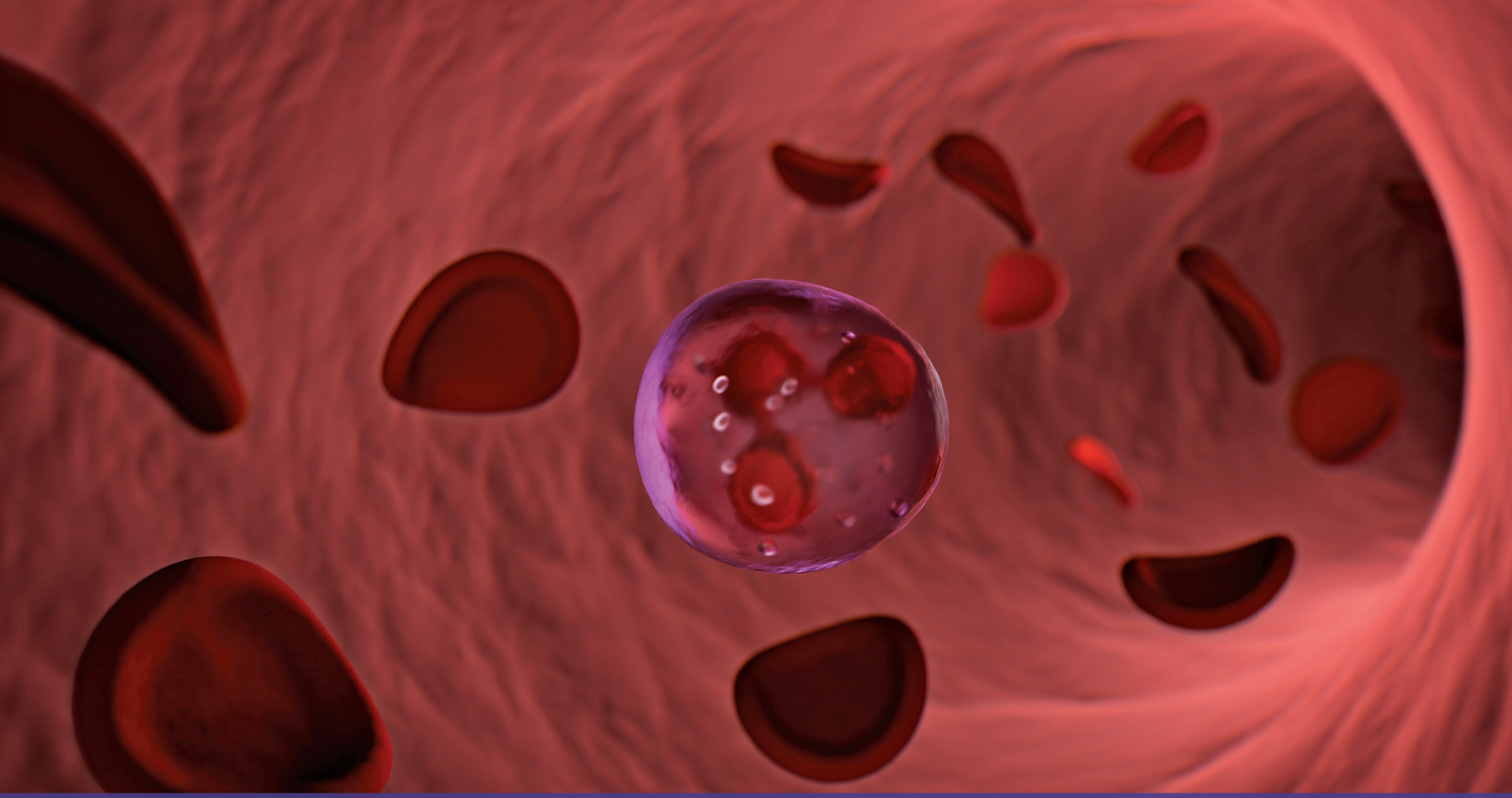


WHITE PAPER - FEBRUARY 2019

BIOTECH SUPPORT GROUP
Sample Prep that Matters

STROMA LIQUID BIOPSY™

Blood-based biomarkers to monitor stromal conditioning in cancer.

WALL STREET SELECT

Patent Application by Biotech Support Group LLC Describes New Cancer Serum Biomarker Panel- Stroma Liquid Biopsy™

U.S. Patent Application No. 15/953,260, entitled "Monitoring Dysregulated Serum Complement, Coagulation, and Acute-Phase Inflammation Sub-Proteomes Associated with Cancer," filed April 13, 2018, published October 25, 2018.

Stroma Liquid Biopsy™ offers methods to monitor and potentially modulate the systemic response to cancer, opens new avenues for early detection, personalized medicine and therapeutic modalities.

Table of Contents……Page

Is there a common systemic response to cancer?
We set out to answer whether there is a common blood response to most if not all cancers, regardless of primary tumor, stage, or metastatic disease.

Yes, the common systemic response is reflected in a biomarker pattern from "wounds that never heal" pathways including:

Coagulation

Acute-phase Inflammation

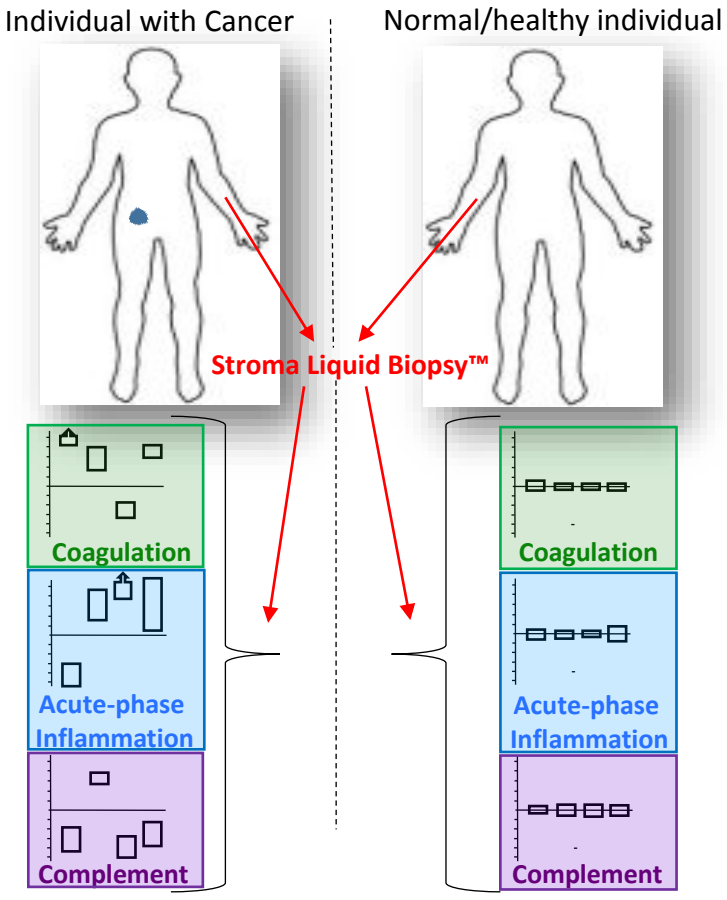
Complement

These pathways are all:
>systemically interconnected
>regulated through proteolytic post-translational modifications (PTMs)

Introduction

The concept of liquid biopsy has generated much scientific and commercial enthusiasm as it starts with a very accessible sample type - a body fluid, typically blood, rather than a surgically extracted tissue. Once available, a liquid biopsy can then be analyzed in a variety of ways to provide for example, a landscape of cancer-associated DNA mutations. Yet, most current liquid biopsy efforts focus on genomic data which relies on a largely reductionist view that tumors form and progress only through the collection of its immortalized cells. These contributions notwithstanding, it is now overwhelmingly apparent that throughout cancer progression, there are necessary adaptive microenvironments to support metastatic disease.

