green), "signaling by FGFR4" (dark green) and "metabolism of nucleotides" (red). The proteins fall within two clusters in the association network with one cluster enriched for immune response and signaling, and the other cluster enriched for nucleotide metabolism. Twenty-six of the 39 dog-specific proteins have known annotation of at least one enriched functional term (ie directly annotated) and eight of the remaining 13 proteins can be assigned a functional term based on association with an annotated protein (ie, indirectly annotated), leaving five proteins neither directly or indirectly annotated (Table 4). Of note, more than one-third (14) of the dog-specific proteins are annotated as signal proteins, of which 11 are also identified as CAPS proteins. Interleukin-8, CEACAM21, and neuropilin-1 isoform X2 have the most enriched functional terms (Table S7) and are all included in the immune response functional cluster. Thirty-two proteins are connected to at least one other protein, and only 7 proteins are unconnected to any other protein. The proteins are identified by means of gene symbol, which for the 39 canine-specific proteins can be identified by protein name in Table 4. The network has been generated by Cytoscape version 3.7.2 and the functional enrichment analysis has been performed by the Cytoscape StringApp. CAPS, canine activated platelet secretome; CEACAM21, carcinoembryonic antigen–related cell adhesion molecule 21

signaling, have not been well defined in dogs. In one study, thrombin-initiated platelet activation and Akt phosphorylation were inhibited by cangrelor,[56] consistent with a role for $P2Y_{12}$ in canine thrombin signaling. Additionally, canine platelets fail to aggregate in response to human PAR1 and PAR4 agonist peptides.[56-58] Exclusive PAR4 signal initiation could account for differences between human and canine secretomes or may reflect differences in protein expression or granule storage.[55] Interestingly, PAR4 is a promising antithrombotic drug target,[59] and canine platelets may enable exploration of PAR4 expression, signal initiation, and cooperative signaling pathways, as a research tool for development of effective antithrombotics targeting PAR4.

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.[26] 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.

RELATIONSHIP DISCLOSURE

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SUPPORTING INFORMATION

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

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