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New Strategies to Categorize Blood for Proteomic Biomarker Discovery

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ABSTRACT

Although much effort has gone into genomic sequencing to define disease, the downstream products of gene sequences-proteins, nevertheless remain the master regulators of biology via their interactions with nucleic acids and other macromolecules. Many proteins are measurable in blood, making it a rich resource for biomarkers. Yet for reasons largely unrelated to analytical limitations, this resource remains largely untapped. In this review, we describe how chronic illness manifests itself in blood and how we might study innate immunity to understand mechanisms that can potentially translate into new biomarkers and therapeutic modalities. We draw upon our own knowledgebase of proteome information reportable after using depletion or enrichment products in LC-MS/MS workflows and how this knowledge can be utilized in new strategies for biomarker discovery from blood samples. We note that BSG's products have simply and efficiently reduced the complexity of the serum proteome allowing for cost-effective workflows, without the use of antibody-based depletion methods. Finally, we discuss how patterns of Serpins, a superfamily of protease inhibitors, may serve as a surrogate measure of the progressive stages of the innate immune systems' response to both infectious and non-infectious disease. This convergence of strategies and LC-MS/MS technologies has made the task immediately available to investigators to now develop the next generation of molecular tests for more precise and personalized treatment of patients.

INTRODUCTION

Blood is the body's vehicle for the accumulative evidence of pathological insults for diseases. Secreted proteins, extracellular vesicles and circulating blood cells mediate individualized homeostasis via intercellular communication, immune responses, vascular and endothelial cell function, tissue remodelling, fluid exchange and nutrient assimilation [1]. Thus, plasma/serum proteins and other circulating factors directly regulate complex processes such as aging and the development of common chronic diseases. Most diseases are multi-factorial with many proteins collectively acting within highly regulated networks-even single gene diseases can be at the centre of larger, complex regulatory networks.

As such, a disease state results whenever this protein network becomes dysregulated over long periods via a confluence of heredity, lifestyle, or environmental stimuli. Because blood mediates coordination between nonadjacent tissues, it is essential to understand how this dysregulation manifests itself, regardless of the underlying causative factors. Quantitative proteomics from blood can help unravel these regulatory elements. Yet, extracting and characterizing functional changes and adaptations to disease for many of even the highest abundance proteins in circulation remains limited. We therefore propose new strategies to support proteomic analysis of blood.

Proteins in blood can be separated into four main compartments-red cells, white cells, platelets and plasma **(Figure 1)**. Serum differs from plasma primarily in the amount of Fibrinogen, however, for purposes of this review, the concepts proposed herein consider that proteome information derived from serum or plasma would be similar.

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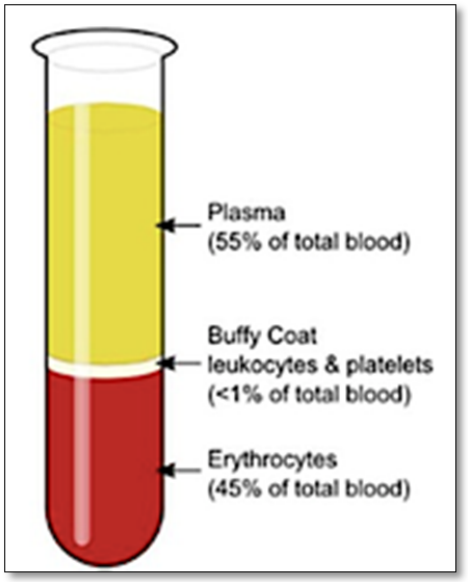

Figure 1. Blood components separated after spin-down in a collection tube.
Blood is the vehicle for the accumulative evidence for all pathological insults–both infectious and non-infectious. Information derived from blood can be compartmentalized into 4 major categories: Plasma, Leukocytes (especially Neutrophils), Platelets, and Erythrocytes.

These compartments do not work independently and are affected by local inflammation. So proteome data from plasma reflects contributions from the cellular compartments.

For that reason, the terms are used interchangeably here.

In this review, we describe how blood cells release inflammatory cargo proteins, disrupting the delicate balance necessary for normal homeostasis. More precisely, information about the function and communication between proteins and blood cells will ultimately be the best possible information that can be derived to understand disease states [1]. Consequently, proteomic biomarker panels from blood will become highly beneficial, as blood is a relatively non-invasive sample type that can be monitored longitudinally throughout a lifetime **(Figure 2)**.

Blood proteomic analysis (e.g., by LC-MS/MS) does however, have inherent challenges. Serum proteomics can be especially challenging for two reasons: 1) the presence of highly abundant proteins, e.g., albumin alone accounts for about 50% of the total protein mass and 2) immunoglobulins, a proteolytically resistant protein family. Several sample preparation strategies are used to address these challenges, most of which employ the use of immuno-affinity depletion to remove one or more high abundance proteins. Common limitations of immuno-affinity however are high costs, regeneration requirements, which may result in a diminished and inconsistent performance and a required marriage of species to antibody. Because of these limitations, Biotech Support Group (BSG) has developed products and methods that are not based on immuno-affinity, but rather are derived from non-biological bead-based chemistries. These have proven advantageous in a variety of LC-MS/MS workflows [2-6].

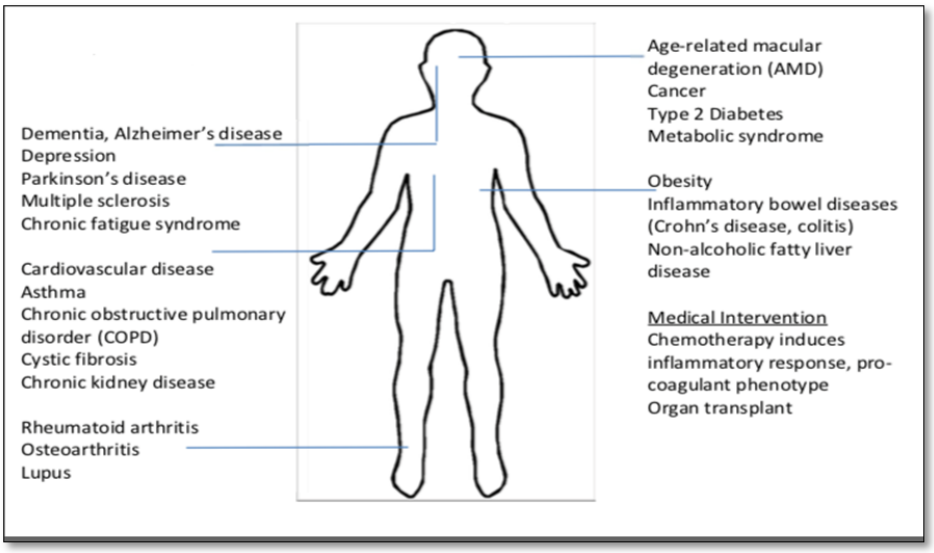

Figure 2. Every disease and illness manifests itself in blood

Blood-borne biomarkers will serve to manage virtually all chronic inflammatory diseases. Unresolved inflammation, driven by mechanisms of innate immunity, is a widely accepted contributor to many progressive debilitating diseases. We propose new strategies to monitor the innate immune response from blood with the goal of developing new biomarker panels.

With some modest adjustments, Viaralet et al. [5] concluded that the BSG product - AlbuVoid™ - proved to be faster and more cost-effective than antibody-based methods to improve quantitative clinical proteomics. Furthermore, BSG's HemoVoid™ proved especially useful for annotating erythrocytes in the human proteome project to identify additional proteins and N-termini; 778 proteins were identified from the cytosolic fraction, 171 of which were not represented in either the soluble non-depleted fraction or the membrane fraction [6]. Based on BSG's internal investigations, we have accumulated data that encompasses greater than 1000 serum proteins that can be observed by LC-MS/MS, and categorically characterized with respect to the proposed strategy described here **(Tables 1, 2 and 3)**.

Table 1. From BSG Serum Knowledgebase Neutrophil & Platelet Releasate Proteins. When differentially quantified from normal/healthy background, these proteins might serve as biomarkers for activation; context dependent states to which can associate to disease.