This whitepaper highlights the importance of these adaptive systems and how they intercommunicate in the vast circuitry of cascading proteolytic events, the predominant mechanism for controlling acute insults in the bloodstream.



What if there was a way to form an early indicator for cancer, possibly before clinical evidence, and with personalized tie-in to therapies?

The heterogeneous nature of cancer's DNA has confounded real progress in detection and treatment. In an American Society of Clinical Oncology and College of American Pathologists Joint Review, the authors found "no evidence of clinical utility and little evidence of clinical validity of ctDNA assays in early-stage cancer, treatment monitoring, or residual disease detection."[1]

As current Liquid Biopsy measurements focus only on remnants from the proliferating cells (the "seed"), they **miss an essential element** of cancer pathogenesis: the tumor-associated microenvironment or **stroma** (the "soil"). This demands a **proteomic approach**.

A New View of Liquid Biopsy

There are a variety of approaches that can characteristically describe a "liquid biopsy". These include detecting tumor cells shed from the primary tumor that become blood-borne, and circulating nucleic acids that are remnants from the tumor cells and must be distinguished from the vast amount of nucleic acids from normal tissue in circulation. Extracellular vesicles are also shed from tumor cells and can be analyzed in the general circulation for tumor specific components. Nevertheless, tumors are more than simply a collection of immortalized cells. Cancers of high-grade malignancy do not arise in a strictly cell-autonomous manner, but rather must be viewed as an entire ecosystem which cannot be fully characterized through the autonomous properties of only proliferating cells[2,3]. The supporting microenvironments or stroma must also be evaluated. Because of this, tumor characterization cannot be sufficiently characterized solely through the analyses of the tumor cell genome – the current emphasis of liquid biopsy platforms.

So because tumors are more than just a mass of proliferating cells, cancer progression is influenced by the multiple cell types and networks of proteins dynamically interacting in active tumorigenesis. These are not simply passive bystanders. Because tumors continually sprout new blood vessels (angiogenesis), accounting for such stromal contributions is possible. We herein present evidence that some of the essential interactions between stroma and proliferating cells can in part, be monitored through the protein response that tracks into the vascularized tumor and re-proportions the extracellular proteins found in the general blood circulation. This rewiring of the blood circuitry is measurable even at early stages of cancer, for many if not most primary tumors, forming the basis of intellectual property[4].

This new observational window will help generate a more comprehensive profile of progressive disease, providing opportunities in monitoring risk factors, early detection, prognosis, recurrence, and guidance for therapeutic decisions

"Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in…the now widely appreciated tumor-promoting consequences of inflammatory responses."
Hallmarks of Cancer: The Next Generation, Cell 144[2]

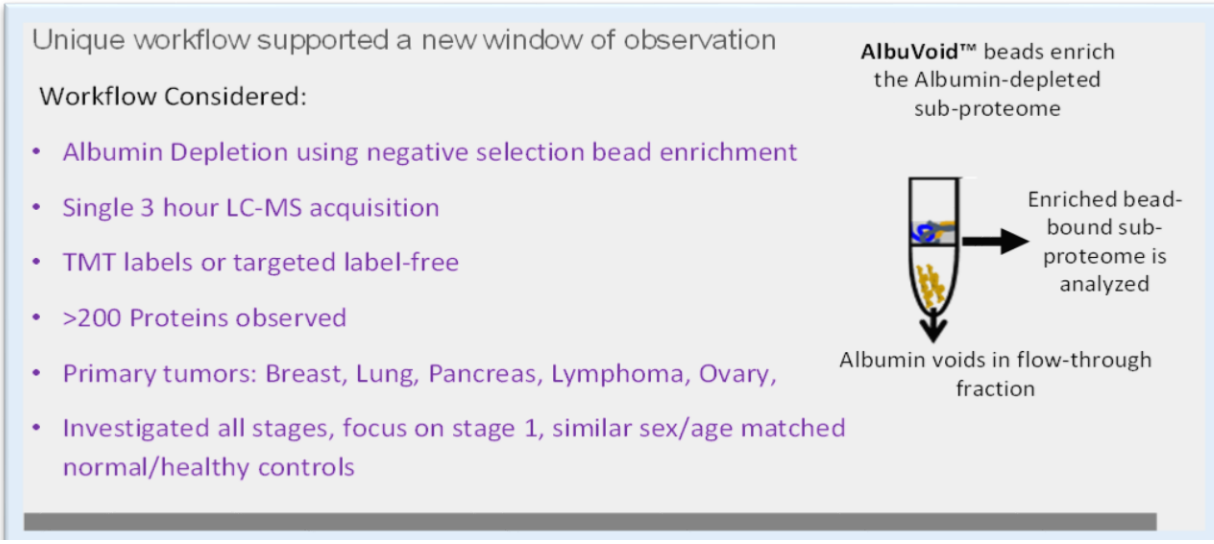
Methods

Discovery of protein biomarkers that can detect cancer early has become an important research area in the proteomics field with the hope of identifying very low abundance (pg/ml range) proteins shed from cancer cells. These efforts all suffer from analysis at the very limits of quantitative LC-MS analysis. Rarely considered but still vitally important, our investigations focused on the serum proteome in the [µg-mg]/ml range. This range by contrast, allows for a targeted LC-MS multiplexed analysis with the further advantage of quantitative precision comparable to current clinical immunoassays[5].

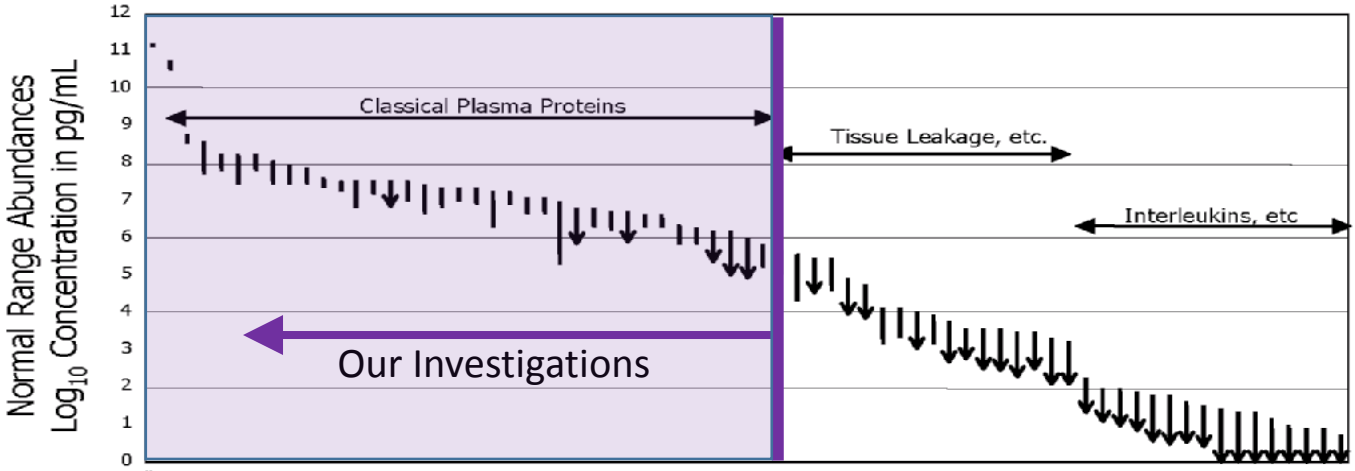
"Our lab works collaboratively with Biotech Support Group to develop robust methods to quantify cancer biomarker proteins/peptides from serum using LC-MS/MS."

