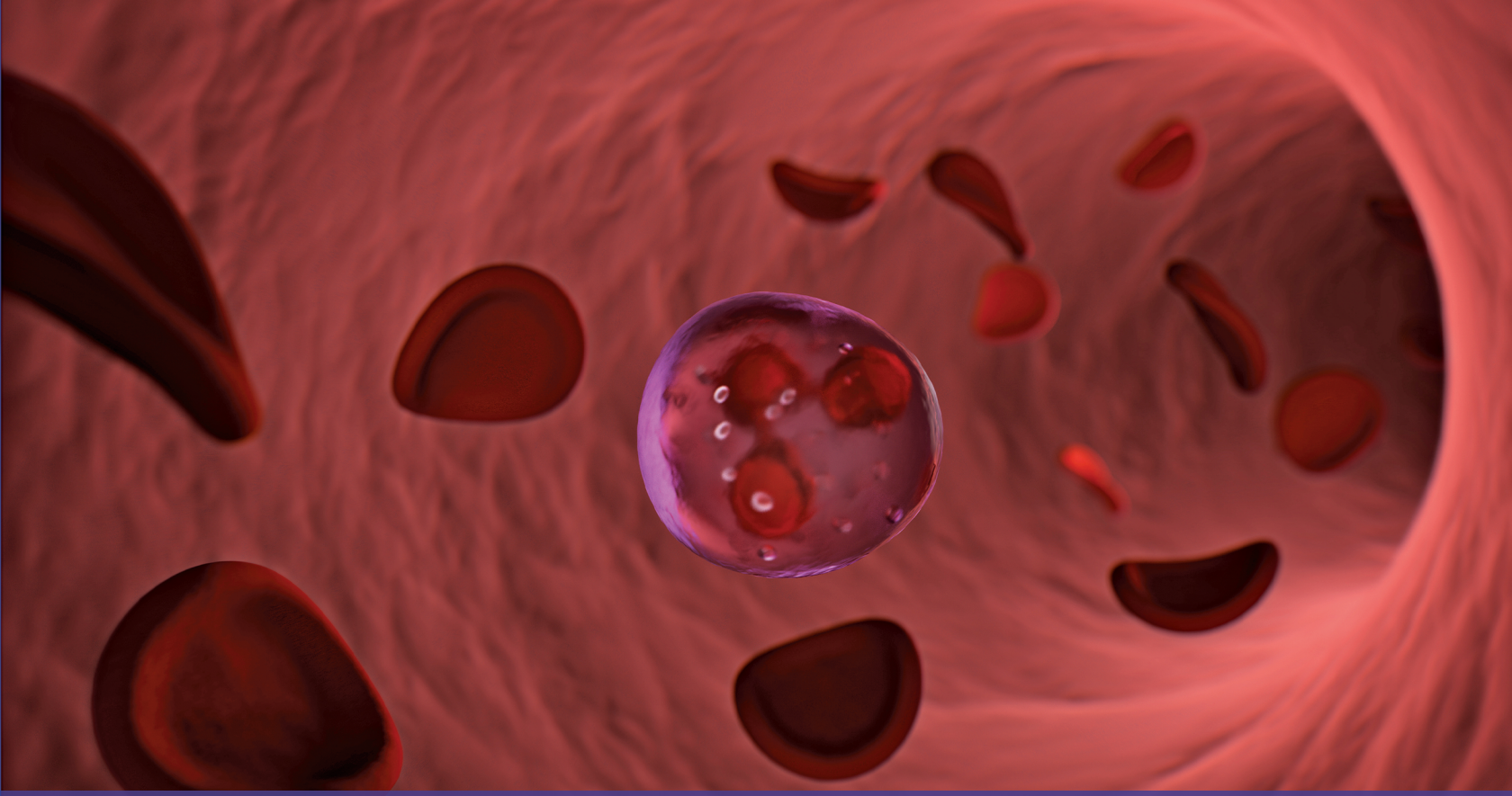


WHITE PAPER - JANUARY 2022 *Version 2*

BIOTECH SUPPORT GROUP
Sample Prep that Matters

STROMA LIQUID BIOPSY™

Substantiating correlations for stromal conditioning using blood biomarkers and tumor tissue profiles

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.

cancers

Journal Article by Leiden University Medical Center and Biotech Support Group Describes a Gene Signature Ratio that Predicts Survival in Colon Cancer

Ravensbergen, Cor J., et al. "The Stroma Liquid Biopsy Panel Contains a Stromal-Epithelial Gene Signature Ratio That Is Associated with the Histologic Tumor-Stroma Ratio and Predicts Survival in Colon Cancer." *Cancers* 14.1 (2022): 163

BSG

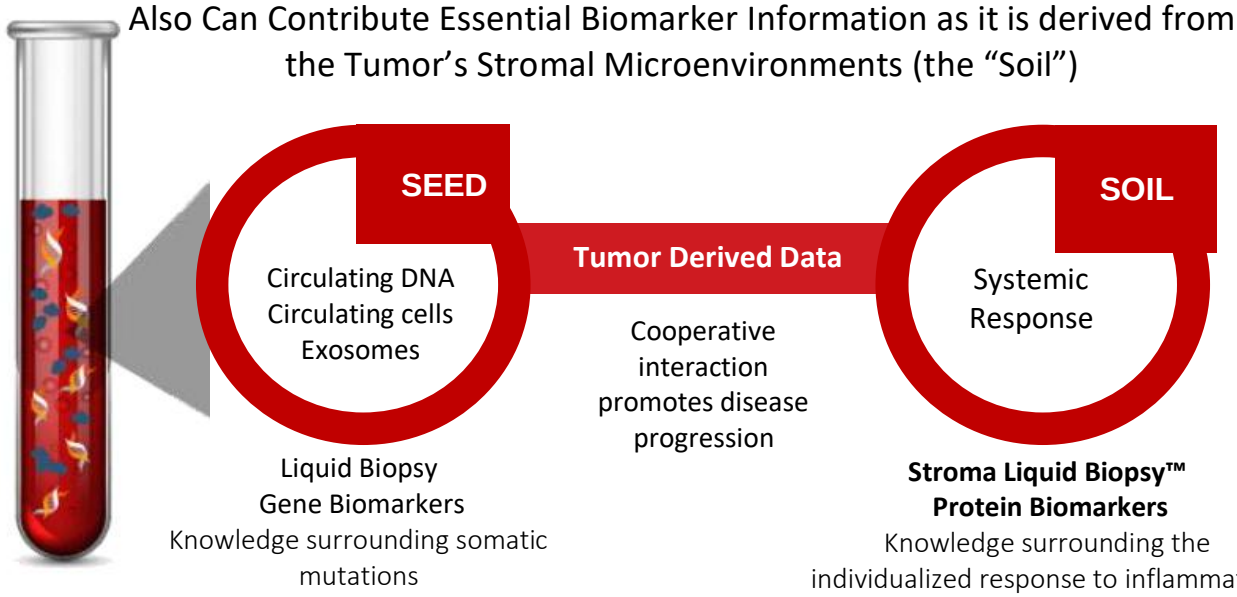
BIOTECH SUPPORT GROUP

Sample Prep that Matters

Authors: Matthew Kuruc (Biotech Support Group); Wilma E. Mesker Ph.D. (Leiden University Medical Center); Cor J. Ravensbergen (Leiden University Medical Center); Devjit Roy MD (Nathan Littauer Hospital)

