Targeted LC-MS Proteomic Methods to Monitor and Quantify Stromal Conditioning in Cancer from Blood

Matt Kuruc[1]; Swapan Roy[1]; Wilma Mesker[2]; Rob Tollenaar[2]

[1]Biotech Support Group LLC, Monmouth Junction, NJ;   [2]Leiden University Medical Center, Leiden, Netherlands

Corresponding Author: mkuruc@biotechsupportgroup.com

Abstract

Commercial enthusiasm continues for the prospects of measuring cancer non-invasively through liquid biopsy biomarkers. However, current biomarkers only serve to report tumor burden, which is not the full picture of progressive disease. The other largely neglected part of tumors, comes from the cooperation of the normal host response to acquired inflammation at the primary site. This stromal conditioning assists the tumor ecosystem and affects resistance to treatment and final outcomes. The innate immune system cooperates in this response, and we now report proteomic methods to monitor that influence from blood. With these methods, we aim to characterize and quantify Stroma Liquid Biopsy™ biomarkers in patient sera, to correlate to Tumor-Stroma Ratio scoring methods developed at Leiden University Medical Center (LUMC).
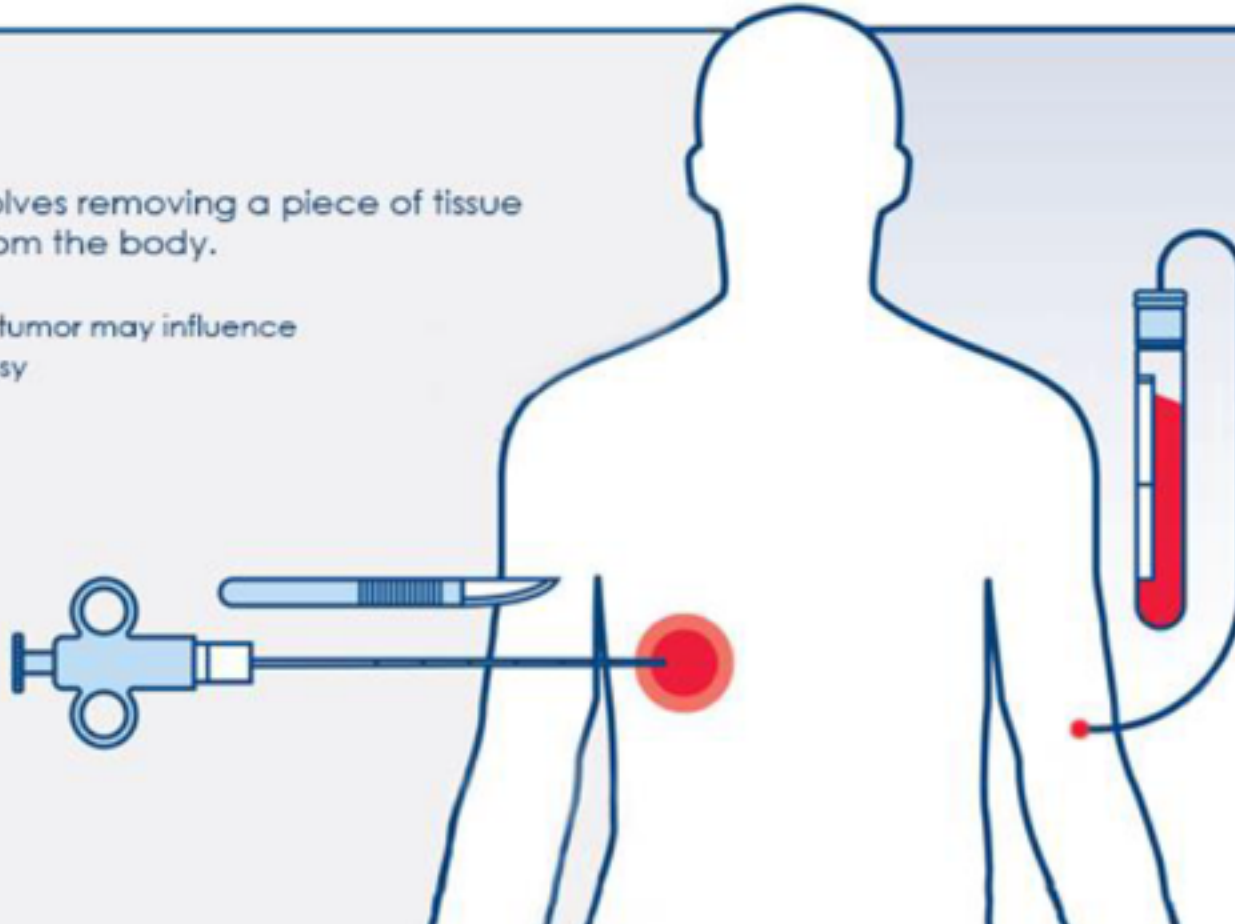
Biopsies - Tissue vs. Liquid

Tissue Biopsy

A procedure that involves removing a piece of tissue or a sample of cells from the body.

