# Stromal conditioning correlations using Stroma Liquid Biopsy™ blood-based biomarkers and tumor tissue profiles

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**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 conventional solid tissue biopsy. Applications for liquid biopsy data range from early detection, residual disease monitoring, and personalized medical decisions. Even so, 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 the current TNM staging system stratifies patients based on the extent of cancer spread, it is now overwhelmingly apparent that throughout cancer progression, tumor promoting inflammation is intrinsic to progressive disease. So although the TMN system provides guidance for clinical management, outcomes from local tumor excision at one end of the spectrum and adjuvant therapy at the other end, vary greatly among the variety of possible treatments within these stages. There remains great need for more personalized biomarkers to differentiate patient sub-populations and treatment options. For this, the supporting tumor microenvironments or stroma, must also be evaluated. Using proteomics and gene expression, we highlight the importance of the systemic inflammatory response to the presence of cancer anywhere in the body, largely derived from an unresolved innate immune response, see Figures 1 & 2. Also, that the tumor-stroma microenvironment is an important prognostic parameter for patients with epithelial cancer types[1]. A detailed review of all these aspects is included in the Stroma Liquid Biopsy™ whitepaper by reference[2].

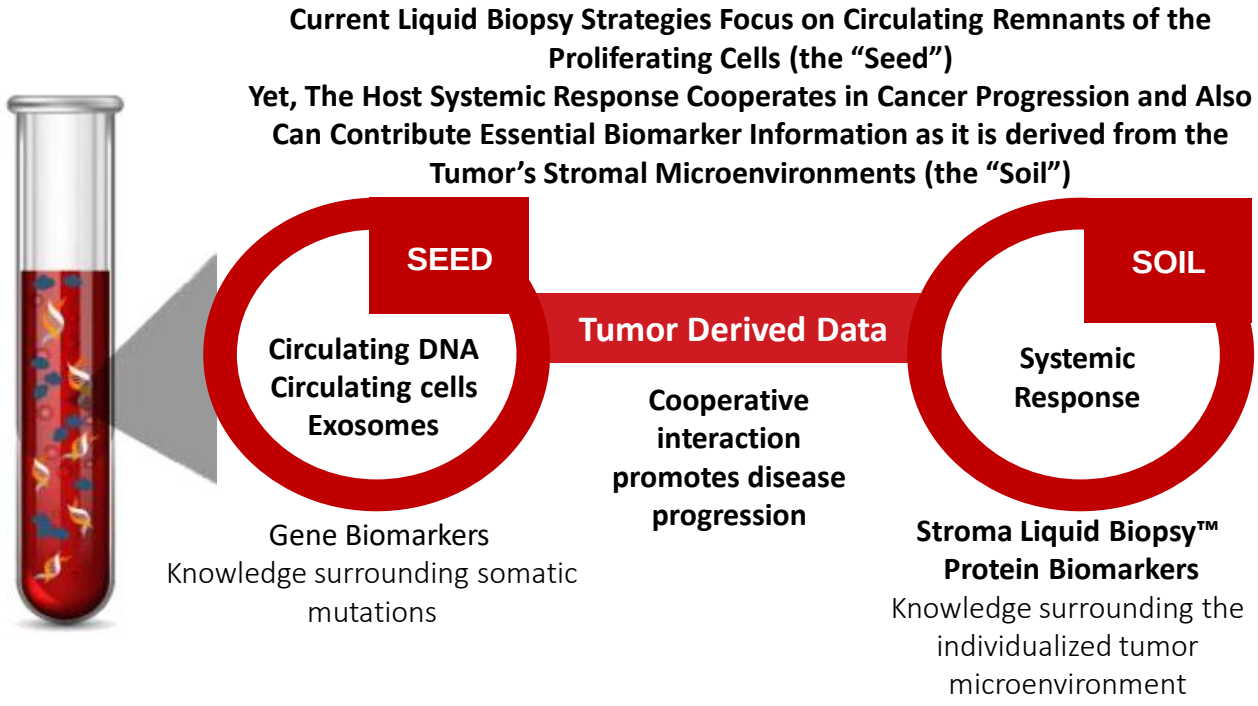


**Figure 1**

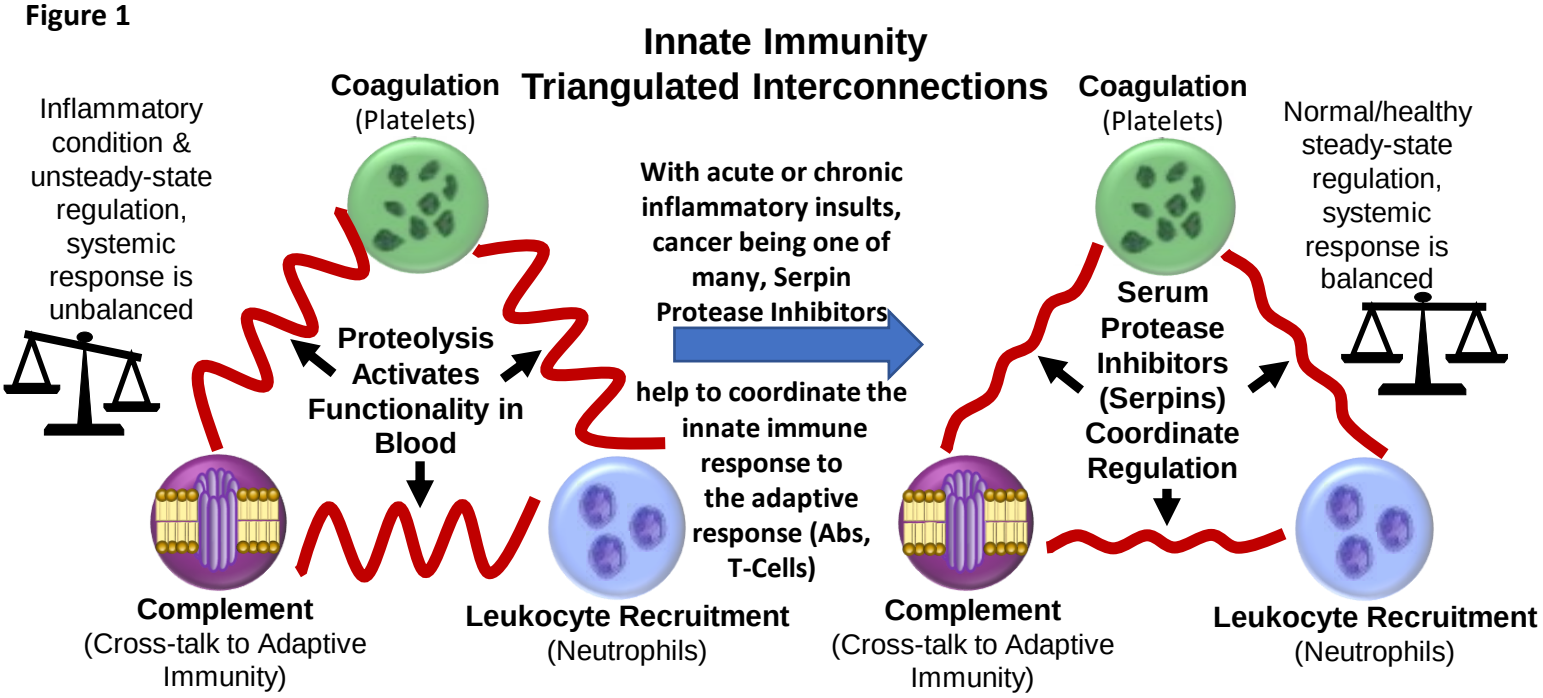
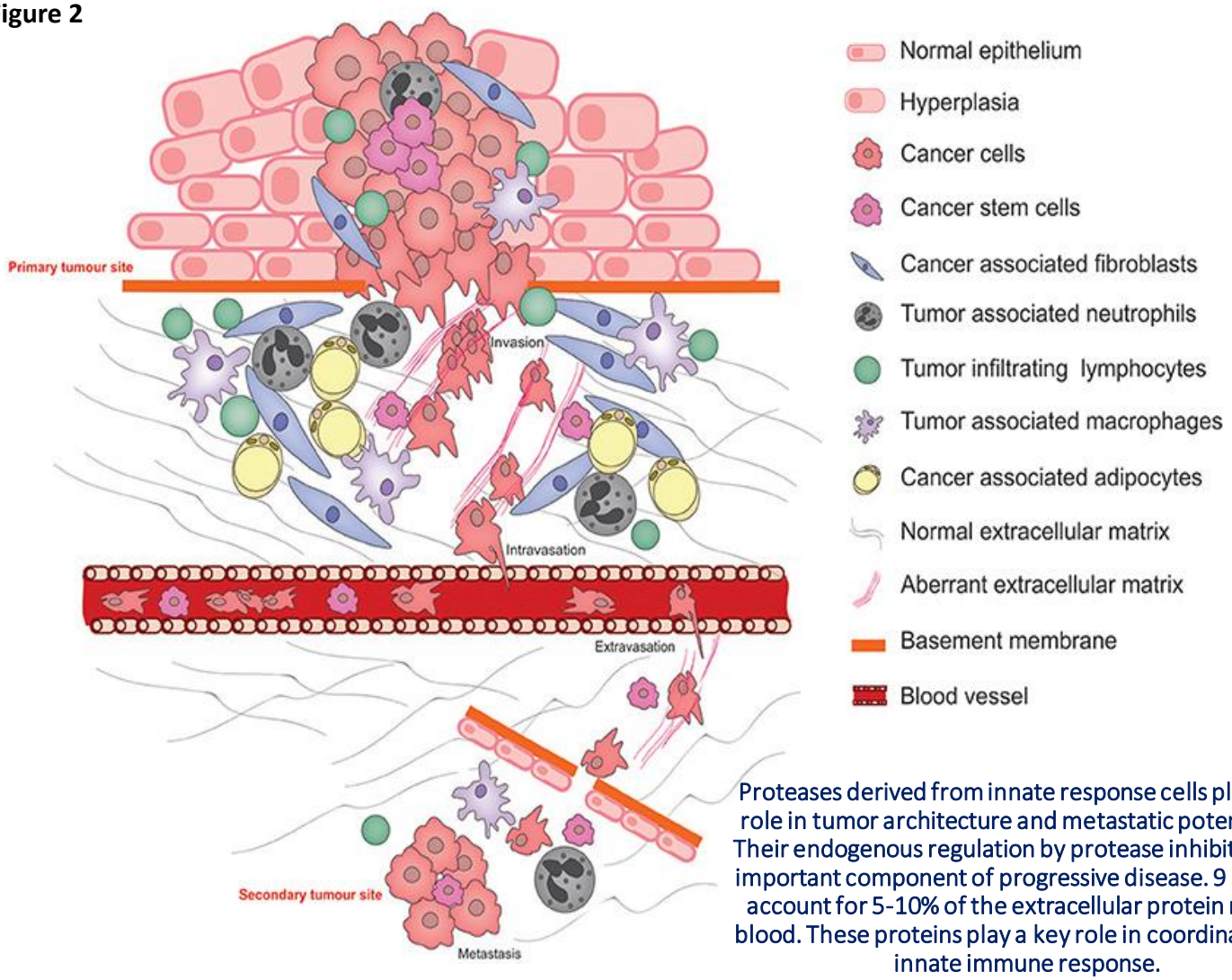