"...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[1]

Current Liquid Biopsy Strategies Focus on Circulating Remnants of the Proliferating Cells (the "Seed")
Yet, The Host Systemic Response Cooperates in Cancer Progression and Also Can Contribute Essential Biomarker Information as it is derived from the Tumor's Stromal Microenvironments (the "Soil")



Liquid Biopsy
Gene Biomarkers
Knowledge surrounding somatic mutations

Stroma Liquid Biopsy™
Protein Biomarkers
Knowledge surrounding the individualized response to inflammatory stimuli, in part hereditary, but foremost derived from lifestyle and lifelong environmental stressors

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. While genomic instability is considered an enabling hallmark of cancer, it is now overwhelmingly apparent that throughout cancer progression, the second enabling hallmark – tumor promoting inflammation, is necessary to support metastatic disease.

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."[2]

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**.

This whitepaper highlights the importance of the systemic inflammatory response to the presence of cancer anywhere in the body. It also describes how the vast circuitry of cascading proteolytic events, requires suitable regulation in order to resolve innate immunity and accommodate the adaptive T-cell response in cancer. From this, new strategies for more durable therapeutic efficacy will be uncovered.

A New View of Liquid Biopsy

"The tumor-stroma microenvironment is an important prognostic parameter for patients with epithelial cancer types. We do know that patients with a high amount of stromal cells in the primary tumor have a bad prognosis and respond worse to current chemo-, radio- and/or immunotherapy regimens. Now with the help of Biotech Support Group's Stroma Liquid Biopsy™ panel, our working hypothesis is showing real evidence for how stromal conditioning impacts survival." Collaborators Drs. Wilma Mesker (Associate Professor) and Cor Ravensbergen (M.D./Ph.D. candidate) of the Leiden University Medical Center

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[1,3]. 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 simply a mass of proliferating cells, cancer progression is influenced by the multiple immune 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 solid primary tumors, forming the basis of intellectual property[4].

Through proteomic data, we will help generate a more comprehensive profile of progressive disease, providing opportunities to monitor risk factors, detect cancer before clinical symptoms appear, prognosticate outcomes, monitor therapeutic response, and provide guidance for medical intervention.

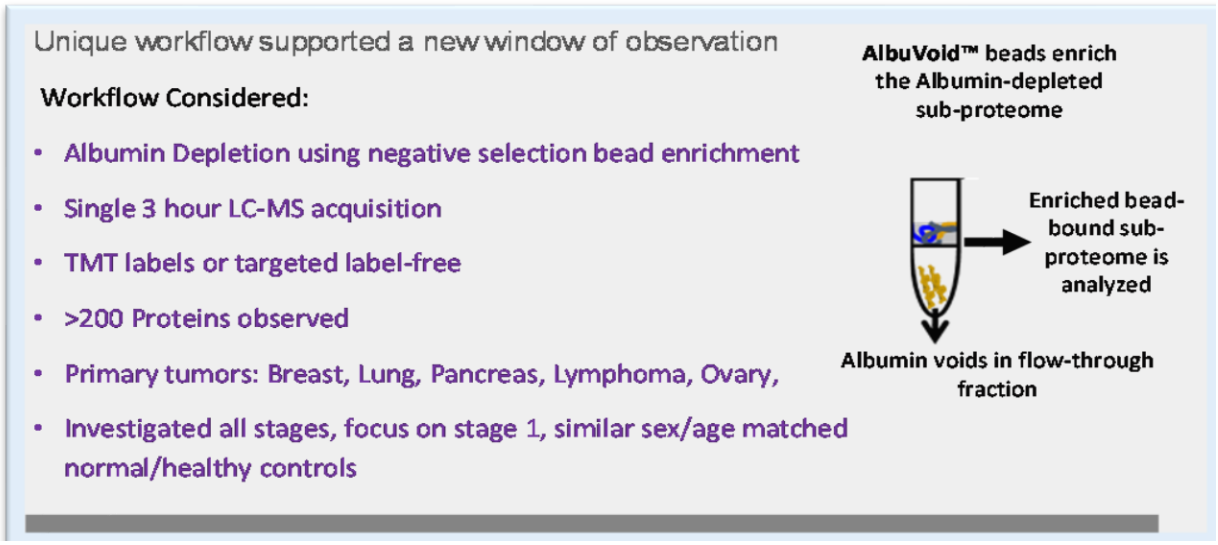
"Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in…the now widely appreciated tumor-promoting consequences of inflammatory responses."
Hallmarks of Cancer: The Next Generation, Cell 144[2]

Methods

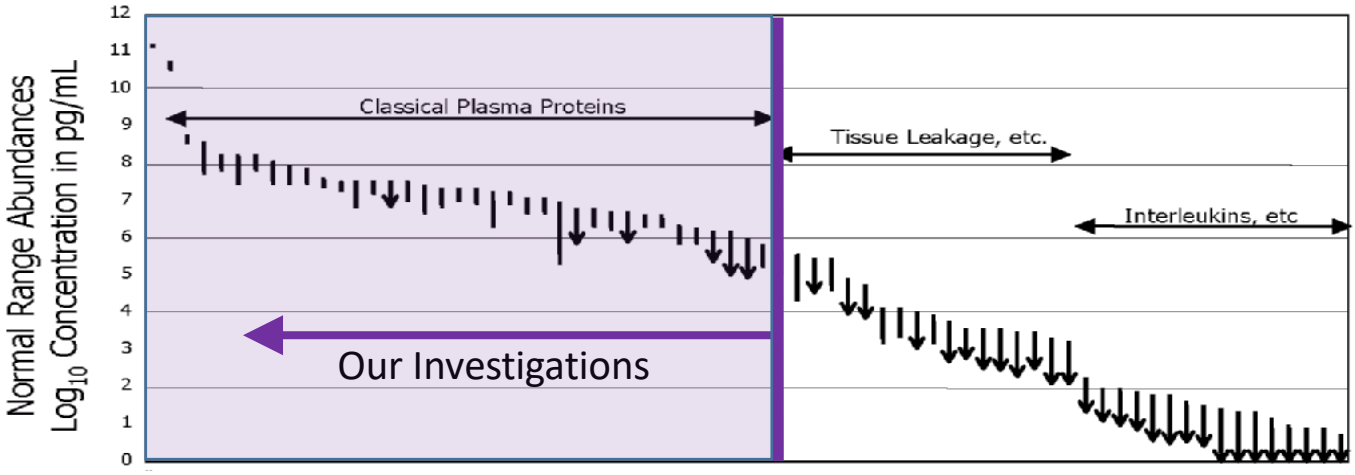
"Our lab works collaboratively with Biotech Support Group to develop robust methods to quantify cancer biomarker proteins/peptides from serum using LC-MS/MS." Haiyan Zheng Ph.D, Rutgers Proteomics Center

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].