UniProtKB	Gene	Protein name	Neutrophil Granule Type(s)[13]	Platelet Granule Types[13]	Function
P08246	ELANE	Neutrophil elastase	Specific, Azurophil		Modifies the functions of natural killer cells, monocytes and granulocytes. Extracellular matrix/Elastin proteolysis. Primary substrate protease for SERPIN A1 (Alpha-1-Antitrypsin) inhibition. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers
P07339	CTSD	Cathepsin D	Specific, Ficolin-1 rich, Tertiary		Aspartyl protease. Involved in the pathogenesis of several diseases such as breast cancer and possibly Alzheimer's disease
P09211	GSTP1	Glutathione S-transferase P	Secretory, Ficolin-1 rich		Catalysis of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
P11142	HSPA8	Heat shock cognate 71 kDa protein	Secretory, Ficolin-1 rich		Pleiotropic. Binds bacterial lipopolysaccharide (LPS) and mediates LPS-induced inflammatory response
P04040	CAT	Catalase	Secretory, Ficolin-1 rich		Protect cells from the toxic effects of hydrogen peroxide
P06396	GELS	Gelsolin	Secretory, Ficolin-1 rich		Calcium-regulated, actin-modulating protein
P00746	CFD	Complement factor D	Secretory, Ficolin-1 rich,	Alpha	Cleaves factor B when the latter is complexed with factor C3b, activating the amplifying C3 convertase of the alternate complement pathway.
P06702	S100-A9	Protein S100-A9	Secretory	Alpha	Calcium- and zinc-binding protein, plays a prominent role in inflammatory processes
P02765	AHSG	Alpha-2-HS-glycoprotein	Secretory		Promotes endocytosis
60814	H2BC12	Histone H2B type 1-K	Core component of nucleosome		Has broad antibacterial activity. Changes from normal background may indicate NET release. No intrinsic specificity for neutrophils
P62805	H4C1	Histone H4	Core component of nucleosome		Changes from normal background may indicate NET release. No intrinsic specificity for neutrophils
P16035	TIMP2	Metalloproteinase inhibitor 2	Specific, Ficolin-1 rich, Tertiary		Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor
P02750	LRG1	Leucine-rich alpha- 2-glycoprotein	Specific, Ficolin-1 rich, Tertiary		Involved in promoting new blood vessel growth
P19652	ORM2	Alpha-1-acid glycoprotein 2	Specific, Tertiary	Alpha	Transports hydrophobic ligands
P61769	B2M	Beta-2-microglobulin	Specific, Tertiary		Involved in the presentation of peptide antigens to the immune system
P27918	CFP	Properdin	Specific, Tertiary		An activation factor for complement, binds to and stabilizes the C3- and C5-convertase enzyme complexes. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
P49913	CAMP	Cathelicidin antimicrobial peptide	Specific, Tertiary		Binds to bacterial lipopolysaccharides (LPS), has antibacterial activity.
P02792	FTL	Ferritin light chain	Azurophil		Stores iron in a soluble, non-toxic, readily available form.
Q8NBS9	TXNDC5	Thioredoxin domain-containing protein 5	Azurophil		Possesses thioredoxin activity.
P02766	TTR	Transthyretin	Azurophil		Thyroid hormone-binding protein.
P68104	EEF1A1	Elongation factor 1-alpha 1	Ficolin-1 rich		Involved in T helper 1 (Th1) cytokine production
P04264	KRT1	Keratin, type II cytoskeletal 1	Ficolin-1 rich		In complex with Complement receptor - C1QBP, is a high affinity receptor for kininogen-1/HMWK
P01034	CST3	Cystatin-C	Ficolin-1 rich, Tertiary		Inhibitor of cysteine proteinases
P02775	PPBP	Platelet basic protein	Tertiary	Alpha	Proteolytic modifications generate up to 10 split products, several of which are neutrophil chemoattractants and activators. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.

P02776	PF4	Platelet factor 4		Alpha	Released during platelet aggregation. Neutralizes the anticoagulant effect of heparin and dermatan sulfate. Chemotactic for neutrophils and monocytes. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
P04275	VWF	von Willebrand factor		Alpha	Important in the maintenance of hemostasis
P07996	THBS1	Thrombospondin-1		Alpha	Pleotropic, Adhesive glycoprotein that mediates cell-to-cell and cell- to-matrix interactions. Binds heparin. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
	KNG1	Kininogen-1		Alpha	Kininogens are inhibitors of thiol proteases
P01344	IGF2	Insulin-like growth factor II		Alpha	The insulin-like growth factors possess growth-promoting activity.
P04196	HRG	Histidine-rich glycoprotein		Alpha	Binds many ligands such as heme, heparin, dermatan sulfate, thrombospondin, plasminogen, and divalent metal ions.
P07225	PROS1	Vitamin K-dependent protein S		Alpha	Cofactor to activated protein C in the degradation of coagulation factors Va and VIIIa.
P12259	F5	Coagulation factor V		Alpha	Central regulator of hemostasis.
P01033	TIMP1	Metalloproteinase inhibitor 1		Alpha	Metalloproteinase inhibitor that functions by binding to their catalytic zinc cofactor. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
P10909	CLU	Clusterin		Alpha	Pleotropic, protects cells against cytolysis by complement
P49908	SELENO P	Selenoprotein P		Dense	Might be involved in the transport of selenium
Q06033	ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3		Dense	May act as a carrier of hyaluronan in serum
Q08380	LGALS3 BP	Galectin-3-binding protein		Dense	May stimulate host defense against viruses and tumor cells.
Q16610	ECM1	Extracellular matrix protein 1		Dense	Stimulates the proliferation of endothelial cells and promotes angiogenesis. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4		Dense	Acute-phase protein (APP) involved in inflammatory responses to trauma.
P05452	CLEC3B	Tetranectin		Dense	Tetranectin binds to plasminogen and to isolated kringle 4.

Table 2. The Fluid-Phase Complement System, reportable in blood serum or plasma.

Protein Name	Uniprot Identity	Apprx. Conc. µg/ml	Action
C1R (Complement C1r subcomponent, Classical)	P00736	100	Serine protease that combines with C1q and C1s to form C1, the first component of the classical pathway of the complement system.
C1S (Complement C1s subcomponent, Classical)	P09871	80	Serine protease that combines with C1q and C1r to form C1, the first component of the classical pathway of the complement system. C1r activates C1s so that it can, in turn, activate C2 and C4.
Complement C1q subcomponent subunit A	P02745	60	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
Complement C1q subcomponent subunit B	P02746	55	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
Complement C1q subcomponent subunit C	P02747	50	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
C2 (Complement C2)	P06681	20	Part of the classical pathway, cleaved by activated factor C1 into two fragments: C2b and C2a. C2a combines with C4b to form C3 convertase (classical, lectin)
Factor D (Complement factor D, Bb fragment)	P00746	3	Cleaves Factor B when the latter is complexed with factor C3b, assists to activate C3 convertase of the alternate pathway
Factor B (Complement factor B, Bb fragment)	P00751	320	Cleavage Product Bb combines with C3b to form C3 Convertase (Alternative). Fragment forms complex with Properdin.
Complement C3	P01024	1,500,000	Central role in the activation of the complement system, multi-functional sub-unit C3b triangulates complex with Properdin and Complement Factor B, proteolytic fragments are chemoattractants to Neutrophils. Many different proteolytic sub-forms exist in circulation. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.

Complement Factor H	P08603	500	Accelerates the decay of the complement alternative pathway C3 convertase (C3bBb), cofactor of the serine protease factor I
Complement Factor H related protein 1 (CFHR1)	Q03591	40	The dimerized forms have avidity for tissue-bound complement fragments and efficiently competes with the physiological complement inhibitor CFH.
Complement Factor H related protein 2 (CFHR2)	P36980	60	The dimerized forms have avidity for tissue-bound complement fragments and efficiently competes with the physiological complement inhibitor CFH
Complement Factor H related protein 3 (CFHR3)	Q02985	0.1	Might be involved in complement regulation
Properdin (CFP)	P27918	25	Properdin is stored in neutrophil granules and is considered a positive regulator of the alternate pathway of complement, binding to and stabilizes the C3- and C5-convertase enzyme complexes. Conformational differences between serum Properdin and leukocyte released Properdin has been suggested. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
Complement C4-A	P0C0L4	100	Non-enzymatic component of the C3 and C5 convertases, propagates the classical complement pathway, derived from proteolytic degradation of complement C4.
Complement C4-B	P0C0L5	365	Non-enzymatic component of the C3 and C5 convertases, propagates the classical complement pathway, derived from proteolytic degradation of complement C4.
Complement C4b-binding protein alpha chain	P04003	300	Cofactor for Factor I accelerate decay of classical pathway C3 convertase. Also, a cofactor for Protein S in the coagulation pathway; Protein S enhances the anticoagulant activity of Activated Protein C. In plasma, 60–70% of protein S is bound to C4b-binding protein. Part of the BSG's Stroma Liquid Biopsy™ panel of cancer biomarkers.
Complement C4b-binding protein beta chain	P20851	0.5	The beta chain of C4b-binding protein binds protein S.
Factor I (Complement factor I)	P05156	35	Serine protease controls complement by cleaving three peptide bonds in the alpha-chain of C3b and two bonds in the alpha-chain of C4b thereby inactivating these proteins. Essential cofactors for these reactions include factor H and C4BP in the fluid phase and membrane cofactor protein/CD46 and CR1 on cell surfaces.
C3-convertase (Classical, Lectin: C4bC2a)			Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C3-convertase (Alternative: C3bBb)			Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C3-convertase (Aqueous: C3:H2O)			Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C5-convertase (Classical: Cell Membrane, C4b2b3b)			Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
C5-convertase (Alternative: Cell Membrane, C3bBbC3b)			Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
C5-convertase (Classical: Fluid, C4b2boxy3b)			Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
MASP 1 (Mannan-binding lectin serine protease 1)	P48740		Activates MASP2 or C2 or C3
MASP 2 (Mannan-binding lectin serine protease 2)	O00187		Cleaves/Activates C2 and C4
MASP 3 (Mannan-binding lectin serine protease 3)	P48740		Cleaves/Activates complement pro-factor D
Complement C5	P01031	60	Activation of C5 by a C5 convertase initiates the spontaneous assembly of the late complement components, C5-C9, into the membrane attack complex (MAC). The C5b-C6 complex is the foundation upon which the MAC lytic complex is assembled.
Complement C6	P13671	60	Constituent of the membrane attack complex (MAC)
Complement C7	P10643	90	Constituent of the membrane attack complex (MAC)
Complement component C8 alpha chain	P07357	50	Constituent of the membrane attack complex (MAC)
Complement component C8 beta chain	P07358	50	Constituent of the membrane attack complex (MAC)
Complement component C8 gamma chain	P07360	20	Constituent of the membrane attack complex (MAC)
Complement C9	P02748	50	Constituent of the membrane attack complex (MAC)

