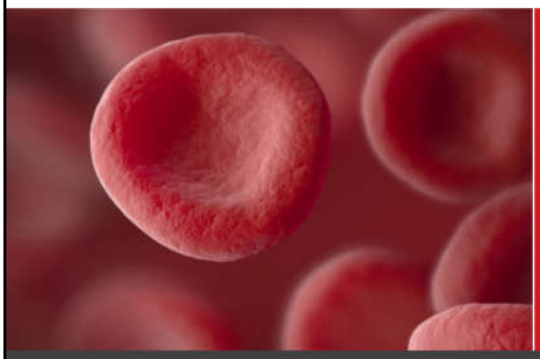
BIOTECH SUPPORT GROUP
Sample Prep that Matters

rakta



Stroma Liquid Biopsy

Biomarkers of the Dysregulation of the Serum Proteome in Cancer

Presented at the 16th Human Proteome Organization World Congress
Dublin Ireland 17-21 September 2017

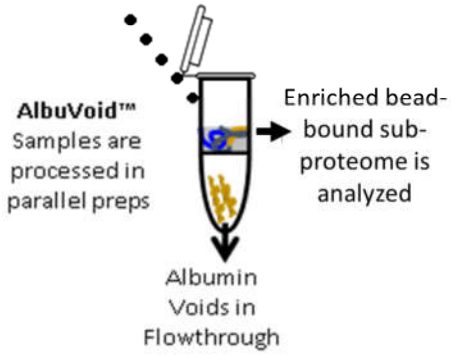
Biotech Support Group applies proteome separations technology to research products and methods. Through investigation of these methods, the company has submitted intellectual property surrounding protein biomarkers in blood that become re-proportioned in cancer. We now plan to commercialize these discoveries as a separate business unit (Rakta) apart from its other business, through partnerships and collaborators.

Introduction:
Is there a common systemic response to cancer?

Unique workflow supported a new window of observation, uncovering a common dysregulated pattern of proteins, across different primary tumors which can be monitored and quantified by LC-MS.

Workflow Considered:

- Albumin Depletion Using AlbuVoid™ bead separation
- On-Bead & Eluate Digestion
- Single 3 hour LC-MS acquisition
- TMT labels or targeted label-free
- >200 Proteins observed
- Primary tumors: Breast, Lung, Pancreas, Lymphoma, Ovary



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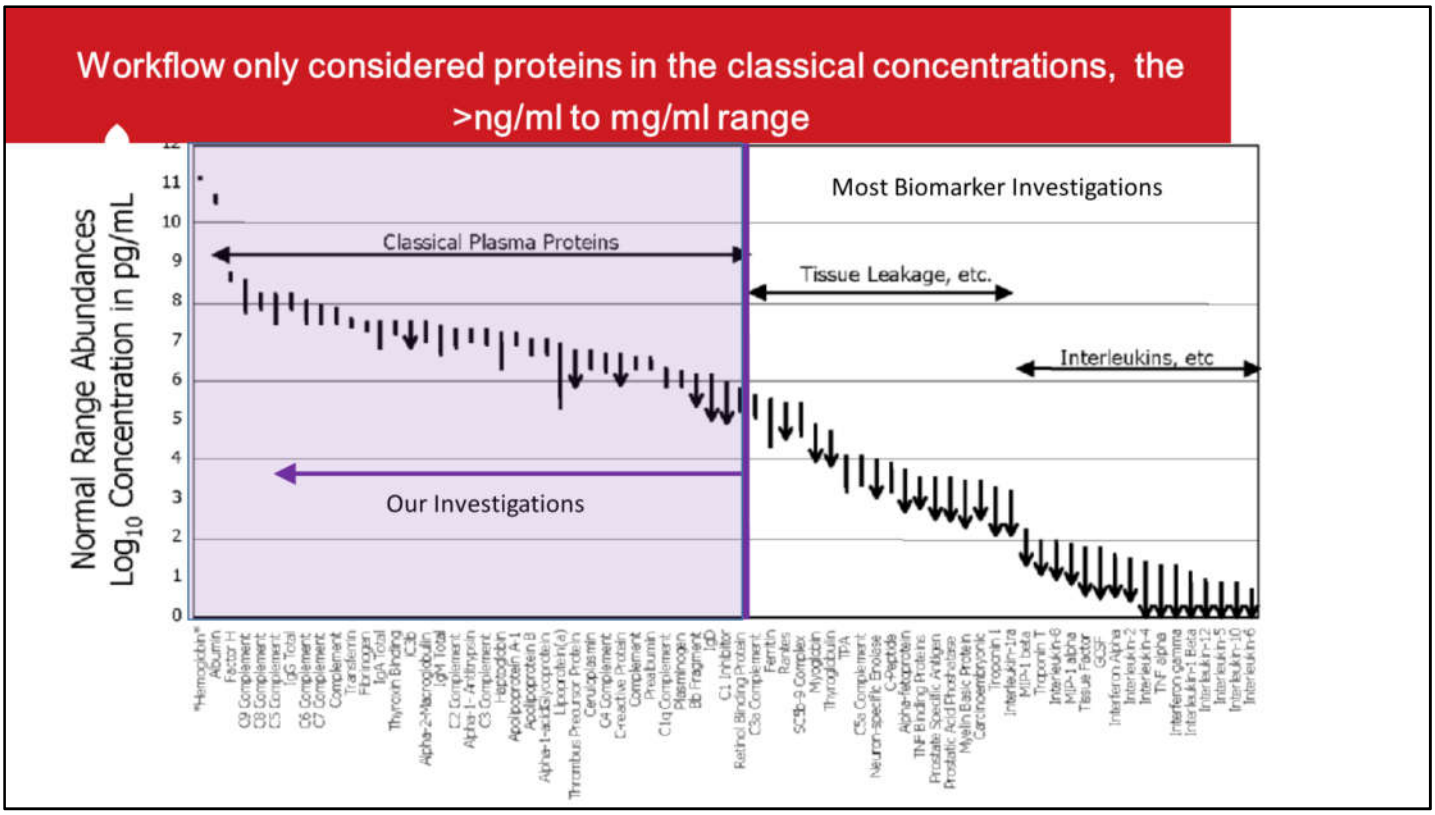
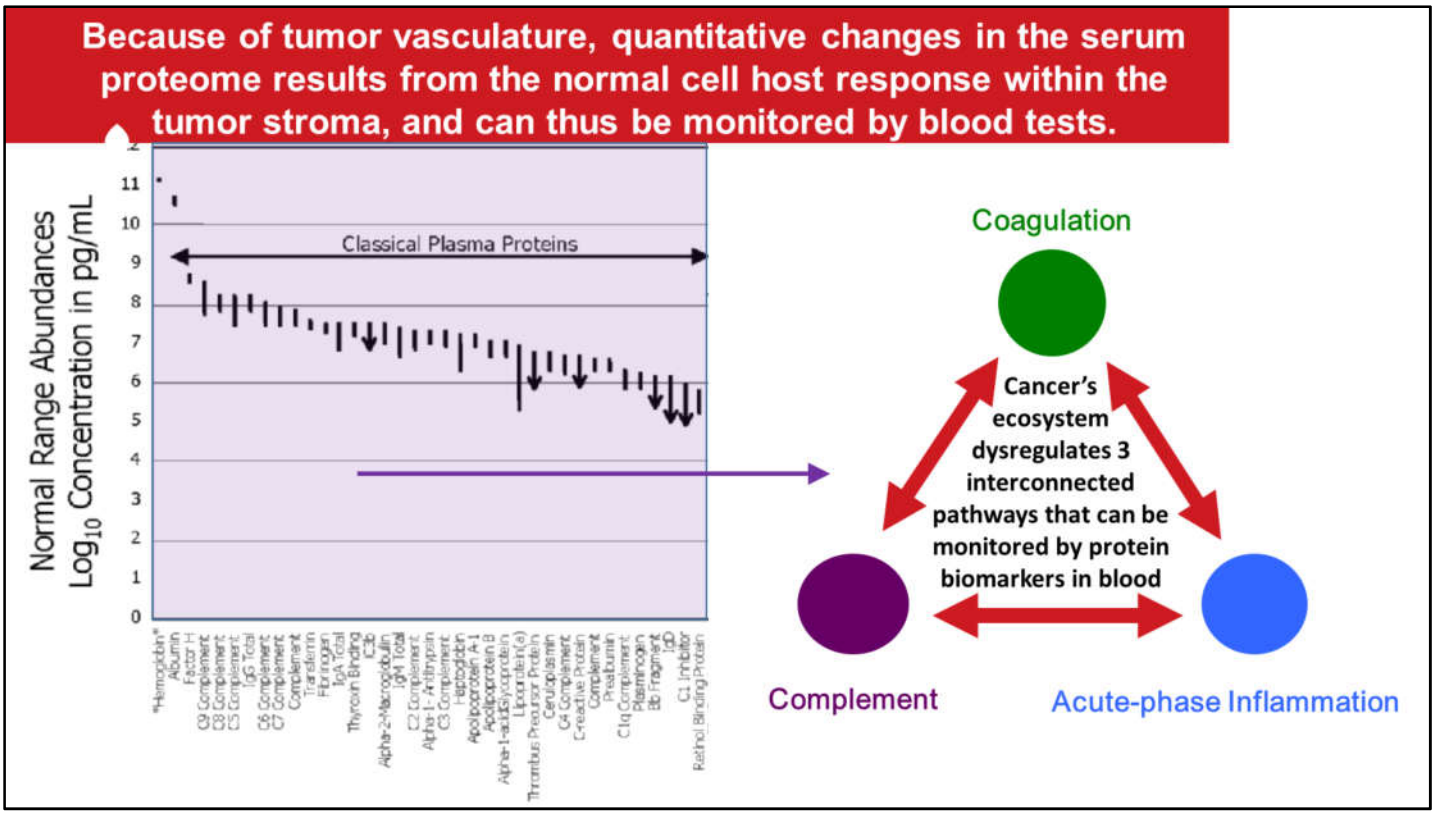
We continuously evaluate our sample prep and enrichment products for proteomic research, so as to improve workflows and provide unique windows of observation. For this study we utilized a selective voidance strategy to greatly reduce the interference from serum Albumin. By incorporating AlbuVoid™, followed by LC-MS/MS analyses, our initial results were pared down to a target panel of serum protein markers. These were then measured label-free by multiple reaction monitoring (MRM), providing evidence for a pattern or signature significantly different in the cancer patient population compared to an approximate sex/age matched normal and healthy control population.