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 resulting from proteolytic regulation. The significance of these proteoforms 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 & Neutrophil recruitment, 2) coagulation, and 3) complement. Noteworthy is that all these pathways interconnected within the innate immune response, and the circuitry of proteolytic events.

This is especially important, as 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, must control aberrant proteolysis in order to maintain normal homeostasis. 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 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.

"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]

Acute-Phase Inflammation & Neutrophil Recruitment

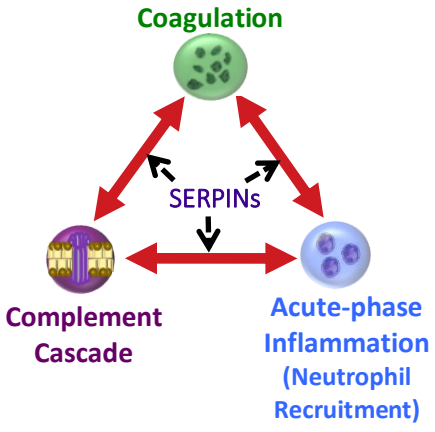
The role of Alpha-1-Antitrypsin (SERPINA1) and Neutrophil Elastase in Cancer

In the course of acute insults such as wound healing and infections, the innate immune system supplies the first responders to inflammatory stimuli. Under textbook circumstances, innate immunity then resolves to adaptive immunity (T Cells, antibodies, etc.) within 10-14 days. However, the innate immune response does not always fully resolve in chronic inflammation, and this lack of resolution is associated with many pathologies including fibrosis, aberrant angiogenesis and cancer. So, at the earliest stages of disease, incipient tumors recruit a host of innate stroma cells with self-sustaining reciprocal influences; recruitment of Neutrophils being one such opportunistic case.

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, 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 and Fibroblasts, severe in many chronic inflammatory conditions, might be rule in/out marker based on severity stratification
Acute-phase Inflammation	CMGA	Chromogranin A	-	↑	Only Lymphoma severely differential from 5 primary tumors tested, may differentiate hematologic and solid tumors
SERPIN Function	SERPIN A1	Alpha-1-Antitrypsin	1,500 µg/ml	(Active sub-form) ↓	Inhibits Neutrophil Elastase, and activated Protein C (a regulator of the coagulation cascade)
SERPIN Function	SERPIN D1	Heparin Cofactor II	60 µg/ml	(Active sub-form) ↓	Inhibits extravascular Thrombin, activated by Heparin cofactors
SERPIN Function	SERPIN A3	Alpha -1-Antichymotrypsin	300 µg/ml	(not all cases) ↑	Inhibits Neutrophil Cathepsin G, Complexes with Prostate Specific Antigen, might be tissue of origin classifier

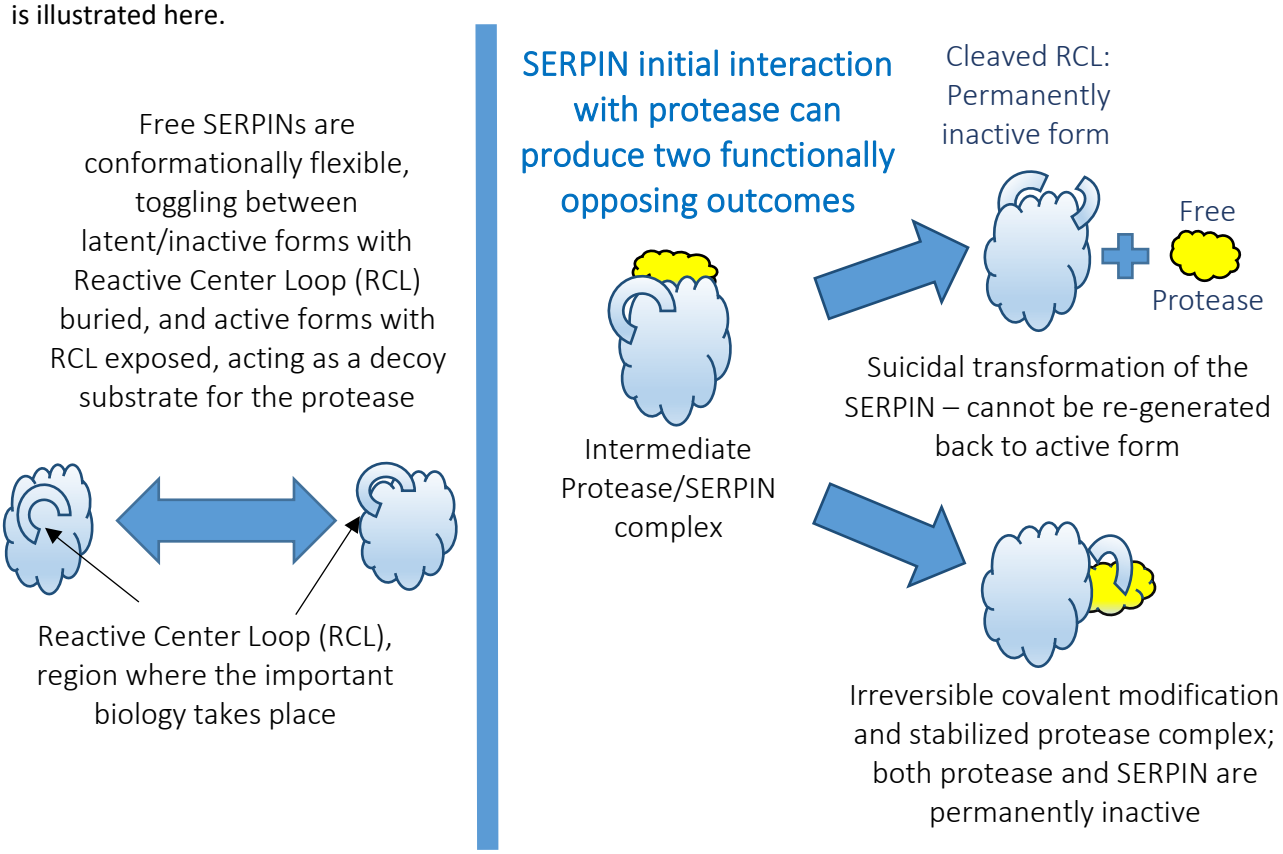
The special significance of this pattern is that serum proteome changes do not act independently. These three host systemic response pathways triangulate in a network of innate immunity interactions. Furthermore, it is especially significant that they all intercommunicate in the vast circuitry of cascading proteolytic events, the predominant mechanism for responding to acute insults in blood. As proteolysis is irreversible, all species of life have evolved molecular mechanisms to regulate proteolytic perturbations to 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[10]. 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[11]. To quantify function, 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 more conventional (Kunitz-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[10]

"… in cancer…a large portion of systemic α1-antitrypsin is biologically not functional"
Abnormal Profile of Serum Proteinase Inhibitors in Cancer Patients. CANCER RESEARCH 44, 2718-2723, June 1984[12]

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

- "ACTIVE" all Serpin sub-populations that report as having inhibitory potential, or
- "INACTIVE" all Serpin sub-populations that report a suicidal transformation and irreversible loss of inhibitory function.