Heterogeneity within the tumor may influence the accuracy of the biopsy
Can be painfuful
invasive

Liquid Biopsy

A new, non-invasive technique that can detect disease biomarkers in blood, urine and other body fluids.

Monitoring of:
Treatment response
Drug resistance
Disease recurrence

Detection of:
Genomic mutations to guide treatment decisions
Various tumors at an early stage

Benefits of Liquid Biopsy
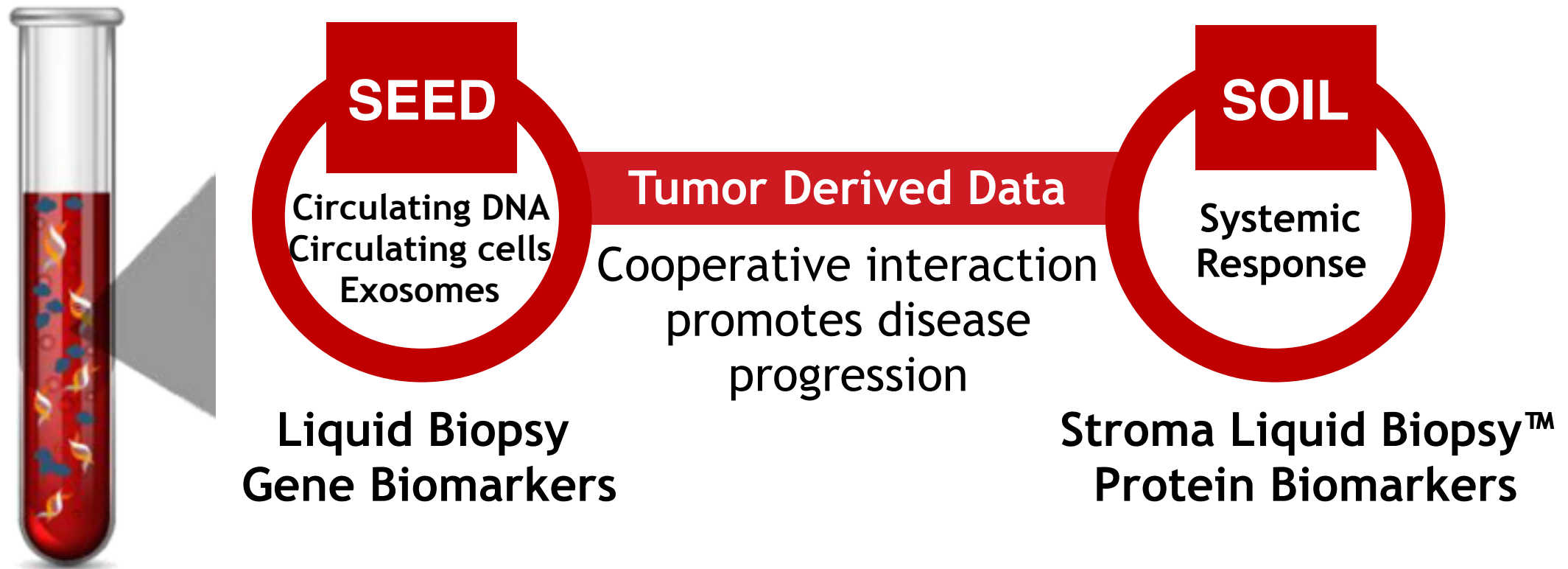Minimally invasive
Screening for early detection
Monitoring disease progression
Determining disease prognosis and selection of treatment