Discovery of protein biomarkers that can detect cancer early and personalize a treatment process has become an important research area in the proteomics field. For this, many proteomics approaches are being implemented in cancer research. Most biomarker investigations focus on very low abundance (pg/ml range) proteins shed from cancer cells, at the very limits of quantitative LC-MS analysis. To discover, characterize and monitor these 'needle in the haystack' biomarkers remains an industry wide challenge.

By contrast, our investigations focused on the serum proteome range often not considered in biomarker investigations. Yet, an important advantage of the use of biomarkers within this window is that they are all highly observable with serum concentrations in the [µg-mg]/ml range. Furthermore, this range has been previously determined to be quantitative by LC-MS/MS with precision comparable to current clinical immunoassays.



We hypothesized that because of tumor vasculature, changes in the serum proteome might result from the host cell response within the tumor-associated stromal microenvironments, and can thus be monitored by blood tests. We can now envision such measurements contributing to a Stroma Liquid Biopsy™ and we adopt this new term here to differentiate our model from previous liquid biopsies.

The localized microenvironments of tumors cooperate to subvert normal homeostatic mechanisms in blood, consistent systemically with the presence of 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.

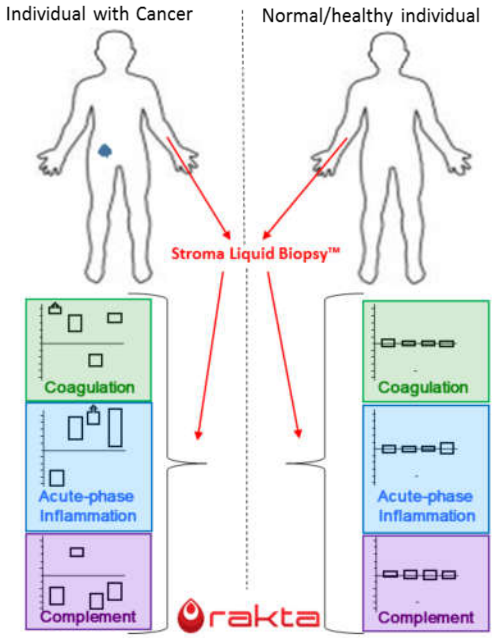
Yes, the common response is reflected in biomarkers for:

- Coagulation
- Acute-phase Inflammation
- Complement Cascade

All of these pathways are

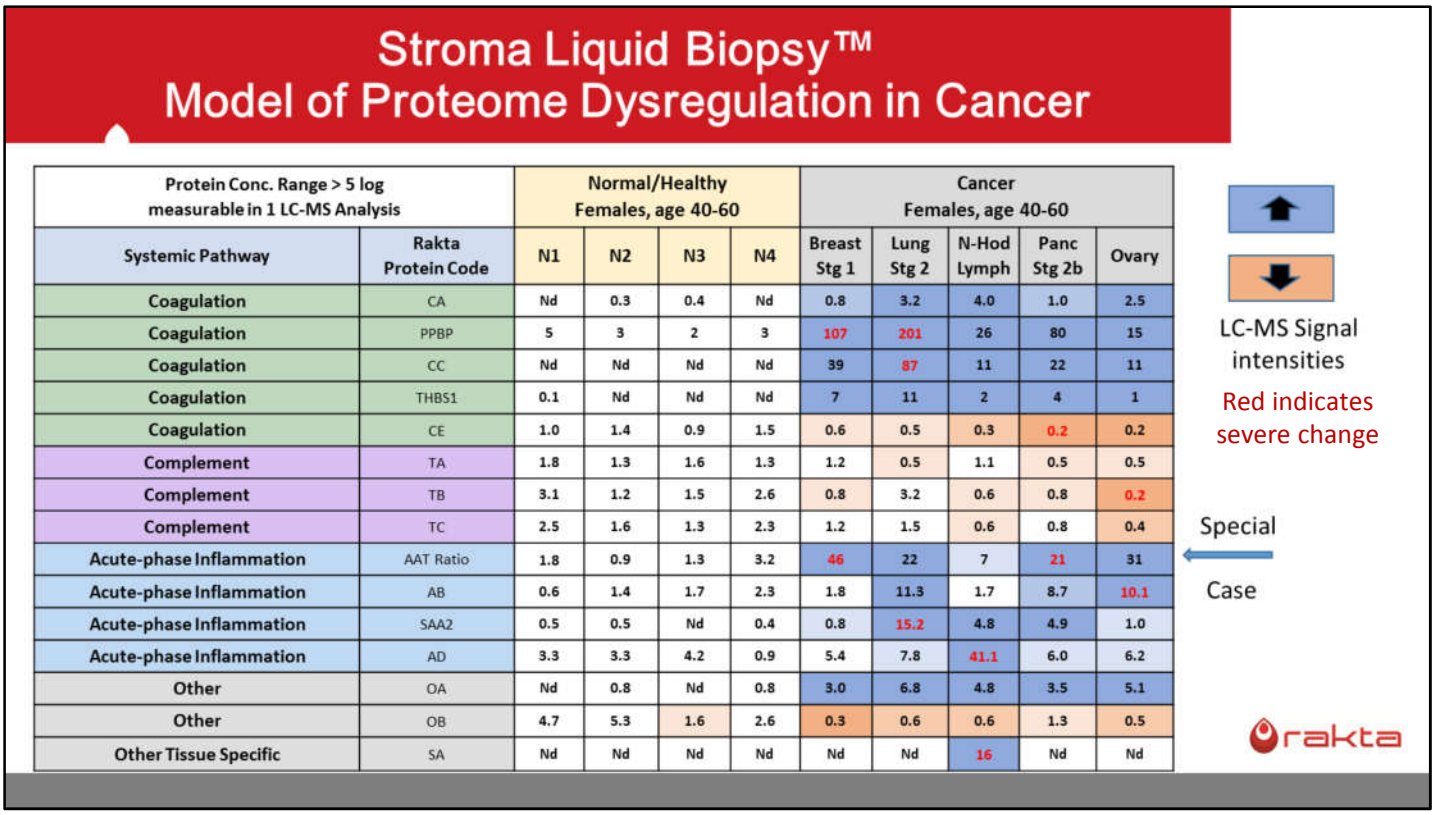
> systemically interconnected

> regulated through proteolytic PTMs



The special significance of this pattern is that serum proteome changes were categorical and primarily contained within these three host systemic response pathways: acute-phase inflammation, coagulation, and the complement cascade.