This is in contrast to the more conventional quantitative approaches which only can 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 ACTIVE AAT sub-populations and an increase in the abundancy of Neutrophil Elastase. Loss of AAT functionality was previously reported, though the suicidal mechanism was not known at the time[12]. To the extent that this imbalance reflects *in vivo* biology, we surmise such dysregulation supports 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.

This has important consequences in cancer. For in a normal and healthy population, there is a sufficient blood reservoir of ACTIVE Serpins to regulate acute insults. AAT for example is the second most abundant protein in plasma. However, because of heredity, lifestyle, environmental exposures, and/or progressive disease, collectively in cancer populations, this SERPIN reservoir becomes depleted. As a result, the body can no longer replenish sufficient quantities of functionally ACTIVE Serpins to regulate the many inflammatory proteolytic mechanisms churning within the tumor. So, without regulation of innate immunity, derangement of the adaptive T-Cell response results. Next, we describe how coagulation participates in these mechanisms.

Past proteomic analyses count all SERPINS as one homogeneous population. Our intellectual property describes methods to differentiate ACTIVE from INACTIVE SERPIN Proteoforms

Our data supports that there are inflammatory proteases supporting tumorigenesis that are 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].

"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 function, one from another, rather than the more common methods that adopt antigen presentation (i.e., ELISA). Our preliminary functional results for SerpinD1 align very well with the LC-MS RCL peptide feature data from BSG." Ingrid Verhamme, PhD, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center

"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[18]

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[19]. Our data corroborates with other reports raising the possibility that a significant portion of thrombin generated *in vivo* escapes inhibition in cancer[20,21].

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[22]. 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[21,23]. 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.

"…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 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[24]

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[25]

The third pathway in our innate immunity model is the complement system; an immediate reaction to inflammatory insults, it is relatively non-specific, and does not change over the course of an individual's lifetime. The complement system consists of over 50 plasma and membrane proteins, most being inactive precursors (zymogens) circulating in blood, that when triggered, become activated through proteolytic cascades. It terminates with the formation of the membrane attack complex (MAC), which punctures the cell membrane. Its function is to support phagocyte and antibody targeting of microbes or infected cells, the clearance of microbes and damaged cells, and to help coordinate other phases of immunity including the adaptive response. Notwithstanding the three textbook activation pathways (classical, lectin, alternate), there are other complement activation mechanisms provided through crosstalk with other innate pathways. Largely under-appreciated is its evolutionarily conserved link to coagulation to eliminate damaged tissues[26-29].

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[30]. Such functions are diverse, cross-communicating 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[31]. Complement is a potent inducer for release of extracellular vesicles; such release being ubiquitous and enhanced in apoptotic and tumor cells. Even so, complement activation within the tumor microenvironment can serve both a positive role and negative influence; at times perpetuating local T-cell immunosuppression and chronic inflammation that promotes metastasis[32]. Because of the often-conflicting duality of complement in chronic inflammatory conditions, a more precise gauge of the state of complement at any given time, will help guidance for many clinical decisions pre- and post- cancer diagnosis. In addition to the current markers in our panel, 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[33]

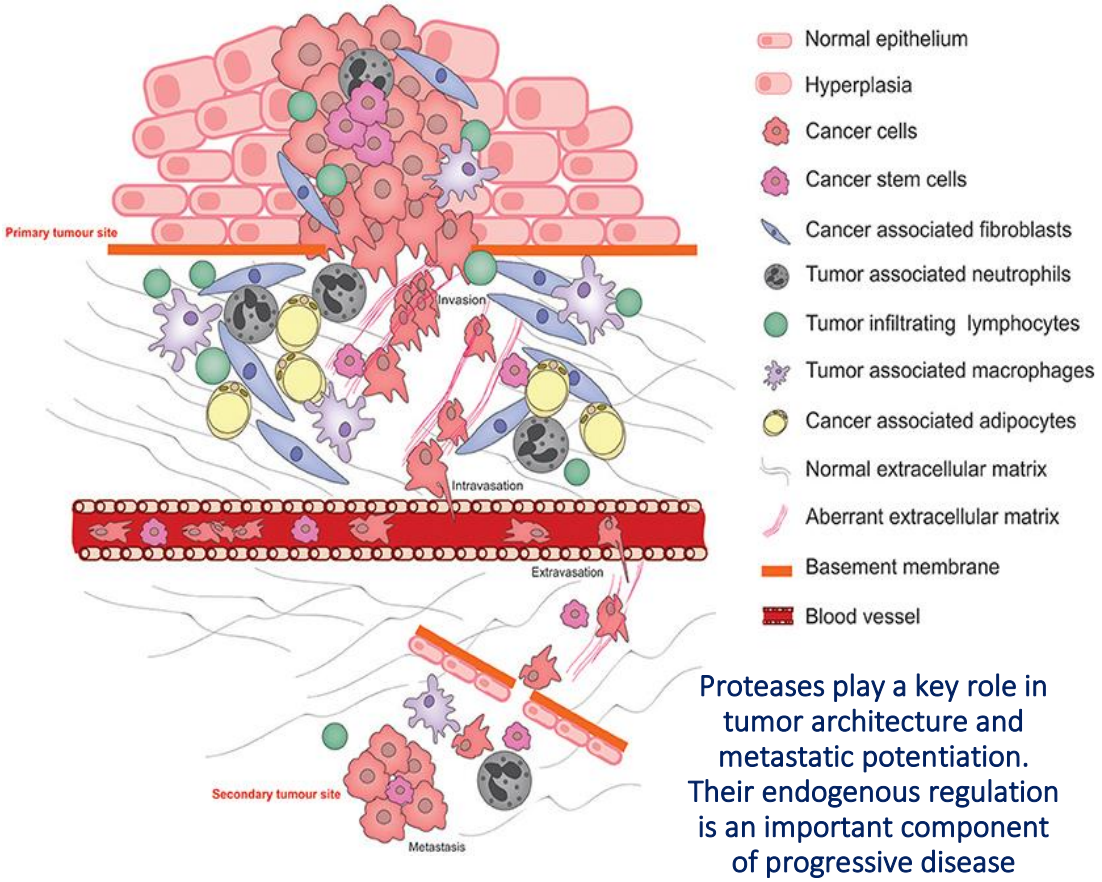
"...intricate interaction among complement activation products and cell surface receptors provides a basis for the regulation of both B and T cell responses.";
The complement system in regulation of adaptive immunity. Nature Immun 2004[34]

