BIOTECH SUPPORT GROUP
Sample Prep that Matters

APPLICATION REPORT
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The Comparison of the Serum Proteome in Individuals with Cancers versus those without Cancer, and its application to Wellness

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Introduction and Objectives

For many diseases, pancreatic cancer for example, long term survival is critically dependent upon early detection. So various strategies for early detectable markers are being investigated. One such strategy is to identify a singular biomarker, derived from genomic analysis, and then determine its derivative protein concentration in blood. This has been challenging as many differentially regulated genes do not generate a differentially regulated protein. An alternative biomarker strategy is to consider panels of proteins as up and/or down regulated biomarkers, which differ in diseased and normal states. Following this strategy, many serum proteomic investigations have analyzed one cancer type or another, but our goal was to consider whether patterns of multiple proteins dysregulated in cancer could be observed regardless of the primary tumor, stage of progression or tumor burden. In this study we adopt a relatively new method, which combines Albumin depletion and on-bead digestion of the depleted serum in a seamless process, called **AlbuVoid™ LC-MS On-Bead**. From this method, we were able to compare labeled quantification of proteins from normal and disease state sera – for this case, breast, lung and pancreatic cancer. The methods spectrally quantify over 200 total proteins, in a cost effective and reproducible manner. No offline peptide level fractionation prior to LC-MS was employed, lowering the LC-MS acquisition time 5-10x compared to common serum proteomic workflows. We describe these workflow advantages applied towards a "wellness" proteome strategy whereby knowledge and data surrounding individual normal and healthy proteomes can be annotated, compared and contrasted to those with a clinically definable disease, in our case the serum cancer phenotype.

For such a strategy to be viable, most proteins from normal/healthy sera should be within a small and consistent variance taking into account both technical and biological variance for the proteins measured. Once established, a panel of proteins can be selected that are observed to be dysregulated (either up, down, or cyclical up/down) within the disease state; defining in our case a cancer phenotype. We cite one report supporting such a strategy using multiple protein markers in a risk prediction model for lung cancer in lieu of stand-alone tests[1]. Yet in almost all previous studies, only one primary tumor site (lung or pancreas for example) was considered in the study. Notably, we found only one report which considers the

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common proteomic patterns of three cancer primary tumor sites - breast, colorectal and lung, revealing distinctive expression of acute-phase proteins[2]. This suggests the feasibility of our goal, revealing a measureable cancer phenotype in serum, without regard to primary tumor of origin, clinical stage of progression, or tumor burden.

In this report, 3 cancer types – pancreatic, breast and cancer, with sera taken from clinically characterized stages I – IV, were compared against a pooled sera from matched 5 normal/healthy individuals of similar age and sex, in this case females, ages 40-60. In like manner, we also considered the variance within these same normal/healthy individuals to account for any combined technical and biological variance with our methods. A discussion of how these observations compare to previous observations by others and areas for future research are tabulated.

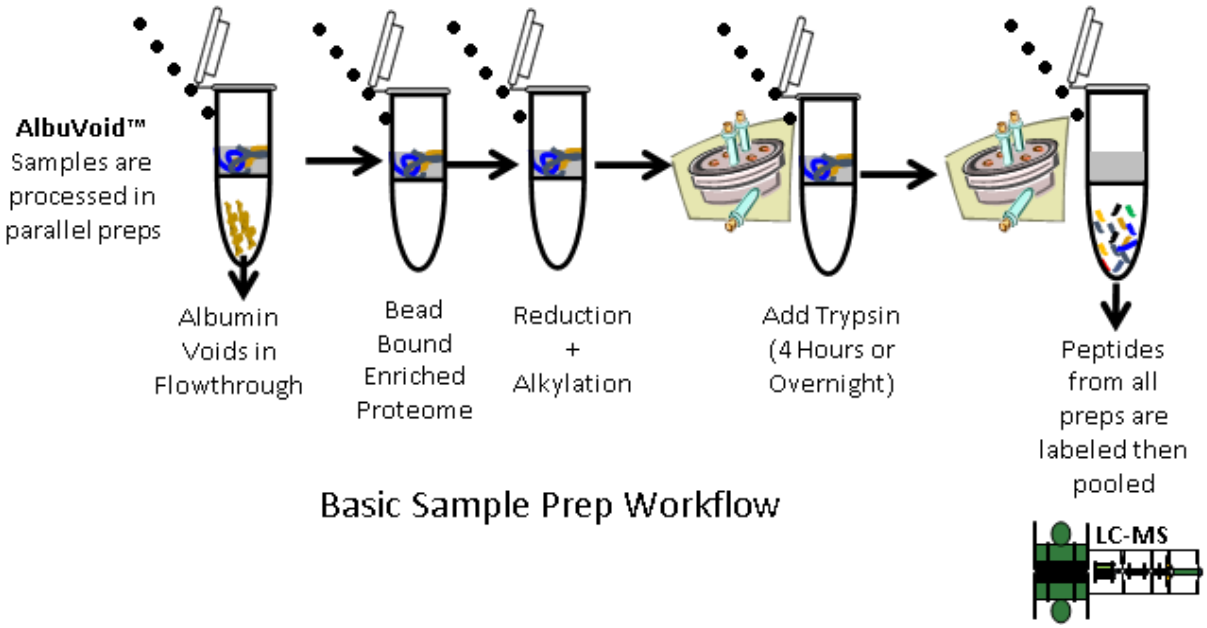
Individual Cancer Sample Types Tested - Females, ages 40-60, (Discovery Life Sciences, CA)
Ratio for all samples were compared to 5 normal/healthy pooled individuals, same age group and sex (Valley Biomedical Products, VA)