Clusterin	P10909	60	A multi-functional protein, Clusterin along with Factor H, C4BP, CFHR1, and Vitronectin limit formation of complement activation products at cell surfaces, binding to lipid bilayers or to bio membranes.
Vitronectin	P04004	115	Inhibits terminal MAC. Vitronectin is a multifunctional glycoprotein present in blood and in the extracellular matrix occupying a role in the earliest events of thrombogenesis and tissue repair. Conformationally variable, it also is a co- factor to plasminogen activation inhibitor-1 (SERPINE1), binds to thrombin–antithrombin III (SERPINC1) and Heparin Cofactor II (SERPIND1) complexes, implicating a pleotropic role in immune response and coagulation, and especially SERPIN function.
C-reactive Protein (CRP)	P02741	Varies, acute-phase reactant	Activates complement via C1q, massively induced as part of the innate immune response to infection and tissue injury. Normal concentrations of CRP vary between 0.8 mg/L to 3.0 mg/L. With sufficient stimulus, CRP levels can rise can rise 10,000-fold from less than 50 µg/l to more than 500 mg/L, making it a clinically useful biomarker for acute inflammation.

Table 3. Inhibitory SERPINS reportable in blood serum or plasma.

Protein ID	Uniprot Identifier	Also Known As [apprx. conc.]	Function	Reactive (RCL) bond site	Notable attributes
SERPINA1	P01009	Alpha-1-Antitrypsin (AAT) [1-2 mg/ml]	Inflammation, Neutrophil elastase inhibition, inhibits activated Protein C in coagulation pathway	Met382-Ser383	Z Variant {Glu366→Lys366} deficiency syndrome, Pittsburgh variant {Met382→Arg382} life-threatening bleeding
SERPING1	P05155	Plasma Protease C1 inhibitor [250 µg/ml]	Pleotropic regulator of complement and coagulation, levels rise ~2-fold during acute inflammation, only known inhibitor of C1r (and, less potently, of C1s). It is an important regulator of the contact pathway of coagulation, acting on factors Xia and XIIa, and on fibrinolysis by inhibiting plasmin	Ala465-Arg466 chymotrypsin, Arg466-Thr467	
SERPINA3	P01011	Alpha-1-Antichymotrypsin [100-500 µg/ml]	Physiological function is unclear. It can inhibit Neutrophil Cathepsin G and mast cell chymase, both of which can convert angiotensin-1 to the active angiotensin-2. Its proposed to help maintain structural integrity of the lower respiratory tract. Forms complexes with Prostate Specific Antigen (PSA).	Leu383-Ser384	
SERPIND1	P05546	Heparin cofactor II [40-80 µg/ml]	Coagulation regulator especially in extravascular space, co-factor activated regulation of Thrombin, and factors IXa, Xa, and Xia	Leu463-Ser464	May inhibit Neutrophil Cathepsin G at alternate RCL cleavage site
SERPINC1	P01008	Antithrombin, ATIII [120 µg/ml]	Inhibits thrombin, factors IXa, Xa, and Xia, regulates coagulation & angiogenesis. Activity enhanced by heparin cofactor, regulates Thrombin in the intravascular space	Arg425-Ser426	mutations/variants can lead thrombosis
SERPINA4	P29622	Kallistatin [20 µg/ml]	Inhibits human amidolytic and kininogenase activities of tissue kallikrein. Heparin blocks its complex formation with tissue kallikrein and abolishes its inhibitory effect. Effects kidney function.	Phe388-Ser389	cleavage at the reactive site by tissue kallikreins
SERPINF2	P08697	α-2-antiplasmin [60-80 µg/ml]]	Fibrinolysis, inhibitor of plasmin and trypsin	Arg403-Met404 plasmin, Met404-Ser405 chymotrypsin	Alanine insertion at the reactive site promotes serious bleeding disorders
SERPINA10	Q9UK55	Z-dependent proteinase inhibitor [1-2 µg/ml]	Coagulation regulation. Inhibits activity of the coagulation protease factor Xa in the presence of Vitamin K-dependent protein Z, calcium and phospholipids. Also inhibits factor Xia in the absence of cofactors.	Tyr408-Ser409	Tyr408→Ala408 loss of inhibition
SERPINA5	P05154	Protein C inhibitor, Plasma serine protease inhibitor [5 µg/ml]	Heparin-dependent, acts as a pro-coagulant and pro-inflammatory factor by inhibiting activated protein C/thrombin/thrombomodulin complex.	Arg373-Ser374	Variants near or at the reactive bond alter inhibition of thrombin activity

While acknowledging some exceptions, notably C-reactive protein (CRP) or antibodies, consequential changes in the blood proteome are not necessarily derived from tissue leakage, but rather from proteolytic modifications, presumably driven in large part by inflammatory release of blood's cellular protein cargo. Within the coagulation/complement axis, many proteins circulate as inactive precursors (*zymogens*) and only become active upon

proteolysis. Once activated, protein sub-forms then become ligands for cell receptors, substrates for other proteases in cascading sequences, or interacting partners for regulating mechanisms involving all blood cells, vessel walls and vasculature, along with other plasma co-factors (i.e., lipids, Heparins, metal cations, etc.). For these reasons, we examine new ways to observe and measure such categorical and functional responses to inflammatory disease and related disorders.

Unlike proteins from tissue, the vast majority of proteins contained in blood, either cellular or humoral (extracellular), are quite constant; blood's cellular content being derived either from cells without nuclei (red cells and platelets) or from those with lobed nuclei with limited new protein production capacity (neutrophils). Thus, differential changes are derived from a host's systemic response to many varieties of environmental stimuli (both infectious and non-infectious), not from altered gene expression. As such, we make the case in this paper for proteomic analysis of blood to become a discipline of ***categorical classification and functional metrics***, rather than a discipline of finding the exceedingly low concentration (<<ng/ml), needle in the haystack type protein(s).

Furthermore, categorical metrics have the advantage of monitoring proteins in a highly observable LC-MS/MS concentration range, ≥ 0.1 μg/ml in most cases.

This new strategy, to represent proteome information within categories, is designed to derive characteristic patterns, rather than single biomarkers, that can support clinical manifestations of disease or response to medical intervention. By **categorical classification**, we hope to gain a much deeper understanding of the molecular relationships that are shared amongst apparently distinct pathological phenotypes. This would help explain, for example, the increased risk of cancer in patients with inflammatory bowel disease, or the apparent link between Rheumatoid arthritis or lupus with increased risk of blood clots. Finally, once we understand these shared relationships, we hope to address therapies that may have been developed for one clinical condition and apply them towards other clinical phenotypes, with biomarkers that can help guide selection and utility.

While important, we have purposely left out the influence of the red cell proteome. This is because there is limited information on the role of erythrocytes, with notable exceptions (i.e., Paroxysmal nocturnal haemoglobinuria), in the central theme of this review, namely, the orchestration of the innate immune response.

INNATE IMMUNITY

Innate immunity refers to first-line, non-specific defense mechanisms that come into play immediately or within hours of a perceived pathogenic insult in the body. The innate immune response consists of physical, chemical and cellular defences against pathogens. Its main purpose is to immediately prevent the spread and movement of foreign or non-self-pathogens throughout the body and to initiate the second line of defence, the adaptive or acquired immune response. As a second-line defense, the adaptive response occurs downstream from the innate immune response and starts transferring immunological longer term memory towards the non-self-pathogens. So a normal resolution of the innate response leads to a productive handoff to the adaptive response. Conversely, an unresolved innate response may delay or confound a suitable adaptive response, with both acute and chronic disease consequences.