Correlating Stroma Liquid Biopsy™ with the Tumor-Stroma Ratio

Leiden University Medical Center

"The Tumor-Stroma Ratio (TSR) has been validated over the years for all types of epithelial tumors. Currently for colorectal cancer we are finalizing a large prospective European study collaborating with 15 countries, to prepare for implementation in guidelines. When I read about the Stroma Liquid Biopsy from Biotech Support Group, I immediately contacted them. This has resulted now in a nice collaboration to evaluate stroma formation in tissue and biomarkers in blood." Dr.Wilma Mesker, Associate Professor, Leiden University Medical Center

The goal of the Leiden collaboration is to better understand the crosstalk between cancer cells and their supporting stroma. This contributes to the functional and structural support of the tumor microenvironment, leading to tumor progression and metastasis. To better characterize this aspect of tumor biology, Leiden University Medical Center (LUMC) has developed a histopathology-based Tumor-Stroma Ratio (TSR) using routinely stained resected tumor tissue. This is reported to be a strong, independent prognostic parameter and continues to be validated in medical research centers around the world for a range of solid epithelial tumors[35]. As of February 2022, the TSR has been mentioned in the title or abstract of more than 1.100 scientific papers. For more information, go to http://watchstroma.com/research/.

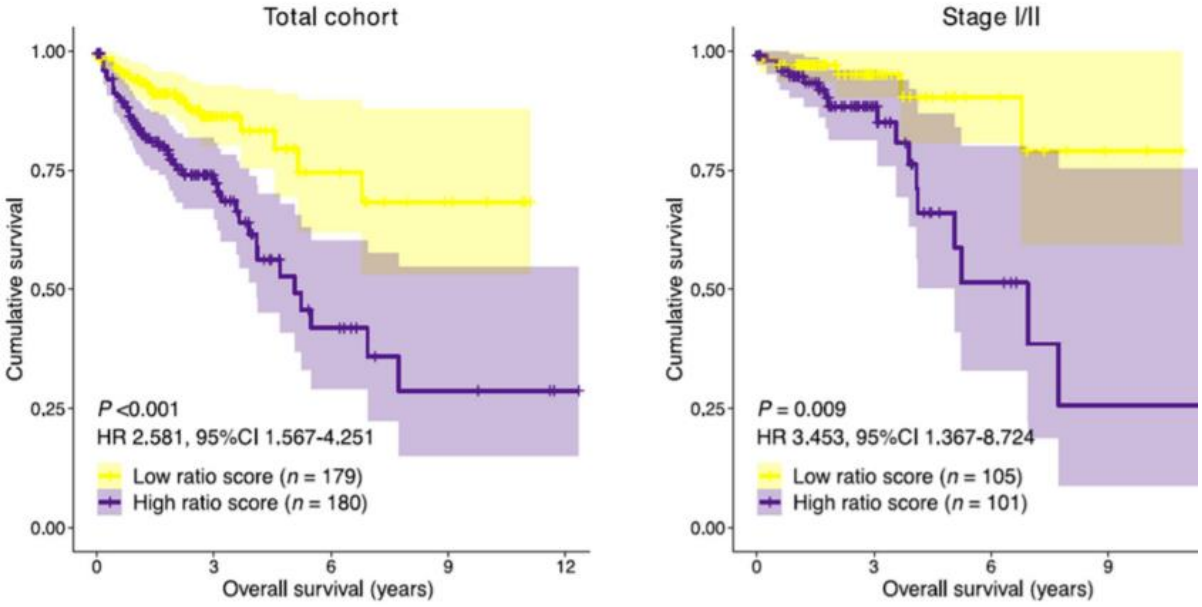


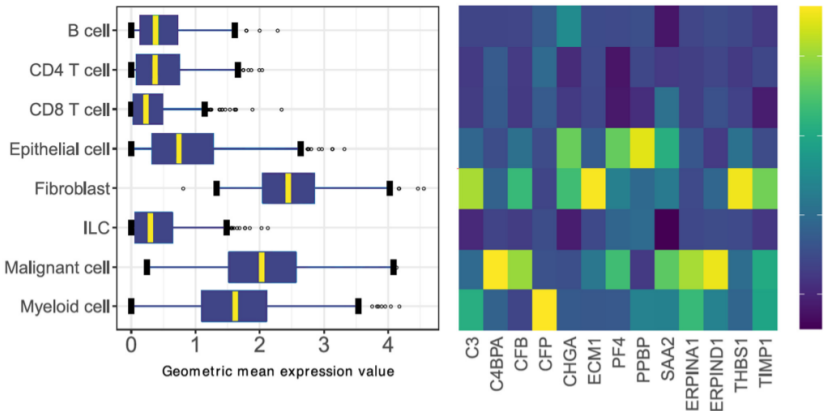
Proteases play a key role in tumor architecture and metastatic potentiation. Their endogenous regulation is an important component of progressive disease

The estimation of TSR is simple and inexpensive, and can be done in routine pathology investigation of the resected tissue. However, the TSR has its limitations, as it is mainly conducted postoperatively, results from preoperative biopsies remain unclear and are currently subject to investigation. In addition, its prognostic value for tumors other than of epithelial origin is also unclear. Finally, its required invasiveness

limits its accessibility and, hence, the ability to monitor the course of disease and cancer treatment response.

A more comprehensive characterization of stromal conditioning would be very valuable in assessing tumor aggressiveness. Our hypothesis is that there are two basic tumor types, stroma-low and stroma-high, that can be classified by a variety of multi-omic signatures. These differences may be observable at the tissue and blood levels to provide a more refined stratification of the tumor, prognosis for the patient and potentially new therapeutic strategies that might beneficially modulate the tumor microenvironment. Blood-based biomarkers, such as those proposed in the Stroma Liquid Biopsy™ panel, would solve many of current TSR limitations and provide deeper and less subjective clinical characterization. To begin supporting this hypothesis, we highlight the current *in-silico* study that provides an explorative gene transcriptomic characterization of the Stroma Liquid Biopsy™ proteomics panel in colon carcinoma, by integrating single-cell and bulk transcriptomics data from publicly available repositories[36].





The **Stromal-Epithelial Gene Signature Ratio**, is based on genes from the Stroma Liquid Biopsy™ panel, and classified by their expression as being derived from stromal-phenotypic or epithelial-phenotypic cells. In this way, it is demonstrated that histologic high stromal content is accompanied by increased gene expression of stroma-associated pathways in comparison to tumors with low stromal content. The gene signature ratio described in this study was found to have a striking prognostic performance; over twice as much cumulative survival for stage I/II tumors with a low stroma ratio score. It also demonstrated remarkably similar performance to previous investigations of

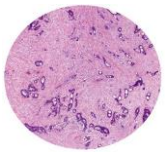
histologic TSR, providing further evidence for the prognostic power of the tumor stroma in clinical practice.

In addition to patient prognosis, high gene signature ratio-risk scores also were associated with an increased proportion of Microsatellite instability (MSI) in comparison to low ratio-risk scores. Given the increased proportion of MSI in the high ratio risk score group, the signature ratio might be predictive of immune checkpoint inhibitor (ICI) therapy response in cancer and should be the subject of future studies in ICI therapy-treated patient cohorts.