Pancreatic Cancer Stage 1	Pancreatic Cancer Stage 1	Pancreatic Cancer Stage 2	Pancreatic Cancer Stage 3	Pancreatic Cancer Stage 4
Breast Cancer Stage 1	Breast Cancer Stage 1	Breast Cancer Stage 1	Breast Cancer Stage 2	
Lung Cancer Stage 2	Lung Cancer Stage 3	Lung Cancer Stage 4		

Methods

The workflow follows the AlbuVoid™ LC-MS On-Bead sample prep method[3]. In brief, 50 µl serum is prepared by adding a binding buffer, then applied to the AlbuVoid™ beads, and washed. All steps are performed within a microfuge spin-filter format. Albumin is specifically voided out, while the majority of the remaining serum proteome is retained on the bead. After the final wash, reduction, alkylation and Trypsin digestion all take place on the bead. After labeling, the peptides were pooled and analyzed with a single LC-MS/MS 3 hour gradient run using nanoRSLC system interfaced with a Thermo Scientific™ Q Exactive™ HF (Thermo Scientific) instrument, using data-dependent acquisition with resolution of 60,000, followed by MSMS scans (HCD 30% of collision energy) of 20 most intense ions, with a repeat count of two and dynamic exclusion duration of 60 sec.

The LC-MS/MS spectral data was searched against the Human Ensembl databases using X!tandem (thegpm. org) with carbamidomethylation on cysteine as fixed modification and oxidation of methionine and deamidation on Asparagine as variable modifications using a 10 ppm precursor ion tolerance and a 20 ppm fragment ion tolerance. The searches were done using an in-House version of X! Tandem with protein filters set based on FPR supplied by the software: valid log(e) < -0.4, ρ = 87, FPR = 0.72%. The peptides were filtered by loge<-2 and protein filtered by minimal number of peptide>2.



Basic Sample Prep Workflow

We used 4 hour digest times for all tests to minimize proteolytic background[4]. TMT-6 labels (Proteome Sciences plc, Surrey UK) were used throughout with individuals reported as a quantitative ratio of the individual cancer report signal compared to a pooled normal/healthy report signal. So, no variance would report as a ratio of 1. Most proteins from normal/healthy individuals reported with variance no greater than about 30%, so we used ≥ 1.5 as an up-regulated outlier, and ≤ 0.7 as a down-regulated outlier. Stronger report variance is also noted on the tables that follow.