**Figure 2**

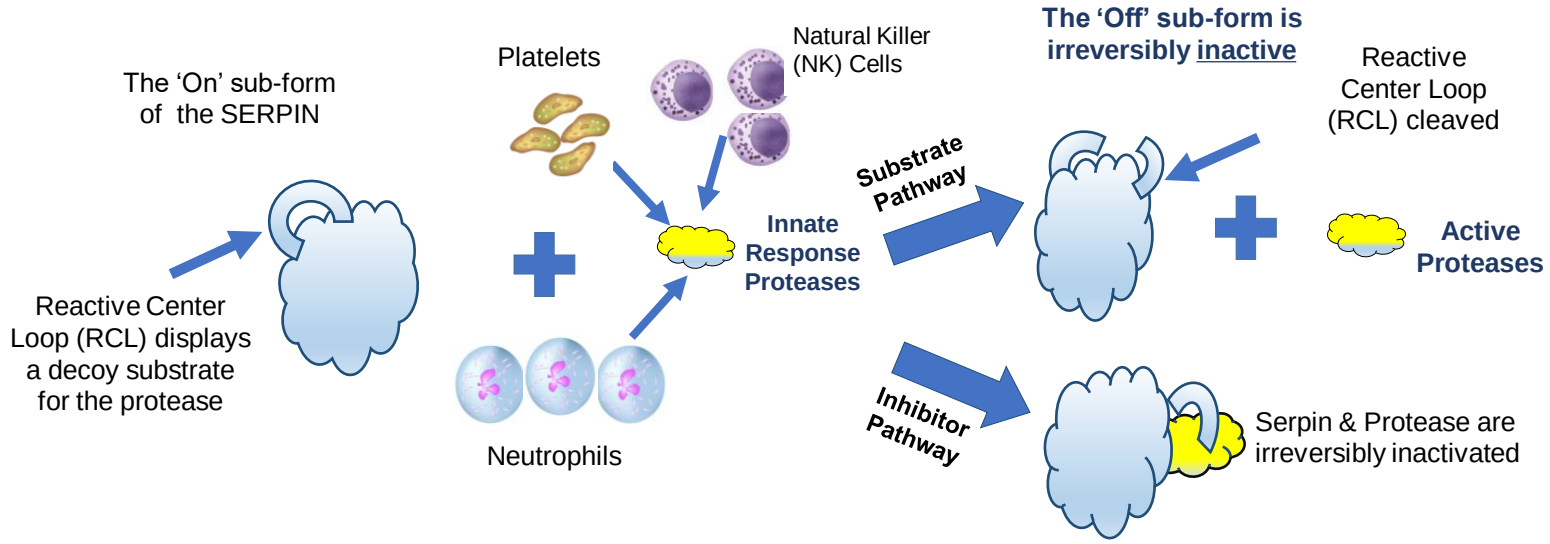



Proteases derived from innate response cells play a key role in tumor architecture and metastatic potentiation. Their endogenous regulation by protease inhibitors is an important component of progressive disease. 9 SERPINS account for 5-10% of the extracellular protein mass in blood. These proteins play a key role in coordinating the innate immune response.

| Protein Conc. Range > 5 log measurable in 1 LC-MS Analysis | | | | | |
|---|---|---|---|---|---|
| **Systemic Pathway** | **Protein Gene Name** | **Protein Description** | **Appx. 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 thresholds |
| **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** | ↓ **varies** ↑ | Inhibits Neutrophil Cathepsin G, complexes with Prostate Specific Antigen, might be tissue of origin classifier |

**Methods.** We report here on a unique sample prep workflow combining Albumin depletion in a negative selection strategy, and on-bead digestion (**AlbuVoid™ LC-MS On-bead**[4]), upfront to targeted LC-MS data acquisition performed by the Rutgers Proteomics Center by methods previously described[5]. Total spectral intensities were normalized to exogenous peptide standards. This was used to profile similar age/sex matched individuals with normal/healthy controls, with different cancers (Non-Hodgkins Lymphoma, Breast Stg 1, Lung Stg 3, Pancreatic Stg 2b), and individuals with other inflammatory diseases (obesity, diabetes, Rheumatoid Arthritis, Crohns, etc.), to determine whether proteomic profiles taken from blood, can differentiate these sub-populations.

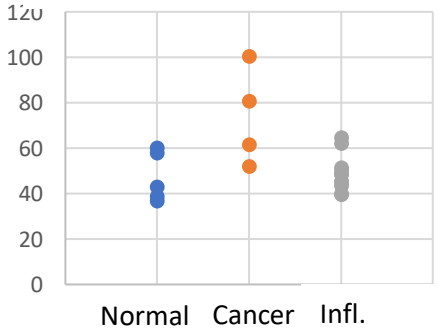
Unlike other protease inhibitors (i.e., Kunitz-type), SERPINs do not function in a strictly competitive concentration-dependent manner. Because of their "suicidal" bifurcated mechanism as shown, three functionally distinct sub-forms can exist. As platelets, neutrophils and natural killer (NK) cells continuously release proteases, the 'On' sub-form needs be sufficiently generated to resolve the innate response. However, a higher amount of 'Off' sub-forms may also occur challenging the measurement dogma of one gene= one function. When the 'On' sub-forms are insufficient, the systemic regulation of innate immunity becomes unbalanced, the 'protease storm' continues unabated, and the adaptive (i.e., T-Cell) response can become delayed, altered or paralyzed.




**Inhibitory Serine Protease Inhibitors (SERPINs) can circulate as three functionally distinct sub-forms. Current proteomic methods do not adequately distinguish these sub-forms, creating analytical bias depending upon the measurement platform. Our patent pending methods using LC-MS analysis, uniquely monitor the most important sub-form for characterizing the innate immune response – the functionally active sub-form[3]. Here we report using a ratio of the non-RCL tryptic peptide regions relative the RCL region uncovers functionality – the higher the ratio the less proportion of functional sub-forms.**