Taken together, the human immune system has evolved to adapt and respond to a variety of physical insults and never-ending exposure to infectious agents to survive. While the emergence of COVID-19 reminds us that infectious insults remain a large healthcare threat, mankind's pharmacological skills (e.g., antibiotics, vaccines, etc.) has largely eliminated many infectious insults of the past. As a result, we are able to live longer than our predecessors. Unfortunately, our pharmacopeia and inflammatory response systems are not sufficiently capable of fending off today's longer-lifetime exposure to environmental and lifestyle insults that we now face. These exposures and insults contribute to chronic inflammation which, over time, are manifested in a variety of pathological conditions, including an important link between the coagulation and innate inflammatory system [7, 8].

Thrombo-inflammation describes a process by which the activation of coagulation assists the function of the innate immune system and, conversely, components of the immune system contribute to thrombosis. Thus, the innate immune system is an integrated triangulated network that when functioning properly provides steady state control of pathways involved with coagulation, complement and leukocyte recruitment (**Table 4 and Figure 3**). Any dysregulation within one affects the others and so collectively, a dysregulated innate immune system can contribute to the genesis of many acute and chronic pathologies. For example, chronic inflammation is widely recognized as a potential contributor to cancer and many progressive debilitating diseases (e.g., inflammation due to H.pylori leads to stomach lesions and cancer). As witnessed with COVID-19, the pre-existing inflammatory status, may be a key factor to the acute severity of disease upon exposure to infectious pathogens.

Because the innate immune system does not change genetically over time, measuring panels of its protein level orchestration is a worthwhile goal of proteomic investigation. Because of the many feedback signals necessary to maintain steady state within the network, therapeutically modulating even one rogue component may ultimately unwind the overall dysregulation and contribute to longer term management of the disease. For these reasons,

we have focused this review on key regulatory elements of innate immunity that can be observed, reported and surveyed by proteomic analysis of serum/plasma. Within this model system, we describe protein contributions from each pathway and new strategies for proteomic categorization, function and disease characterization.

White blood cells (WBCs, also called leukocytes) are the cells of the immune system that are rapidly recruited to protecting the body against both infectious disease and perceived environmental insults. When anti-coagulated whole blood is centrifuged in collection tubes, the white blood cells (WBCs) form a thin, white layer of cells (the *buffy coat*;) between the sedimented red blood cells (erythrocytes) and plasma (see tube in **Figure 1**). WBCs make up approximately 1% of the total blood volume in a healthy adult, substantially less numerous than the red blood cells (about 45%). Having nuclei distinguishes WBCs from the enucleated red blood cells (RBCs) and platelets, yet, even with nuclei, most are terminally differentiated and do not undergo cell division in the bloodstream. White blood cells are composed of differentiated constituents, each having specific functions. Neutrophils are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes and they thus contribute to the observable blood proteome much more so than the rest of the white blood cell constituents combined. Neutrophils have extensive crosstalk with each of the major blood cell subsets (megakaryocyte/platelets, myeloid and lymphoid) and other extracellular soluble proteins contained in plasma, further magnifying their importance in health and disease.

Table 4. Classification of immune system.

The immune system contains two branches	
Innate	**Adaptive**
• Non-specific alarm to pathogens, damaged or stressed cells. • First responders, clinically called acute inflammatory response, dissipates in 1 to 14 days, the common cold serves as model example • No immunological memory	• Lag time, days between exposure and maximal response • Immunological memory, response dissipates in years not days, and can vary dependent upon the initial insult
Cell mediators Leukocytes (white blood cells) Neutrophils 60-70% Innate lymphoid cells	**Cell mediators** B-cell & T-cell lymphocytes
Protein mediators Complement cascade; the conduit for communication between the innate and adaptive branches	**Protein mediators** Antigen/Antibodies

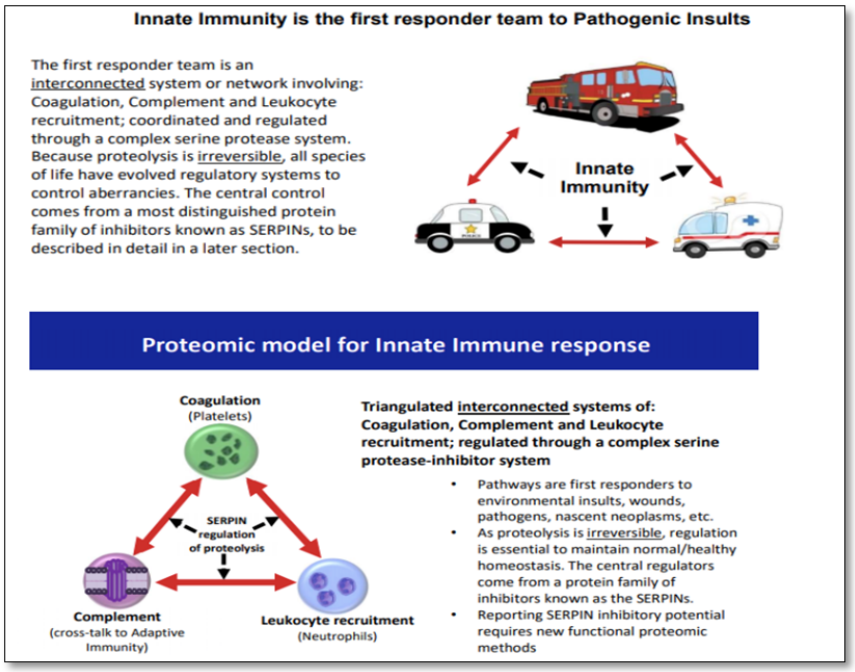

Figure 3. Protease model for Innate Immune response.

Leukocytes: Constituents, Functions, Recruitment Neutrophils

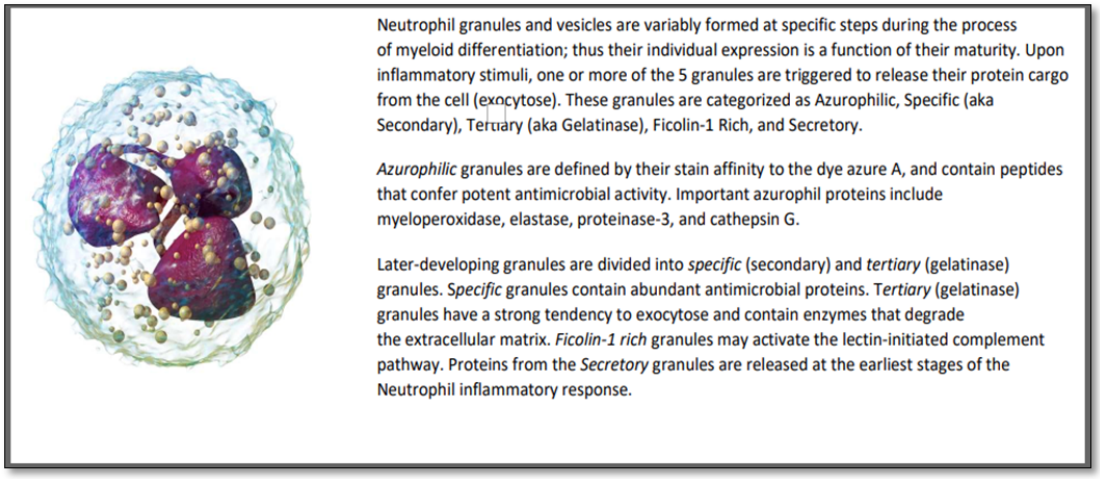

Figure 4. Neutrophils: The large maroon vesicles are lobed nuclei characteristic of neutrophils [9].

For example, changes in circulating leukocytes including the ratios of neutrophils-to-lymphocytes (NLR), monocytes-to-lymphocytes (MLR) and platelets-to-lymphocytes (PLR), have been used to predict tumor occurrence and prognosis [10]. During the beginning (acute) phase of inflammation, neutrophils migrate to the site of inflammation, particularly as a result of infection, environmental stimuli, or onset of cancer. Inflammation follows chemotactic signals such as Interleukin-8 (IL-8), C5a from Complement activation, as well as other peptides and small molecules. Neutrophils, as "first responders" release a variety of proteases (i.e., Elastase) to remodel the extracellular matrix of the tissues to which they migrate.

