Invited lecture/Review

Serum, Saliva, and Liver Proteome Indices Associated with Platelet Biology during Inflammatory Conditions in Different Animal Species

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Abstract:
The understanding of platelet biology stepped out of the (patho)physiology of hemostasis long ago. Currently, platelets are acknowledged as effective sentinels against pathogens and powerful regulators of inflammatory processes. While accomplishing these tasks, their structural and physiological features undergo constant changes, often associated with the proteomics indices in the tissues and biofluids. Assessing these associations in different animal species provides a substantial comparative benefit. Nevertheless, the *sine qua non* for the reliable interpretation of the obtained data is a comprehensive understanding of the applied analytical and bioinformatics methods.

Keywords: Tissue; Body fluids; Proteomics; Platelet biology; Animals; Infections

1. Introduction

In general, two main perceptions provide the rationale for the multidisciplinary research interest in the platelet biology features as part of the immunity-related network. The first is the evolutionary perspective, as the platelet immunocompetency in vertebrates occurs as a relict from the predecessor in the invertebrates–hemocyte, the cell in control of the hemolymph volume homeostasis and the immune response. The second concept emerged some ten years ago, with the description of immunothrombosis, a powerful intravascular innate immunity phenomenon, conjoining coagulation with the immune mechanisms, both cellular and humoral (Carestia et al., 2022).

An extensive repository of experimental results allows consideration of platelets as sentinels against pathogens and acknowledges their involvement in shaping the immune response against them. Platelets accomplish these tasks either alone or via crosstalk with immunocompetent cells, such as monocytes, neutrophils, and lymphocytes (Magraf and Zarbock, 2019; Carestia et al., 2022). An analogy is evident regarding the platelets' roles in hemostasis and immunity. First, their involvement requires a trigger signal. During hemostasis, vascular injury initiates the platelet aggregation cascade. In terms of immune properties, the presence of pathogen- or damage-associated molecular patterns, recognized via their corresponding receptors, generates their activation. The analogy further expands to effector molecules secreted from platelet granules. Depending on the trigger responsible for platelet activation, the individual components of the platelet secretome converge their activities towards hemostatic outcomes or pathogen elimination. Finally, both hemostatic and immune/inflammatory activities require tight control mechanisms to prevent their transformation from protective to severely detrimental phenomena such as thrombosis, systemic inflammatory response syndrome, or chronic inflammation (Margraf and Zarbock, 2019). Plasticity characterizes the platelet functional phenotype, thus facilitating adjustment to real-time (micro) environmental requirements. Among the numerous factors, reliable sensing of these requirements depends on a cluster of host-originating proteins (Margraf and Pareti, 2022). Therefore, a comprehensive analysis of the different proteome compartments, such as tissue(s) and body fluids, is an approach of choice for deciphering the interactions between platelets and their environment.

The interspecies differences in the morphological and functional features of platelets are among the essential factors guiding the interpretation of experimental data about their biology (Weiss, 1999; Margraf and Zarbock, 2019). In this regard, gathering the evaluation of these features in different animal species yields a substantial comparative advantage. As the best outcome, this generated research knowledge allows for reliable translation between animal and human biology models.

The article will start with a brief outline of the biology of platelets, with an emphasis on the features that make them immunocompetent. This review will continue with a compendious overview of the proteomics workflow. After that, four examples will present recent results illustrating the association between host proteome indices and platelet biology within the inflammatory scope in different animal species. The first two examples provide results on experimental inflammation and natural infection in pigs. In the third example, the focus will be on natural parasitic infestation in deer, while the fourth will bring the data obtained in the study on dairy cows with postpartum infection. A summary of the applicability of the current results and directions for further research are presented.

2. The biology of platelets-a brief outline

The first description of platelets, the small cytoplasmic fragments of megakaryocytes, dates back to the second half of the 19th century. The bone marrow hubs for platelet production; the lungs also represent a suitable environment for their maturation. Thrombopoietin monitors the number of circulating platelets. The concentration of this protein in the blood increases in parallel with a reduction in platelet count. The spleen and liver are the main organs that remove platelets from circulation, while the lungs, brain, and macrophages can also contribute. Numerous factors influence platelet count and functionality, such as hemorrhage or inflammatory challenges, age, sex, and circadian variation (Margraf and Zarbock, 2019).