H. Zheng Ph.D.
Rutgers Proteomics Center

By using a novel enrichment separations strategy – AlbuVoid™ to deplete Albumin without immuno-affinity, we anticipated that this new workflow would generate a unique window of observation. This was a successful approach as we found several dysregulated proteins common to all sera from the 5 primary tumors tested. The discovery methods have been reported previously[6,7].



The classical plasma proteins are required for normal body function, but can become dysfunctional with acute-phase and chronic stimuli



We filtered our initial results to a target panel of serum protein markers; evidence for a pattern or signature from three host systemic response pathways. Although many of the proteins in our panel have been previously described in the literature as potential biomarkers for select primary tumors, our pattern profile defines the interconnections between three pathways common to most if not all primary tumor origins. Furthermore, some of the biomarkers in the pattern are not based on the fully intact gene product, but rather truncated proteoforms of Serpins resulting from proteolytic regulation. These can now be monitored by our patent pending methods. The significance of Serpins is described in detail later in this report.

The Systemic Response to Cancer

In Table 1, we list the panel of proteins that we can monitor for three essential systemic responses to the presence of cancerous tissue. These protein markers are associated with 1) acute-phase inflammation (blue) 2) coagulation (green), 2) and 3) complement (burgundy). However, all these pathways intercommunicate in the vast circuitry of the protease web.

Unlike most chemical and biological reactions which are subject to equilibria between the reactants and products, proteolysis is irreversible. Because of this, all organisms have evolved a complex system of regulation whereby multiple factors, both macromolecules and small molecules, control aberrant proteolytic events. In blood, these regulating events are overlapping with multiple pathways and regulating mechanisms, all subject to periodic insults which may perturb this very delicate balance[8]. Once disturbed however, this network web of dysregulation can foster microenvironments suitable for the seeds of neoplastic cells to continue to grow unabated and metastasize. One such opportunistic disturbance is inflammation, which impacts every single step of tumorigenesis, from initiation through tumor promotion, all the way to metastatic progression[9]. In the sections which follow, we briefly highlight how some of these essential dysregulated interactions play a role throughout cancer pathogenesis.

"In the setting of systemic inflammation, activation of the coagulation cascade, is accompanied by a profound activation of the complement system…";
Molecular Intercommunication between Complement and Coagulation Systems, J Immunol 2010[10]

Acute-Phase Inflammation

The role of Alpha-1-Antitrypsin (SERPINA1) and Neutrophil Elastase in Cancer

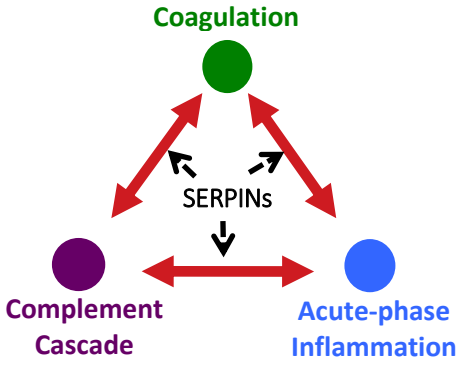
In the course of acute insults such as wound healing and infections, immune inflammatory cells appear transiently and then disappear. However, their persistence in sites of chronic inflammation has been associated with many pathologies including fibrosis, aberrant angiogenesis and cancer. At the earliest stages of disease, incipient neoplasias recruit a host of stroma cells with self-sustaining reciprocal influences. Inflammatory recruitment of Neutrophils is one such case.

"The infiltration of neoplastic tissues by cells of the immune system… supports the association of sites of chronic inflammation with tumor formation, and to the observation that tumors could by portrayed as wounds that never heal.";
Hallmarks of Cancer: The Next Generation, Cell 144[2]

Table 1 – The Stroma Liquid Biopsy™ panel of biomarkers for cancer

Protein Conc. Range > 5 log measurable in 1 LC-MS Analysis					
Systemic Pathway	Protein Gene Name	Protein Description	Serum Conc. In normal/ healthy	Spectral Intensities cancer relative to normal	Comments
Coagulation	PF4	Platelet Factor 4	10 ng/ml	↑	Severe, released from platelets
Coagulation	PPBP	Pro-platelet basic protein	5 µg/ml	↑	Severe, released from platelets, activates Neutrophils
Coagulation	TIMP1	Tissue inhibitor of metalloproteinases-1	100 ng/ml	↑	Severe
Coagulation	THBS1	Thrombospondin 1	200 ng/ml	↑	Released from platelets, multifunctional with some sequence and functional similarities to Complement regulating protein – Properdin below
Complement	C3	C3	1,500 µg/ml	↓	Complement cascade function & regulation is multi-faceted, Coagulation protein Thrombin activates C3
Complement	C4BPA	Complement Component 4 binding protein alpha	300 µg/ml	↓	Complement cascade function & regulation is multi-faceted
Complement	PROP	Properdin	25 µg/ml	↓	Released from Neutrophils, some sequence and functional similarities to coagulation protein THBS1
Acute-phase Inflammation	SAA2	Serum Amyloid 2	5 µg/ml	↑	Near limits of detection with current methods
Acute-phase Inflammation	ELANE	Neutrophil Elastase	250 ng/ml	↑	Near limits of detection with current methods
Acute-phase Inflammation	ECM1	Extracellular Matrix Protein 1	800 ng/ml	↑	Released from Platelet dense granules, severe in many chronic inflammatory conditions, might be rule in/out marker based on severity stratification
Acute-phase Inflammation	CMGA	Chromogranin A	-	↑	Only Lymphoma but severely differential from 5 primary tumors tested, below limits of detection for all others and all normals
SERPIN Function	SERPIN A1	Alpha-1-Antitrypsin	1,500 µg/ml	↓	Inhibits Neutrophil Elastase, and activated Protein C (a regulator of the coagulation cascade)
SERPIN Function	SERPIND 1	Heparin Cofactor II	60 µg/ml	↓	Inhibits extravascular Thrombin, Neutrophil Cathepsin G
SERPIN Function	SERPIN A3	Antichymotrypsin	300 µg/ml	inconclusive	Might be Tissue Specific? Complexes with Prostate Specific Antigen