Once arrived, neutrophils then release granule proteins and/or chromatin (DNA & histones) to form an extracellular fibril matrix known as Neutrophil extracellular traps (NETs). Such NET release has been observed to occur not only during acute (bacterial or viral) inflammation but also in numerous pathological conditions, such as autoimmune diseases, vascular diseases, and cancer [11]. In addition to NETs, neutrophil granules can be secreted extracellularly (released cargo) in response to localized immunological stimuli. As neutrophils have limited capacity for de novo protein synthesis, this released cargo thus becomes a subset proteome that collectively reflects a weighted average of cell maturities and the inflammatory stimulus that triggered the release. These released proteins can be observed and reported in the general blood circulation (many in our BSG serum knowledgebase, see **Table 1**).

Platelets

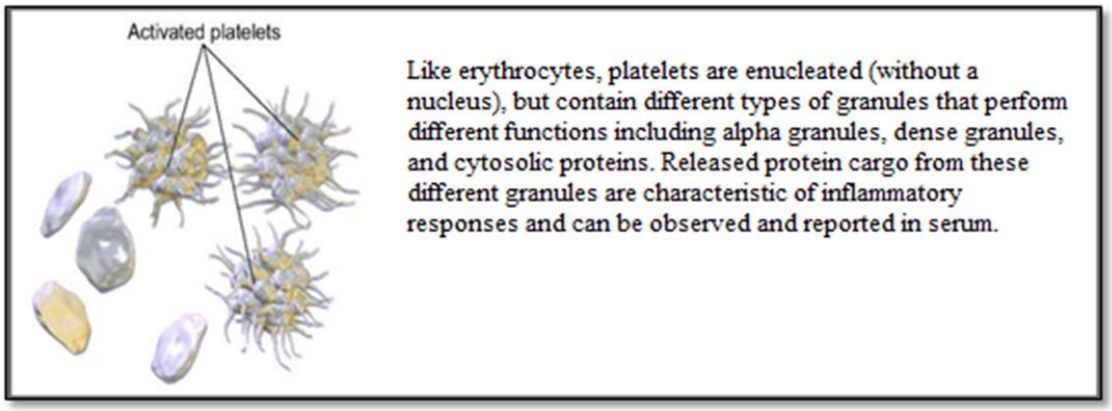

Figure 5. Platelets-description.

Platelets are rapidly deployed to sites of injury or infection, and can modulate inflammatory processes by interacting with neutrophils, forming platelet-neutrophil aggregates. Platelets play a central role in innate immunity, initiating and participating in multiple inflammatory processes, operating in parallel with their better known clotting function. Platelets contain dense granules, alpha granules and cytosolic proteins; granule secretion being pivotal to establishing and controlling the microenvironment at the local inflammatory site.

Granule cargo release is both contextual and kinetically controlled, mediating early activation, or persisting long after the initial stimuli. Persistence of Platelet-released along with Neutrophil-released cargo in unresolved chronic inflammation, necessitates a systemic response to regulate these effects, proteolysis being the most deleterious [12].

Granule characteristics

1. α granules (alpha granules) – contents include insulin-like growth factor 1, transforming growth factor beta (TGF-β), platelet factor 4, and other clotting proteins (such as thrombospondin, fibronectin, factor V, and von Willebrand factor) [13].

2. δ granules (delta or dense granules) – contents include adenosine di- & tri-phosphates, proteins, and the majority of platelet calcium, an essential co-factor in the coagulation cascade [13].

Like neutrophil granule cargo, this released cargo thus becomes a subset proteome that collectively reflects the inflammatory stimulus that triggered the release. Released protein cargo from these different granules thus forms a signature characteristic of inflammatory responses that can be observed in serum and reported (**Table 1**). Differences in quantitative elements from one granule vs. another may be informative as to the relative weight that one granule type cargo has to a particular disease phenotype. The released cargos from both neutrophils and platelets simultaneously imparts their net effects on the third component of the triangulated network – the complement cascade.

COMPLEMENT CASCADE

The complement cascade is a major component of the immune system that provides powerful host surveillance and protection from invading microbes. Comprising about 5% of the total protein mass in plasma (**Figure 1 and Table 2**), most complement proteins circulate in blood as inactive precursors (zymogens); when triggered, these zymogens become activated through proteolytic cascades. These cascades enhance the ability of antibodies and phagocytic cells to clear microbes and damaged cells as well as promote local inflammation.

The complement system links the innate immune system to the adaptive immune system. This is a critical juncture; a delicate balance must be maintained to allow activation when necessary to counteract foreign or modified self/host surfaces, while concurrently protecting healthy self/host tissue [14]. Protection is achieved systemically through the concerted action of activators and inhibitors - about 50 membrane-bound and soluble proteins - that ensure cell and tissue integrity essential for normal health and well-being. When dysregulated, pathological conditions can arise, including neurodegenerative diseases, cancer, age-related macular degeneration (AMD), membranoproliferative glomerulonephritis, systemic lupus erythematosus, transplant rejection, paroxysmal nocturnal haemoglobinuria (PNH), ischemia-related conditions and autoimmune disease, to name a few [14,15].

The three best characterized branches of complement activation are: the classical, lectin, and alternative pathways, as shown in **Figure 6**.

1. The **classical pathway** is initiated by the C1 complex binding to multimeric antibody complexes, leading to cleavage of C4 and C2 components and formation of the classical C3 convertase, C4bC2a.

2. The **lectin pathway** is activated by binding of mannan-binding lectin (MBL), or ficolins (secreted, lectin- type pattern recognition receptors) to carbohydrate or acetylated groups on target surfaces. MBL and ficolins interact with MBL-associated serine proteases (MASP) leading to cleavage of C4 and C2 and formation of the classical C3 convertase, C4bC2a [16]. Noteworthy is that one of the neutrophil granules previously described, is *Ficolin-rich*; another example of the many network associations between cellular and extracellular events occurring within the innate immune response.

3. **Alternative pathway** activation involves interaction of $C3(H_2O)$ and/or previously generated C3b with factor B, which when cleaved by factor D, generates the alternative C3 convertases $C3(H_2O)Bb$ and/or C3bBb. Complement related proteins are always present in the blood and a small percentage spontaneously activate (**Table 1**). At about 1.5 mg/ml concentration in serum, C3 is by far the most abundant. Notably, low-level hydrolysis of the C3b thioester bond - also known as the C3 'tick-over' notated as C3 (H_2O) - keeps the alternative pathway in constant alert to pathogen challenge.

All three pathways converge upon generation of the C3 convertase complexes, facilitating the proteolytic cleavage of C3 to generate split forms opsonin C3b and anaphylatoxin C3a. C3b covalently binds to proteins distributed across the target cell surface. This is followed by an amplification reaction that generates additional C3 convertases and deposits more C3b at the local site. C3b can also bind to C3 convertases switching them to C5 convertases, which then cleaves C5. Thus, by whichever initial means of activation, the complement cascade system leads to one or more important final outcomes:

1. Opsonization of pathogens or damaged-self cells to enhance phagocytosis,
2. Production of anaphylatoxins C3a & C5a involved in the acute inflammatory response,
3. Recruitment of leukocytes to the inflammatory site,
4. B- and T-cell stimulation, and
5. The terminating end of the cascade – assembly of the membrane attack complex (MAC) on the cell surface.

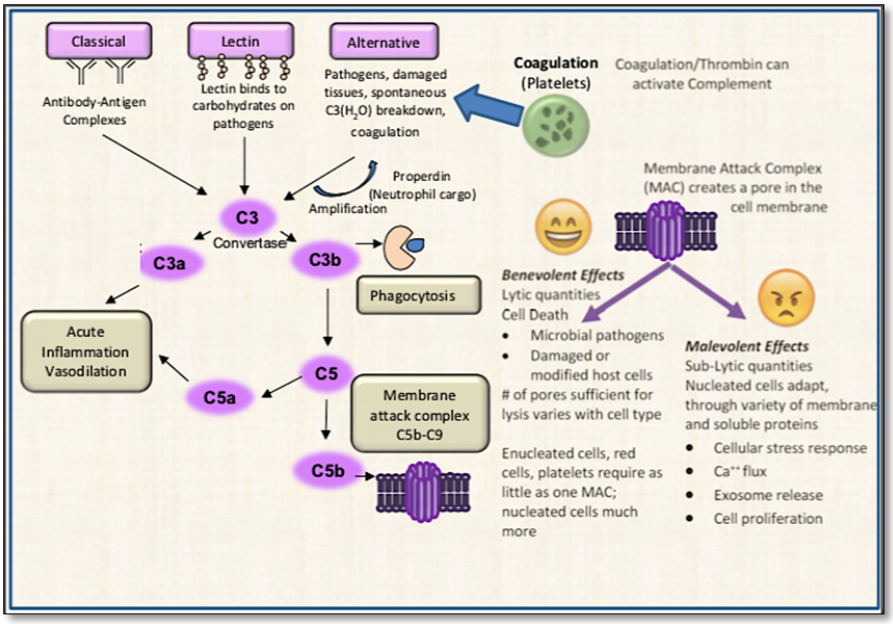

Figure 6. Branches of complement activation.