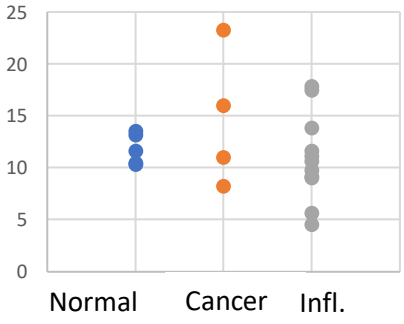
**Serpin Functionality**

Alpha-1-Antitrypsin
[SERPIN A3] Ratio non-RCL/RCL

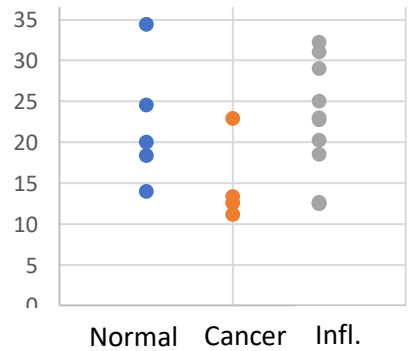



Normal & Inflammatory are very consistently and tightly aligned

Alpha-1-Antichymotrypsin
[SERPIN A3] Ratio non-RCL/RCL

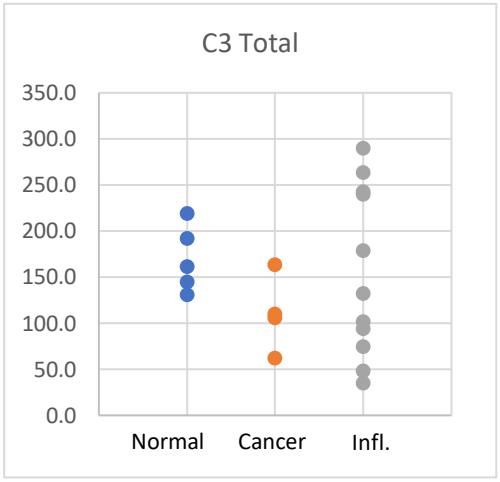


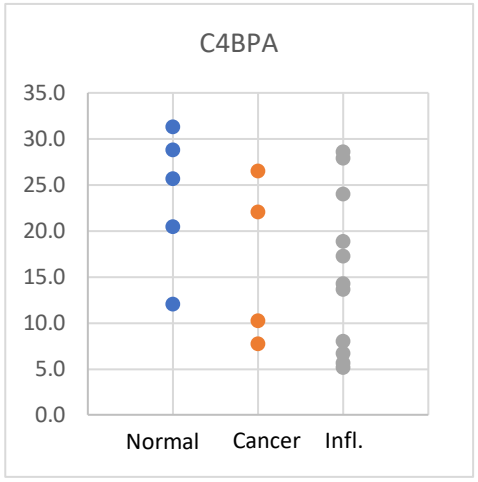

Heparin Cofactor II [SERPIN D1] RCL

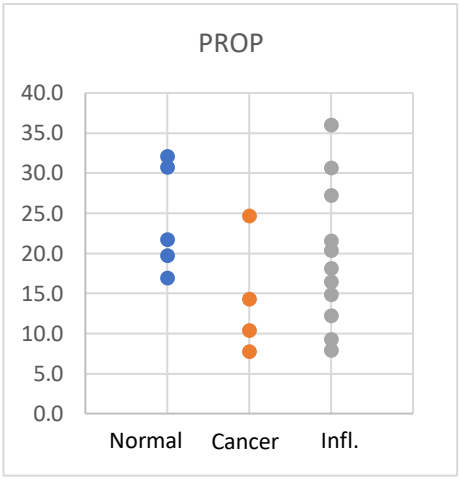



**Taken as a set, though not for all in all cases, pattern profiles of Serpin RCL regions hold promise to distinguish between normal, cancer, and other inflammatory conditions.**

## Complement Proteins

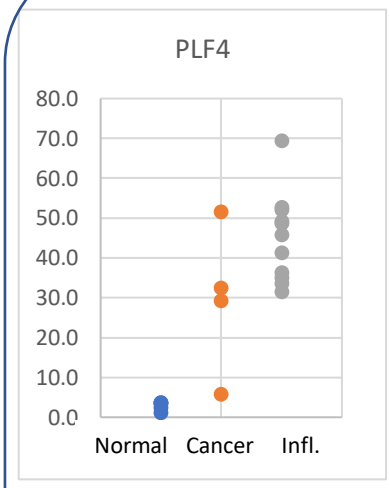


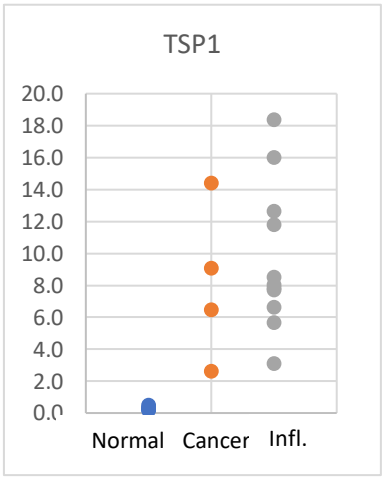



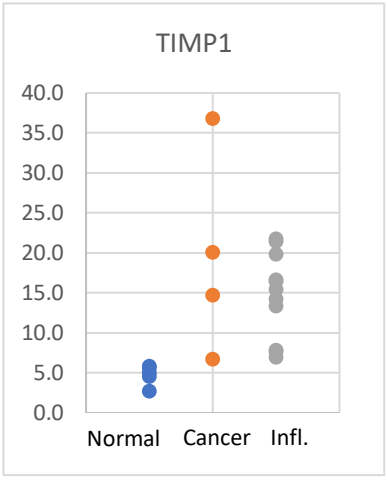



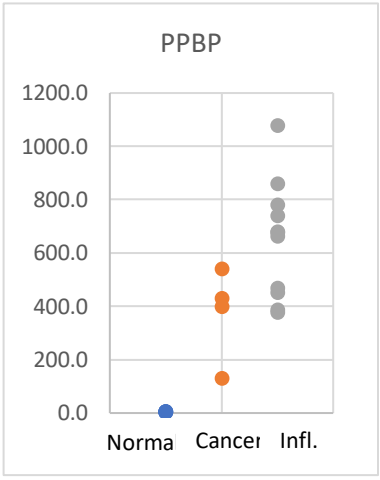

**Compared to Normal, one or two Complement proteins go down in all 4 cancers, other inflammatory generates a wide range of data.**