Finally, the current report provides a first theoretical framework for proteomic signatures to potentially serve as an indicator for tumor-stroma content when applied to liquid biopsy. Ultimately, the stromal conditioning protein blueprint, as captured by the Stroma Liquid Biopsy™ panel, may encompass a more discreet stratification of the tumor and patient prognosis, and offer new insights into therapeutic strategies that might beneficially modulate the tumor-microenvironment.

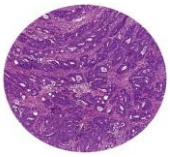
Proposed tumor characteristics

- **High tumor-stroma ratio (TSR)**
- (histology high stroma content)



- **High Stro-Epi signature ratio**
- High infiltration of innate cells, especially Neutrophils
- High platelet degranulation
- Unsteady-state/dysfunctional regulation of Complement
- Insufficiencies of functional Serpins A1 & D1 proteoforms
- Increased proportion of Microsatellite instability
- Incompetent Adaptive T Cell Response / 'Cold' Immunotherapy tumor type

- **Low tumor-stroma ratio (TSR)**
- (histology low stroma content)



- **Low Stro-Epi signature ratio**
- Low infiltration of innate cells
- Low platelet degranulation
- Steady-state regulation of Complement
- Sufficiencies of functional Serpins A1 & D1 proteoforms
- Competent Adaptive T Cell Response / 'Hot' Immunotherapy tumor type

"Tumor tissue is only available on selected time points during the course of disease/treatment. So that limits its clinical use in terms of a tool for follow up and monitoring of disease. Ideally, by developing our liquid biopsy approach, we want to create this easy-to-use and patient friendly clinical tool that provides relevant information about the state of the tumor microenvironment at numerous time points during the course of disease. Hopefully, we will eventually be able to accurately quantify intra-tumoral stromal content via a simple blood draw."

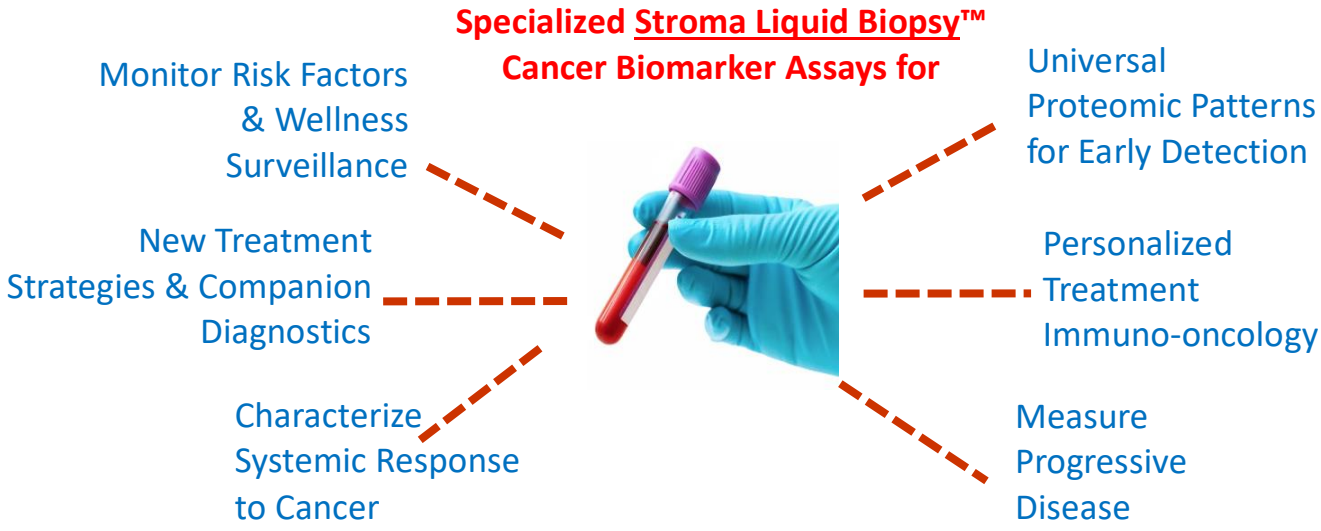
Cor Ravensbergen, M.D./Ph.D. candidate, Leiden University Medical Center

"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[35]

Future Directions

We welcome collaboration and partnership inquiries to engage our intellectual property to support the many commercial opportunities it offers.



Monitor risk factors and wellness surveillance

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). 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, also expressed in Fibroblasts. 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 that it might offer a risk factor surveillance profile for cancer though certainly would not be diagnostic. However, under clinical guidance, it may help determine whether or not to rule out cancer as a possible diagnosis when other risk factors or pre-existing conditions 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 method development. Taken together as patterns however, the differentiation of a variety of chronic inflammatory conditions are distinguishable from cancer with the Stroma Liquid Biopsy™ panel. 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. While this still needs

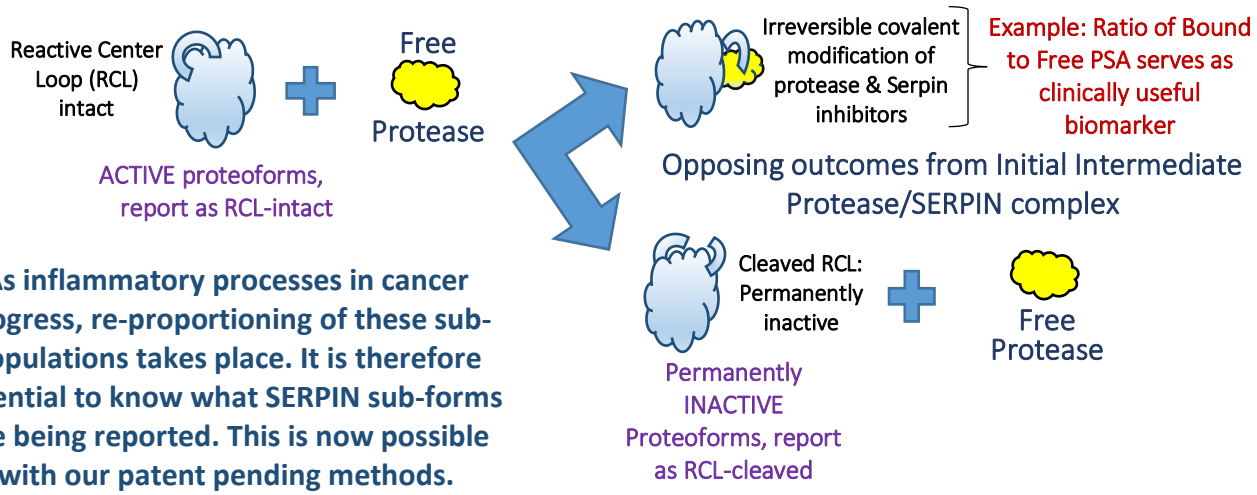
further investigation, one measure of this is already established in the clinic; the ratio of bound to free Prostate Specific Antigen (PSA).

