Advancing precision medicine: Current and future proteogenomic strategies for biomarker discovery and development
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Advancing precision medicine: Current and future proteogenomic strategies for biomarker discovery and development

About the cover: The multiomics human, represented by the interconnected biomarkers making up a human form. Variations in genes and proteins define individuality and set both the challenge and solution for advancing precision medicine.

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A worthwhile goal

It is difficult to pick up a scientific publication these days, or a newspaper for that matter, without seeing those ubiquitous buzzwords “precision medicine.” This term describes the ability to tailor diagnosis, prognosis, and therapy—ideally to individual patients, but at the very least to stratified patient groups. But what is really needed to reach this laudable goal? There is good agreement among clinicians and researchers that robust, accurate, and sensitive biomarkers are critical to this endeavor.

At the start of the genomics era when the first human genome became available, it was thought that knowing our DNA code would provide sufficient biomarkers to get us a good way toward our precision medicine goal. Unfortunately, the underlying complexity of the genome (and epigenome) proved to be far more intractable than many researchers expected. It soon became clear that genomics was not the panacea we sought, but required complementation from other ‘omics, including (but not limited to) proteomics, metabolomics, and transcriptomics. We are now at the point where genomics discovery tools (mostly high-throughput, rapid-sequencing technologies) are reaching maturity, while proteomics and other technologies are on the upswing. The articles in this booklet describe recent advances in proteomics technologies and how they are enabling the identification of new biomarkers that researchers are optimistic will advance us well along the track to realizing our objective for precision medicine.

Much work remains to be done, but there is little doubt that the wind is at our backs and the scientific discoveries coming out of this multi-omics era will benefit patients in measurable ways.

Sean Sanders, Ph.D.
Editor, Custom Publishing
Science/AAAS

The start of a new proteomics era

Dear Reader,

Olink Proteomics has supported the publication of this supplement, through Science/AAAS and in collaboration with senior researchers in the field, with the goal to inspire scientists with a vision of how a modern proteomics tool could drive the implementation of precision medicine.

This publication provides a summary of the current state of the field and a point of reference for future study designs, considerations, and interpretation of results across many research areas.

With emerging technologies, we see strong trends in the recent expansion to multi-omics studies and an increasing shift toward proteomics—going straight to the heart of biology that represents actual disease state and progression.

Another strong trend we have noted is the casting of a broad net of proteomics targets, with the goal of defining a protein signature for diagnostic, stratification, and prognostic purposes, as well as to gain insight into the pathophysiology of disease and to identify proteins that are causally associated with disease, providing new targets for effective drug development.

The team at Olink Proteomics is dedicated to supporting the research community with a rapidly growing high-quality, validated protein target library that, within the next couple of years, will cover the complete plasma proteome. We hope that we can be an integral part of your next important proteomics project.

I trust you will find this booklet beneficial to your work. We thank all participants who were essential to making this publication a reality. Happy reading!

Jon Heimer
CEO
Olink Proteomics
Biomarkers in precision medicine: Challenges and opportunities

Erik Ingelsson

The scientific premises of proteomics studies can be broadly considered a useful tool for assessing causality in biomedicine. Using this method, one can assess the likelihood that biomarkers are causally related with disease, and consequently whether perturbation of the biomarker is likely to affect disease development or progression. This is especially straightforward for protein biomarkers, given that there are usually strong protein quantitative trait loci (pQTLs) for protein levels that can be measured in the body or its products and influence or predict the incidence of outcome or disease (3). With such a broad definition, it is hardly surprising that the idea of using biomarkers to promote precision medicine is an obvious choice, as most measurable human characteristics qualify as biomarkers. Yet most would agree that the greatest promise for advancing precision medicine using biomarkers lies in circulating biomarkers (i.e., hormones, cytokines, and metabolites) that can be assessed in blood, urine, or other bodily fluids using minimally invasive methods.

Over the past decade, a paradigm shift from single-marker studies to ‘omics approaches has taken place. This shift has been most obvious in genomics, where genome-wide association studies have provided thousands of novel genotype-phenotype associations for complex traits, many in regions previously not known to be involved in these traits. The successes of this agnostic approach—where the whole genome is searched for associations—have inspired other ‘omics studies, such as transcriptomics, proteomics, and metabolomics. Among different types of circulating-biomarker and ‘omics methods, those using protein biomarkers and proteomics are of particular interest due to their close relation to gene function and expression, enzyme function, and disease states. Given recent rapid technological advances, we are now at the brink of truly groundbreaking discoveries using proteomics approaches.