Furthermore, this study signifies the importance of these pathways intercommunicating in the vast circuitry of cascading proteolytic events, the predominant mechanism for controlling acute insults in the bloodstream. Because proteolysis is irreversible and therefore highly regulated, the pathways in our model cannot be viewed as separate independent cascades, but rather as one interdependent system with extensive cross-talk, mutually fine-tuning their functional status.



Coagulation

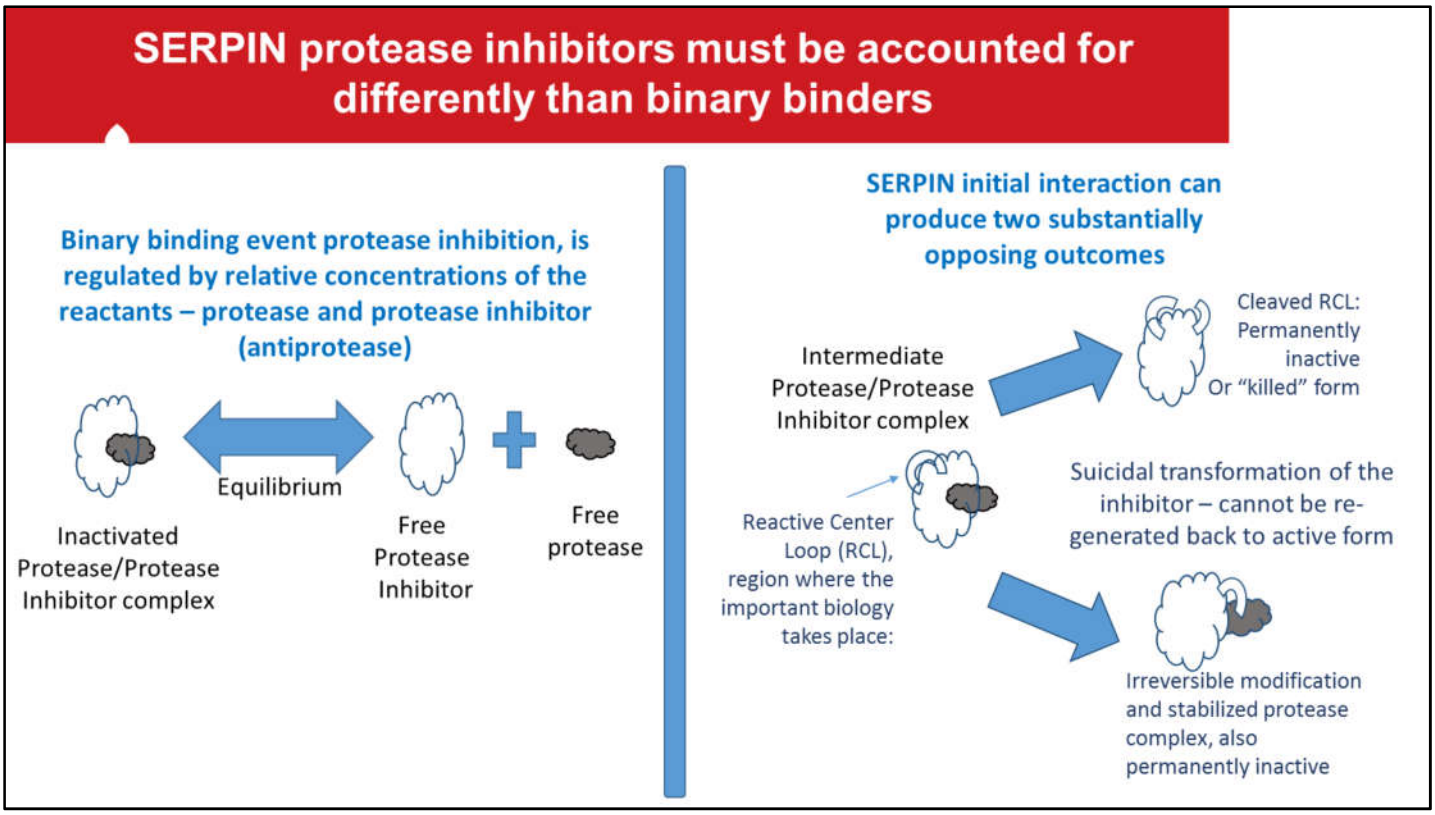
Patients with malignancy display a hyper-coagulation state that include the production of pro-coagulants directly from the tumor, as well as some general systemic responses of the host to the tumor, notably from inflammation and angiogenesis. That the coagulation system conspires in support of cancer progression serves to illustrate a normal homeostatic function being dysregulated in cancer pathogenesis.

Complement Cascade

Complement provides a powerful defense mechanism against pathogens as well as an endogenous danger sensor. However, dysregulated complement activation can have a significant role in both acute and chronic inflammatory conditions. Though its role in cancer is complex and remains inconclusive, activated complement component proteins are abundantly dispersed throughout the inflammatory tumor microenvironment.

Acute-Phase Inflammation

The functional relationship between inflammation and cancer is not new and is now generally accepted that many cancers arise from sites of infection and chronic inflammation. Acute inflammatory diseases are usually self-regulating but persistent inflammation, comes in play as a failure of mechanisms to resolve acute inflammatory response. One such special inflammatory case in our proteomic biomarker pattern is Alpha-1-Antitrypsin (AAT).



Alpha-1-Antitrypsin or SERPINA1, belongs to the SERPIN family of suicidal serine protease inhibitors. SERPINs play an integral role in regulating a wide variety of biological activities, and represent 2-10% of circulating plasma proteins. They regulate coagulation, hormone transport, complement, inflammation, angiogenesis, and blood pressure along with many other pathways. This unique family of protein inhibitors have been associated with progression or remission of cancer and so they may become valuable biomarkers for therapeutic or diagnostic use.

Unlike more conventional binary binding inhibitors, the initial interaction of the protease to the Reactive Center Loop (RCL) of inhibitory SERPINS, produces two seemingly opposing outcomes:

1. An irreversible covalent interaction, inactivating the protease through the stabilized protease complex, or
2. A suicidal transformation of the inhibitor, cleaving the RCL region and permanently inactivating its inhibitory potential.