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[37]

PSA is a tissue Kallikrein, a protease initiator of the coagulation pathway. There is extensive crosstalk between the coagulation, complement and inflammation cascades, regulated through serine proteases in peripheral blood. Representing 5-10% of circulating plasma protein mass, functionally ACTIVE SERPINs are necessary to regulate this complex network of serine proteases in order to maintain normal homeostasis. Select Serpins have been associated with progression or remission of cancers, making them valuable for therapeutic, prognostic or potentially diagnostic use.

[SERPIN proteoforms functionally reportable by BSG intellectual property]



As inflammatory processes in cancer progress, re-proportioning of these sub-populations takes place. It is therefore essential to know what SERPIN sub-forms are being reported. This is now possible with our patent pending methods.

Preliminary data also supports that Serpin dysregulation forms a profile for cancer different from other chronic inflammatory conditions (i.e., Diabetes, Obesity, etc.). Profiles of ACTIVE relative to INACTIVE Serpin ratios, along with more established tumor burden biomarkers (i.e., CEA, CA125) may also help narrow the possible primary tissues of origin, or establish hematologic vs. solid, etc., in a clinical setting. More investigation is warranted in these areas.

"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[38]

"The clotting process engages several cellular elements of the circulating blood and impacts both local and systemic aspects of cancer. Understanding how the coagulome and tumor microenvironment influence each other offers exciting new prospects for predicting hemostatic complications and boosting the effectiveness of cancer treatment." Coagulome and the tumor microenvironment: an actionable interplay." Trends in Cancer (2022)[39]

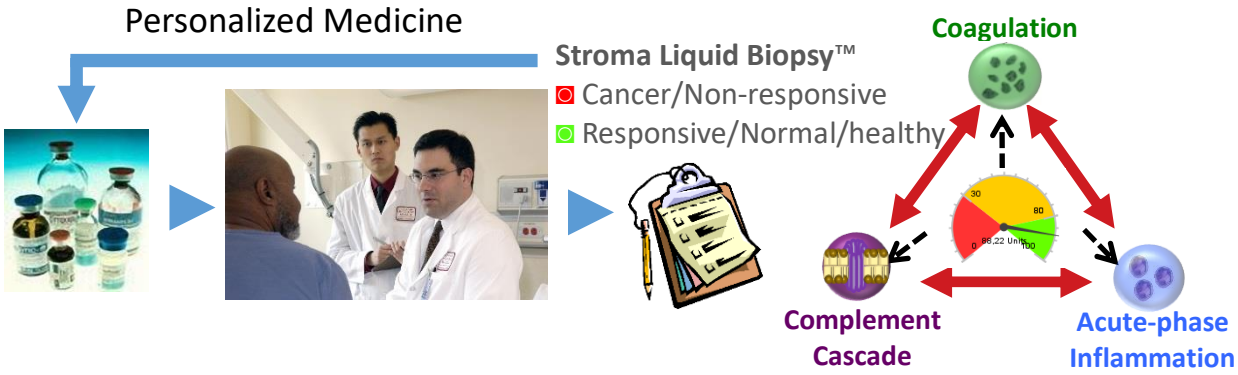
New treatment strategies & companion diagnostics

Genomic instability has confounded therapies targeting gene re-arrangement. Yet, the innate immune response remains a more unwavering therapeutic target. As such the variety of potential new therapeutic strategies that can be monitored by the Stroma Liquid Biopsy™ panel is exceptionally varied and too numerous to report here. Nevertheless, 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 solicit that extravascular (though not necessarily intravascular) Thrombin inhibition is insufficiently controlled in cancer. This reinforces either neutralization of Protease Activated Receptor (i.e., PAR1) activation, or allosteric modulation of extravascular Thrombin as therapeutic strategies.
- 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.

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.

"…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]



Although immune monitoring studies in peripheral blood would be preferable …, an understanding of the tumor microenvironment would first be necessary…Many studies have shown that tumor-infiltrating T cells correlate with clinical outcomes to immune checkpoint therapy … other studies have indicated that certain genes or pathways may exclude T cells from the tumor microenvironment thereby leading to resistance to immune checkpoint therapy." Immune Checkpoint Therapy and the Search for Predictive Biomarkers, *Cancer J.* 2016[40]

Personalized Treatment Immuno-oncology

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. Immune checkpoint therapy can be highly effective, but only about 15-20% of patients respond to it. Our premise is that the innate immune response impacts the adaptive T-Cell response, the latter being necessary for successful immune checkpoint therapy. Inadequate resolution of the innate response can partially exclude the adaptive response and conversely, therapeutic modulation of the innate response may re-engage the adaptive response. This necessitates pre-defining phenotypes before treatment, and monitoring the change in phenotypes upon treatment, to establish long-term responsiveness and survival benefits.

To maximize therapeutic benefit and avoid unnecessary toxicity, the establishment of predictive biomarkers to guide treatment decisions is warranted. 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 therapies. 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. This will help establish patient sub-populations, monitor progressive disease, and, dosing and combination strategies which may best utilize 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 …";
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, common across different primary tumors and associated with categorical mechanisms of coagulation, complement, and acute-phase inflammation & Neutrophil recruitment. 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.

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 circulating DNA, circulating tumor cells, extracellular vesicles, or more established tumor burden biomarkers (i.e., PSA, CEA). Our data supports that these proteomic biomarkers are measurable even at very early stages of cancer, for many if not most primary tumors.

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 response upon treatment. This will establish long-term survival benefits for immuno-oncology and related therapies, all with relatively easy, and 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 (SERPIN A1), Heparin Cofactor II (SERPIN D1) and α-1-Antichymotrypsin (SERPIN A3), acting as critical nodes in the tumor and systemic microenvironments promoting carcinogenesis.

"It is very rewarding to see how, through the use of one of our enrichment products - AlbuVoid™, because of its unique selection properties, we observed something that others did not. Our experimental design was at first quite simple. Using proteomic data, we wanted to determine whether a systemic response was measurable in blood, to most if not all cancers, regardless of primary tumor, stage, or metastatic disease. This discovery research ultimately led to our patent pending panel of Stroma Liquid Biopsy™ biomarkers. From this, the genes from this panel have now been investigated through our collaboration with Leiden, providing a first theoretical framework for the panel's suitability in liquid biopsy. We welcome commercial partnership opportunities so that we can advance the clinical utility for proteomic characterization of stromal conditioning in cancer. This can be an objective way to stratify patients towards the best treatment options, and personalize bedside decisions, which ultimately can prolong survival." Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

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

- With few exceptions, of relative high abundance in serum and measurable by LC-MS
- all highly differentiated – many severely, in the cancer population, and very stable in the normal/healthy population
- pleotropic and determinately linked to innate immunity
- in part, functional sub-forms, that can now be monitored by our patent pending methods, and which cannot be monitored by antigen presentation, aptamer or like binding motifs.