AlbuVoid™ On-Bead Digestion Method Reveals Altered Cancer Associate Protein Levels Categorized into 4 Separate Biomarker Panels, presented in Tables 1-4

1. Proteins Supported by Other Investigations Not Using AlbuVoid™
   - Classical serum proteins, (10-1000) µg/ml range
   - Generates confidence that our discoveries are valid as others have made similar discoveries
2. Proteins common to serum proteomes but which have not been observed to be dysregulated in cancer to the best of our knowledge
   - Classical serum proteins, (ng - µg)/ml range
   - Many proteins in categories 1 & 2 are acute phase response proteins indicative of dysregulation from wellness but not necessarily specific to cancer dysregulation
3. Proteins rarely or never annotated to serum proteomes and never observed as dysregulated in cancer
   - Unknown concentration, estimate ≤ng /ml range
   - Possible new cancer-specific biomarkers, formerly buried in the proteolytic noise, revealed by our methods
4. Conflicting quantitative evidence with other investigations
   - SERPIN family protease inhibitor with variant sub-populations
   - Methods may have bias towards sub-populations; these sub-populations may be dysregulated

The contributions from all four categories demonstrate AlbuVoid™ On-bead methods as presenting a unique discovery engine.

Table 1 - Classical Serum Proteins Supported by other cancer investigations

Protein ID	Protein Description	Plasma Proteome Annotation (approx. conc.)	TMT Report Ratio Threshold	# Individuals Threshold/ All	Cancer Types	Evidence in Cancer Sera by (Others)	Comments
ITIH3/4 (at least one subunit chain)	Inter alpha trypsin inhibitor, heavy chain ¾	40 µg/ml	> 1.5 (up-regulated)	7/13	Pancreatic, Breast, Lung All Stages	Up-regulated (3)(4)(5)(6)	4 Sub-unit protease inhibitors, suspected role in inflammation
APOA1	Apolipoprotein A I	1 mg/ml	< 0.7 (down-regulated)	9/13	Pancreatic, Breast, Lung All Stages	Down-regulated (7)	major protein component of high density lipoprotein (HDL)
APOC3	Apolipoprotein C III	100-200 µg/ml	< 0.7 (down-regulated)	5/13 With APOA1 11/13	Pancreatic, Breast, Lung 4/5 Stage 1	Up-regulated (8)	a component of very low density lipoprotein (VLDL) Our results conflict with other reports, maybe APOC3 reports as cyclical up & down dysregulation
CRP	C-reactive protein	1-2 µg/ml	> 1.5 (up-regulated)	7/11	Pancreatic, Breast, Lung All Stages	Up-regulated (9)	Acute phase response protein, may scavenge nuclear material released from damaged circulating cells
PF4	Platelet Factor 4	5-10 ng/ml	> 1.5 > 2.0 (up-regulated)	13/13 9/13	Pancreatic, Breast, Lung All Stages	Up-regulated (8)(9)(10)	promotes blood coagulation, may play role in wound repair and inflammation
PPBP	Beta thromboglobulin aka CTAPIII/NAP-2	5-10 µg/ml	> 1.5 > 2.0 (up-regulated)	13/13 9/13	Pancreatic, Breast, Lung All Stages	Up-regulated (1)	Platelet activation Elevated levels predated lung cancer diagnosis by 29 months, with cyclical levels upon surgical resection and recurrent disease (1).
CLU	Clusterin	100 µg/ml	> 1.5 (up-regulated)	12/13	Pancreatic, Breast, Lung All Stages	Up & down-regulated (11)	Multiple isoforms, associated with clearance of cellular debris and tissue remodeling. Up & down regulation have been described for many cancers, may be isoform specific (11)
PIGR	Polymeric immunoglobulin receptor	25 ng/ml	> 1.5 (up-regulated)	3/5	Pancreatic, All Stages	Up-regulated (4)(12)	mediates transcellular transport of polymeric immunoglobulins
TTR	Transthyretin	100-400 µg/ml	< 0.5 (down-regulated)	4/5	Pancreatic, All Stages	Down-regulated (5) (7) (13)	carrier of the thyroid hormone thyroxine (T4)
THBS1	Thrombospondin I	200 ng/ml	> 1.5 (up-regulated)	3/3	Lung, All Stages	Up-regulated (14)	an antiangiogenic, blood vessel constriction clinically associated with smoking >5 fold iTRAQ ratio in pooled breast cancer plasma (14)
SAA2	Serum amyloid A2	5 µg/ml	= 10 1 spectrum, 2 tests, quantitation may be misleading	5/5	Pancreatic, Breast, Stage 1	Up-regulated (7)	Amyloid As (SAA) are rapid response acute phase markers, not clear how computational assignments distinguish SAA2 from the more common SAA2-SAA4

Table 2 - Classical Serum Proteins Dysregulated in Cancer, not previously reported