When an endothelial injury occurs, the adhesion of platelets to the subendothelial structures initiates the hemostatic cascade. It continues via platelet activation and aggregation until the establishment of a thrombus as a barrier against the further extravasation of blood. The underlying mechanisms are receptor-mediated and involve changes in platelet morphology coupled with conformational changes. As a result, platelets release granular content, thus increasing the local levels of secondary hemostatic mediators such as von Willebrand factor, adenosine diphosphate, calcium ions, biogenic amines, platelet factor 4, and chemokine ligand 5 (Sandmann and Köster, 2016; Estevez and Du, 2017; Margraf and Zarbock, 2019).

The circulating platelets are also constantly patrolling with a "mission" of protecting from pathogens. Their molecular "equipment" for this purpose is versatile, with a plethora of pattern recognition receptors reactive against the diverse pathogen-associated molecular patterns (such as lipoproteins, lipopolysaccharides, or nucleic acids) or the damage-associated molecular patterns (Shevchuk et al., 2021). When sensing the bacterial presence, the platelet membrane receptors interact with the molecules on the bacterial surface or secreted from the bacterial cell, directly or indirectly, using the host-originating proteins for the "bridging". The platelet glycoprotein Ibα, Fc, complement, or toll-like receptors are the representative structures involved in these interactions (Kerrigan, 2015; Shannon, 2015). The resulting effects on the platelet activity depend on the numerous bacterium- and host-related factors (McNicol, 2015). The mechanisms underlying the platelet-virus interactions largely resemble those occurring in the case of bacteria. The type of platelet receptors "in charge" (like toll-like receptors, Fc receptors, or DC-SIGN) can vary between the viruses. The net effect of these interactions is the "adsorption" of the virus cells on the layer formed of platelet, leading to their activation and joint removal with the adsorbed virus cells. A deleterious side effect of this otherwise protective mechanism is protruding thrombocytopenia, which can be multicausal. Besides the overactivation, it can originate also from the viral penetration into the megakaryocytes or the overconsumption if the infection of endothelial occurs. Malaria represents a suitable model to study platelet-parasite interactions. Generally, during Plasmodium spp. Infection platelets function in the "Yin and Yang" mode. On one side, they elicit the initial immune response or even act cytotoxically, thereby contributing to parasite clearance. Opposite to these beneficial effects, platelets can also mediate the binding of the infected erythrocytes to the endothelium, resulting in the formation of heterogenous multi-structural aggregates and consequentially to the cerebral sequel (Alonso and Cox, 2015).

In the next step, platelets join the cross-talk with the other pathogen-recognizing cells - neutrophils and monocytes, and the traditional innate immunity mechanisms are initiated (Shevchuk et al., 2021). Analogous to other immune cells, the role conferred to platelets is tightly balanced between pro- and anti-inflammatory effects. The cross-talk with neutrophils, mediated via the membrane glycoproteins and so-called kinocidins (like platelet factor 4 or antimicrobial peptides), strengthens the recruitment and transmigration of the immune cells, formation of neutrophil extracellular traps and immune-thrombosis. As the counterbalance, the activated platelets prevent the inflammation-associated hemorrhage or cell death, while via the CLEC2-mediated mechanisms potentiate the anti-inflammatory phenotyping changes of the macrophages (Nicoali and Massberg, 2020; Carestia et al., 2022). Finally, the platelet research field in the Immunobiology area is expanding towards new aspects like interferon signaling, T-cell response, or host homeostasis restoration (Nicolai and Massberg, 2020).

The classification of platelets into the different functional phenotypes can rely on the combinations of their morphological, hemostatic, inflammatory, and immunomodulatory characteristics. During platelet senescence, besides the decrease in their volume, the impairment in the response upon stimulation occurs, together with the proteomic changes indicating apoptosis. On the contrary, immature platelets tend to show more pronounced pro-inflammatory properties (Margraf and Pareti, 2022). Also, the functional effects depend on the site of maturation. For example, the megakaryocytes in bone marrow supply the platelets for hemostatic purposes. Megakaryocyte populations in the lungs express an "immune phenotype"-those located on the blood vessel releases platelets, and the extravascularly positioned ones share features with the lung dendritic cells. The continued migration of megakaryocytes between these two structure-functional compartments is the

basis of platelet phenotype plasticity, an emerging exclusive research area (Boilard and Machlus, 2021).