The terminating end of the complement cascade is derived from the C3 Convertase proteolytic product - C5b, which engages the sequential recruitment of C6, C7, C8, and C9, assembling the MAC. Also known as the "terminal complement complex", it results from the coordination of C5b-7 insertion in the membrane, which then captures C8, inducing polymerization of a C9 ring – to as many as 18, C9's per pore. Terminal MACs punch a hole (pore) in the membrane of the invading pathogen or target cell, and when a sufficient number of MAC pores form, the cell dies by osmotic lysis.

However, when insufficient quantities of MAC or when sufficient inhibitors of MAC are present, sub-lytic conditions persist that are non-lethal, and unable to kill the cell. Instead, these conditions promote other cellular stress adaptations thereby setting up the duality of outcomes imposed by Complement activation. At sub-lytic doses, the complement MAC has wide ranging effects that differ based on the cell types, leading to a variety of cellular responses, such as extracellular vesicle secretion, aggregation and chemotaxis. Sub-lytic Complement can also induce increased cell resistance to lytic doses of Complement [17]. These can all conspire to have pathogenic consequences. Complement regulators expressed at high levels on malignant cell membranes form an escape mechanism from MAC, setting up sub-lytic concentrations that can activate intracellular signals leading to malignant cell proliferation [18, 19].

While traditionally described as three activation pathways, an additional, largely under-appreciated pathway is Complement's evolutionarily conserved link to coagulation to eliminate damaged tissues [20]. C1 Inhibitor (Serpin G1; see next section for further details) serves as one model protein mediator of this linkage, as it acts to inhibit both complement activation and coagulation initiation. As another example, C4b-binding protein is a cofactor for both Factor I (a Complement regulator) and Protein S (a Coagulation regulator). Also, Complement may activate platelets or facilitate biochemical and morphological changes in the vascular endothelium, thus potentiating coagulation and contributing to haemostasis in response to injury [21, 22]. Conversely, Complement can be activated from proteolytic enzymes derived from coagulation and fibrinolysis that can cleave both C3 and C5 [23, 24]. Consequently, the Complement system adapts to, and is influenced by many different proteolytic triggering events.

Such events can also be influenced by competing factors from coagulation and neutrophil recruitment. For example, in mouse models of fatal injury deprived of neutrophils, amplification of complement can occur, which suggests infiltrating inflammatory cells participate in localized Complement activation [25]. Likewise, platelets adhered to injured vessel walls form strong adhesive substrates for leukocytes, a major event in thrombo-inflammation. This series of responses is mediated by Complement and specifically to alternative Complement activator Properdin, derived in part from neutrophil granule cargo [8].

It has also been proposed that the release of Properdin from neutrophils is both a major determinant of local Complement activity and behaves differently from endogenous serum Properdin [26]. In like manner, Properdin released from neutrophils, is structurally related to Thrombospondin-1 (THBS1), a protein released from platelet cargo, both containing conformationally diverse Thrombospondin Type

1 Repeats (TSRs) [27,28]. It is interesting to speculate how TSR binding site competition might influence Complement regulation (one example being THBS1 interaction with Alternative Complement regulator-Factor H [29]). Significantly, several reports suggest Complement-mediated interactions between Neutrophils and Platelets [30, 31]. Regardless of the underlying mechanism, the end result may well be a tug-of-war between platelets and neutrophils for the localized control and regulation of Complement [32] (**Figure 7**).

These triangulated pathways cooperatively form an intensified cycle resulting in local inflammation, thrombosis, and tissue damage. Chen et al. [33] illustrates how this all may play out in disease. In that study, proteomic analysis classified tumor tissues of over 500 cancers into just 10 subtypes, regardless of the primary tumor of origin. Out of the 10, four subtypes were based solely from the host's stromal character at the local microenvironment, unrelated to cellular mutations. Of the four stromal subtypes, two reflected immune components: the first by presence of immune T cells, while the second immune subtype, was highlighted by complement activation [32].

Taken together, these studies illustrate how the complement system orchestrates host defense by sensing a danger signal, translating the signal into specific cellular responses, and concurrently, communicating with other biological pathways ranging from adaptive immunity to hemostasis [34]. Such importance notwithstanding, there are limited tests available to assess these conditions in clinical practice. C3 and C4 are most frequently measured; total complement activity (CH50 lytic assay) can be measured if a deficiency is suspected. These tests, however, are non-specific and none can monitor sub-lytic activity, which can often be the most malevolent outcome of complement activation. Clearly, a better characterization of the total picture of Complement activity is needed. Proteomics can serve that need as it can identify and develop biomarkers to capture and report virtually all potentially competing influences, context dependencies and duality of outcomes imposed by Complement. In this regard, we propose here the functional profiling of the protease inhibitor family of Serpins as an important way to address this unmet need.

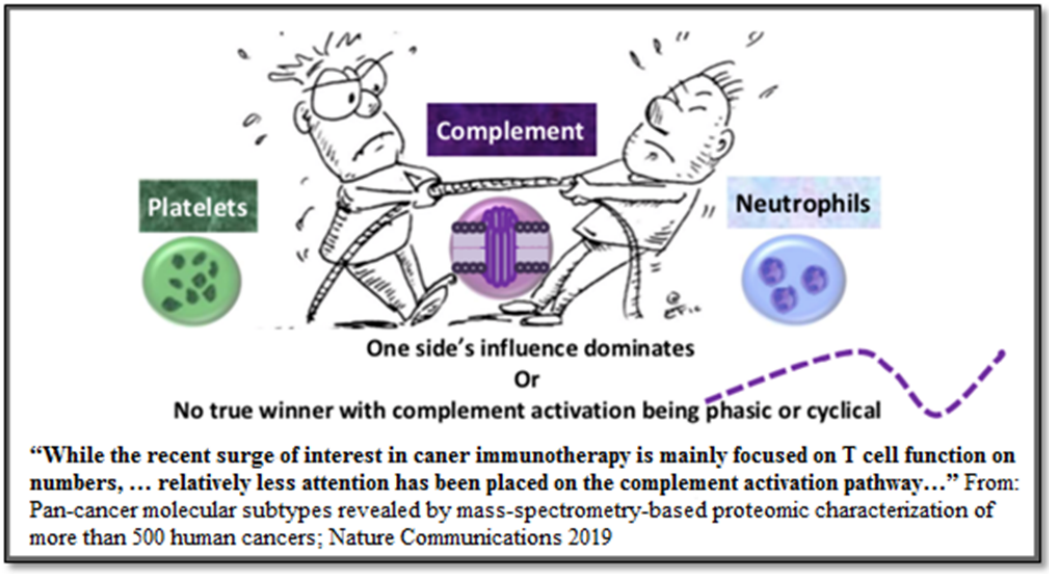

Figure 7. A tug-of-war between platelets and neutrophils for the localized control and regulation of Complement [32].

SERPIN Function and Regulation

Motivation

As proteolysis is irreversible, there is an essential balance and regulation of proteolytic cascades necessary to keep aberrancies controlled. Central to this maintenance are the Serpins. This unique family of protease inhibitors serves the central control function of innate immunity, in some way regulating all of its proteolytic mechanisms.

Our goal is to show how patterns of Serpins, a superfamily of protease inhibitors, may serve as a surrogate measure of the progressive stages of the innate immune systems' response to a disease.

BACKGROUND

Serpins are a superfamily of proteins with similar structures found in all kingdoms of life. They were first identified for their protease inhibition activity (proteolysis regulation). Although some proteins with Serpin sequence annotation are not protease inhibitors, but instead perform diverse functions such as hormone transport, we focus in this section on only serum Serpins with inhibitory activity.

Serpins are an extensively studied family of both intercellular and extracellular proteins that exhibit conformational adaptabilities and a most unique functional mechanism. Often called suicidal inhibitors, inhibitory Serpins are in sharp contrast to the more conventional competitive mechanism for protease inhibitors that bind to and block access to the protease active site in a concentration dependent (Michaelis-Menten type) equilibrium. Such is not the case with Serpins, as the initial interaction starts through a decoy process (an intermediate Michaelis complex). Here, the protease sensing the Serpin as a suitable substrate, initially binds to a peptide region of the Serpin – known as the reactive center loop (RCL) -that transiently protrudes from the core of the Serpin body. From this intermediate complex, one of two possible final outcomes are produced, called, respectively, the Substrate and Inhibitor Pathways, as depicted in **Figure 8**, and summarized as: follows:

In the inhibitor pathway, the protease selectively cleaves a peptide bond in the reactive center loop (RCL) of the serpin. The resulting acyl-enzyme undergoes an extensive and rapid conformational change, with a 180 degree translocation of the attached protease. During this process the active site is deformed, and hydrolysis cannot be completed. This process results in an irreversible covalent complex. Trapped in this suicidal covalent embrace, both the protease and the Serpin are irreversibly modified and cannot be regenerated back to their active forms.