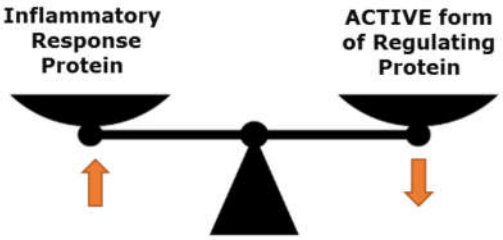
Proteolysis controls systemic dysregulation in Cancer – Protease Inhibitors control Protease Regulation

Column A	Description	Suicidal Cleavage Site	Normal/Healthy Females, age 40-60				Cancer Females, age 40-60				
			N1	N2	N3	N4	Breast Stg 1	Lung Stg 2	N-Hod Lymph	Panc Stg 2b	Ovary
sp|P01009|A1AT_HUMAN Total x100	SERPINA1		12.2	9.9	9.8	4.7	4.7	11.0	28.4	8.5	2.6
AAT RCL peptide x10000	GTEAAGAMFLEAIPMSIPPEVK		6.8	10.8	7.5	1.5	0.1	0.5	4.0	0.4	0.1
Ratio: AAT/[AAT RCL]			1.8	0.9	1.3	3.2	45.7	21.5	7.1	20.7	30.6

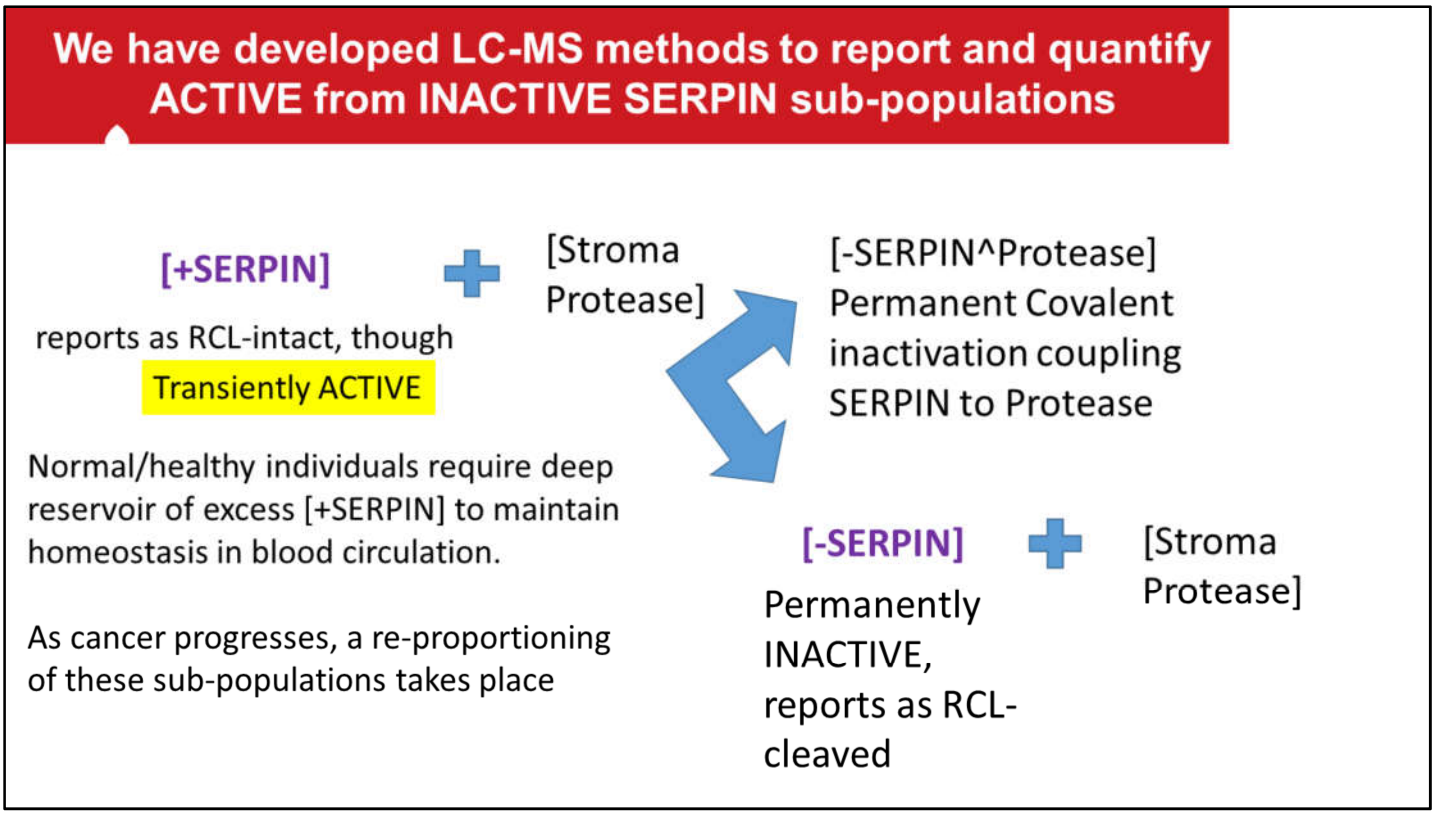
- Unmasking a cancer distinguishing ratio of 2 sub-populations of a highly abundant house-keeping protein, previously quantified as one homogeneous population, which would otherwise not be observable by conventional proteomic methods
- By ratio reporting metrics, we now have methods to observe that ACTIVE form is severely downregulated in cancer

AAT belongs to a gene family of suicidal protease inhibitors called SERPINS

Past proteomic analyses count all AAT as one homogeneous population

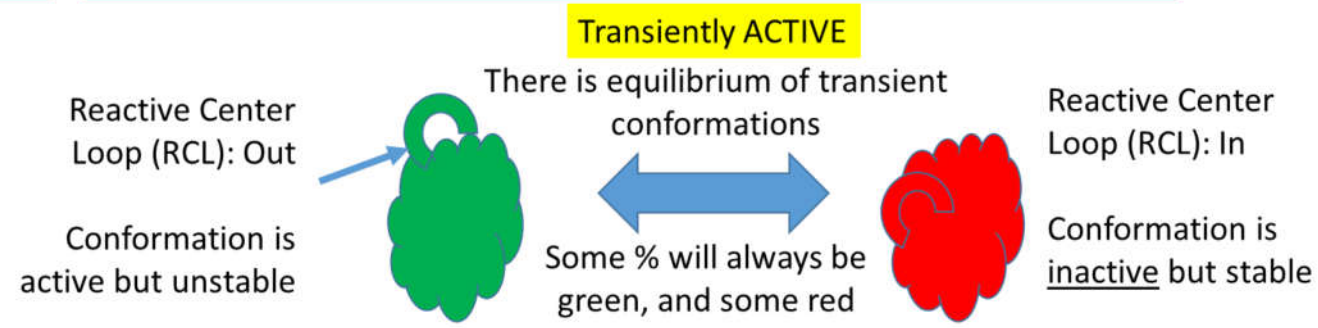


It may be this inability to switch off the acute response that makes chronic inflammation a contributing factor in a variety of cancers despite it having many of the same mediators (e.g. cytokines and free radicals) as those generated during acute inflammation. **In our special case,** Alpha-1-Antitrypsin (AAT), because of its unique characteristic as a SERPIN suicidal protease inhibitor, exhaustion of its inhibitory capacity may in part be one of the mechanistic failures to resolve the acute response and contribute to chronic inflammation. Many investigators have reported AAT as an alarming factor in malignancy and suggested there may be an imbalance between Alpha-1-Antitrypsin (AAT) & Neutrophil Elastase (NE) activities that could play a role in the progression of cancer. Using LC-MS reporting metrics, we now can observe peptide features distinguishing the 2 variant sub-populations of AAT of opposite function; reporting either as an [inhibitory active proteoform] or an [inactive proteoform]. We find that the cancer sera signature generally follows a decline in the abundancy of [inhibitory active] AAT sub-populations relative to the total population.



In the vast circuitry of cascading proteolytic events, protease inhibition is an essential mechanism for regulating acute insults within the bloodstream. So chronic dysregulation will contribute to many pathologies. As the cleavage products of SERPINs can now be reported efficiently by LC-MS analysis, it will be advantageous to report not only their relative abundances but whenever possible, the cleavage sub-forms and how these sub-form ratios modulate in blood throughout cancer progression.