3. A compendious overview of the proteomics workflow

Proteomic research in animal and veterinary science offers advantages in experimental design, sample selection, and preparation because of the broad availability of diverse biological samples, like tissues, cells, and fluids, such as serum, plasma, urine, saliva, milk, or semen (Bilić et al., 2018). Mass spectrometry (MS) -based proteomics includes two broad groups of techniques: "top-down" proteomics, measuring the intact proteins, and "bottom-up" proteomics, which analyzes peptides derived from proteolytic digestion. Tandem MS/MS, in combination with nanoflow liquid chromatography (LC), has become the analytical technique of choice for comprehensive analysis of complex samples.

An optimized sample preparation protocol is a prerequisite for any robust and sensitive bottom-up proteomics workflow. Drafting of these standardized protocols involves a detailed explanation of the sampling, handling, and storage conditions. To ensure comparable and reproducible results, the protocols also need to address the selection of collection tubes and additives and specific issues such as the influence of variations in clotting time, allowable lag time before centrifugation, hemolysis, and repeated freeze/thaw cycles. Significant attention has also been paid to strategies to minimize sample heterogeneity and disruption (Rai et al., 2005; Hsieh et al., 2006).

Sample preparation for protein profiling using MS requires multiple steps. The workflow includes the extraction of proteins and their denaturation, reduction, and alkylation, after which proteins are digested into peptides with a site-specific protease (Bodzon-Kulakowska et al., 2007; Switzar et al., 2013; Vandermarliere et al., 2013). Optionally, the protocols include the depletion of the highly abundant or enrichment of target proteins (Marco-Ramell and Bassols, 2010; da Costa et al., 2017) or clean-up procedures, removal of salts, denaturing agents, and other interfering substances with filtered assisted sample preparation (FASP) (Wiśniewski et al., 2009).

To quantify abundance changes in proteome's, labeling approaches, such as those based on isobaric mass tags, have been used for over a decade. The tandem mass tag (TMT) labeling approach utilizes chemical derivatization (Rauniyar and Yates, 2014). Isobaric mass tags are isotope-coded molecules with the same chemical structure and molecular weight that are used to differentially label peptides without introducing mass differences and sample complexity. The isotopically derivatized peptides displayed a single peak in the MS spectrum and yielded a series of low-mass reporter ions for quantification upon fragmentation in tandem mass spectrometry. Quantitative results are obtained from the direct correlation between the relative intensities of the reporter ions and peptides selected for MS/MS fragmentation (Rauniyar and Yates, 2014).

After protein identification and quantification, characterization and bioinformatics analysis follow to determine gene ontology. In addition, the pathway enrichment analysis is indisposed in identifying the biological pathways associated with the observed proteome changes. Unique challenges appear when the available bioinformatic repositories do not include data on the studied animal species (Heck and Neely et al., 2020). In such a case, the usual first step is assigning protein sequences to their model species' equivalent using the "Basic Local Alignment Search Tool" (BLAST) analysis.

4. The examples illustrate the association between host proteome indices and platelet biology within the inflammatory scope in different animal species

4.1. Case No. 1: Pigs with septic and non-septic inflammation

The interconnection between the proteomic features and platelet biology during an infection can depend on the intensity of the triggered inflammation, as evidenced by López-Martínez et al., 2022). Serum and salivary proteome were compared, using pigs as the experimental model, during the time course of the septic (induced in five pigs via intramuscular administration of lipopolysaccharide (LPS) from *Escherichia coli*) and non-septic inflammation (triggered in five pigs after subcutaneous injection of turpentine oil). Table 1 brings the platelet-associated proteome changes which occurred in the saliva and serum proteome after the first six hours of the experiment.