## Coagulation Proteins



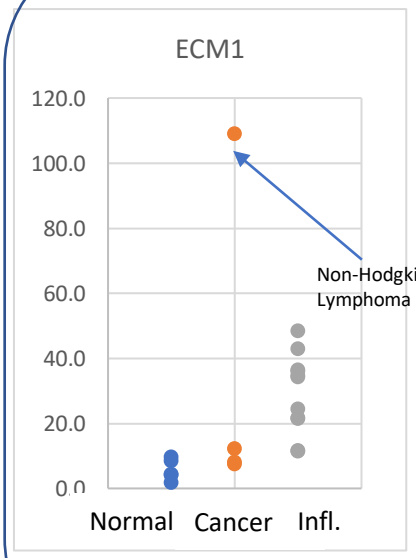



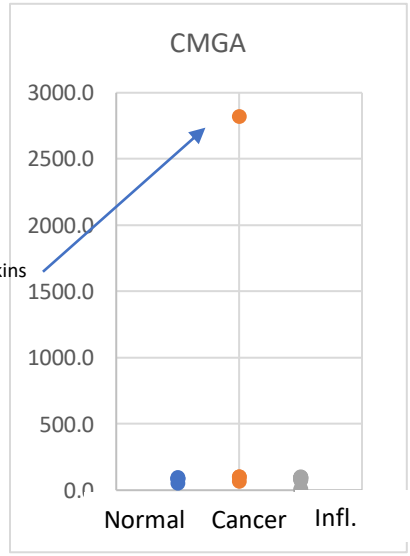



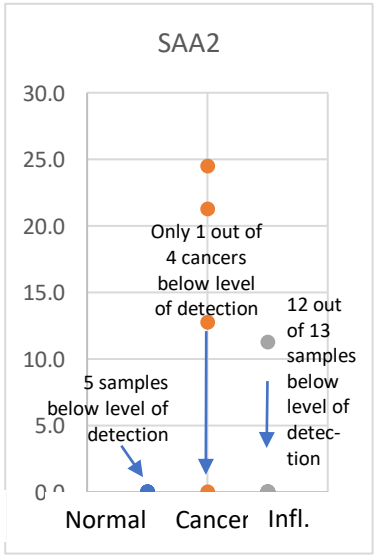




**Cancer and other inflammatory conditions are highly distinguishable from normal with the coagulation panel of proteins. Other inflammatory are also highly up relative to normal.**

## Other Acute Phase Proteins from the Stroma Liquid Biopsy™ panel

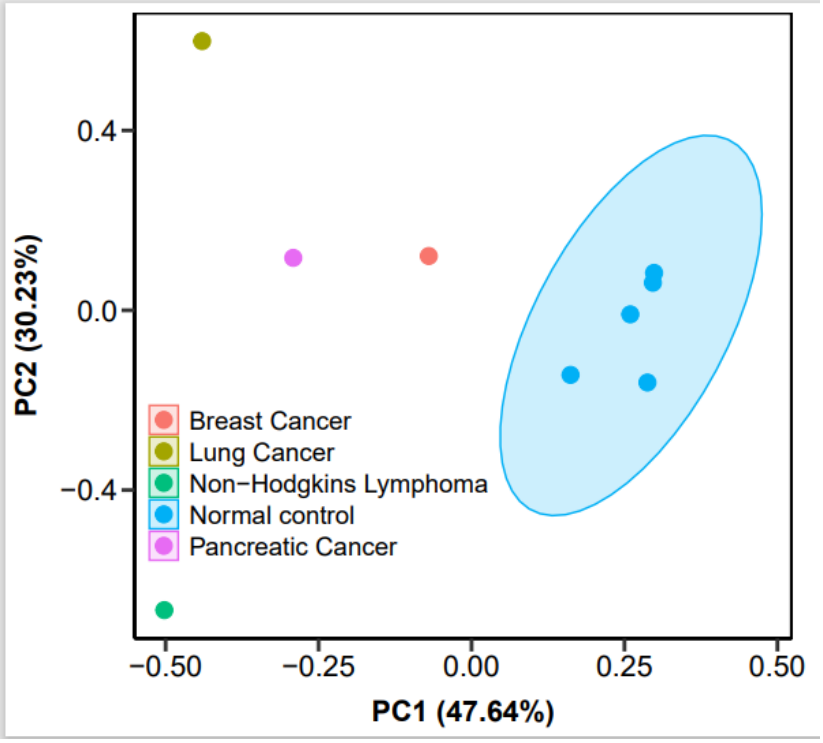



**ECM1 goes up in cancer but to greater extant in the other inflammatory, so there may be thresholds to distinguish cancer from other inflammatory. This needs further investigation. The same for ECM1 and CMGA, while drastically elevated in only Lymphoma, may be a strong biomarker for Lymphoma or hematologic cancers vs. solid tumors. This also needs further investigation. Noteworthy is that SAA2 holds promise as a biomarker to distinguish all three sub-populations. Neutrophil Elastase, though part of the Stroma Liquid Biopsy™ panel is not reported here as it is very low abundance and optimal methods for measurement separate from those used here, needs development.**