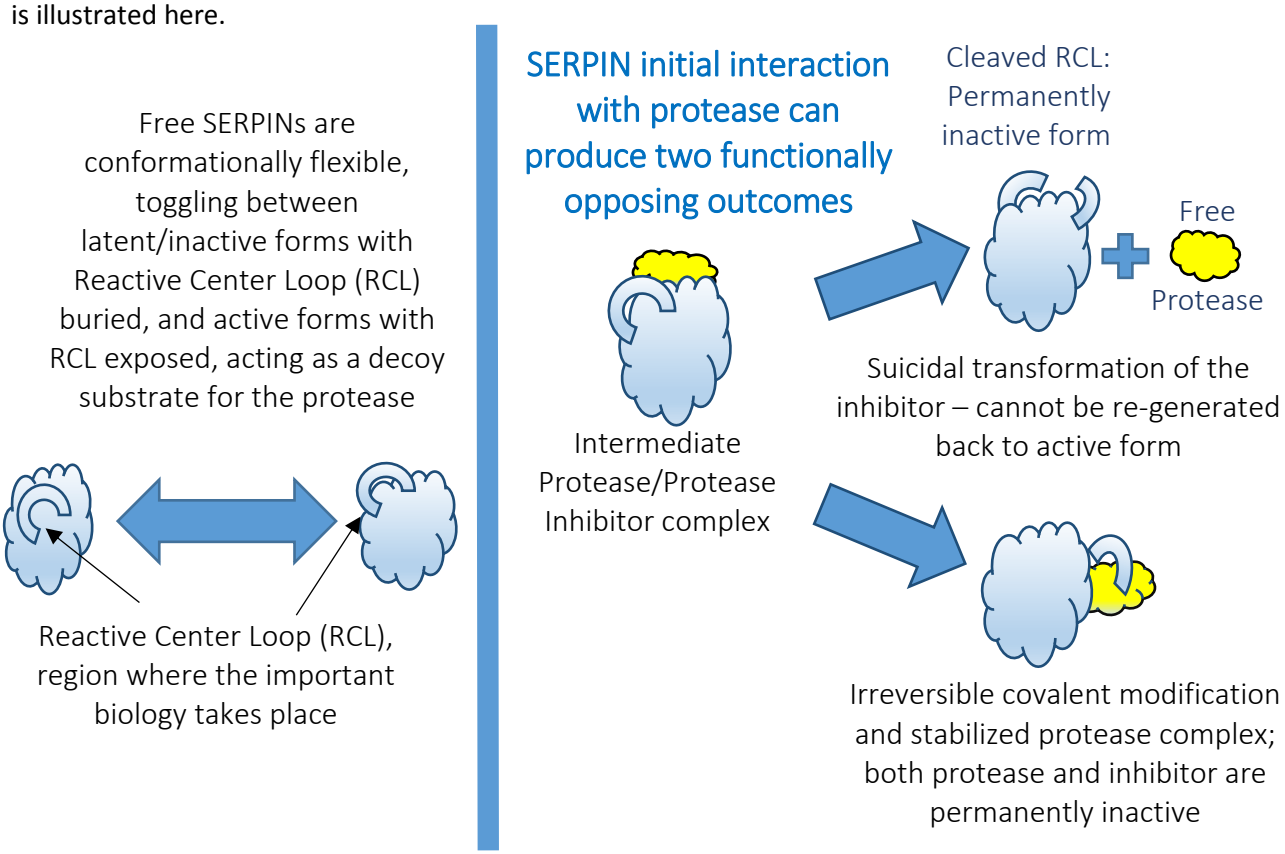
The special significance of this pattern is that serum proteome changes do not act independently as these three host systemic response pathways triangulate in a network of interactions. Furthermore this report signifies the importance of the pathways intercommunicating in the vast circuitry of cascading proteolytic events, the predominant mechanism for responding to acute insults in the bloodstream. As proteolysis is irreversible, all species of life have evolved molecular mechanisms to regulate perturbations and maintain homeostasis. The most distinguished is a protein family of suicidal serine protease inhibitors known as SERPINs.



Functional SERPIN Dysregulation

In a 2004 Review article, clinical oncologists Drs. Sun & Yang suggested there may be an imbalance between α-1-Antitrypsin (AAT) & Neutrophil Elastase (NE) activities that could play a role in the progression of cancer[11]. α-1-Antitrypsin (AAT) deserves special attention because of its unique characteristic as a suicidal protease inhibitor belonging to the serine protease inhibitor SERPIN family. Although the primary function of AAT (SERPINA1) is in lung tissue, where it protects the insoluble elastic biomolecule – Elastin, from destruction by Neutrophil proteases, published reports describe AAT as an alarming factor in malignancy. Yet the standard measurements in blood account for only the complexed NE-AAT amounts by ELISA with no measurements of NE activity. Past reports also do not take into account the conformational features of AAT that divide the total population of AAT into 2 very distinctive and functionally opposite sub-populations. Our patent pending reporting methods now can do that.

AAT belongs to the SERPIN gene family of suicidal protease inhibitors[12]. To quantify their functions, their suicidal mechanism of action demands much different proteomic accounting methods than if their functions were simply stoichiometric, as would be the case if they were not suicidal-type inhibitors. This is illustrated here.



"...a deficiency in α1-antitrypsin is associated with increased risk of liver, bladder, gall bladder, lymphoma and lung cancer. "Normally in the general population, the concentration and activity rates of α1-antitrypsin and neutrophil elastase are in balance. It is the imbalance between these two or a net effect of excess neutrophil elastase that might cause various pathological consequences.";

Role of imbalance between neutrophil elastase and α1-antitrypsin in cancer development and progression, The Lancet Oncology 2004[11]

We have developed methods to make this critical distinction by measuring Liquid Chromatography-Mass Spectrometry (LC-MS) traceable peptide features within the RCL region of Serpins. These distinct sub-populations we now can measure and observe as:

- "ACTIVE" or [+] all Serpin sub-populations that report as having transient inhibitory potential
- "INACTIVE" or [-] all Serpin sub-populations that report a suicidal transformation with permanent loss of inhibitory function.

This is in contrast to the more conventional quantitative approaches which only observe the total population of Serpins by antigen presentation (i.e., ELISA). Such measurements not only discount the significance of whether the Serpin is functional or not, but can lead to misinterpretation of pathway effector mechanisms. Such is the case in cancer, when Serpins are observed by total populations rather than functional sub-populations. Unlike in many previous reports on AAT populations in cancer, we observe that in cancer sera, there is a decline in the abundancy of [+] AAT sub-populations and an increase in the abundancy of NE. To the extent that these ratios are reflecting *in vivo* biology, we advance that such dysregulation may support a progressive cycle in cancer whereby Neutrophil Elastase activity and likely other proteases (i.e., Thrombin) are not sufficiently regulated within the tumor microenvironment, and track into the general blood circulation.

