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# The building blocks of successful translation of proteomics to the clinic

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Recently, the first two multiplexed tests using selective reaction monitoring (SRM-MS) mass spectrometry have entered clinical practice. Despite different areas of indication, risk stratification in lung cancer and preterm birth, they share multiple steps in their development strategies. Here we review these strategies and their implications for successful translation of biomarkers to clinical practice. We believe that the identification of blood protein panels for the identification of disease phenotypes is now a reproducible and standard (albeit complex) process.

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## Introduction

The test development path from discovery to clinical practice is long, complex and challenging [1••]). Critical requirements include employing a systems-driven strategy to identify the candidate biomarkers; establishing clinical and analytical validation as well as clinical utility; developing a solid health economics basis for the test; gaining reimbursement; achieving intellectual property coverage; being in regulatory compliance with multiple agencies; gaining acceptance by practitioners and eventual inclusion in the appropriate medical guidelines; and obtaining funding to do all the above. Blood biomarkers will play a critical role in personalized and precision medicine to improve healthcare by distinguishing normal from diseased individuals, stratifying patients into drug responders and non-responders; stratifying diseases into distinct subtypes and developing biomarkers that are prognostic of disease outcomes.

While single analyte tests are currently more prevalent, clinical practice now includes multiplexed genomic tests (e.g. Oncotype DX [2]) and proteomic tests (e.g. Vectra DA [3]), the latter relying predominately on immunoassay platforms. The multiplexed panels of proteins make sense by virtue of the fact that in disease multiple biological networks become disease perturbed [4,5] and hence relevant proteins from these networks can contribute to the power of the diagnostic determinations. Multiplexed immunoassay test development is challenged by technological limitations, including availability of reagents, interference, cross-reactivity and lack of specificity [6]). Cross-reactivity is an enormous challenge, especially in complex mixtures of analytes such as blood. In contrast, SRM-MS has been proposed as a technology platform that can overcome many of these challenges, primarily due to its high specificity, high multiplexing capabilities and low assay development costs [7]. Nevertheless, the appearance of diagnostic tests based on SRM-MS technology in the clinic has lagged. The first two multiplexed SRM-MS diagnostic tests used in clinical practice are Xpresys® Lung [8•,9] and PreTRM® [10•,11], launched in 2013 and 2015, respectively.

Xpresys Lung is a blood test for assessing the cancer risk of lung nodules discovered by radiology such as CT scans. The national program for annual CT screening for lung nodules in high-risk individuals was initiated in 2015 largely based on the National Lung Screening Trial [12]. Each year approximately 1.6 million lung nodules are detected in the US alone [13] and that is expected to increase strikingly as the national screening program is fully implemented. The majority of these nodules are benign with an estimated 15–25% being malignant. Lack of precise diagnostics, however, results in 35–42% [14,15]) of benign nodules being over-treated with invasive procedures such as biopsies and surgeries. Xpresys Lung measures the relative expression of eleven proteins by SRM-MS, five being diagnostic and six used for normalization of signal, and uses this information to generate a probability estimate that a lung nodule is benign, providing molecular evidence for whether or not invasive procedures can be avoidable. Interestingly all five of these proteins are expressed in disease-perturbed networks found in lung cancer. A detailed analysis of the economic impact of the Xpresys Lung test is that it has the potential to save the American healthcare systems multiple billions of dollars a year by avoiding unnecessary procedures and surgeries.

PreTRM is a blood test that reports an individualized risk of spontaneous preterm birth (sPTB) in asymptomatic women in the middle of pregnancy (at 19–20 weeks) [10]. PTB is a leading cause of infant mortality and morbidity worldwide. In the U.S. it is the leading cause of neonatal death and death in children before age 5 years and the health-economic impact was estimated by the National Academy of Medicine (formerly Institute of Medicine) in 2005 to be in excess of $26 billion per year [16]. Prior to the development of PreTRM, intense research into the development of predictive algorithms based on clinical and demographic factors or using measured serum or vaginal biomarkers did not result in clinically useful tests. PreTRM measures the relative level of two proteins by SRM-MS. These measurements are combined into a risk estimate that a pregnancy will end in sPTB. High-risk pregnancies can then be treated with interventions such as progesterone and/or high intensity case management.