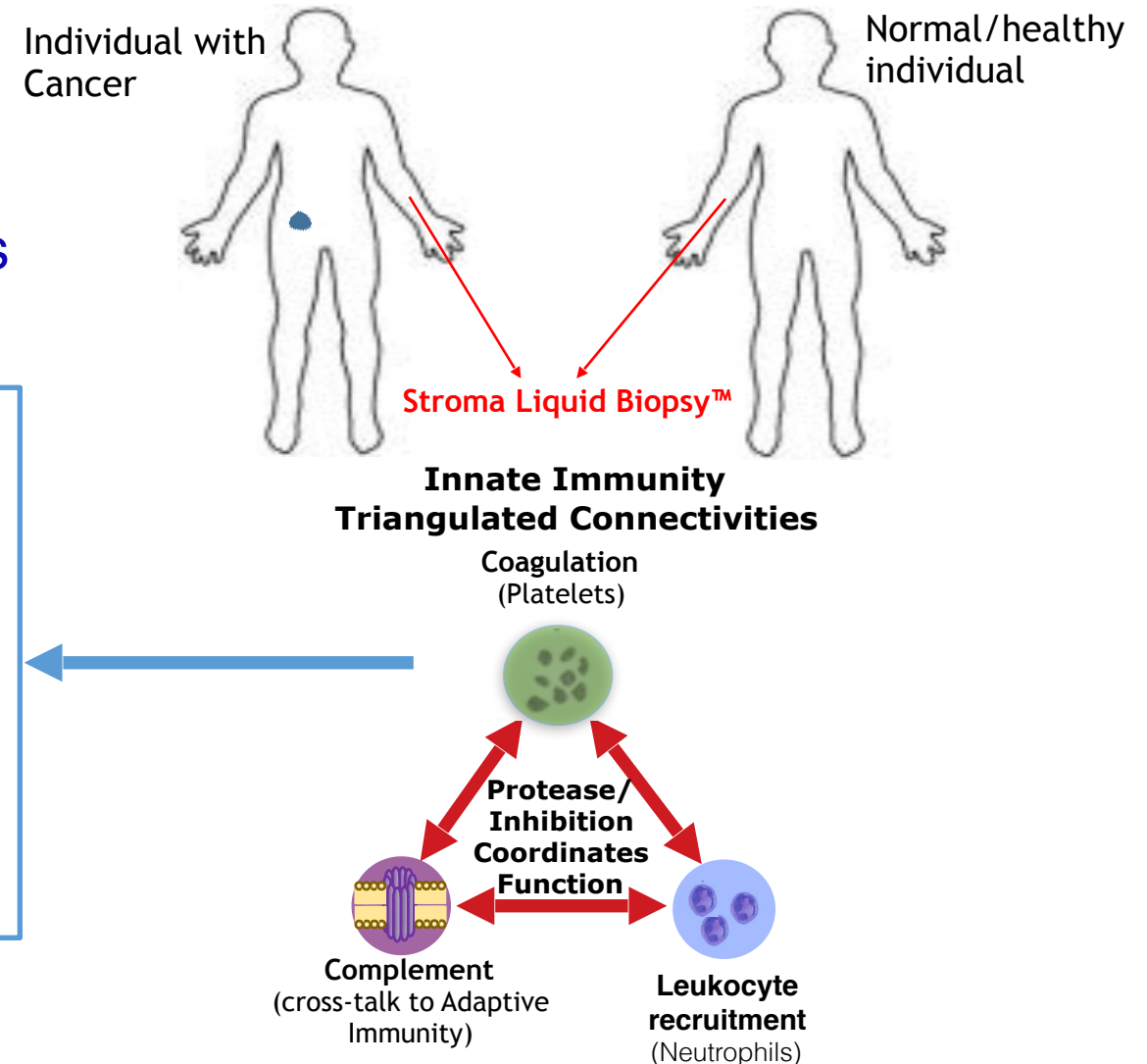# Liquid Biopsies Are Based On Monitoring Biomarkers
Current Strategies Focus on Circulating Remnants of the Proliferating Cells (the "Seed")
The Host Systemic Response Cooperates in Cancer Progression and Also Can Contribute Essential Biomarker Information as it is derived from the Tumor's Stromal Microenvironments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

SEED

Circulating DNA
Circulating cells
Exosomes

Tumor Derived Data

Cooperative interaction promotes disease progression

SOIL

Systemic Response

**Liquid Biopsy
Gene Biomarkers**

**Stroma Liquid Biopsy™
Protein Biomarkers**

Using an Albumin Removal product - AlbuVoid™, designed to enrich the low-to-mid abundance serum proteome prior to quantitative LC-MS/MS, we set out to answer whether there is a common systemic response measurable in blood sera, to most if not all cancers, regardless of primary tumor, stage, or metastatic disease.

Yes, the common response is reportable in biomarkers that reflect differential changes from normal/healthy individuals to individuals with cancer:

Individual with Cancer

Normal/healthy individual

Stroma Liquid Biopsy™

Innate Immunity Triangulated Connectivities

Coagulation (Platelets)

Protease/ Inhibition Coordinates Function

Complement (cross-talk to Adaptive Immunity)

Leukocyte recruitment (Neutrophils)

🔺 Coagulation/Platelet activation

🔺 Leukocyte Recruitment & activation

🔺 Complement Cascade

All of these pathways are

> derived from innate immunity

> systemically interconnected

> regulated through proteolytic PTMs

Chronic/reoccuring inflammation, driven by mechanisms of innate immunity, is a widely accepted contributor to cancer and many acute and progressive diseases

Innate immunity refers to first-line, non-specific defense mechanisms that come into play immediately after a perceived pathogenic insult in the body. When functioning properly, its main purpose is to immediately prevent the spread and movement of foreign or non-self pathogens throughout the body, and to initiate the second line of defense, the adaptive or acquired immune response. As a second-line defense, the adaptive response (e.g., T Cells) occurs downstream from the innate immune response and starts transferring immunological longer term memory towards the non-self pathogens. So a normal resolution of the innate response leads to a productive handoff to the adaptive response. Conversely, an unresolved innate response may delay or confound a suitable adaptive response, with both acute and chronic disease consequences.

Because the innate immune system has limited differential gene expression over time, protein level orchestration is the differentiator. As we learn how key systemic cross-talk mechanisms may go awry, therapeutically modulating even one rogue component may ultimately unwind the overall dysregulation and contribute to longer term management of disease. For these reasons, we have focused on key regulatory elements of innate immunity that can be observed, reported and surveyed by proteomic analysis of serum/plasma: Neutrophils, Platelets, the Complement cascade, and the protease inhibitor family called the SERPINs[1].