The intersection of proteomics and biomarkers

Proteomics can be applied for many different reasons, but the scientific premises of proteomics studies can be broadly separated into two main categories: establishing biomarkers for prediction, diagnosis, or prognostication; and discovering novel biology and drug targets. Biomarkers that can improve a diagnosis or help in risk prediction are relevant in essentially all disease areas, and there are many unmet needs and clinical situations where better patient stratification would lead to earlier detection of disease, improve patient survival, and decrease suffering and costs for patients, their relatives, and society. New biomarkers may be used in combination with established risk predictors and should add clinical value to existing algorithms. Overall, there is a high bar for new biomarkers to enter clinical care. The evaluation of a new biomarker in a clinical decision-making process includes: assessment of analytical validity (it should provide reproducible results, be safe and acceptable to the patient, and be simple to interpret), clinical validity (it should have good performance characteristics, such as sensitivity and specificity; high predictive accuracy; and consistency across different study samples), and clinical utility (it should refine global risk assessment and guide management). For a new biomarker to be deemed useful, it must positively impact the management of patients, for example by leading to quicker and/or better diagnosis, or by aiding in selection of therapy. In addition, there are other factors that should be considered, such as economic costs for measurement, need for re-education of clinicians and the public, the invasiveness of the measurement, and ethical considerations (such as consent and confidentiality).

For discovery of novel biology and drug targets, proteomics is a promising approach that can provide new insights into the pathophysiology of different diseases. By design, proteomics approaches can interrogate a larger number of proteins and pathways than single-marker studies, and therefore be more likely to discover new pathways previously not implicated in disease. Along the same lines, this technology is potentially a very powerful means for the discovery of novel drug targets, particularly when combined with genomics. Over the past few years, Mendelian randomization (MR) has been established as a useful tool for assessing causality in biomedicine. Using this method, one can assess the likelihood that biomarkers are causally related with disease, and consequently whether perturbation of the biomarker is likely to affect disease development or progression. This is especially straightforward for protein biomarkers, given that there are usually strong protein quantitative trait loci (pQTLs) for protein levels that can be used for these studies. Indeed, we and others have performed large MR studies that provide evidence for a causal role for several risk factors in the development of coronary heart disease, such as body mass index (4) and levels of low-density lipoprotein cholesterol (LDL-C) (5), triglycerides (6, 7), interleukin-6 receptor (8), lipoprotein[a] (9, 10), and interleukin-1 (11), while the causal role of other risk factors such as C-reactive protein (CRP) (12), homocysteine (13), high-density lipoprotein cholesterol (HDL-C) (5), and lipoprotein-associated phospholipase A2 (Lp-PLA2) (14), have been rejected. Interestingly, the latter two examples are consistent with several large, randomized clinical trials, where HDL-C-increasing drugs and Lp-PLA2 inhibitors have failed to show reductions in cardiovascular disease events (15-18).

The appeal of biomarkers

Apart from the use of biomarkers in patient care—diagnosis, risk prediction, and prognosis for stratification and treatment decisions—and in novel drug-target discovery, there are...
also other traditional and emerging uses for biomarkers. The pharmaceutical and biotech industry is interested in biomarkers that can be used as surrogate markers in clinical trials, as well as in the development of novel biomarker assays for diagnostic purposes. Further, there is increased interest from the wellness and direct-to-consumer industries for using biomarkers, particularly the application of proteomics and other large-scale measurements for their tracking. Interest in the “quantified self” is increasing, and ‘omics methods can potentially be a complement to wearables and other measurements in this development, as exemplified by the “Snyderome” released in 2012 (19). In this project, a single individual was followed over a 14-month period using repeated genomic, transcriptomic, proteomic, metabolomic, and autoantibody measurements in an integrative personal ‘omics profile that revealed actionable health information, including newly developed type 2 diabetes. The hope (or hype) is that large-scale assessments of biomarkers in healthy individuals can lead to earlier detection and intervention, although this remains to be established in scientific studies.