A Proteogenomic Model now can consider the transient nature of SERPINs



[+SERPIN] reports all transient conformations with RCL intact
But Cancer Progression and Risk Factors Can Change these proportions

- SERPIN genetics are known cancer risk factors
- Age
- Progressive Disease
- Co-pathologies, ie. smoking

We suspect that in a normal and healthy population, there would be a sufficient blood reservoir of [inhibitory active] SERPINs. However, because of heredity, environmental risk factors, or progressive disease, in cancer populations, this reservoir becomes depleted, and the body can no longer replenish sufficient quantities of [inhibitory active] SERPINs to sustain inhibition of stromal proteases. In our special case, this would be primarily the exhaustion of [inhibitory active] AAT to sufficiently neutralize its primary substrate Neutrophil Elastase. Indeed if this model of dysregulation proves correct *in vivo*, it could contribute to a variety of therapeutic and detection strategies for the management and treatment of cancer.

A Proteogenomic Model now can consider the risk factors of SERPINs

Represented graphically
Percent Transient
Populations

Active, Unstable
Inactive, Stable

Normal/Healthy

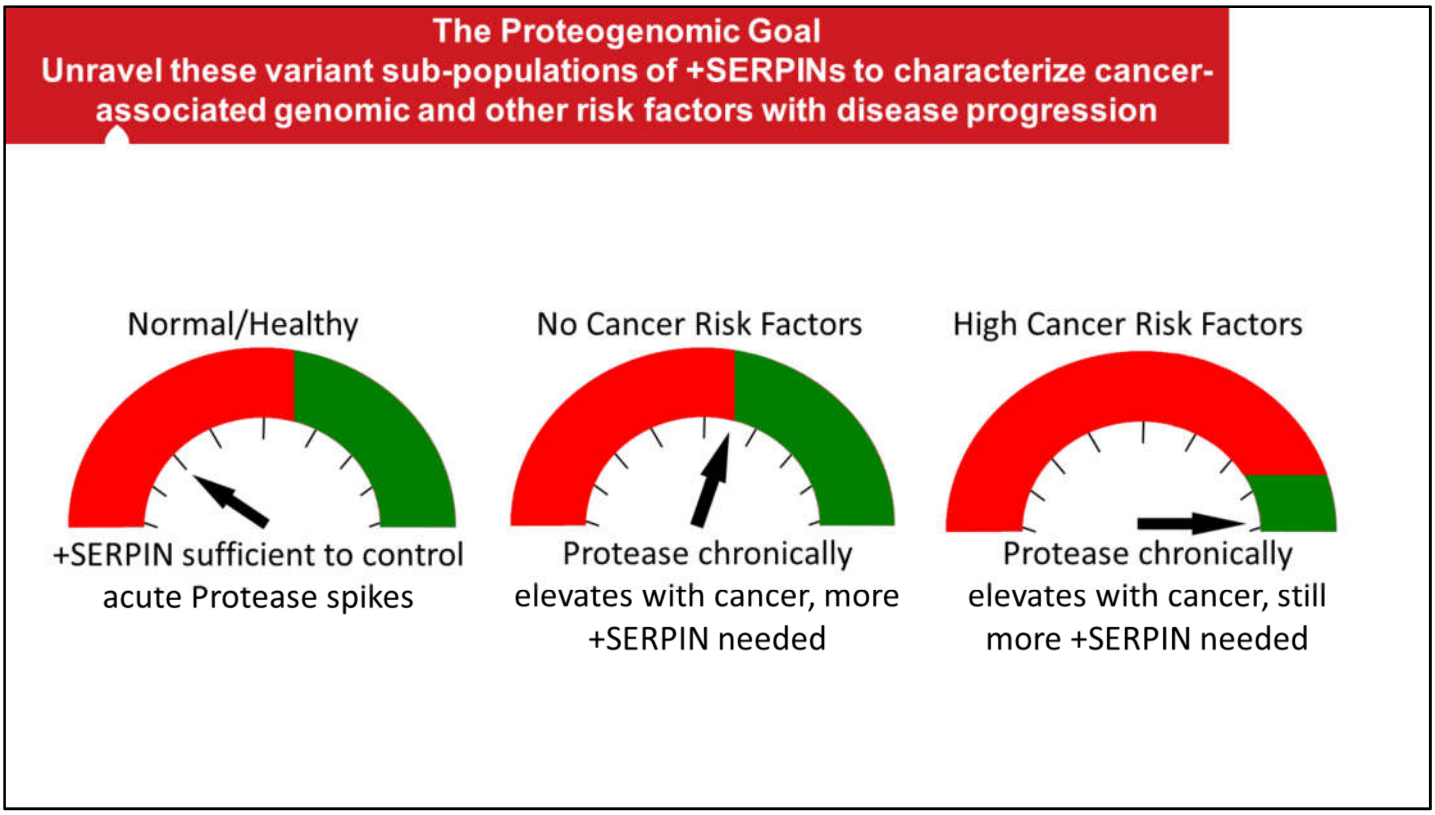
Higher % Populations associate with ACTIVE functions