There is consequence to this dysregulation in cancer. In a normal and healthy population, there is a sufficient blood reservoir of [+] Serpins to regulate acute insults. However collectively, because of heredity, lifestyle, environmental exposures, and/or progressive disease, in cancer populations this reservoir becomes depleted, and the body can no longer replenish sufficient quantities of functional Serpins to regulate the many inflammatory proteolytic mechanisms involved with wounds that do not heal. This can be profound as serine proteases facilitate the irreversible cascading events of the three interconnected pathways in our biomarker panel.

Past proteomic analyses count all SERPINS as one homogeneous population. Our patent application discloses methods to differentiate ACTIVE from INACTIVE SERPIN Proteoforms

Our data supports that there are inflammatory proteases supporting tumorigenesis not sufficiently regulated due to chronic exhaustion of ACTIVE proteoforms of SERPIN protease inhibitors!

Coagulation

Interconnected with acute-phase inflammation are coagulation and complement cascades, the other two legs of the **Stroma Liquid Biopsy™** triangulation model.

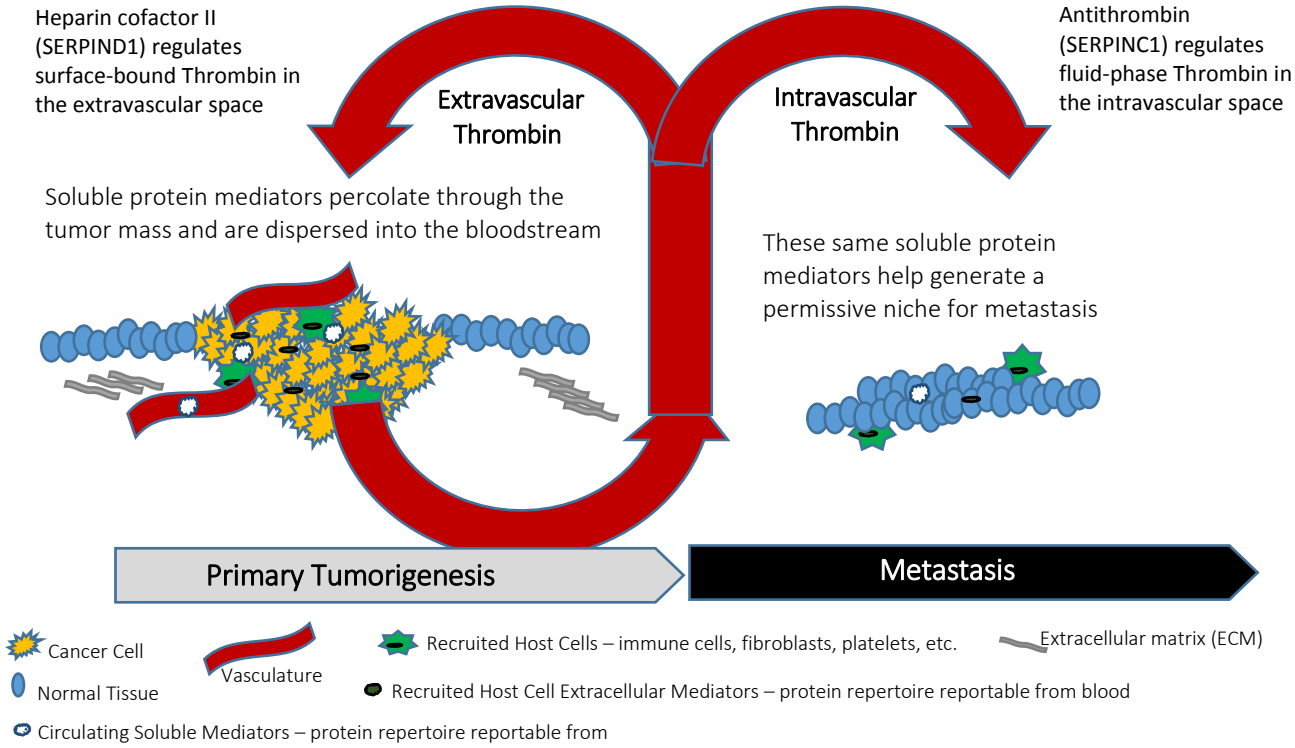
Patients with malignancy have a hyper-coagulable state due to the ability of almost all type of cancer cells to activate the coagulation system, the process by which blood changes from a liquid to a gel, forming a clot. High platelet count is associated with poor prognosis across multiple cancers[13-15]. Coagulation factors in cancer include the production of pro-coagulants directly from the tumor, along with general systemic responses of the host to the tumor, notably from inflammation and angiogenesis[16,17].

"…platelets play an important role in the hostile microenvironment of the bloodstream, where they directly interact with tumor cells and enhance survival. These platelet-tumor cell clusters thereby provide an additional layer of immune evasion, which may contribute to disease progression.";
Microenvironmental regulation of tumor progression and metastasis, Nat Med 2013[3]

In circulation, platelets form platelet-cancer cell aggregates to aid and shield migrating cancer cells by several mechanisms promoting metastasis. In addition, it has been suggested that platelet released cargo provides instructive signals that induce a transitory epithelial-to-mesenchymal transition and metastatic potential[18]. Our data corroborates with other reports raising the possibility that a significant portion of thrombin generated *in vivo* escapes inhibition in cancer[19,20].

Heredity also plays a role. Mouse knockout models developed to modulate thrombin production up or down demonstrate the congenital susceptibility for thrombosis to potently up-regulate the metastatic capacity of tumors, compared to wild-type. In the same study, the anti-coagulation/hemophilic phenotype offered protection against metastases, compared to wild-type[21]. Others reports support over production of Thrombin, and/or biomarker dysregulation within Serpins - notably two in our panel, Alpha-1-Antitrysin (SERPINA1) and Heparin Cofactor II (SERPIND1), as strong influential factors in childhood cancers[20,22]. Such dysregulation in children cannot be attributed to aging, lifestyle or environmental stimuli. Consequently this evidence strongly supports that a highly engaged platelet activation system, along with insufficiently regulated Thrombin production, occurs in cancer and most importantly serve as strong effectors in the survival crisis of metastases. At least in part, some of the aggravating impact of excess and un-regulated Thrombin production comes from its interconnection with Complement activation.

"Thrombin plays important roles in many (patho)physiological conditions that reach far beyond its well-established role in stemming blood loss and thrombosis, including...inflammatory processes, complement activation, and even tumor biology.";
Pathologies at the nexus of blood coagulation and inflammation: Thrombin in hemostasis, cancer, and beyond, J Mol Med 2013[23]



Complement Cascade

"The complement system orchestrates the host defense by sensing a danger signal and transmitting it into specific cellular responses while extensively communicating with associated biological pathways ranging from immunity and inflammation to homeostasis and development."; www.reactome.org[24]

The third pathway in our model is the complement system, part of the innate immune system which in contrast to the adaptive immune system, does not change over the course of an individual's lifetime. The complement system consists of over 50 serum and membrane proteins, most being inactive precursors (zymogens) circulating in blood, that when triggered, become activated through proteolytic cascades culminating in the release of effector molecules with multiple biological functions. Notwithstanding the traditionally described three activation pathways, there are multiple complement activation mechanisms provided through crosstalk with other blood-based networks. Largely under-appreciated is its evolutionarily conserved link to coagulation to eliminate damaged tissues[25-28].