## Principal Component Analysis (PCA) of Stroma Liquid Biopsy™ data

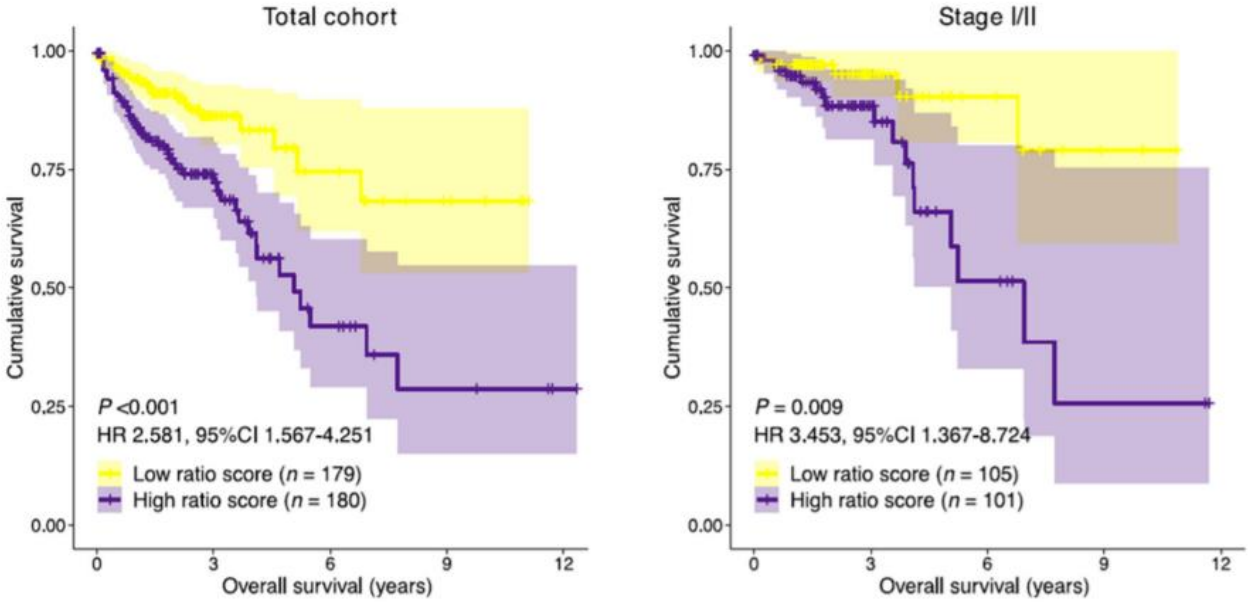


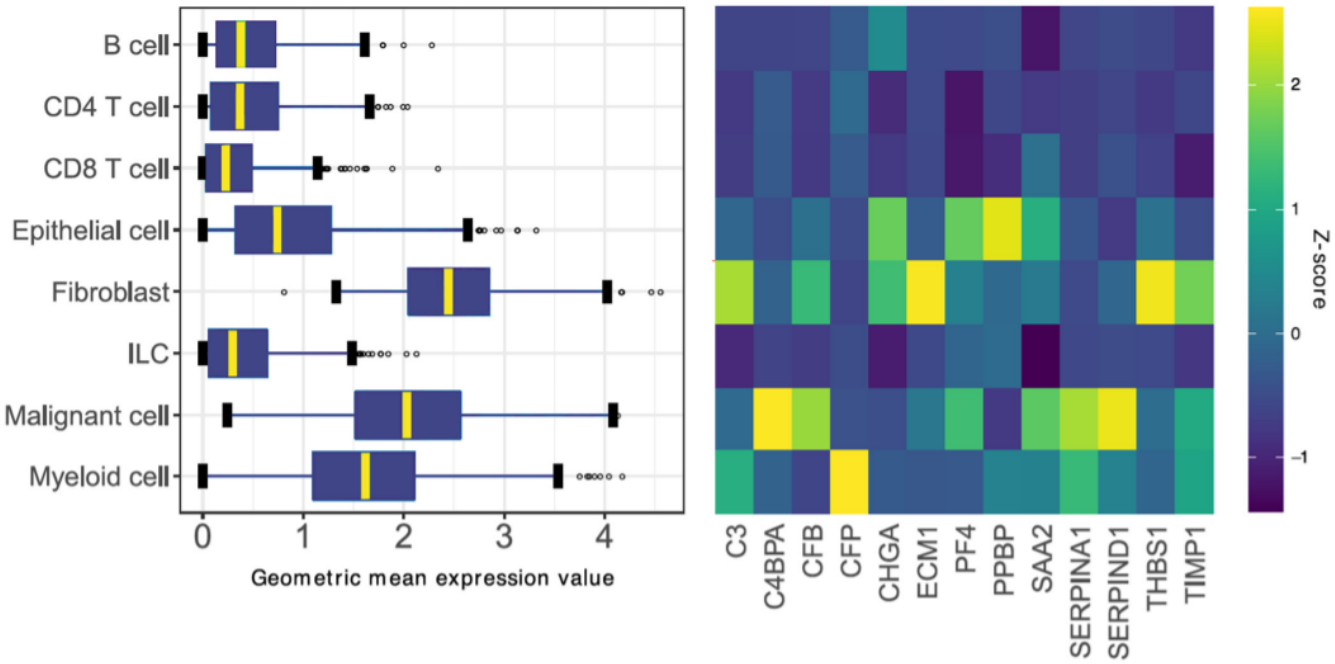

PCA tries to capture the variance of multidimensional data (i.e. multiple variables like our protein concentrations) into a 2D plot to determine clusters. The percentages at the X and Y axis represent how well the PCA was capable to explain the variance in the data and thus find clusters.

In the plot there are significant differences in sample protein concentrations between the healthy controls and the four cancers. Based on PC1 and PC2, the PCA was not able to make distinct clusters of the inflammatory conditions and cancer samples. However, this does not necessarily mean there are no differences between the two groups, just that the PCA was simply not able to detect them given the state of variance within this data. As such, further methods and thresholds development using exogenous heavy labeled peptides in an MRM workflow is planned for future studies; the adjacent workflow reported here. In addition to that, combinations with multi-omic markers (protein, gene, metabolite), holds promise in a clinical decision strategy to rule in/rule out cancer from other inflammatory conditions.

To better characterize tumor biology, Leiden University Medical Center has developed a histopathology based tumor-stroma ratio (TSR) using stained resected tumor tissue. This is reported to be a strong, independent prognostic parameter for a range of solid epithelial tumors[1]. 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/. Using gene expression, we describe a first theoretical framework that associates a gene expression signature, called the Stromal-Epithelial (Stro-Epi) Gene Signature Ratio to the protein panel known as Stroma Liquid Biopsy™. To begin supporting this hypothesis, we highlight an *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[6].