RCL-intact report features do not distinguish these two SERPIN sub-types

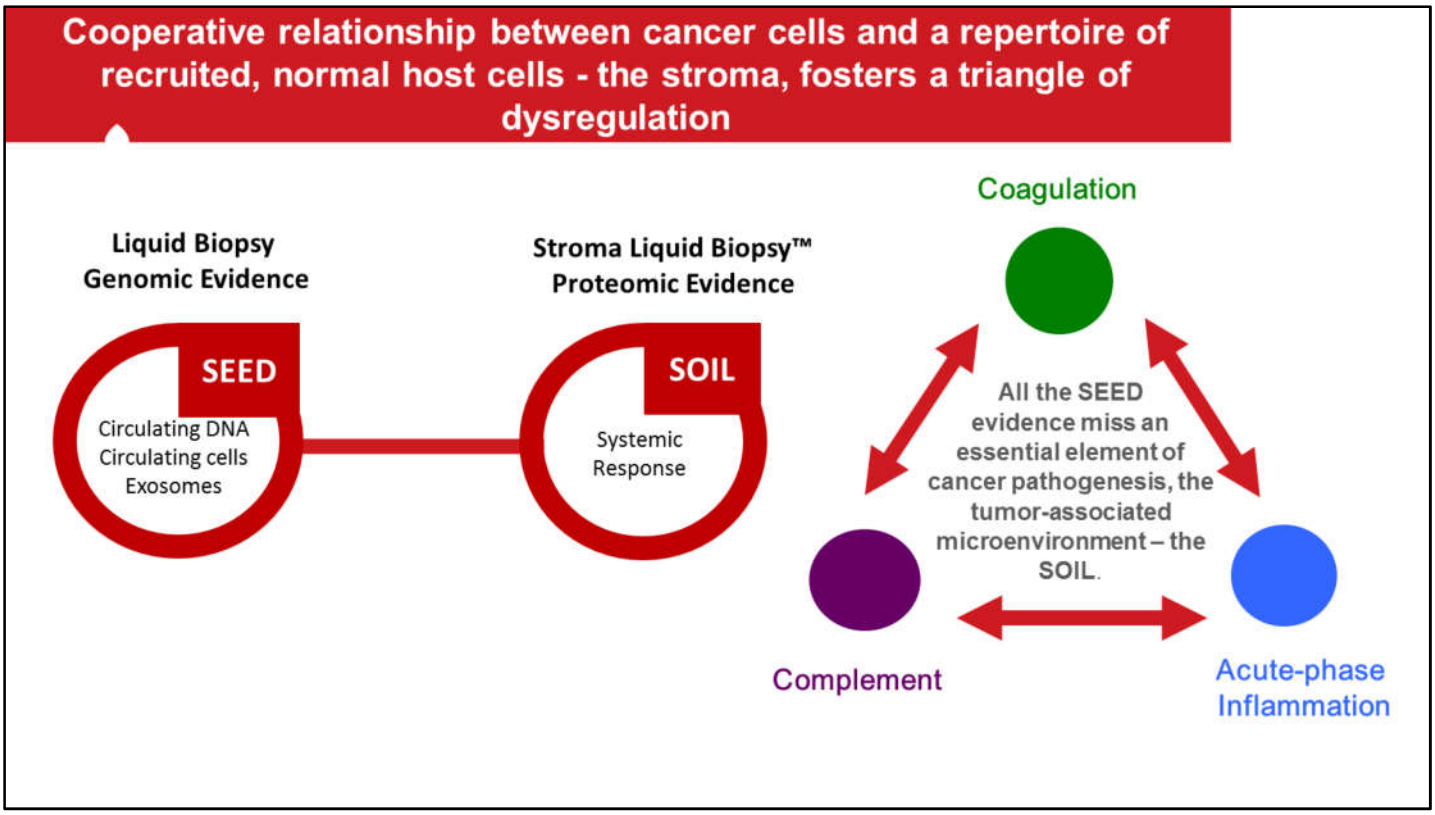
High Cancer Risk Factors

Less % Populations associate with ACTIVE functions

Hereditary risk factors associated with SERPIN inhibitors may also play a role in carcinogenesis. As an example, hereditary dysfunction of SERPINA1 (Alpha-1-Antitrypsin), has been previously determined as a risk factor for cancer. The aberrant form is found in the plasma of chronic smokers, and persists after smoking is ceased. As many proteins within the SERPIN family proteins are of moderate to high abundance quantities in serum, depleted functionality would impose severe modulation to the three interconnected pathways in our **Stroma Liquid Biopsy™** model – Coagulation, Complement and Inflammation.



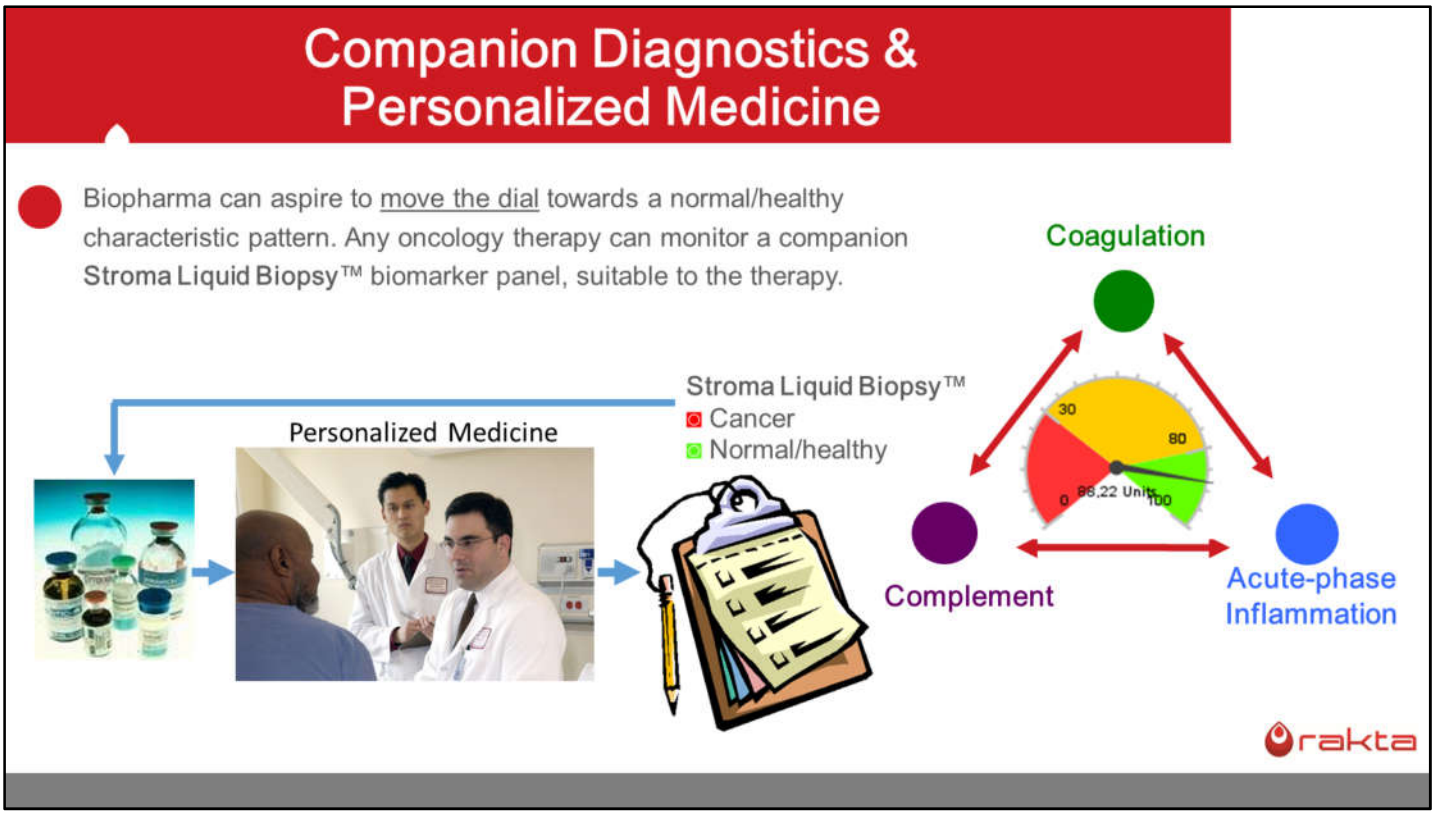
Notably, several of the key regulators in the Tissue Factor Coagulation Pathway such as SERPINA10 (Z-dependent Proteinase Inhibitor) and SERPINA5 (Protein C Inhibitor), have genomic variants that alter their inhibitory function. These might therefore be risk factors for cancer, and so hereditary proteogenomic factors associated with SERPINs need further investigation.



The liquid biopsy field is engaged to advance measurable biomarkers that can define a disease, patient sub-populations, and therapeutic strategies. For now, the prospects for liquid biopsies are dominated by the genomics field. While commercial enthusiasm surrounds genomic approaches, such strategies will always suffer the problem of shooting after a moving target; cancer's hallmark feature of genomic instability has confounded real progress in detection and treatment.