Yet regardless of the initial activation, in a normal setting when complement activation occurs at low levels, the dysfunction of a single component can be tolerated or compensated for by many regulators – both fluid-phase and receptor. However, during chronic localized inflammation the complement cascade is constantly on, requiring the concerted action of recruited regulators for the protection of bystander host cells from complement-mediated functions[29]. Such functions are multifactorial and cross-communicate with other immune response pathways including coagulation and tissue repair. As complement proteins account for about 5% of the total protein content in plasma, dysregulated complement activation has a significant role in many acute and chronic inflammatory conditions, especially cancer.

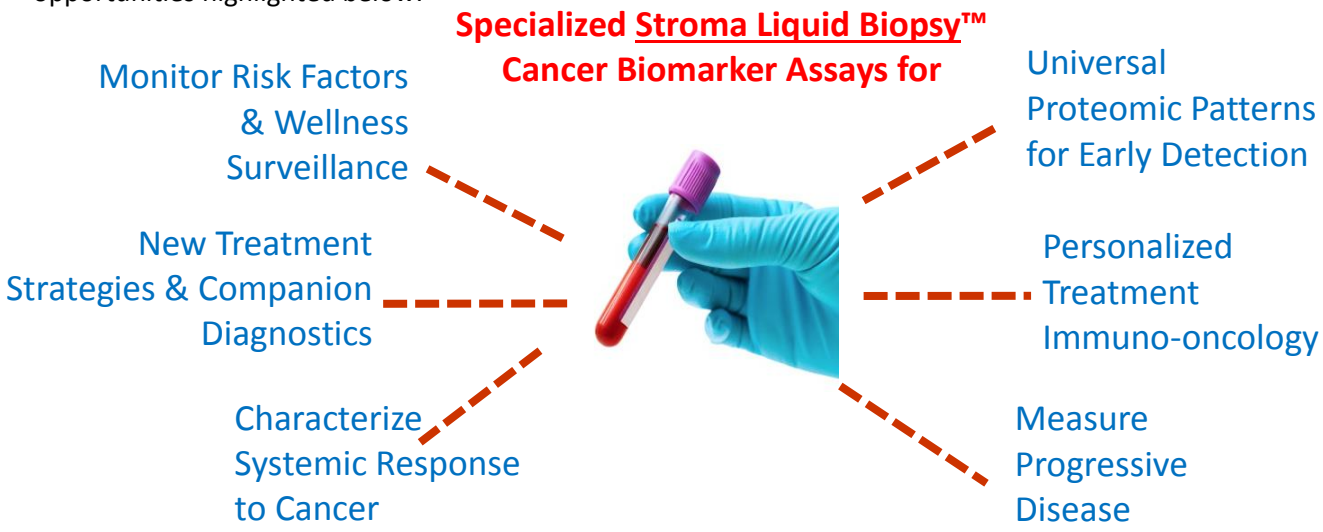
As major participants in the inflammatory milieu surrounding neoplastic tissue, activated complement proteins are abundantly dispersed throughout the extracellular matrix surrounding tumors[30]. Complement is a potent inducer of release of extracellular vesicles; such release being ubiquitous and enhanced in apoptotic and tumor cells. However, complement activation within the tumor microenvironment can serve both a positive role and negative influence as complement can also perpetuate local T-cell immunosuppression and chronic inflammation that promotes metastasis[31]. Because of the often conflicting duality of complement in chronic inflammatory conditions, a more precise characterization of complement at any given time, will likely help guidance for many clinical decisions pre- and post- cancer diagnosis. We are exploring new methods for a more refined functional characterization of the Complement sub-proteome for this purpose.

"Complement protein C5a is a strong chemotactic agent for neutrophils…also plays an important role in the function of neutophils…as it primes them for enhanced functional responses"; Complement: An overview for the clinician, Hematol Oncol Clin North A. 2016[32]

"…intricate interaction among complement activation products and cell surface receptors provides a basis for the regulation of both B and T cell responses.";
The complement system in regulation of adaptive immunity. Nature Immun 2004[33]

Future Directions

We welcome opportunities to engage our intellectual property to support the many commercial opportunities highlighted below.



Monitor risk factors and wellness surveillance

We considered that many of the inflammatory biomarkers in our panel, might be also involved with other chronic inflammatory conditions. Though more investigation is needed, the coagulation biomarkers in particular were severely differentiated in serum samples from patients with diabetes, clinical obesity, Rheumatoid Arthritis and Crohn's Disease, compared to normal/healthy in about the same relative abundances as in the cancer patient samples. There was one exception, the dense granule cargo platelet protein - Extracellular Matrix Protein 1. This biomarker appears to stratify inflammatory conditions differentially from cancer. That is, the relative quantity goes up much more so in the non-cancer inflammatory conditions than it does in cancer. This suggests these biomarkers might offer a risk factor surveillance profile for cancer though certainly would not be diagnostic with current methods. However, under clinical guidance, such biomarkers will generate objective measures whether or not to rule out cancer as a possible diagnosis when other risk factors are accessed. These important decisions are now often based on very imprecise clinical evidence; the consequences of which are either the patient must undertake costly and invasive testing, or early detection is missed and survival is compromised.

Universal proteomic patterns for early detection

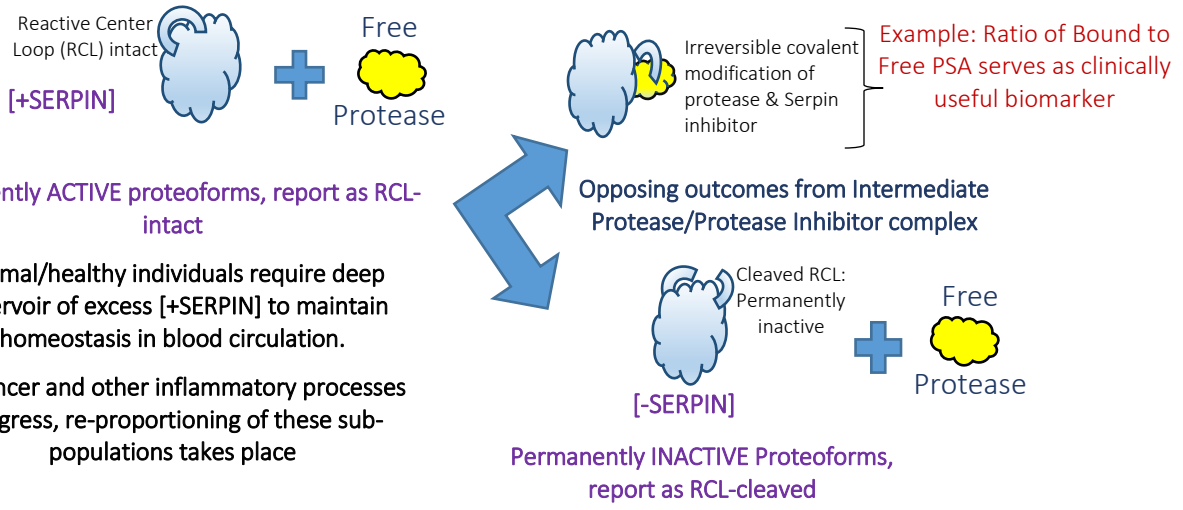
Besides coagulation, the other biomarkers in our cancer panel appear dysregulated in some but not all inflammatory conditions and with different profiles. Several of the biomarkers are at the limits of detection with current methods and so need further investigation. Taken together as patterns however, the differentiation of a variety of chronic inflammatory conditions are distinguishable with our Stroma Liquid Biopsy™ panel, and therefore distinguishable between cancer, normal/healthy and many chronic pathologies. The statistical validation of these preliminary observations remains for future investigation. The SERPINs in particular may offer an especially attractive differentiator for not only the presence or absence of cancer, but also as a way to narrow down the primary tissue of origin. It can serve as a pattern profile yardstick to rule in or rule out tissues of origin. This also needs further investigation.