Protein ID	Protein Description	Plasma Proteome Annotation (approx. conc.)	TMT Report Ratio Threshold	# Individuals meeting Threshold/ All	Cancer Types	Comments
AGT	Angiotensinogen Aka SERPINA8	40-60 µg/ml	<0.7	5/5	Pancreatic, All Stages	precursor to angiotensin II, blood pressure regulation. The renin-angiotensin system may play role in carcinogenesis (15) (16) (17)
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Common peptides in Peptide Atlas	> 1.5 (up-regulated)	12/13	Pancreatic, Breast, Lung All Stages	protease inhibitor, levels rise ~2-fold during inflammation
CPN1	Carboxypeptidase N, serum	35 µg/ml	> 1.5 (up-regulated)	10/13	Pancreatic, Breast, Lung All Stages	metallo-protease
IGLV3-27	Ig-like	Common peptides in Peptide Atlas	< 0.7 (down-regulated)	9/10	Pancreatic, Breast, Lung 5/5 Stage 1	
IGHV1OR15-1	Ig-like	Common peptides in Peptide Atlas	< 0.7 (down-regulated)	2/3	Lung All Stages	
IGHV3-53	Ig-like	Common peptides in Peptide Atlas	< 0.7 (down-regulated)	3/3	Breast, Lung Stages 1	
IGKV1D-33	Ig-like	Common peptides in Peptide Atlas	> 2.0 (up-regulated)	3/3	Breast, Lung Stages 1	
SHBG	Sex hormone binding globulin	100-200 ng/ml	> 1.5 (up-regulated)	5/5	Pancreatic, All Stages	
SEMA3D	Sema domain immunoglobulin secreted 3D	Annotated to Plasma Proteome, no concentration data	< 0.7 (down-regulated)	3/3	Lung All Stages	neural development, may have role in immune function
C4BPB	Complement component 4 binding protein, beta	200-500 µg/ml	> 1.5 (up-regulated)	4/5	Pancreatic, All Stages	large glycoprotein (500 kDa), known to interact with C-reactive protein (CRP)

Table 3 - Potential New Cancer Serum Biomarkers Rarely or Never Observed in Serum

Protein ID	Protein Description	Plasma Proteome Annotation (approx. conc.)	TMT Report Ratio Threshold	# Individuals meeting Threshold /All	Cancer Types	Comments
CFAP61	Cilia- and flagella-associated protein 61	Observed tissue only, not observed in Peptide Atlas for Plasma	< 0.5 (down-regulated)	7/8	Pancreatic, Breast, All Stages	
PFKM	Phosphofructo-kinase 1	Cytosolic protein, few observations in Peptide Atlas for Plasma	> 1.5 (up-regulated) > 2.0 (up-regulated)	5/5 3/5	Pancreatic, Breast, Stage 1	key regulatory and rate limiting step of glycolysis (Warburg effect), not expected and not often observed in serum/plasma
SPRED2	Sprouty related EVH1 domain containing 2	Observed tissue only, not observed in Peptide Atlas for Plasma	> 2.0 (up-regulated) > 4.0 (up-regulated)	4/5 3/5	Pancreatic, Breast, Stage 1	Tyrosine kinase substrate that inhibits growth-factor-mediated activation of MAP kinase, not expected nor so far observed in serum/plasma
C18orf63	No annotation, simply called Uncharacterized	Only 2 tissue observations in Peptide Atlas	> 5.0 (up-regulated)	Inconsis-tently observed	Pancreatic, Breast, Lung All Stages	Not consistently observed with low spectral counts, but outliers are highly up-regulated
CH17-224D4.2	No annotation	Not observed in Peptide Atlas, No annotation in Uniprot	> 1.5 (up-regulated)	3/5	Pancreatic, All Stages	Ensembl gene annotation: Immunoglobulin (Ig) and T-cell receptor (TcR)
CTD-2007N20.1	No annotation	Not observed in Peptide Atlas, No annotation in Uniprot	< 0.5 (down-regulated)	6/8	Pancreatic, Breast, All Stages	Ensembl gene annotation: Immunoglobulin (Ig) and T-cell receptor (TcR)

Table 4: Potential New Cancer Serum Biomarkers – Sub-populations of High Abundance Protein AAT