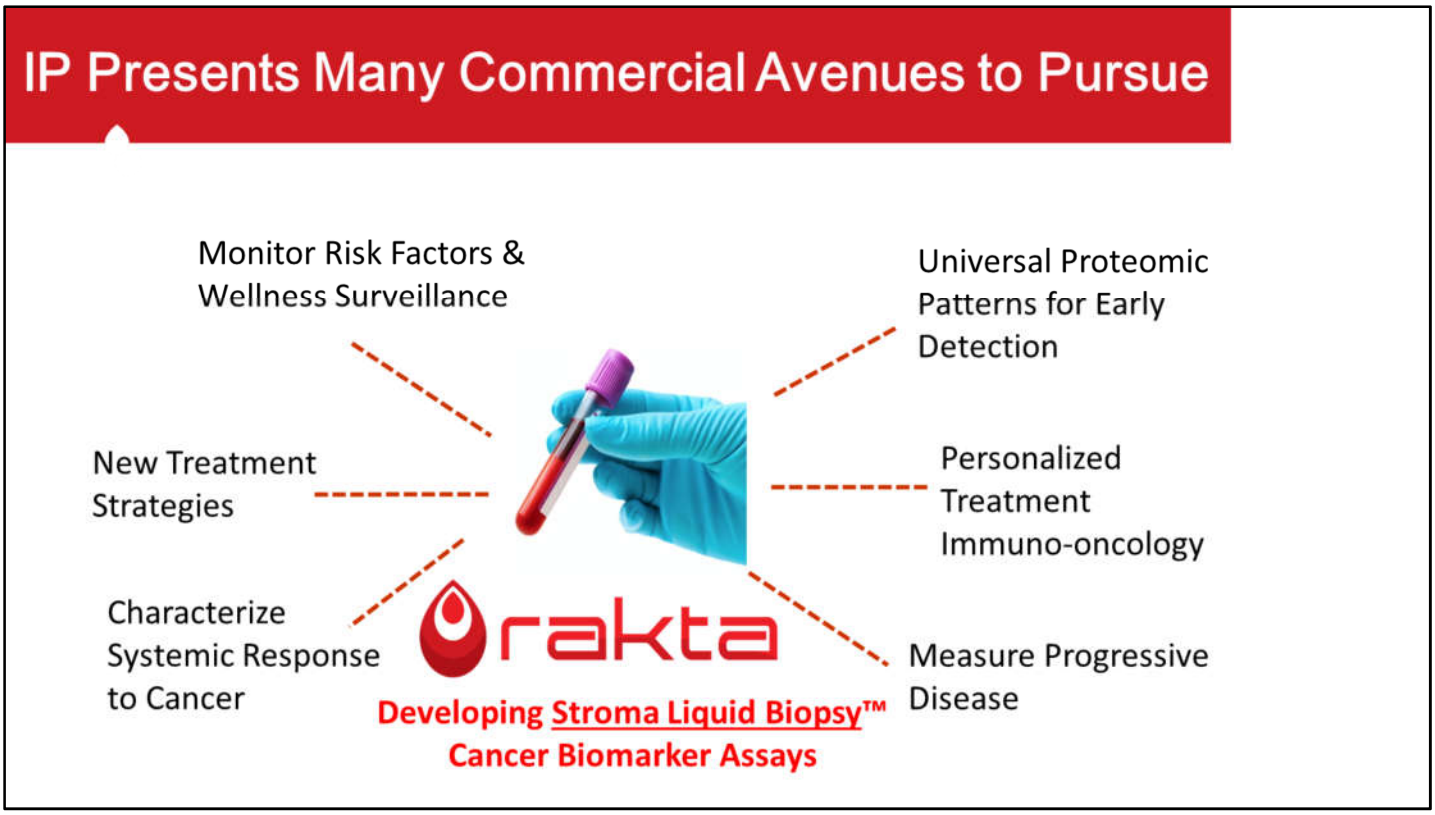
Notwithstanding these features, the cancer phenotype ultimately displays an individualized interplay between the host systemic response and the tumor. This individualized response is derived from a multitude of factors from the patient host not predicated upon the genomic variation of proliferating malignant tissue, but derived from the systemic response of the supporting microenvironments.

Cancer cells modify the surrounding microenvironments to serve its needs for nutrients, immune evasion, and space. Our observations corroborate other reports in the field that categorical mechanisms of coagulation, complement, and acute-phase inflammation are characteristic of cancer. However, with our panel of biomarkers, we now have quantifiable evidence for these pathways.



We solicit that there is great advantage to the variety of highly observable and quantifiable proteins that form a serum pattern of normalcy in a healthy person without cancer. By contrast this same panel of proteins is dysregulated by the localized subversion of normal homeostatic mechanisms within the tumor microenvironments. Such dysregulation forms a cancer-specific serum pattern of great novelty; that of activation of acute-phase inflammation and coagulation, and down-regulation of the early initiators of the complement cascade.

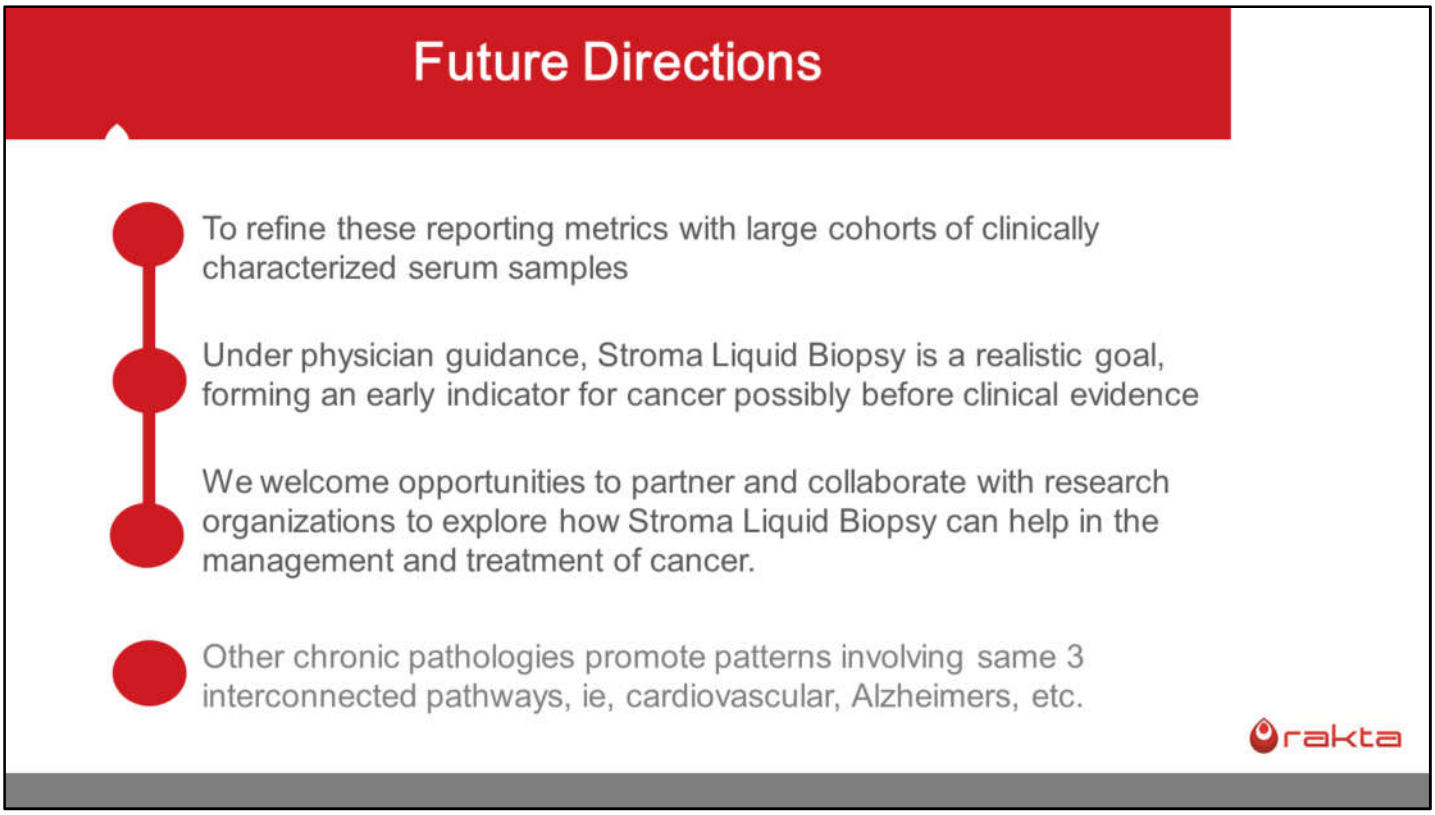
Our data suggests that this pattern is measurable even at very early stages of cancer, for many if not most primary tumors. Therefore it reflects in part, the individualized density and soluble mediators of the cellular composition of the tumor stroma. Consequently, using proteomic data from the proposed 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, to 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 and to manipulate the tumor microenvironment for therapeutic effect, may also be established.



We have submitted intellectual property disclosing data on numerous proteins re-proportioned in blood from cancer patients when compared to a normal/healthy population. This systemic response forms a characteristic cancer "rewiring" pattern, involving three interconnected systemic pathways, measurable even at early stages of cancer for many if not most primary tumors. These discoveries are the basis for **Stroma Liquid Biopsy™**.