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 learn more about how
Stroma Liquid Biopsy™ can help your biomarker or translational research?
Contact: Matt Kuruc, VP Business Development, mkuruc@biotechsupportgroup.com or
Tel: 732-274-2866

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
2. Merker, Jason D., et al. "Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review." *Archives of pathology & laboratory medicine* (2018).
3. Quail, Daniela F., and Johanna A. Joyce. "Microenvironmental regulation of tumor progression and metastasis." *Nature medicine* 19.11 (2013): 1423-1437.
4. Roy, Swapan, Devjit Roy, and Matthew Kuruc. "Monitoring Dysregulated Serum Complement, Coagulation, and Acute-Phase Inflammation Sub-Proteomes Associated with Cancer." U.S. Patent Application No. 15/953,260.
5. Anderson, Leigh, and Christie L. Hunter. "Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins." *Molecular & Cellular Proteomics* 5.4 (2006): 573-588.
6. Zheng H, Zhao C, Roy S, et al. The Comparison of the Serum Proteome in Individuals with Cancers versus those without Cancer, and its application to Wellness. Poster reprint first presented at 12th Annual US HUPO 2016 Conference, held March 13 – 16, 2016 Boston, MA, USA.
7. Zheng H, Zhao C, Roy S, et al. The Commonality of the Cancer Serum Proteome Phenotype as analyzed by LC-MS/MS, and Its Application to Monitor Dysregulated Wellness. Poster reprint first presented at AACR Annual Meeting 2016 Conference, held April 17-20, 2016 New Orleans, LA USA.
8. van Kempen, Léon CL, Karin E. de Visser, and Lisa M. Coussens. "Inflammation, proteases and cancer." *European journal of cancer* 42.6 (2006): 728-734.
9. Grivennikov, Sergei I., Florian R. Greten, and Michael Karin. "Immunity, inflammation, and cancer." *Cell* 140.6 (2010): 883-899.
10. Sun Z, Yang P. Role of imbalance between neutrophil elastase and α1-antitrypsin in cancer development and progression. *The LANCET Oncology* Vol 5 March 2004.
11. Law, Ruby HP, et al. "An overview of the serpin superfamily." *Genome biology* 7.5 (2006): 216.
12. Abnormal Profile of Serum Proteinase Inhibitors in Cancer Patients. CANCER RESEARCH 44, 2718-2723, June 1984
13. Brockmann, Marc A., et al. "Preoperative thrombocytosis predicts poor survival in patients with glioblastoma." *Neuro-oncology* 9.3 (2007): 335-342.
14. Brown, Kimberly M., et al. "Increased preoperative platelet count is associated with decreased survival after resection for adenocarcinoma of the pancreas." *The American journal of surgery* 189.3 (2005): 278-282.
15. Taucher, Susanne, et al. "Impact of pretreatment thrombocytosis on survival in primary breast cancer." *THROMBOSIS AND HAEMOSTASIS-STUTTGART-* 89.6 (2003): 1098-1106.
16. De Cicco, Marcello. "The prothrombotic state in cancer: pathogenic mechanisms." *Critical reviews in oncology/hematology* 50.3 (2004): 187-196.
17. Caine, Graham J., et al. "The hypercoagulable state of malignancy: pathogenesis and current debate." *Neoplasia* 4.6 (2002): 465-473.
18. Danckwardt, Sven, Matthias W. Hentze, and Andreas E. Kulozik. "Pathologies at the nexus of blood coagulation and inflammation: thrombin in hemostasis, cancer, and beyond." *Journal of Molecular Medicine* 91.11 (2013): 1257-1271.
19. Labelle, Myriam, Shahinoor Begum, and Richard O. Hynes. "Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis." *Cancer cell* 20.5 (2011): 576-590.
20. Falanga, A., et al. "The hypercoagulable state in cancer patients: evidence for impaired thrombin." *Blood Coagulation and Fibrinolysis* 5.1 (1994): S19-S23.
21. Mitchell, L., et al. "Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency." *Blood* 83.2 (1994): 386-391.
22. Brüggemann, Lois W., et al. "Experimental melanoma metastasis in lungs of mice with congenital coagulation disorders." *Journal of cellular and molecular medicine* 12.6b (2008): 2622-2627.
23. de Souza Cavalcante, Marcio, et al. "A panel of glycoproteins as candidate biomarkers for early diagnosis and treatment evaluation of B-cell acute lymphoblastic leukemia." *Biomarker research* 4.1 (2016): 1.
24. Amara, Umme, et al. "Molecular intercommunication between the complement and coagulation systems." *The Journal of Immunology* 185.9 (2010): 5628-5636.
25. Fabregat, Antonio, et al. "Reactome graph database: Efficient access to complex pathway data." *PLoS computational biology* 14.1 (2018): e1005968.
26. Dzik, Jolanta M. "The ancestry and cumulative evolution of immune reactions." *Acta Biochimica Polonica* 57.4 (2010): 443-466.
27. Markiewski, M. M., B. Nilsson, K. N. Ekdahl, T. E. Mollnes, J. D. Lambris. 2007. Complement and coagulation: strangers or partners in crime? *Trends Immunol.* 28: 184–192.
28. Esmon, C. T. 2004. Interactions between the innate immune and blood coagulation systems. *Trends Immunol.* 25: 536–542.
29. Krisinger, Michael J., et al. "Thrombin generates previously unidentified C5 products that support the terminal complement activation pathway." *Blood* (2012): blood-2012.
30. Zipfel, Peter F., and Christine Skerka. "Complement regulators and inhibitory proteins." *Nature Reviews Immunology* 9.10 (2009): 729.
31. Rutkowski, Martin J., et al. "Cancer and the complement cascade." *Molecular Cancer Research* 8.11 (2010): 1453-1465.
32. Reis, Edimara S., et al. "Complement in cancer: untangling an intricate relationship." *Nature Reviews Immunology* 18.1 (2018): 5.
33. Varela, Juan Carlos, and Stephen Tomlinson. "Complement: an overview for the clinician." *Hematology/Oncology Clinics* 29.3 (2015): 409-427.
34. Carroll, Michael C. "The complement system in regulation of adaptive immunity." *Nature immunology* 5.10 (2004): 981
35. van Pelt, G. W., et al. "Scoring the tumor-stroma ratio in colon cancer: procedure and recommendations." *Virchows Archiv* 473.4 (2018): 405-412
36. Ravensbergen, Cor J., et al. "The Stroma Liquid Biopsy Panel contains a Stroma-Epithelial Gene Signature Ratio that is associated with the histologic Tumor-Stroma Ratio and predicts survival in Colon Cancer." *Cancers* 14.1 (2022): 163..
37. Bergamaschi, A., et al. "Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome." *The Journal of pathology* 214.3 (2008): 357-367.
38. Janciauskiene, Sabina. "Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles." *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1535.3 (2001): 221-235.
39. Galmiche, Antoine, et al. "Coagulome and the tumor microenvironment: an actionable interplay." *Trends in Cancer* (2022).
40. Immune Checkpoint Therapy and the Search for Predictive Biomarkers. *Cancer J*. 2016 ; 22(2): 68–72.

.