Protein ID	Protein Description	Plasma Proteome Annotation (approx. conc.)	TMT Report Ratio Threshold	# Individuals meeting Threshold /All	Cancer Types	Comments
SERPINA1	Alpha-1-antitrypsin (AAT)	1 mg/ ml	< 0.7 (down-regulated) < 0.5 (down-regulated < 0.3 (down-regulated)	13/13 12/13 9/10 Pancreatic, Breast	Pancreatic, Breast, Lung, All Stages	Concentration can rise manyfold upon acute inflammation. Reports of up-regulation in cancer (18), and mutation driven down-regulation in emphysema and cancer (19) We observe strong down-regulation, severe in Breast and Pancreatic; suspect we are measuring sub-populations and not total populations

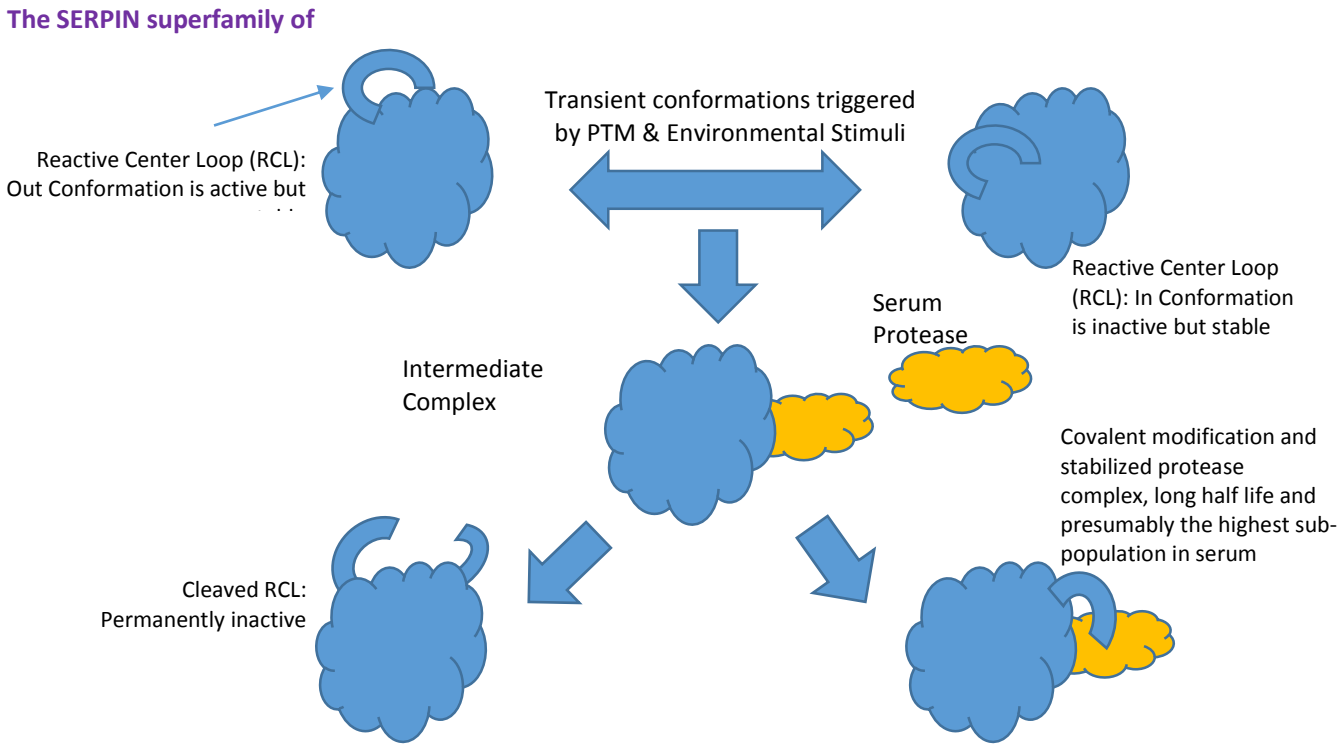
The SERPIN family of proteins (8 observed) were exceedingly non-variant with respect to normal/healthy female populations, with only 1 out of 40 proteins (5 individual X 8 proteins) observed as an outlier, and that was up - not down-regulated. In males, similar patterns are observed with only a few (mostly up) outliers in the SERPIN family. This suggests that the SERPIN family of proteins is tightly regulated in normal/healthy individuals and as 3 SERPINs were on the list for dysregulation in cancer, an important family of proteins to consider within the strategy for key wellness proteins.