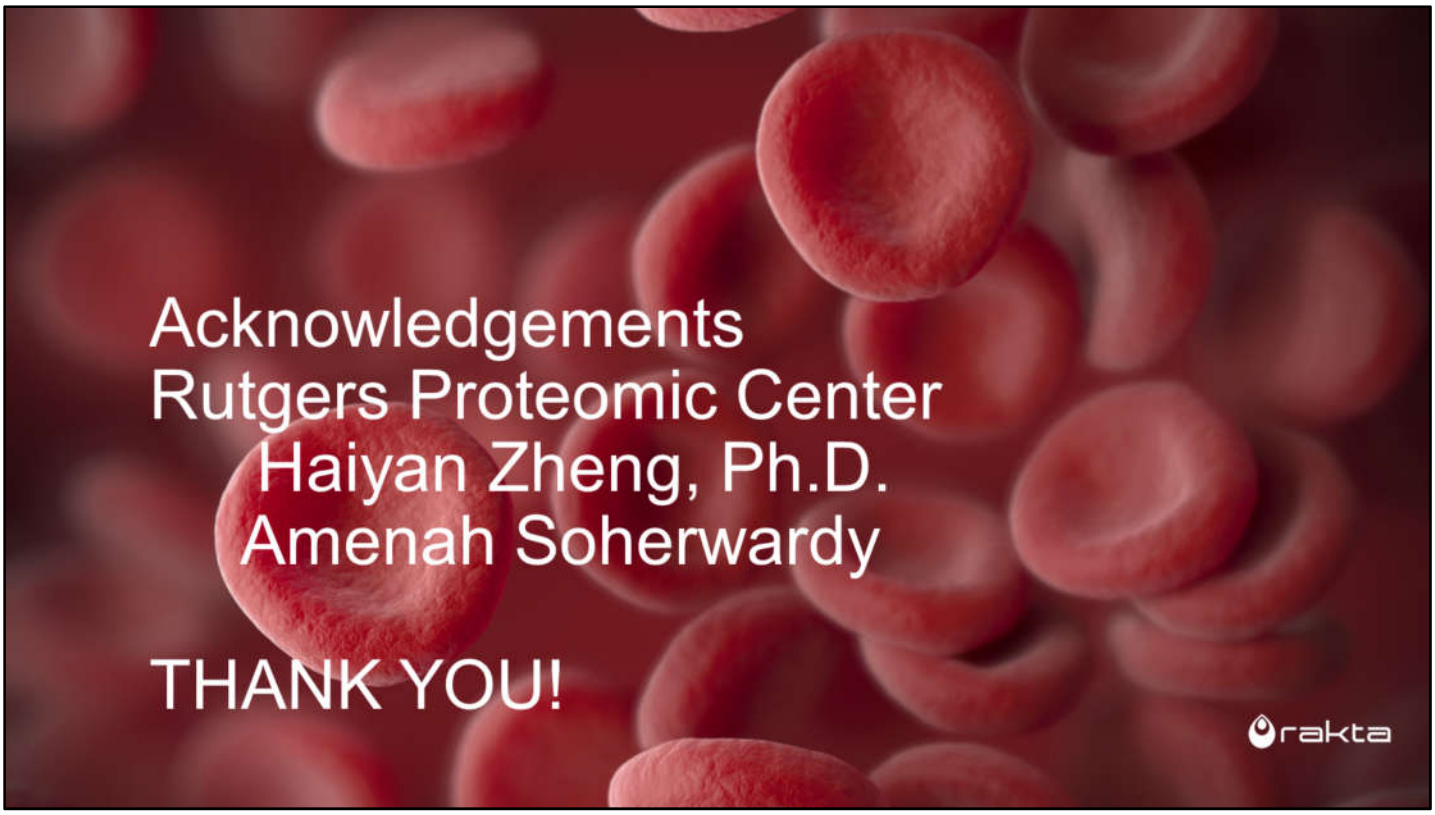
Our selection of biomarkers offer key advantages as they are:

- all highly observable, of relative high abundance in serum
- 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



This presentation serves only as an introduction to the prospects for compiling **Stroma Liquid Biopsy™** proteomic data. In future investigations, we hope to refine these reporting metrics with large cohorts of samples, so we might find patterns for early cancer dysregulation and response to therapies, regardless of the primary tumor. Under the guidance of a physician, the concept for **Stroma Liquid Biopsy™** might then be a realistic goal. It would generate objective measures whether or not to rule out cancer as a possible diagnosis when risk factors are accessed, or even screen for early detection.

Other chronic pathologies might also benefit by considering the pathways and biomarkers we have under investigation.



We thank Haiyan Zheng, Ph.D., and Amenah Soherwardy at the Rutgers Proteomic Center for their guidance on experimental design, and the acquisition of the LC-MS data.

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Sample Prep that Matters

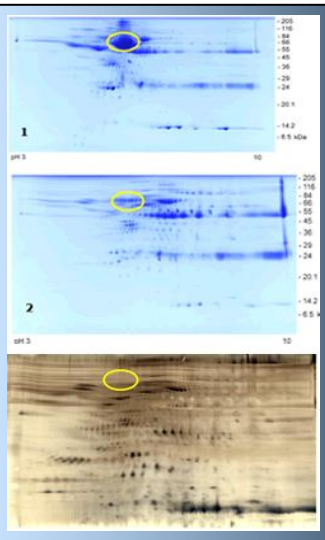
Albumin Removal Kits

• Unique surface chemistries, no antibodies • depletes Albumin 90-95%

AlbuVoid™

Selectively Voids Albumin, Binds Low Abundance Proteome

- Albumin voids in flow-through, >95%
- <30 minute protocol
- Low abundance enrichment equivalent or better than hexa-peptides or antibodies
- On-bead digestion protocols, efficient LC-MS workflows
- Disposable, cost-effective, no column regeneration or cross-contamination
- Mild elution maintains native structure with retained enzymatic, functional & bio-activities
- Species agnostic



2DE analysis of AlbuVoid™ treated sheep serum. Samples were reduced, alkylated and total protein normalized. The circled regions indicate the albumin zone. 1: Control. 2: AlbuVoid™ Eluate. Bottom: AlbuVoid™ Eluate silver stain.

AlbuSorb™

Selectively Binds Albumin

- Removes serum albumin >90%
- Economical small ligand surface architecture (not dye-based), bio-affinity performance
- Consumable, cost-effective, no column regeneration or cross-contamination
- Species agnostic
- Compatible with
  - LC-MS
  - Microarrays
  - Functional assays

Non-reduced SDS-PAGE profiles

Left lane: Normal human pooled serum control

Right lane: Flow-through from **AlbuSorb™**

AlbuVoid™ LC-MS On-Bead

Selectively Voids Albumin, Binds Low Abundance Proteome before LC-MS use

- Seamless workflows
- Label, label free & glyco- compatible
- Unique proteolytic efficiencies

Total LC-MS/MS Proteins identified at 2 digestion times: 4 hours (4h) & overnight (O/N), single 3 hour LC gradient.

AlbuSorb™ PLUS

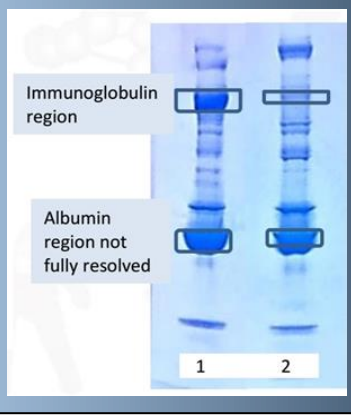
Selectively Binds Albumin & Immunoglobulin

- 400 µg total serum protein mass per prep
- > 85% Albumin, 85% IgG depleted from 25 µl serum

AlbuSorb™ combines with an optimized immobilized Protein A to create **AlbuSorb™ PLUS.** Unlike immuno-affinity, the surfaces utilized are disposable eliminating cycle to cycle variance and cross-contamination.

Lane 1: Human Serum Control

Lane 2: Serum after treatment with **AlbuSorb™ PLUS**



AlbuTrial™ Kit

Don't know which one to try? Try both. The AlbuTrial kit has 1 gram of **AlbuSorb™**, and 5 preps of **AlbuVoid™**

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