Neutrophils - the most abundant white blood cell, constituting 60-70% of the circulating leukocytes, and they thus contribute to the observable blood proteome much more so than the rest of the white blood cell constituents combined. Neutrophils have extensive crosstalk with each of the major blood cell subsets (megakaryocyte/platelets, myeloid and lymphoid), and release into circulation, cargo proteins from 5 different granule types in coordinated response to inflammatory insults, further magnifying their importance.
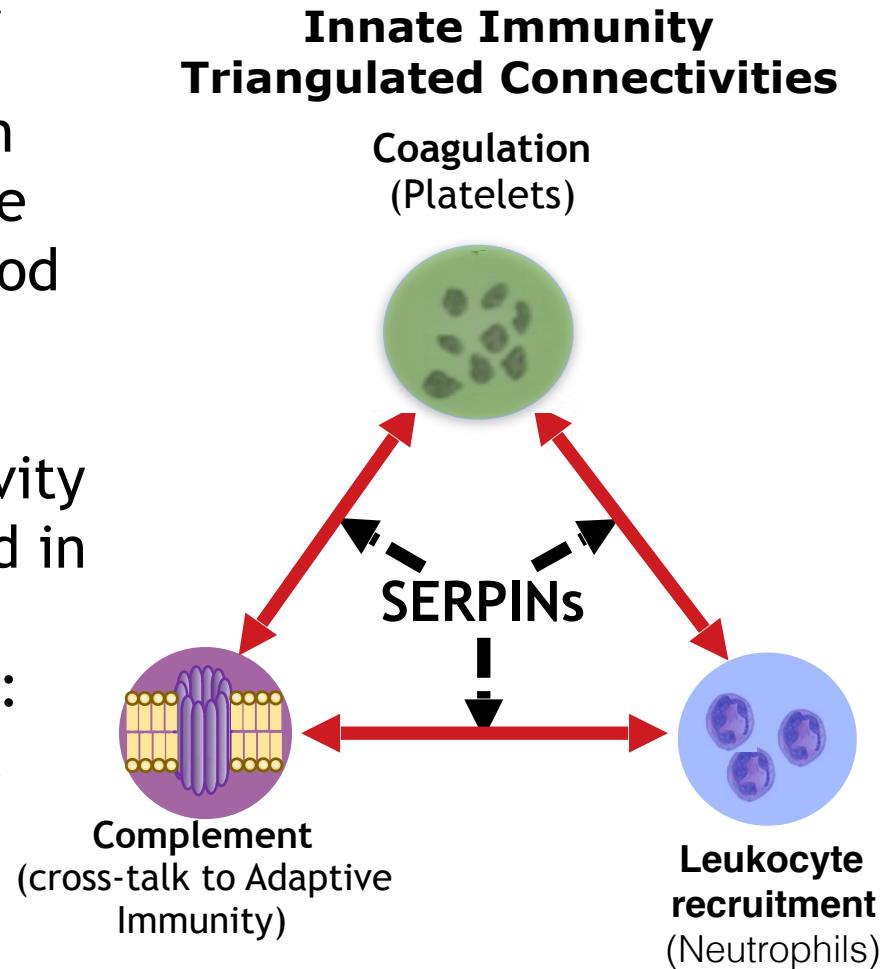
Platelets - do not have a nucleus, and contain dense and alpha granules; granule secretion being pivotal to establishing and controlling the microenvironment at the local inflammatory site. Platelets release contextually appropriate or kinetically controlled cargo that influences the entire immune spectrum, operating in parallel with their better known clotting function. Whereas rapidly released cargo can mediate early activation necessary for hemostasis, prolonged release can persist long after the initial stimuli. Persistence of Platelet/Neutrophil-released cargo in unresolved chronic inflammation, results in an ensuing 'protease storm'.

The Complement cascade - a major component of the immune system that provides powerful host surveillance and protection from invading microbes. Comprising about 5% of the total protein mass in plasma, most complement proteins circulate in blood as inactive precursors (zymogens); when triggered, become activated through proteolytic cascades. Cargo proteins from Neutrophils and Platelets regulate Complement activation in a tug-of-war fashion imposing a duality of positive and negative outcomes, in acute and chronic conditions[1].

The basis of Stroma Liquid Biopsy™, comes from innate immunity response and the cooperative relationship between cancer cells and a repertoire of recruited, normal host cells - the stroma, promoting an interdependent triangle of dysregulation in cancer[2]

For cancer, Stroma Liquid Biopsy™ reports key protein biomarkers in these pathways from blood

Serpin imbalance with protease activity is well documented in cancer, and used clinically, example: bound vs. free PSA

Innate Immunity Triangulated Connectivities

Coagulation
(Platelets)

SERPINs

Complement
(cross-talk to Adaptive Immunity)

Leukocyte recruitment
(Neutrophils)

As proteolysis is irreversible, there is an essential balance and regulation of proteolytic cascades necessary to keep competing influences from the triangulated pathways controlled. Central to this maintenance are the inhibitory Serpins, as they collectively serve the central control function of innate immunity. There are 9 major inhibitory Serpins reportable from blood and collectively account for about 5-10% of the protein mass in serum. However, conventional antigen-based assays fail to report their bifurcated functional pathways. BSG methods can now allow proteomic investigators to observe patterns of 'On' vs. 'Off' Serpin function[3,4].