Protein	Sample/Inflammation			
	Saliva/Nonseptic	Saliva/Septic	Serum/Nonseptic	Serum/Septic
Fructose-biphosphate aldolase	ø change	↑	ø change	ø change
Alpha-2-macroglobulin	↑	↑	ø change	ø change
Fibrinogen alpha chain	ø change	ø change	ø change	↓
Fibrinogen beta chain	ø change	ø change	ø change	↓
Fibrinogen C-terminal domain-containing	ø change	ø change	ø change	↓
Fibronectin	ø change	ø change	ø change	↓
Transferrin	↑	ø change	ø change	ø change
Albumin	↑	ø change	ø change	ø change
Histidine-rich glycoprotein	↑	ø change	ø change	ø change

Table 1. The platelet-associated proteome changes which occurred in the saliva and serum proteome after the first six hours of the experiment. Changes in the relative abundance basal conditions vs. After 6 hours of experiment: ↑-increase, ↓-decrease.

Under the non-septic conditions, several platelet-associated changes appeared in the salivary proteome, while none emerged in serum. In the septic environment, the alterations occurred in serum; nonetheless, their decreased nature limited their practical applicability. On the other side, saliva offered a biomarker candidate for early recognition of sepsis–the increased relative abundance of fructose-biphosphate aldolase, further verified by the increase in the enzyme activity. This glycolytic enzyme shows adhesin-like and immunostimulatory properties (Elhaik Goldman et al., 2016; Pirovich et al., 2021). Notwithstanding, aldolase participates in the platelet cytoskeletal (re) organization, accompanying their activation (Arias-Salgado et al., 2008).

4.2. Case No. 2: Pigs with meningitis caused by Streptococcus suis

The proteomic-based model for assessing the interplay between the serum and saliva proteomic indices related to platelet biology, established during the experimental infection in pigs (López-Martínez et al., 2022), required verification in natural infection settings. In this context, pigs with meningitis caused by *S. suis* were an attractive study cohort (López-Martínez et al., 2022). The authors compared the differences in salivary and serum proteome compositions between diseased and healthy pigs, with each group containing 10 animals. The relative abundance of 21 salivary proteins differed between the two groups, of

Which 7 were associated with platelet biology (Table 2). Similarly, the pathway enrichment analysis allocated to platelet biology showed six out of 20 proteins identified to have different serum relative abundances between the infected and healthy pigs (Table 2).

Table 2. Proteins differing between healthy and pigs with meningitis by the relative abundance in serum and saliva which were allocated to the platelets' biology.

Protein	Relative abundance meningitis vs. healthy	
	Saliva	Serum
Transferring	lower	lower
Apolipoprotein A-I	lower	lower
Histidine-rich glycoprotein	lower	lower
Extracellular matrix protein 1	higher	ø difference
Alpha-1B-glycoprotein	lower	ø difference
Vinculin	higher	ø difference
Fructose-biphosphate aldolase	higher	ø difference
Albumin	ø difference	lower
Clusterin	ø difference	lower
SERPIN domain-containing protein (LOC100156325)	ø difference	higher

Both the serum and salivary pattern were the comprehensive "puzzle", consisting of the proteins showing potentially opposing effects on the platelet activity. Therefore, it might be very challenging to stand out whether they were "platelet activating". Regardless, two very important features were evident. The first was that the salivary and serum patterns had only three proteins in common, which might imply that the indices of these two proteome compartments provide somewhat different but complementary contexts regarding platelet biology.

The second feature brings a practical upgrade. Namely, the proteomic investigation within the natural infection environment verified the higher relative abundance of the fructose-biphosphate aldolase in the infected pigs exclusively in saliva. In this manner, the salivary biomarkers' potential for the effective management of swine infections further increases (Cerón et al., 2022).

4.3. Case No. 3: Infestation of red deer with the giant liver fluke

The analysis of the proteome of the pathogen's target organ represents an intriguing research task. The study of Šimonji et al. (2022) brought insights into the liver proteome qualitative and quantitative traits associated with the giant liver fluke (*Fascioloides magna*) infestation in the red deer (*Cervus elaphus*). Relative quantification analysis revealed the differences in the abundance of 234 proteins between the infected and healthy deer. Further pathway enrichment analysis linked 12 (Table 3) with the molecular response to elevated calcium ion levels in the platelet cytosol and platelet activation, signaling, aggregation, and degranulation.