Characterize systemic response to cancer & measure progressive disease

"One set of tumours, … had a favourable outcome and was defined by the overexpression of a set of protease inhibitors belonging to the serpin family";
Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome, J Pathol 2007[34]

There is extensive crosstalk between the coagulation, complement and inflammation cascades, mostly controlled through 15-20 serine proteases. Functionally ACTIVE SERPINs are necessary to regulate this complex network of serine proteases in order to maintain normal homeostasis. Representing 2-10% of circulating plasma proteins, SERPINs play an essential role influencing diverse biological activities, especially within our three key pathways – coagulation, complement & inflammation. Select Serpins have been associated with progression or remission of cancers, making them valuable for therapeutic or diagnostic use. One measure of this is already established in the clinic; the ratio of bound to free Prostate Specific Antigen (PSA). PSA is a tissue Kallikrein, a protease initiator of the coagulation pathway.

[SERPIN proteoforms functionally reportable by BSG intellectual property]



"The majority of immunologically identifiable human prostate derived proteases, used clinically to monitor patients with prostate cancer, is found in complex with ACT (SERPINA3)";
Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles, BBA 2001[35]

As a group, preliminary data supports that Serpin dysregulation forms a profile for cancer different from other chronic inflammatory conditions. Profiles of ACTIVE relative to INACTIVE Serpin ratios will help narrow the field in clinical guidance to identify the primary tumor of origin. More investigation is needed in both these areas.

New treatment strategies & companion diagnostics

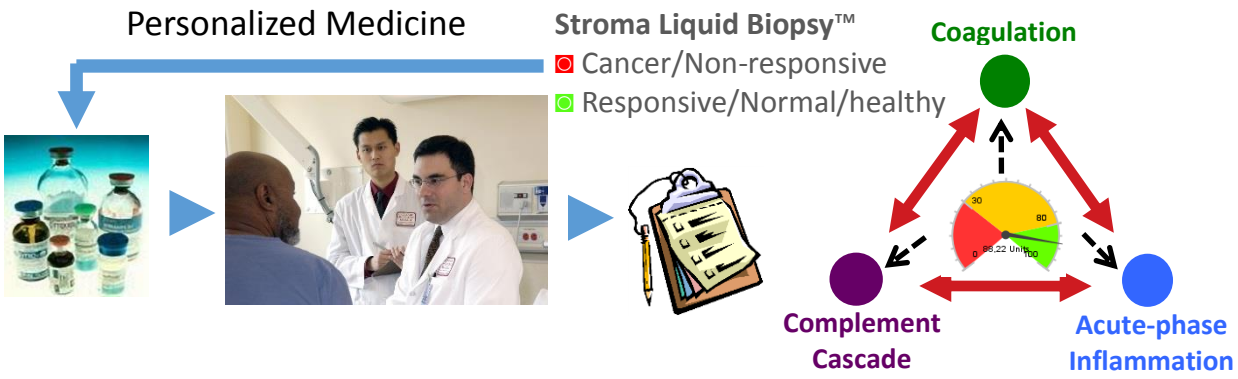
The variety of potential new therapeutic strategies that can be monitored by the Stroma Liquid Biopsy™ panel is too numerous to report here. We highlight that:

- Relationships between elevated neutrophil elastase (NE) activity in cancer tissue samples and poor prognoses have been established, and there are reports that therapeutic modulation of NE can reduce tumor burden. NE's principal inhibitor α-1-Antitrypsin, along with SERPINA5 (Protein C Inhibitor), play roles in regulating coagulation through inhibition of activated Protein C, a feedback system necessary to control overactive Thrombin production.
- A variety of anticoagulants have been clinically tested, with some benefits especially for very early stage cancers. From our model, we can solicit that extravascular (though not necessarily intravascular) Thrombin inhibition is insufficiently controlled in cancer. This reinforces the notion to neutralize Protease Activated Receptor (i.e., PAR1) activation as a therapeutic strategy. In an ideal personalized setting, inhibiting Thrombin's cleavage of Complement C3 to its activated fragments – C3a (anaphylatoxin) & C3b (Initiator of MAC - Membrane Attack Complex), might also be advantageous. Thus, specific allosteric modulation of extravascular Thrombin is warranted as a therapeutic modality.
- The complement system exerts an important influence on the adaptive immune response by acting synergistically with antibodies as well as promoting B- and T-cell stimulation. Sub-lytic doses of MAC induce dramatically different effects than lytic doses, including cell cycle activation, Ca^{2+} flux, and extracellular vesicle release. Therapeutic strategies can thus target the many regulating factors, both fluid-phase and membrane receptors, that inhibit terminal MAC assembly.
- The pleiotropic role of Serpins, especially α-1-Antitrypsin (SERPINA1) and Heparin Cofactor II (SERPIND1) act as critical nodes in the tumor and systemic microenvironments promoting carcinogenesis. Reactive center peptides derived from Serpins have shown potential for immunomodulatory functions, and a precise tuning of Serpin stromal modulation might be possible. This will greatly impact the management and treatment of cancer owing to the systemic metastatic potentiation derived from coagulation activation.

"...disrupting the extracellular environment surrounding and infiltrating tumors may provide an additional level of therapeutic intervention. ...in early stages of disease, patients my benefit from therapeutic intervention that aims to disrupt the premetastatic niche before cancer cells arrive.";
Microenvironmental regulation of tumor progression and metastasis, Nat Med 2013[3]

"My lab studies Serpin function and different co-factors that affect their functions. We have a special interest in SerpinD1, otherwise known as Heparin Cofactor II. Using different cofactors, we have assays that directly assess Serpin functions, one from another, rather than the more common methods that adopt antigen presentation (i.e., ELISA). While preliminary, our functional results nevertheless align very well with the LC-MS RCL peptide feature data from BSG. This opens up an exciting new area in the field; the imbalance of functional SERPIN sub-forms in cancer, reportable from blood. Still more exciting is the possibility that extravascular (but not necessarily intravascular) Thrombin inhibition is <u>not</u> sufficiently regulated in cancer. Specific targeting of extravascular Thrombin activity, or its consequential effects (C3 activation for one) are therefore warranted as potential therapeutic modalities." Ingrid Verhamme, PhD, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center

With the evidence presented to characteristically define a cancer phenotype in serum, Biopharma can now use objective metrics that differentiate a cancer profile from one of a normal/healthy characteristic pattern. This will guide therapies that can move the dial away from a cancer pattern towards a more normal/healthy characteristic pattern. For this purpose, companion Stroma Liquid Biopsy™ biomarker panels, adapted to personalize medicine will be beneficial for survival.