In the Substrate Pathway however, the protease releases from the complex and remains active (the 'On' sub-form), but not so for the inhibitor as the RCL region is cleaved and the inhibitor becomes permanently inactive (the 'Off' sub-form) [35, 36].

Nine inhibitory Serpins account for ≥ 5% of the protein mass contained in serum (**Table 4**, see also top fraction in **Figure 1**). With such a large regulating influence in blood, Serpins are themselves subject to many co-factors (e.g., Heparin) that act upon their inhibition efficiency. Any aberrations in this finely tuned mechanism can lead to progressive loss of functionally active forms or the accumulation of inactive forms, **Figures 8 & 9**. Insufficient Serpin control of irreversible and inflammatory proteolytic activity can lead to systemic dysregulation of innate immunity and progression of disease.

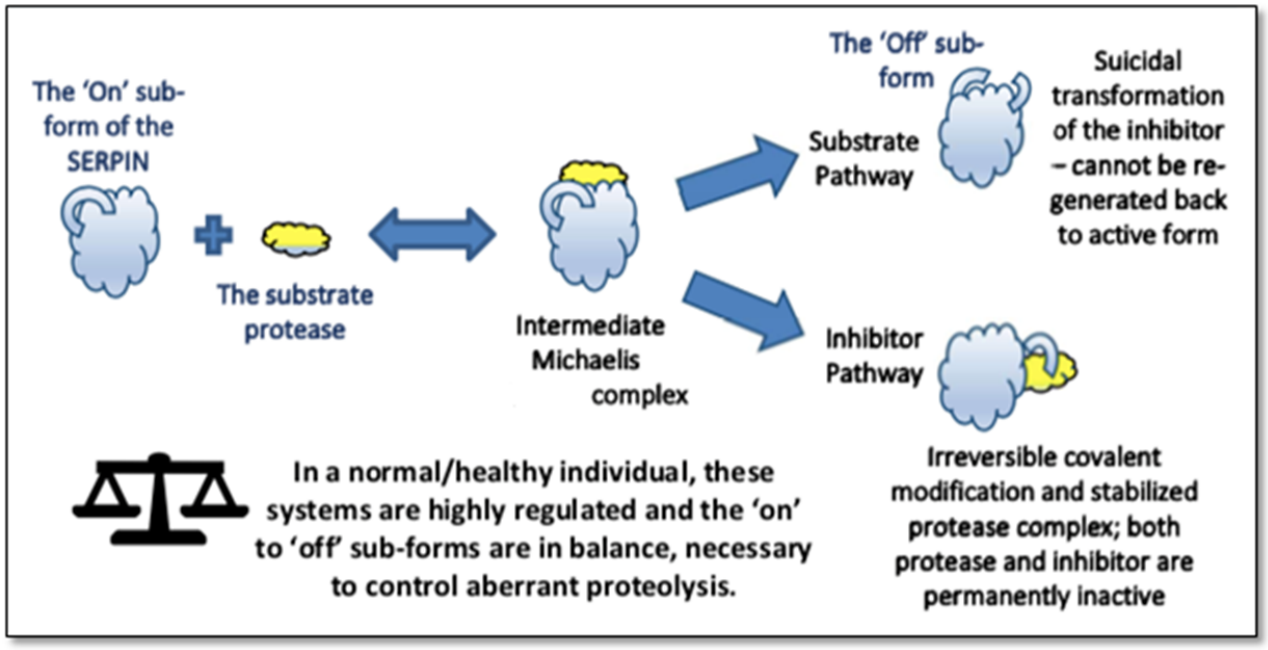

Figure 8. SERPIN initial interaction with protease can produce two functionally opposing outcomes.

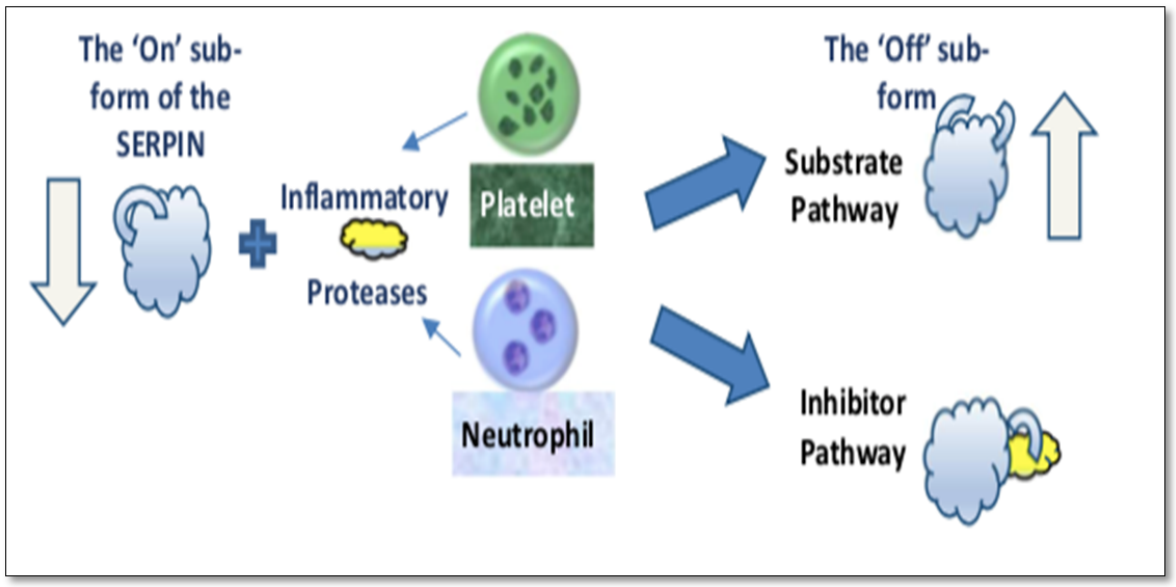

Figure 9. Chronic inflammation-platelets and neutrophils continuously release proteases gradually consuming the 'On' and generating more 'Off' sub-forms.

Further confounding the significance of this bifurcated inhibition mechanism is that measurements of Serpins have conventionally relied on immunological (ELISA)-type assays that count all sub-forms in aggregate and thereby assume homogeneous populations. This can lead to erroneous conclusions about their functional role in disease. Fortuitously, the different active vs. inactive Serpin sub-forms are reportable from biofluids using LC-MS/MS analysis [37]. In our own analysis for cancer, we have identified two critical nodes in this network relating to the progressive loss of functionally-active Serpins: (A1) Alpha-1-Antitrypsin and (D1) Heparin Cofactor II [38]. These proteins are included as part of Biotech Support Group's Stroma Liquid Biopsy™ [39] panel of cancer biomarkers.

Other reports corroborate these results that tumorigenesis can systemically be characterized by chronic exhaustion of inhibitory active Serpins and resulting increased protease activity (**Figure 10**).

Context Dependent Proteomic Categorization of Disease

In the course of this review, we have provided a brief description of the three key innate immunity pathways, how they interact with each other, and how they are all regulated through protease activation balanced by protease inhibition, most notably through the Serpin family. As noted in our review, these immune responses are extremely context-dependent, e.g., the vasculature at inflammatory sites differs significantly from the surrounding tissue. Many factors contribute to create the context in such environments: platelets attached to vessel walls, vasodilation, shear sensing receptors and the relative amounts of diffusive to axial transport of solutes. Thus, the activation of cells and the release of their cargo proteins at the local site offers the means for a categorical deconstruction of the inflammatory elements of disease. Potential markers may now be reported from blood through the protein cargos released from neutrophils and platelets; these cargo proteins are readily observable through quantitative LC-MS/MS analysis. We highlight these proteins in the BSG knowledgebase in **Table 2.**

Finally, this context-specific, released cargo enters the general circulation and simultaneously influences another tier of response, namely, the highly observable and measurable Complement cascade and Serpin regulation **(Tables 3 and 4)**. All these various, systemic responses can be monitored and categorized by quantitative proteomic analysis: Platelet-released cargo, Neutrophil-released cargo, complement components and Functional 'On' & 'Off' sub-forms of Serpins **(Figure 11)**.

Through this new strategy, we posit that biomarker panels can be derived from these categorical proteins which increase or decrease in characteristic patterns from pathological conditions. In turn, these panels can then be used to assess patients for actionable decisions.