Transitioning from Tissue to Liquid Biopsy

Objectives are to characterize and quantify BSG's Stroma Liquid Biopsy™ biomarkers so as to correlate to the Tumor-Stroma Ratio (TSR) scoring methods developed by Leiden University

Tumor-Stroma Ratio (TSR) in epithelial carcinoma

The Tumor-Stroma Ratio (TSR) is a primary tissue based prognostic score. Stroma-high tumors are associated with worse overall and disease-free survival. The prognostic value has been shown for a range of solid epithelial tumors[5,6].
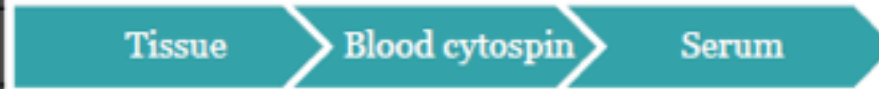
Primary Tissue availability severely limits the clinical utility of TSR. **Stroma Liquid Biopsy™** solves this problem and provides deeper and less subjective clinical characterization.

Our hypothesis: there is a difference in systemic response between stroma-low and stroma-high tumors. These differences may be observable at the tissue and blood levels to provide a more refined characterization of the tumor and prognosis for the patient.

We selected 3 biomarkers Stroma Liquid Biopsy™ biomarker panel to investigate whether there is correlation between tissue and serum.

Protein Gene Name	Protein Description	Serum Conc. In normal/healthy	Spectral Intensities cancer relative to normal	Expression levels found in literature
PF4	Platelet Factor 4	10 ng/ml	⬆	⬆
PPBP	Pro-platelet basic protein	5 µg/ml	⬆	⬆
TIMP1	Tissue inhibitor of metalloproteinases-1	100 ng/ml	⬆	⬆
THBS1	Thrombospondin 1	200 ng/ml	⬆	⬆
C3	C3	1,500 µg/ml	⬇	⬆
C4BPA	Complement Component 4 binding protein alpha	300 µg/ml	⬇	⬆
PROP	Properdin	25 µg/ml	⬇	⬇
SAA2	Serum Amyloid 2	5 µg/ml	⬆	⬆
ELANE	Neutrophil Elastase	250 ng/ml	⬆	⬆
ECM1	Extracellular Matrix Protein 1	800 ng/ml	⬆	⬆
CMGA	Chromogranin A	-	⬆	-
SERPIN A1	Alpha-1-Antitrypsin	1,500 µg/ml	⬇	⬆
SERPIND 1	Heparin Cofactor II	60 µg/ml	⬇	⬆