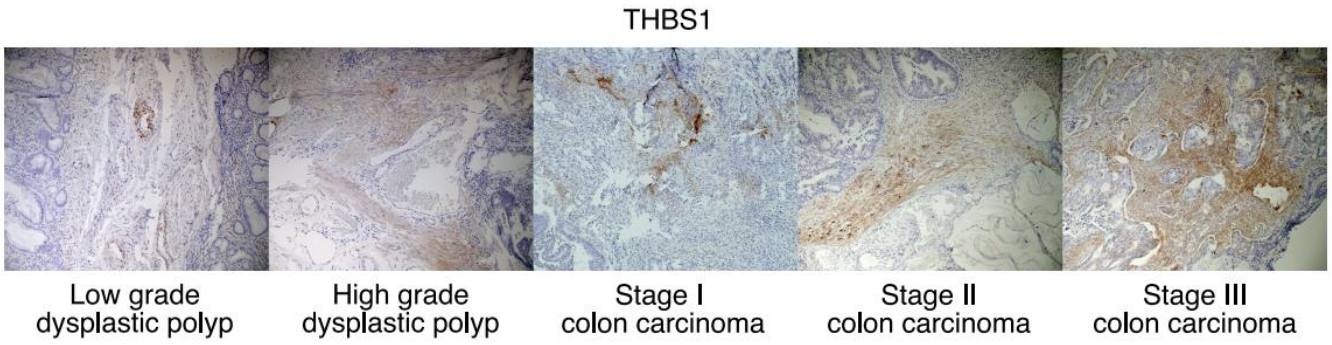
The **Stromal-Epithelial Gene Signature Ratio**, is based on genes from the Stroma Liquid Biopsy™ panel, and classified by their expression as being derived from stromal-phenotypic or epithelial-phenotypic cells.






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. This report provides a first theoretical framework for proteomic signatures to potentially serve as an indicator for tumor-stroma content when applied to liquid biopsy.

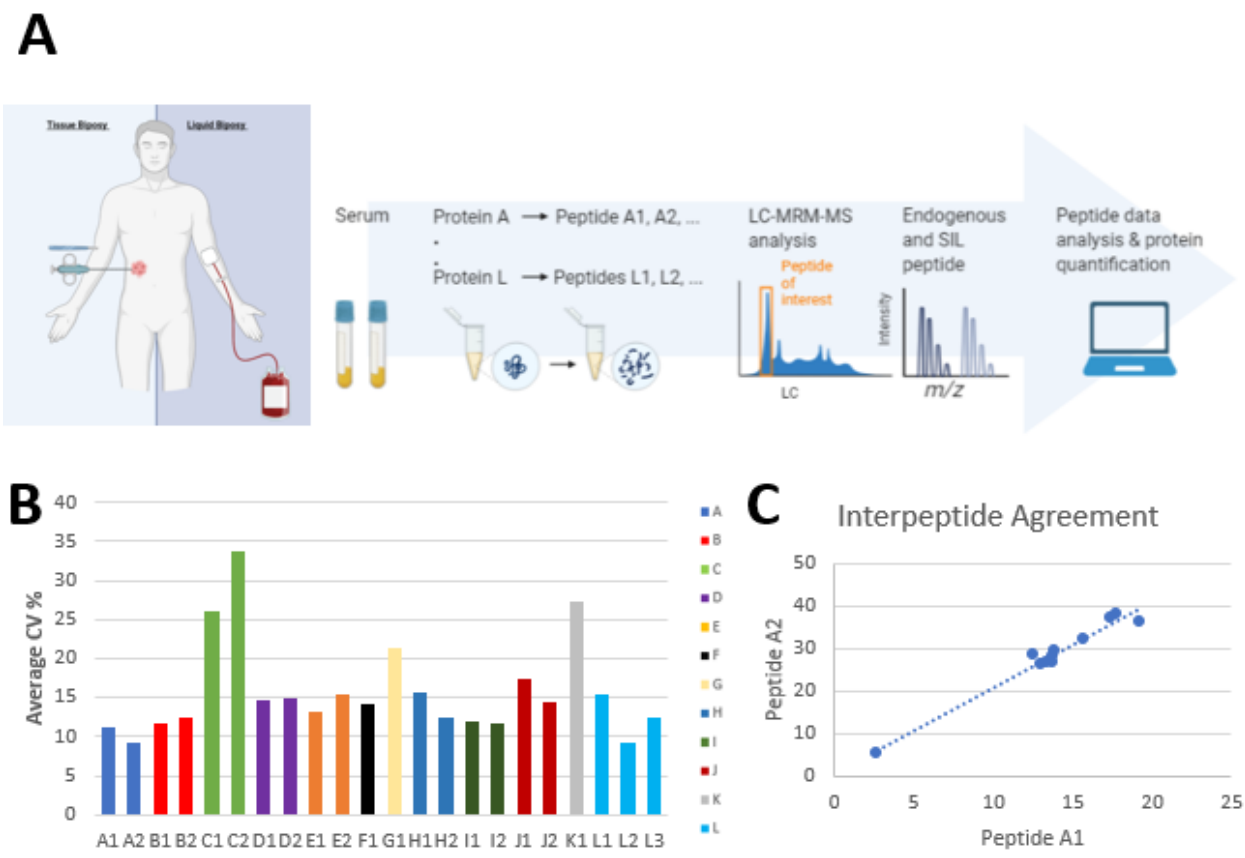
Immunohistochemistry images that depict Stroma Liquid Biopsy™ marker -THBS1 protein expression across the different stages of tumor progression. Basically, everything staining brown is protein expression, while blue is background.




For THBS1, we noticed a significant increase in **stromal** expression in successive tumor stages, starting at pre-cancerous low grade colonic polyps up to stage III colon carcinoma.