In comparing the development paths for Xpresys Lung and PreTRM we identify six shared strategies that contributed to their successful translation into clinical use.

- Adoption of systems-biology techniques for enhancing the likelihood of success in the selection of a large multiplexed panel of potential biomarkers (hundreds).
- Adherence to the National Academy of Medicine best practices [1••] for test development to mitigate risks such as overfitting as well as applying analyses to multiple genetically distinct populations to correct for the noise arising from human polymorphisms.
- Early attention to analytical performance in test development to ensure the test will be robust over time and reproducible over hundreds of thousands of tests to be performed.
- Avoidance of 'loss in translation', an inherent risk in translating a test from a discovery-grade technology platform to a commercial-grade technology platform.
- Utilization of expression correlation techniques for efficient large-scale SRM-MS assay development.
- Normalization techniques that address various sources of analytic and pre-analytic variation.

In what follows we will elaborate on the six strategies shared in the successful development and translation of Xpresys Lung and PreTRM to the clinic.

## Systems biology approach to selecting the initial candidates

Although SRM-MS technology allows for high proteomic multiplexing, it does not reach the comprehensive coverage of genomic technologies. Therefore a set of initial candidate proteins must be selected. For example, discovery stage SRM-MS assays for the Xpresys Lung and PreTRM tests began with 371 proteins and 242 proteins, respectively. The implication is that discovery stage SRM-MS assays must be designed to span specific subproteomes that hold the most promise for diagnostic discovery, such as for lung cancer or pre-term birth proteomes. This focus can be facilitated by systems-biology techniques that harness multiple lines of evidence, including relevant existing high-throughput data analyses and sub-proteome sets that span relevant disease-perturbed networks and that are likely to be reliably detectable in the blood.

For Xpresys Lung, proteins were identified that were likely to be blood-based biomarkers of lung cancer. Literature searches and empirical studies were designed to identify cell-surface (membrane proteins are often cleaved and released into the blood) and secreted proteins differentially expressed by lung cancer cells [8•]. These proteins were then filtered using public resources such as the Peptide Atlas or previous detection in blood. Finally, proteins were prioritized for inclusion on the discovery assay based on specificity to lung tissue.

It turned out that 190/371 discovery proteins could be routinely detected in the blood. These 190 proteins were scored according to their ability to distinguish between samples of individuals with 72 benign and 72 malignant tumors. Thirty-two of these proteins performed significantly better than the others.

For PreTRM, protein biomarker candidates were selected by likelihood of being detectable in blood and for their presence in molecular networks implicated in pregnancy complications. Annotation as either cell-surface or secreted proteins guided selection from pregnancy relevant literature searches, and preliminary *de novo* serum proteomic discovery studies, by both mass spectrometry and an immunoassay panel screen (rules-based medicine), focused on evidence of dysregulation in preterm birth and preeclampsia. Protein candidate filtering and surrogate proteotypic peptide selection utilized public (protein/peptide atlas) and private databases, and full-scan MS/MS data from shotgun proteomic studies [10•].

A second application of a systems approach is the identification and prioritization of "cooperative" proteins. Whereas discovery-stage studies often rank analytes by univariate methods, as described above, cooperative proteins are ranked based on performance on protein panels (i.e. the best "team players") rather than on individual performance. This strategy is motivated by the intent to capture the integrated behavior of proteins within disease-perturbed networks.

In the case of Xpresys Lung, computational methods were utilized to identify cooperative proteins that appeared most frequently on the best performing protein panels by sampling the combinatorial possibilities. This was executed by creating a million panels of 10 from the