Tissue ＞ Blood cytospin ＞ Serum   TIMP1 ⬆

Prior literature does not report split-product proteoforms

Tissue ＞ Blood cytospin ＞ Serum   Properdin ⬇
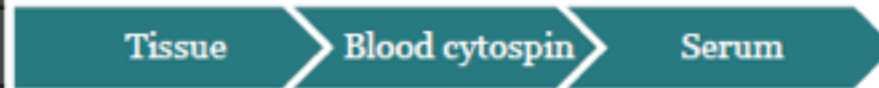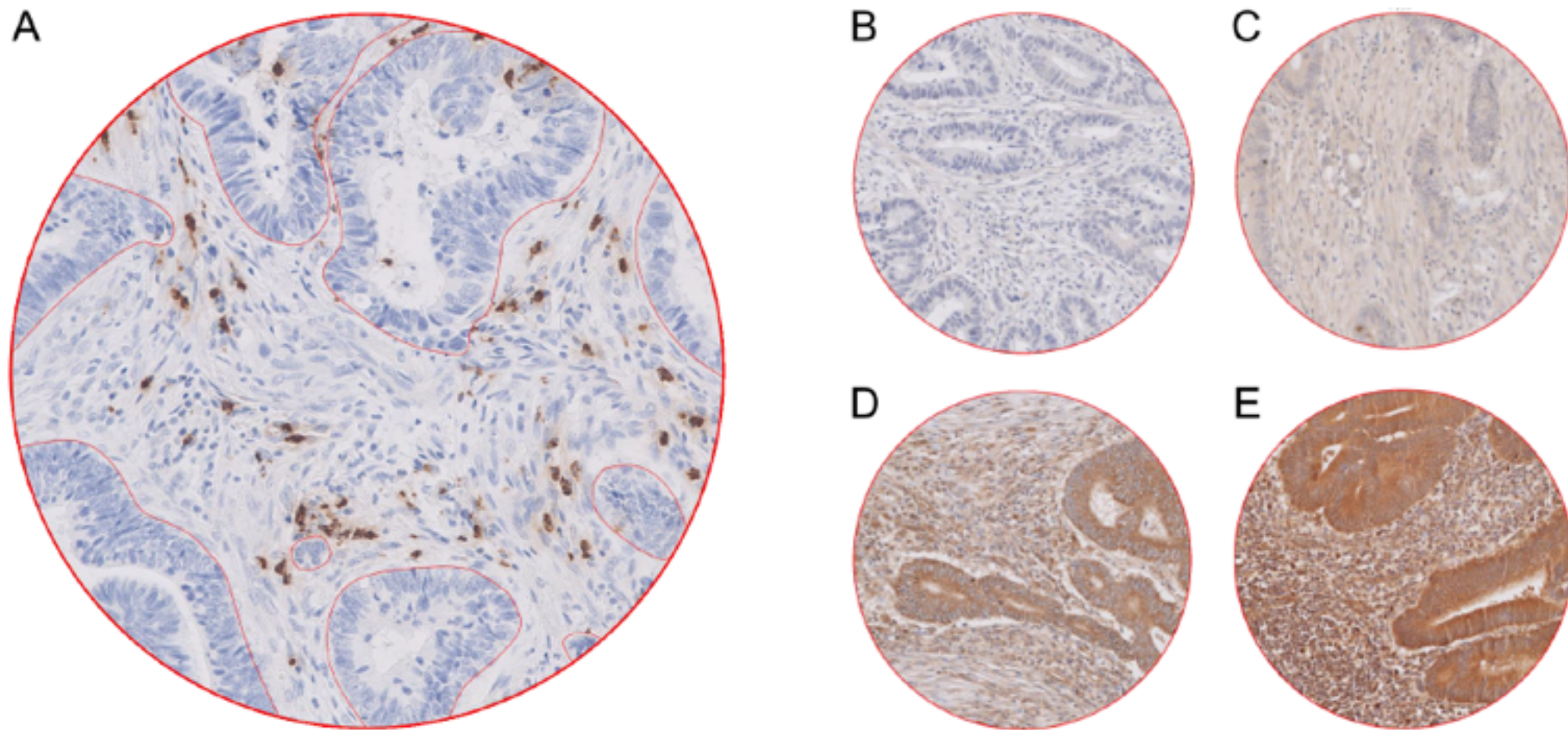
Tissue ＞ Blood cytospin ＞ Serum   ELANE ⬆

Prior literature does not report split-product proteoforms

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Quantative and qualitative scoring methods

Examples of scoring criteria. **A**) Tumor cells, encircled by the red lines, are excluded from *analysis*. Staining intensity is scored according to: 0 corresponds to no staining (**B**), 1 to faint staining (**C**), 2 to moderate staining (**D**) and 3 to intense staining (**E**).

ELANE (Neutrophil Elastase) expression is higher in the stroma-high patients (worse prognosis) compared to stroma-low patients