Table 3. Proteins differing between the liver of the red deer with *F. magna* infestation and healthy deer.

Protein	Infested vs. healthy
Fibrinogen alpha chain	higher
Fibrinogen beta chain	higher
Transferrin	higher
Apolipoprotein A-I	lower
Superoxide dismutase	lower
Calmodulin	lower
Alpha-1-acid glycoprotein	lower
Saccharopine dehydrogenase-like oxidoreductase	higher
Lysosome-associated membrane glycoprotein 1	lower
Annexin	higher
Alpha-actinin-4 isoform X3	higher
Acyl-CoA synthetase medium chain family member 3	lower

The integrative interpretation of the observed differences needs caution and merits additional studies by cause of at least two reasons. The first is that the proteins which showed the platelet-associated differences were also allocated to the other (patho) biological pathways occurring in the liver. Another one appeared from the bioinformatics algorithm, which, in cases of the identified proteins without gene ID for *Cervus elaphus* or the uncharacterized proteins, had to include BLAST analysis and the replacements with the bovine orthologue (Šimonji et al., 2022).

4.4. Case No. 4: Cows with the retained placenta

Extensive research has been conducted to determine the structural and functional characteristics of bovine platelets. Their diameter under non-activated conditions is 1–5 μm, which is approximately two times less than that of companion animals and pigs. The most prominent element in their functional morphology is the absence of the open canalicular system, which probably causes a lower collagen adhesion potential when compared to other animals. Based on these features, an opinion appeared, suggesting that bovine platelets evolved from thrombogenic to inflammatory functions due to the expulsion of granule content (Weiss, 1999).
A recent study on serum proteome alterations associated with the retained placenta (RP) in dairy cows (Beletić et al., 2023) provided experimental evidence of the proinflammatory features of bovine platelets. RP is common and has a significantly negative impact on dairy management. RP risk factors are associated with diverse immunometabolic, obstetric, inherited, and environmental conditions. The pathophysiological hallmark is uncontrolled amplification of low-grade inflammation present in cows with physiological puerperium (Bradford et al., 2015; Dervishi and Ametaj, 2017).

According to Beletić et al. (2023), the extent of serum proteomic changes is positively correlated with the intensity of the acute phase reaction in RP. Using a bioinformatics enrichment tool, they allocated the three proteins with altered relative abundance (fibrinogen alpha chain (FGA), inter-α-trypsin inhibitor heavy chain 4 (ITIH4), and tetranectin) to the following pathways: platelet degranulation, response to elevated calcium ion levels in the platelet cytosol, and platelet activation, signaling, and aggregation. Establishing the corresponding pathobiological correlates was an easy task, as the literature supports an association between an increase in platelet activation with a combination of higher fibrinogen (Frojmović et al., 1996), increased ITIH4 levels (Koch et al., 2021), and decreased tetranectin concentration (Chen et al., 2020; McDonald et al., 2020). Unexpectedly, the validation results for FGA and TNCT, obtained using bovine-specific immunometric methods, did not confirm the LC-MS/MS results. These findings were presumably due to the presence of

FGA and TNCT isoforms, differing in pathophysiological implications and immune reactivity (Dardé et al., 2010; Shang et al., 2019), which merits further investigation.

5. Conclusions

Reliable data support the associations between the indices in various proteome compartments and the main platelet functions in animals with various infections. Quite expectedly, these associations were dependent on the nature of the pathogen, infection severity, and the type of specimen analyzed. In creating molecular correlates, caution is necessary owing to analytical and bioinformatic specificities. Sample complexity, the presence of isoforms, or the discrepancy between LC-MS/MS and validation results are just some examples of potential analytical issues. In addition, bioinformatic assessment bears challenges, such as the choice of the background genome, database coverage, or the requirement for BLAST-ing. Nevertheless, current achievements provide a reliable and encouraging rationale for further research targeting methodological upgrades, (patho)biological significance, and translational potential.

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