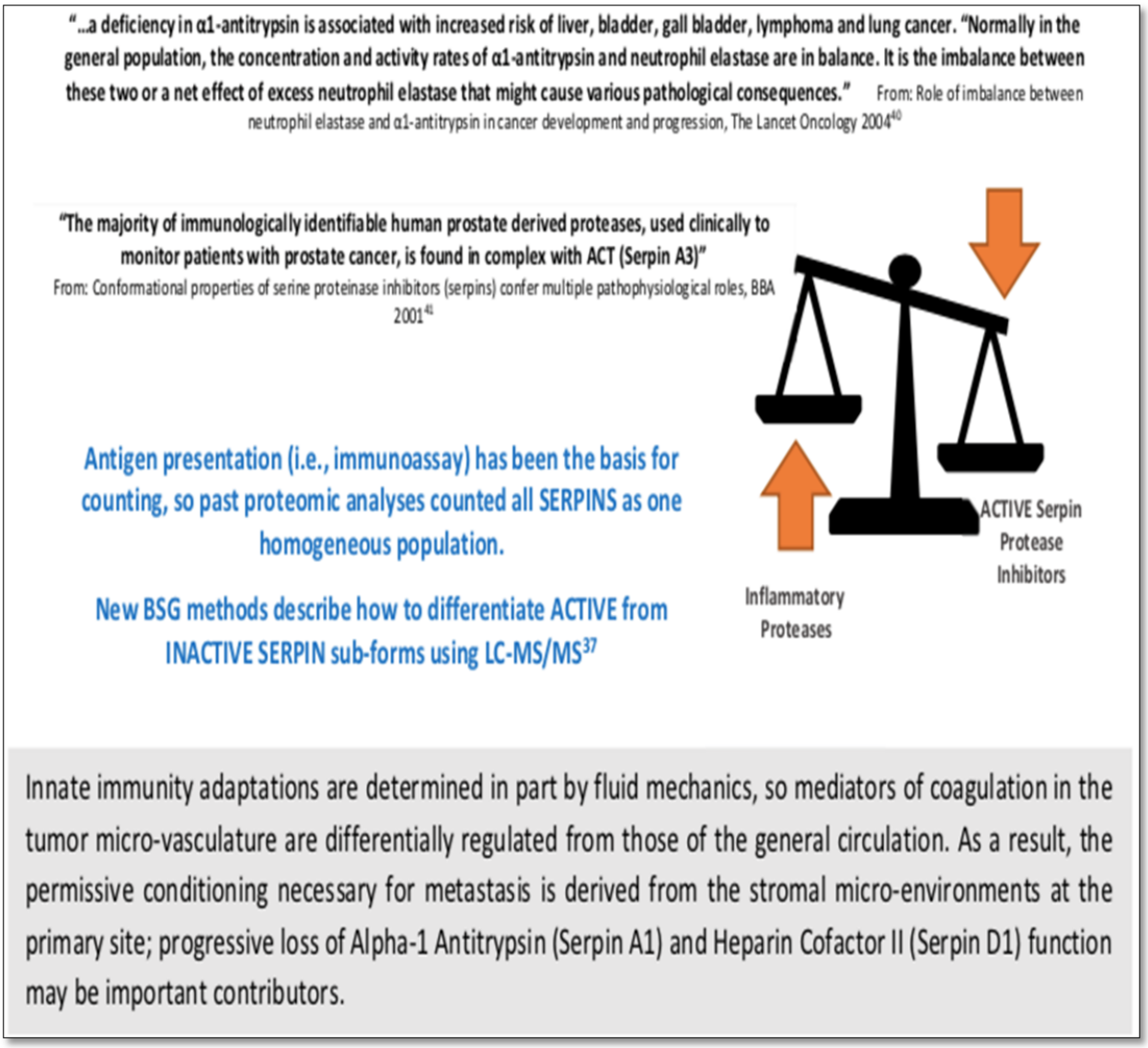

Figure 10. Serpin imbalance with protease activity is well documented in cancer and used clinically, bound vs. free PSA.

This ground truth protein data creates a survey of the function and communication between plasma and cells in blood. With advancements in proteomics and bioinformatics for chronic diseases, we can aspire to bring blood-based protein biomarkers to the clinic. Unlike single markers such as HbA1c for diabetes management, future advances in protein biomarkers will not come from one protein assay or even a small set of protein assays, but from patterns of protein changes. The good news is that these potential protein patterns are highly observable using LC-MS/MS and this capability has become even more precise and comprehensive with improvements in instrument speed and resolution. Advancements in the quantification of multiple proteins at once, in both targeted label and label-free analysis; coincide with the use of internal standards and simple efficient sample prep workflows. This convergence of technologies has made the task immediately available to those who, like us, seek to develop the next generation of molecular tests for more precise and personalized treatment of patients.

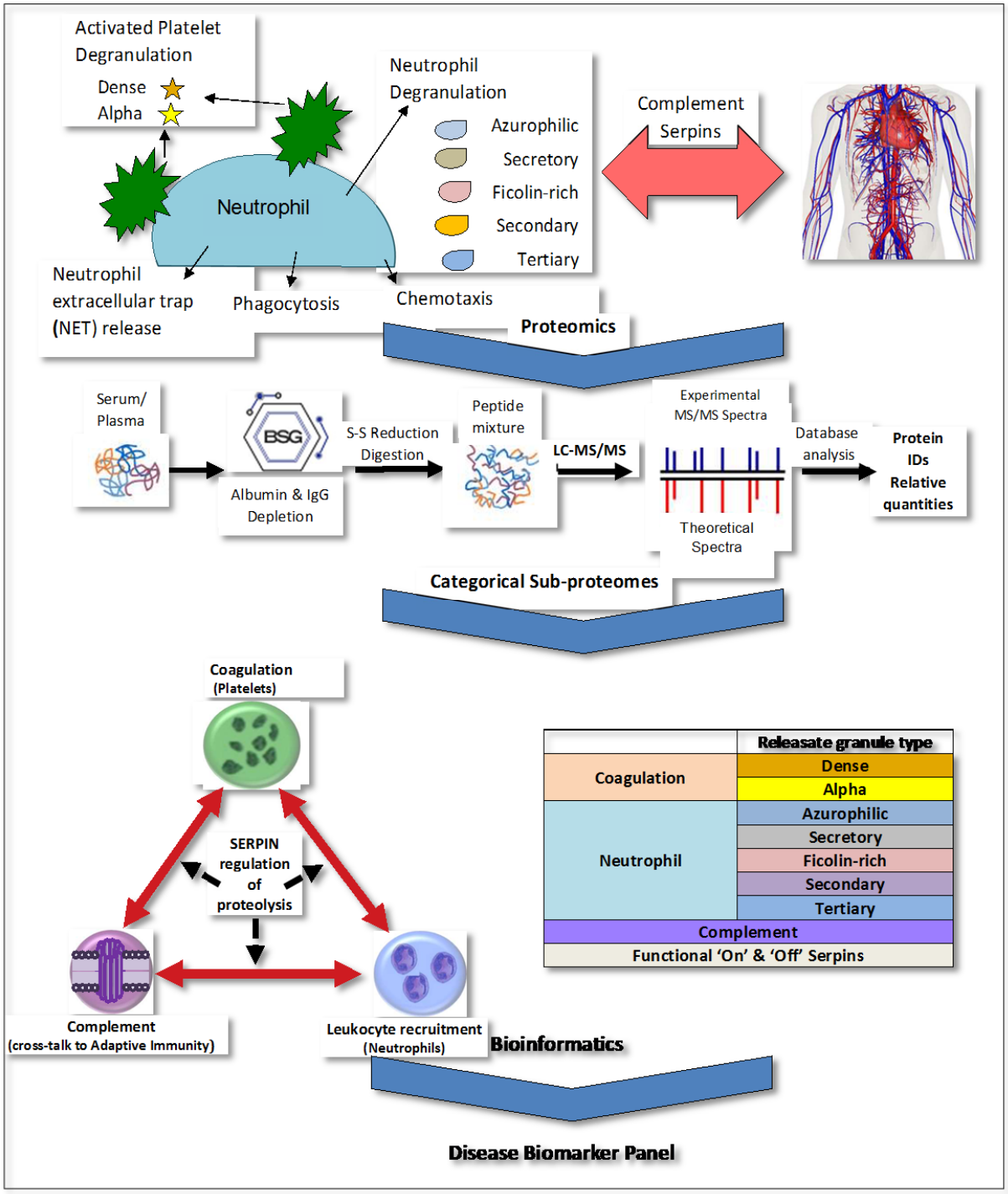

Figure 11. Platelets adhered to inflamed blood vessel walls interact with neutrophils. So in a context dependent manner, coagulation factors derived from degranulation of activated platelets interact directly or indirectly with neutrophils to regulate neutrophil degranulation, NET release, and phagocytic and chemotactic activities at the local inflammatory site. This simultaneously influences protein factors in the general circulation, especially Complement proteins and Serpins.

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