Highest ELANE scores: stroma-high vs stroma-low
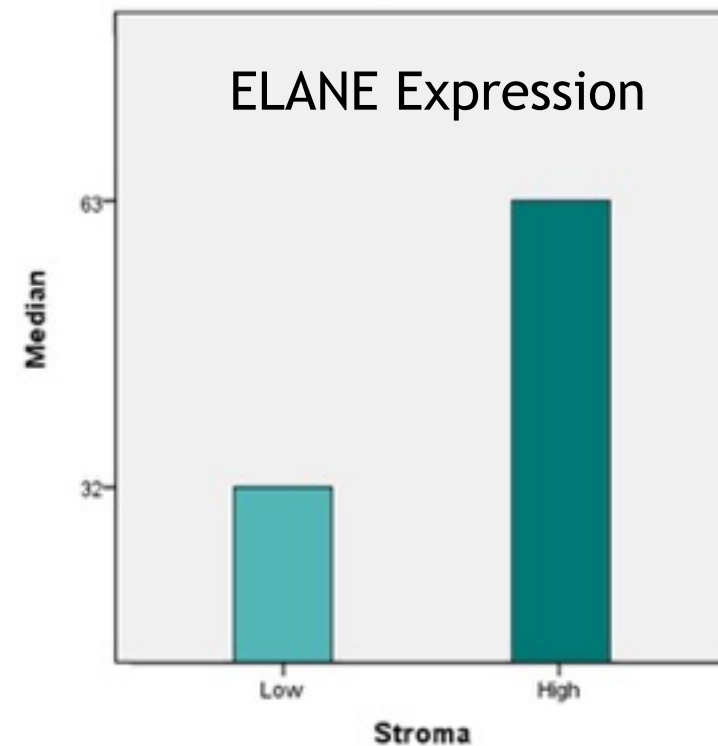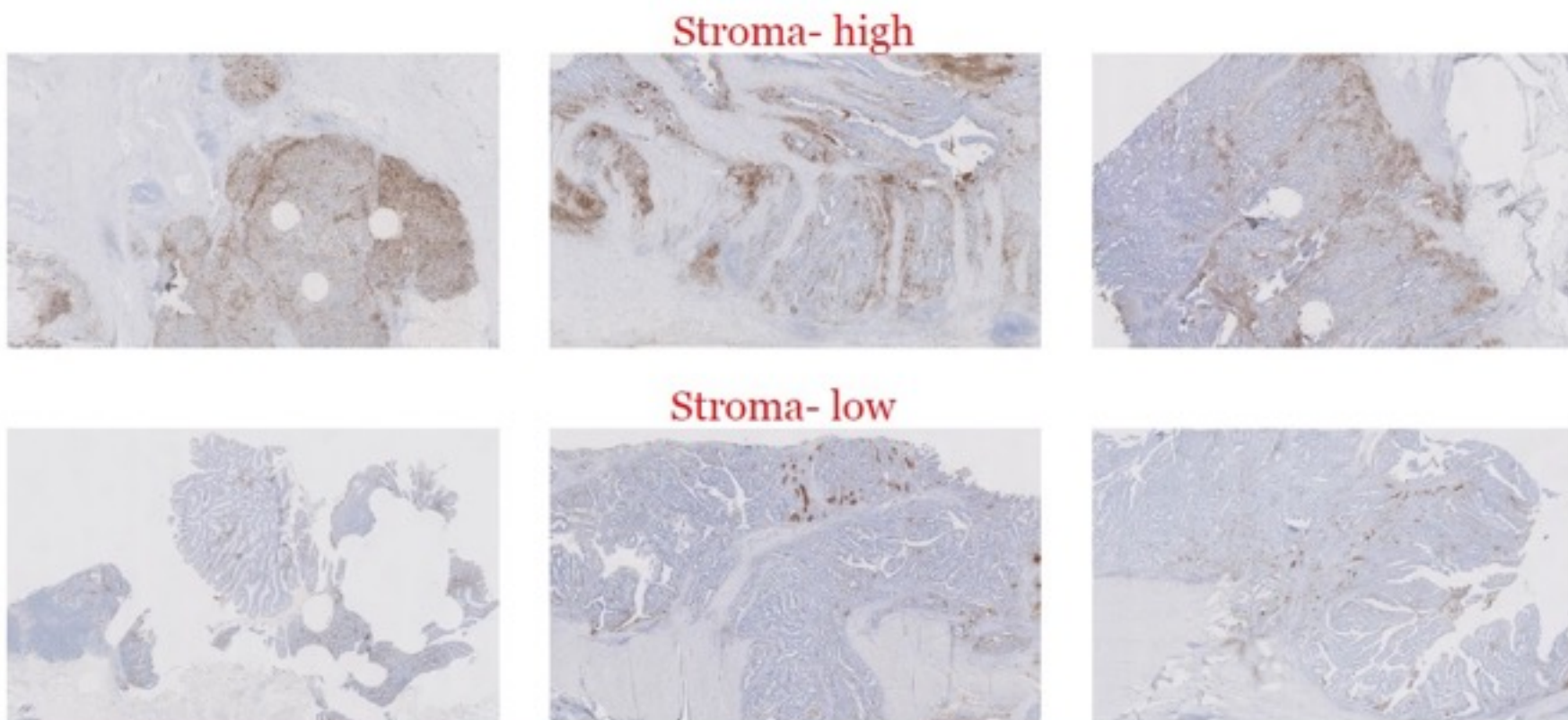
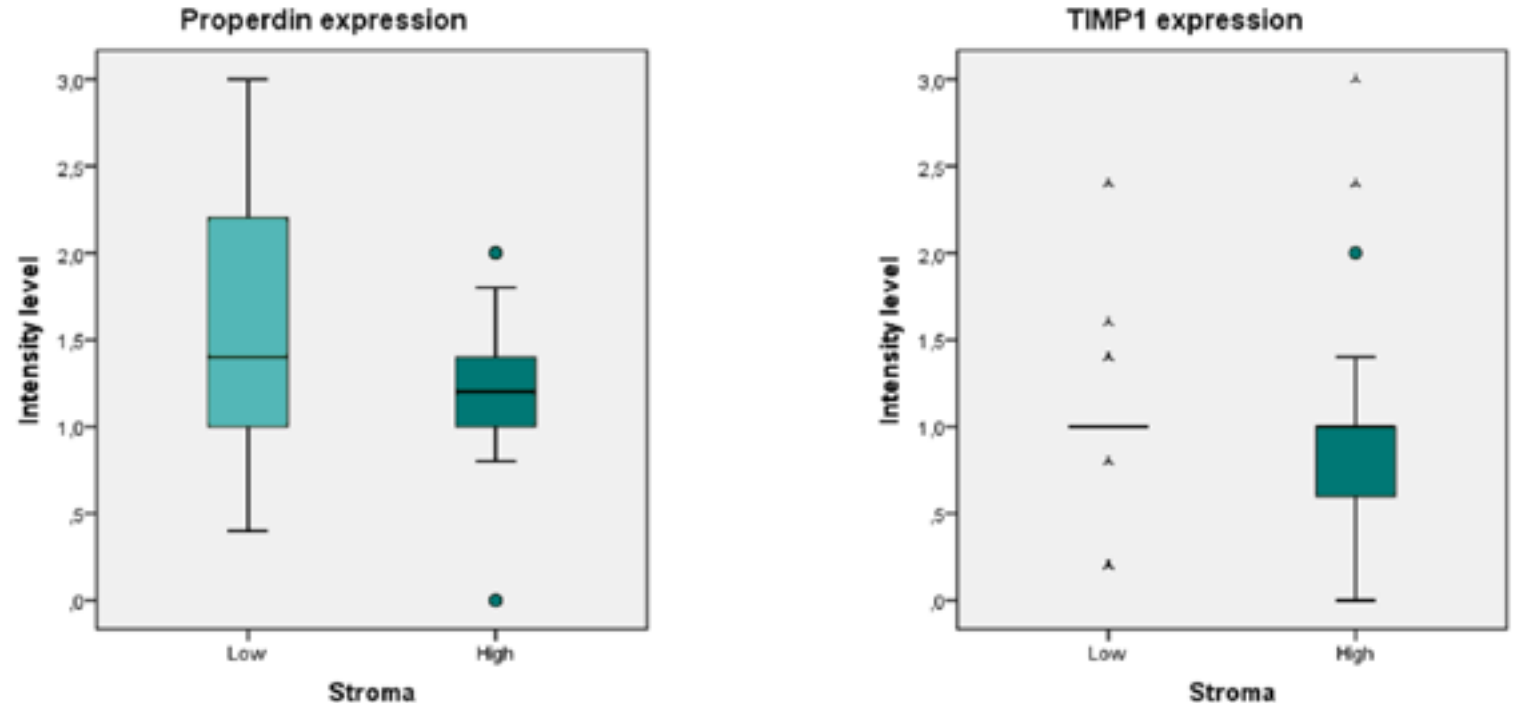**Preliminary Conclusions from tissue immunohistochemistry staining:**