We suspect AlbuVoid™ On-bead methods bias towards certain sub-populations of inhibitor/protease complexes. This demands further study. High abundance proteins, now immuno-depleted or reported as one homogeneous population miss the exquisite regulation of protease inhibitors, and therefore methods to more discriminately observe and measure these sub-population variants are important new areas to investigate. In future work, we shall consider more discreet peptide level annotation and functional proteomics to support such efforts.

Need to Count Variant Sub-populations more discriminately in proteomic workflows

Tissue remodeling is an essential feature of cancer, and proteases play a key role. Consequently, the balance and regulation of proteolytic activity is essential to biomarker discoveries and possibly to therapeutic intervention. Many of these regulating proteins fall under the SERPIN superfamily of protease inhibitors. One such inhibitor Alpha-1-Antitrypsin (ATT), has several isoforms observed in plasma using 2-DE[26]. Others report specific forms of ATT having multiple effects on tumor cell viability and diverse roles in tumorigenesis, suggesting such isoforms may form a specific basis for diagnosis of cancer[24,27]. Yet, most often in proteomics, all sub-populations are rolled into and counted as one homogeneous population, or as in the case of AAT immuno-depletion, simply ignored.

As a result, the regulation, balance and dynamism within these systems and its impact on disease progression cannot be properly investigated. Key sub-populations of the SERPIN superfamily of protease inhibitors are represented here.



Tissue remodeling is essential for cancer progression regardless of tumor origin, and is controlled by the myriad regulating mechanisms of protease inhibition. Current proteomic methods inadequately count the many differential sub-populations of key protease inhibitors in serum. Bead-based enrichments such as AlbuVoid™ may help unravel these sub-populations.

Conclusions

> Whole new fields of cancer biomarker discoveries rests in the data-rich features of the diverse variety of conformational and isoform variants associated with classical high abundance proteins like the SERPIN superfamily (i.e., Alpha-1-Antitrypsin), Clusterin and even Immunoglobulins. Being nothing short of an interference biomolecule, AAT for example, is quite often immuno-depleted prior to LC-MS. More generally, mid to high abundance proteins are quantified as one homogeneous population, negating the disease specificity that can be obtained through the discreet quantification of the multiple sub-populations available to measure. Future investigations will consider the reporting features at both the peptide and protein level, to create sub-population signatures of Alpha-1-Antitrypsin in cancer.

> Individual variance within the AlbuVoid™ On-bead methods are nominal (about 30%), taking into account combined technical and biological variance. This raises confidence that outliers are disease related and not just a result of technical or normal biological variance.

> AlbuVoid™ On-Bead methods using short digest times (4 hours) reduce proteolytic noise uncovering peptide features only rarely or in some cases never before annotated to normal and/or cancer sera previously. Many of these biomarkers are not in the public domain and therefore poorly characterized. They are right at the limit of detectability with current methods, as they were not consistently observed in all tests. As potential new cancer biomarkers, follow on methods to observe these proteins with more consistency are necessary.

> We note two reports that a collection of blood based biomarkers measuring inflammatory and acute phase response proteins, measured within a panel rather than singular tests, can model early detection of cancer 2 to 3 years prior to clinical evidence[1,8]. Our study supports others that describe common dysregulated functions within the cancer phenotype, regardless of the primary tumor or tumor burden[2]. With the exception of glycolysis which may be expected to be proportional to tumor burden, we have found inflammatory, coagulation, and tissue remodeling biomarkers which are common to the 3 primary tumors tested and likely common to the majority of primary tumors. Such functional dysregulation within blood may serve an enabling microenvironment necessary for cancer progression.

> So we solicit that there is a measurable serum cancer phenotype that can be modeled with categorical proteins taken from inflammation, blood coagulation, tissue remodeling and glycolysis. Within this framework, we suggest it feasible to baseline monitor normal/healthy individuals and determine a dysregulation pattern associated with cancer generally but not necessarily for a particular primary tumor. Under the guidance of a physician, such dysregulated patterns may serve as a 'liquid biopsy', forming an early indicator for cancer before clinical evidence. These same patterns may be prognostic and even offer therapy guidance. More refined algorithms for each of these purposes: early detection, prognosis and therapeutic options, can be made with a more detailed investigation in the future. We welcome inquiries in this regard.

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