Ongoing challenges

Some of the main challenges associated with ‘omics studies in general, and proteomics in particular, include technical issues such as reproducibility, validation, sensitivity, specificity, and the dynamic range of assays, as well as factors related to the biobanking of samples, including limited sample volume, the range of sample types, inconsistent preanalytical handling of samples, and repeated freeze-thaw cycles (especially in the oldest, most precious samples). In addition to the technical and biobank-related challenges, it is also important to note that it is often difficult for biomedical researchers applying proteomics to strike the right balance between coverage and cost. Although in an ideal world a proteomics experiment could assess all proteins present in a sample for a complete snapshot, the biobanking of samples, including limited sample volume, the range of sample types, inconsistent preanalytical handling of samples, and repeated freeze-thaw cycles (especially in the oldest, most precious samples). In addition to the technical and biobank-related challenges, it is also important to note that it is often difficult for biomedical researchers applying proteomics to strike the right balance between coverage and cost. Although in an ideal world a proteomics experiment could assess all proteins present in a sample for a complete snapshot of the proteome, this is usually in conflict with high per-sample cost and low throughput. In addition, the more proteins assessed per individual, the larger the sample needed.

This booklet includes a range of articles discussing different aspects of the role for proteomics in precision medicine. Drs. Price and Hood present their work on precision wellness, where they have generated and analyzed personal, dense, dynamic data clouds for 108 individuals over 9 months. Repeated proteomic profiling was integrated with whole-genome sequencing, metabolomics, microbiomes, clinical tests, and activity measurements to improve understanding of scientific wellness and discover early warning signs for human diseases. Dr. Hood—regarded by many as the father of systems biology—is also profiled in an interview by writer Kendall Powell. Drs. Gyllensten and Enroth discuss their efforts in understanding causes of individual variation in plasma protein levels, and how these biomarkers relate to biological aging. Dr. Mälarstig provides an overview of how genomics in combination with large-scale proteomics can build a solid foundation for precision medicine by maximizing information on drug targets. Drs. Siegbahn and Wallentin discuss the role of protein biomarkers in their large cardiovascular studies, comparing proximity extension assays with traditional immunoassays, and illustrating how large-scale proteomics can discover novel biomarkers for resilience against cardiovascular disease and cancer. Drs. Blennow and Zetterberg highlight the need for fluid-based biomarkers in neurodegenerative disease, to help with stratification of patients into subtypes of neurodegenerative disorders and to signal when patients respond to treatments. Dr. Zannad highlights the need for biomarkers in personalized treatment of heart failure, and the potential role for proteomics in this context, while Dr. Uhlén and colleagues give a broad overview of the field of plasma proteomics, and discuss how it can enable precision medicine, and help with development of new drugs and diagnostics.

Even if the term “precision medicine” is new, the idea that medical decisions should be tailored more specifically to the individual patient based on their predicted response is not—indeed, the concept of individualized or personalized medicine has been around for several decades. However, clinical care for the most part still adheres to a one-size-fits-all approach, based on strategies that have been developed for the “average” person, with less consideration of individual differences. That inevitably leads to the questions: why these concepts have yet to be established in clinical practice; what is needed for it to happen; and who has the power and capacity to change this situation? Hopefully, this supplement will provide some ideas and inspire solutions that elucidate how a modern proteomics approach can fit into the precision medicine paradigm, and how we can move closer toward truly individualized care and a more efficient drug development process.

Disclaimer: Dr. Ingelsson wrote this article as a consultant for Olink Proteomics; it was not part of his duties and responsibilities at Stanford University.