Neutrophil Elastase (ELANE) expression is higher in the stroma-high patients compared to stroma-low patients. This is consistent with serum observations comparing normal/healthy to cancer populations, with Stroma Liquid Biopsy™ biomarkers. Neutrophil Elastase may be a stratification biomarker for prognosis at both the tissue and blood levels. Further investigation is warranted.

Properdin expression is lower in the stroma-high patients compared to stroma-low patients. This is consistent with serum observations comparing normal/healthy to cancer populations, with Stroma Liquid Biopsy™ biomarkers. Properdin may be a stratification biomarker for prognosis at both the tissue and blood levels. Further investigation is warranted.

Expression of Properdin and TIMP1

There is no difference in TIMP1 expression between stroma-high and stroma-low patients. Another measure of Coagulation and platelet released protein cargo at the tissue level needs further investigation.

Properdin and Neutrophil Elastase are both released from activated Neutrophils. Properdin released from Neutrophil granules is a major determinant of local Complement activation. A tug-of-war between platelets and neutrophils for the localized control and regulation of Complement has been proposed[1]. This may be an important component of the stromal conditioning at local tumor microenvironments, and further investigation of its prognostic relevance is warranted.

Future Testing

Samples
The matched tissue and blood samples are from population screening in the Netherlands, aiming for earlier detection of colorectal cancer. The patients were iFOBT test positive; an immunochemical fecal occult blood test, that also detects occult (hidden) blood in the stool. The individuals with a positive iFOBT test were further examined in the clinic by colonoscopy. Note that the iFOBT test results in many false positives that were further included in the study as control individuals. The true positives were diagnosed with mostly stage 2 and 3 colon cancer. From these blood samples, it will be interesting to see which patients had more aggressive disease.

Sample Prep - Albumin & IgG Depletion
To reduce interferences from Albumin & IgG, and to enrich the remaining serum proteome, we will use the BSG product AlbuVoid™ PLUS, following the manufacturers standard protocol[7]

LC-MS/MS Data Acquisition
An Agilent 1290 ultra-high-performance LC system equipped with a 20 µL sampling loop will be used in combination with an Agilent 6495 triple quadrupole mass spectrometer (QQQ-MS, Agilent Technologies, Santa Clara, CA), operating in positive ionization mode. We will quantify proteotypic peptides of the Stroma Liquid Biopsy™ protein panel, along with special peptides that report split-product proteoforms - notably from C3, SerpinA1 & SerpinD1, by standard MRM and SIL-peptide approaches.

Joint Goals
Developing Stroma Liquid Biopsy™ Cancer Biomarker Assays For Many Purposes

BSG
BIOTECH SUPPORT GROUP
Sample Prep that Matters

Leiden University Medical Center

Monitor Efficacy
- Prognostic Indicators
- Alarm bells to attending physician to off-target effects
  - bleeding
  - pulminary
  - acute distress

Patient Stratification
- Stromal Conditioning
- Hereditary Factors
- Clinical Stage
- Primary Tumor

Therapy Options

Decide
- Mono
- Combination

Dosing
- how much
- when

References

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7. AlbuVoid™ PLUS product sheet. http://www.biotechsupportgroup.com/v/vspfiles/templates/257/pdf/AlbuVoid%20PLUS%20Product%20Sheet%2004122019MK.pdf