Personalized Treatment Immuno-oncology

There is great interest to define biomarkers that can predict the groups of patients and the types of cancer that will respond to immunotherapy. Given the great potential of cancer immunotherapy and the fact that the immune system can sometimes have opposing roles in cancer, it is imperative to delineate mechanisms, and biomolecules that are required for an effective anticancer response.

The cancer immune response is a multi-step process involving interactions between the tumor and microenvironment including the many host cells and soluble mediators functioning at different times, at different tissues, and throughout the tumor stroma and vasculature. This necessitates pre-defining these phenotypes before treatment, and monitoring the change in phenotypes upon treatment, to establish long-term responsiveness and survival benefits.

Biomarkers that reflect stromal conditioning will be especially useful to follow the mechanism of action for different agents and drug combinations that help to unwind the microenvironments detrimental to immuno-oncology efficacy. So **Stroma Liquid Biopsy™** biomarkers can be an important way to correlate and represent the density of stroma, its phenotype signature, and - albeit indirectly, the array of host cell populations within the tumor microenvironment at the earliest stages. The immune evasive role of platelets may be one such important contributor. This will help monitor progressive disease, and establish patient sub-populations, dosing and combination strategies which may best respond to immuno-oncology therapy.

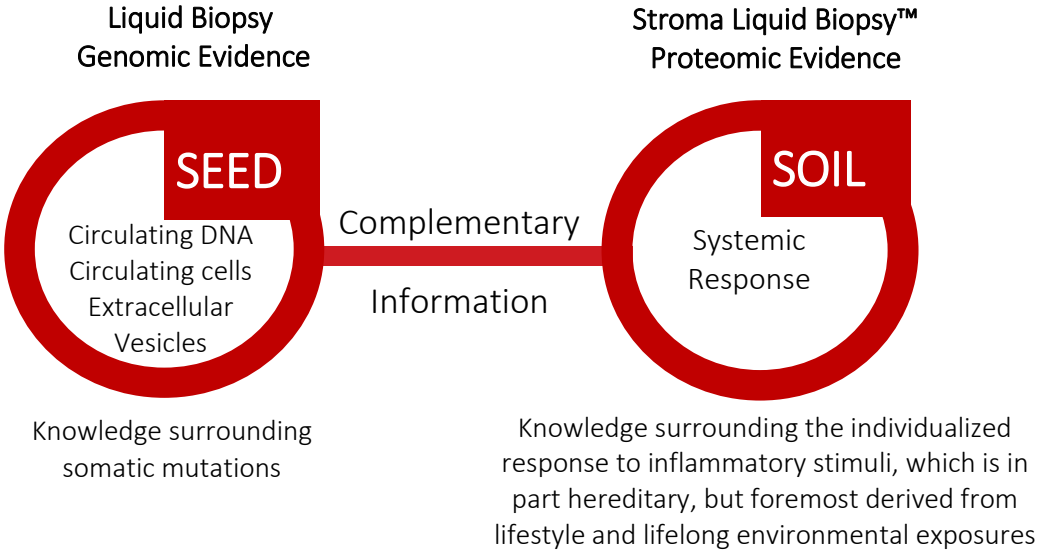
"...it is now evident that tumors are also diverse by nature of their microenvironmental composition, and stromal cell proportions or activation states. ...re-educationg a dysfunctional tumor microenvironment could yield striking results in cancer control and remission as evidenced by the accumulating success stories in the cancer immunotherapy field.";
Microenvironmental regulation of tumor progression and metastasis, Nat Med 2013[3]

Conclusion

We now have evidence that there are measurable protein biomarkers found in blood that are common across different primary tumors and are associated with categorical mechanisms of coagulation, complement, and acute-phase inflammation. These pathways are all interconnected and cross-communicate with each other participating in a triangulated vortex of dysregulation necessary for cancer progression and metastasis. Our observations corroborate other reports that many of these same mechanisms are common to most primary tumors. A persistent inflammatory response observed in or around developing neoplasms has been shown to regulate many aspects of tumor development, from initiation all the way to metastatic progression. Also, that clinical outcome is strongly related to stromal characteristics.

"...biology of tumors can no longer be understood simply by enumerating the traits of the cancer cells but instead must encompass the contributions of the "tumor microenvironment" to tumorigenesis.";
Hallmarks of Cancer: The Next Generation, Cell 144[2]

"The tumor-stroma ratio (TSR) has been reported as a strong, independent prognostic parameter ..., based on stained histological sections, ... It links tumors with high stromal content to poor prognosis.";
Scoring the tumor-stroma ratio in colon cancer: procedure and recommendations, Virchows Archive 2018[36]



This whitepaper serves only as an introduction to the prospects for compiling **Stroma Liquid Biopsy™** proteomic data. Such data will support a different yet very complementary purpose from other Liquid Biopsy analyses, based on nextgen sequencing, circulating tumor cells, extracellular vesicles, or more conventional tumor burden biomarkers (i.e. PSA, CEA). Our data supports that these biomarkers are measurable even at very early stages of cancer, for many if not most primary tumors. Therefore it reflects in part, the individualized density and soluble mediators of the cellular composition of the tumor stroma.

Consequently, using proteomic data from the panel here, we envision to match patients most likely to benefit and least likely to experience adverse events, and ideally to monitor the change in phenotypes upon treatment. This will establish long-term responsiveness and survival benefits for immuno-oncology and related therapies, all with relatively easy, non-invasive measurements.

In much the same manner, methods to monitor new therapeutic strategies to modulate interconnected proteolytic events are possible. Most significantly, we entertain the pleiotropic role of SERPINs, especially α-1-Antitrypsin (SERPINA1) and Heparin Cofactor II (SERPIND1), acting as critical nodes in the tumor and systemic microenvironments promoting carcinogenesis. Consequently, we advance especially that extravascular Thrombin inhibition modalities to manipulate the tumor microenvironment for therapeutic effect, may also be beneficial for survival:

-- As monotherapy to relieve metastatic potential; for maintenance after primary tumor burden debulking, or prophylactic for high risk individuals, or

-- In combination therapy to enhance other modalities, especially for later stages when risk of internal bleeding may be more likely

-- Stroma Liquid Biopsy™ companion biomarkers will be especially useful to monitor both situations.

Finally, our selection of patent pending **Stroma Liquid Biopsy™** biomarkers offer key benefits as they are:

- all highly observable, most of relative high abundance in serum and measurable by a variety of proteomic platforms
- all highly differentiated – many severely, in the cancer population, and very stable in the normal/healthy population
- multi-functional and determinately linked to the systemic interconnections between the three pathways, many of which cannot be monitored by antigen presentation alone.

By using Stroma Liquid Biopsy™ biomarkers in blood, our collaborators and partners will gain invaluable information central to understanding how individuals are uniquely predisposed to cancer, how individuals uniquely adapt to the presence of cancer anywhere in the body, and how individuals uniquely respond to medical intervention.

Want to know more about how
Stroma Liquid Biopsy™ can help your biomarker or translational research?

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