**Development of LC-MRM-MS protein quantification methods to determine the tumor-stroma ratio in a liquid biopsy**

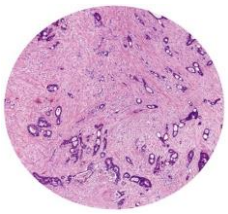
Full conversion of a protein into (proteotypic) peptides is not necessarily needed for *identification* purposes, however in the case of *quantification* this step requires careful attention. Whereas for protein identification purposes a confident assignment of one, or often two peptides is sufficient, multiple (proteotypic) peptides need to be evaluated for each single protein for quantification purposes. This evaluation starts with the determination of peptide intensities and their variations in replicate measurements without internal calibration with SIL-peptides (exemplified in Figure 1B). The peptides that are hardly visible or have intensities that vary in different experiments are likely to fail for the final selections. A next step in the evaluation concerns the variation in digestion. When each protein of a certain identity is fully converted into the peptide of interest this is referred to as equimolar digestion of that specific protein, but this does not always happen in practice. The evaluation of so-called interpeptide agreement at different protein concentrations provides a first clue in how efficient a protein is digested (Figure 1C). For quantification, SIL-peptides are synthesized peptides with identical structural formula as the endogenous peptides of interest but differ in mass due to the inclusion of specific amino acids that contain 13C and 15N, *i.e.* heavy amino acids. A SIL-peptide is spiked into the samples of interest as early as possible during the sample preparation process in order to correct for losses of the corresponding target peptide. Using this workflow, we have successfully performed feasibility on the Stroma Liquid Biopsy™ panel on cancer patient samples, and will soon be reporting on larger patient cohorts.



**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, through our liquid biopsy approach, we can create an 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. From this, we will be able to accurately quantify intra-tumoral stromal content via a simple blood draw.**

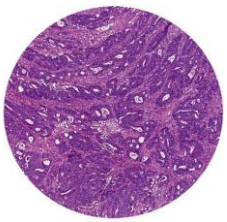
**Proposed tumor characteristics**

**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 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

**Discussion**

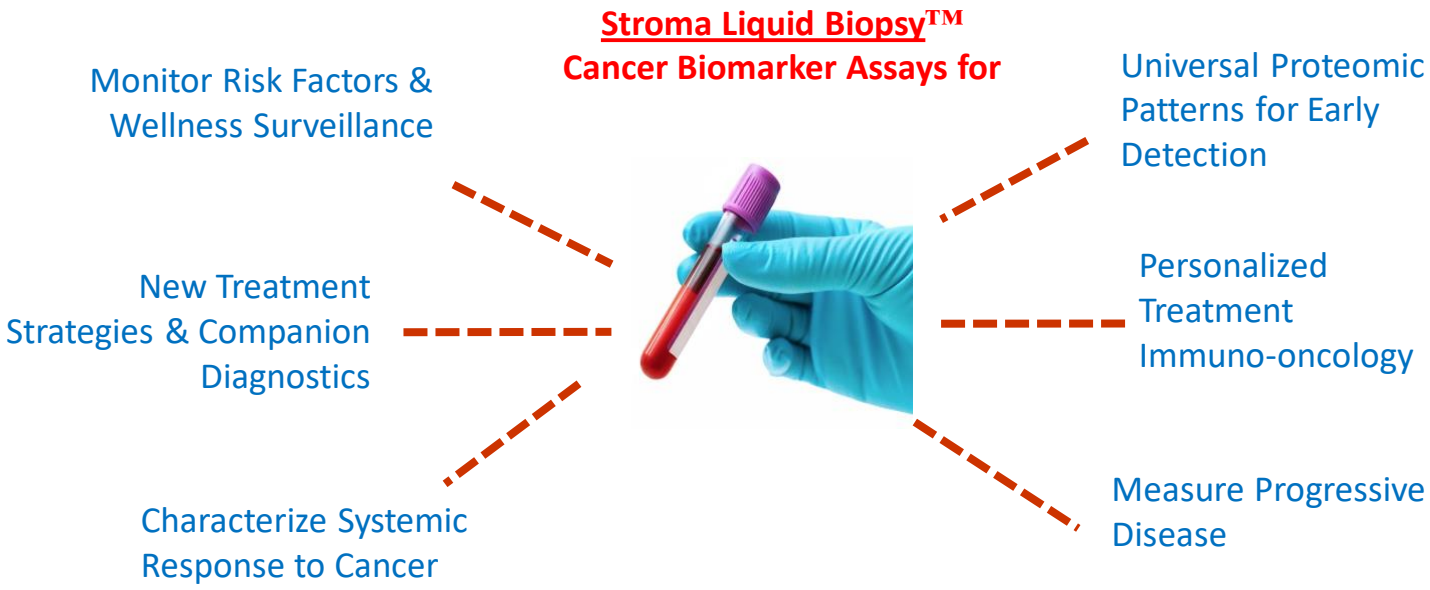
We considered that many of the inflammatory biomarkers in our proteomic panel, might also be involved with other chronic inflammatory conditions. Though preliminary, the Stroma Liquid Biopsy™ coagulation biomarkers in particular were severely differentiated in serum samples from patients with cancer and other inflammatory conditions, compared to normal/healthy. Acute-phase proteins ECM1 and SAA2 offer possible differentiators between cancer and other inflammatory diseases. Most promising is that three protease inhibitors (SERPINs A1, D1, A3), as measured by functional sub-forms, may offer an especially attractive differentiator for not only the presence or absence of cancer, but also a profile of stromal conditioning for those with cancer. Such profiles will help in clinical decisions to rule in or rule out cancer as a possible diagnosis, and to consider treatment options based on stromal considerations. Our observations here corroborate other reports showing that persistent inflammation in or around developing neoplasms regulates many aspects of tumor development, from initiation all the way to metastatic progression. Also, that clinical outcome is strongly related to stromal characteristics. Such stromal character is not being monitored by current TNM staging or by other gene-based liquid biopsy platforms. Our new evidence supports that there are two basic tumor types, stroma-low and stroma-high, that can be classified by a variety of histopathology methods and multi-omic signatures, and have important phenotypic characteristics that associate with long-term survival.

**Conclusion**

We conclude that the stromal conditioning protein blueprint, as captured by the Stroma Liquid Biopsy™ panel, encompasses a more discreet stratification of the tumor and patient prognosis, and offers new insights into therapeutic strategies that might beneficially modulate the tumor-microenvironment.

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 proteomic platforms.




**We welcome commercial partnership opportunities to 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**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