References

A wellness study of 108 individuals using personal, dense, dynamic data clouds*

Nathan D. Price1,2,6,7, Andrew T. Magis2,6, John C. Earls2,6, Gustavo Glusman1, Roei Levy1, Christopher Lausted5, Daniel T. McDonald1,4, Ulrike Kusebaum1, Christopher L. Moss1, Yong Zhou1, Shizhen Qin1, Robert L. Moritz1, Kristin Brogaard2, Gilbert S. Omenn1,3, Jennifer C. Lovejoy1,2, and Leroy Hood1,5,7

In order to understand the basis of wellness and disease, we and others have pursued a global and holistic approach termed “systems medicine” (1). The defining feature of systems medicine is the collection of diverse longitudinal data for each individual. These datasets can be used to unravel the complexity of human biology and disease by assessing both genetic and environmental determinants of health and their interactions. We refer to such data as “personal, dense, dynamic” data clouds: personal, because each data cloud is unique to an individual; dense, because of the high number of measurements; and dynamic, because we monitor longitudinally. The U.S. health care system invests 97% of its resources on disease care (2), with little attention to wellness and disease prevention. Here we investigate scientific wellness, which we define as a quantitative, data-informed approach to maintaining and improving health and avoiding disease.

We report the generation and analysis of personal, dense, dynamic data clouds for 108 individuals over the course of a 9-month study that we call the Pioneer 100 Wellness Project (P100). Our study included whole-genome sequences; clinical tests, metabolomes, proteomes, and microbiomes at 3-month intervals; and frequent activity measurements (i.e., wearing a Fitbit). An increased scale of personal, dense, dynamic data clouds in the future holds the potential to improve our understanding of scientific wellness and delineate early-warning signs for human diseases.

Data collection

108 individuals (ages 21–89+ years; 59% males, 41% females; 89% of European descent) participated in this study. Each individual had their genome sequenced in full. Blood was collected in clinics every 3 months. Additionally, participants completed at-home collections of saliva, stool, and first morning-void urine every 3 months. We called each of these three collection periods “rounds.” For each participant in each round we carried out 218 clinical laboratory tests, measured up to 643 metabolites and 262 proteins, and measured the abundance of 4,616 operational taxonomic units in the gut microbiome using 16S ribosomal RNA sequencing. We used the whole-genome sequence to calculate 127 polygenic scores for disease risks and quantitative traits based on previous studies selected from the National Human Genome Research Institute Genome-Wide Association Study (GWAS) catalog (3).

Community structure in the correlation networks

We built two age- and sex-adjusted correlation networks based on Spearman correlations across our cohort of individuals (Figure 1). Cross-sectional correlations were calculated from mean measurements of analytes calculated using all three rounds (mean A is correlated with mean B across all individuals). Delta correlations were calculated on the change in analytes between rounds (the change in A between time points is correlated with the change in B across all individuals). In these networks, vertices (V) correspond to analytes, and an edge (E) exists between two vertices if and only if a significant \( p_{adj} < 0.05 \) correlation was observed after correction for multiple hypotheses (4). The inter-omic cross-sectional correlation network contains 766 nodes and 3,470 edges. The majority of edges involved a metabolite (3,309) or a clinical laboratory test (3,366), with an additional 20 edges involving the 130 genetic traits tested, 46 with microbiome taxa or diversity score, and 207 with quantified proteins. The inter-omic delta correlation network contained 822 nodes and 2,406 edges. 375 of the edges in the delta correlation network were also present in the cross-sectional network.

We identified clusters of related measurements from the cross-sectional inter-omic correlation network using community analysis, an approach that iteratively prunes the network to reveal densely interconnected subgraphs (communities) (5). The communities often represented a cluster of physiologically related analytes, as described below. We describe below only a few communities in this abbreviated article.

The largest community (246 V; 1,645 E) contains many clinical analytes associated with cardiometabolic health, such as C-peptide, triglycerides, insulin, homeostatic model assessment-insulin resistance (HOMA-IR), fasting glucose, high-density lipid (HDL) cholesterol, and small low-density lipid (LDL) particle number. The four most-connected clinical analytes by degree (the number of edges connecting a particular analyte) were C-peptide (degree 99), insulin (88), HOMA-IR (88), and triglycerides (75). The four most-connected proteins measured using targeted mass spectrometry or Olink proximity extension assays by degree were leptin (18), C-reactive protein (15), fibroblast growth factor 21 (FGF-21) (14), and inhibin beta C chain (INHBC) (10). Leptin and C-reactive protein are indicators for cardiovascular risk (6, 7). FGF-21 is positively correlated with the clinical analytes C-peptide, triglycerides, HOMA-IR, insulin, and small LDL particle number, and is a recently reported biomarker for cardiometabolic disorders (8). INHBC, a member of the transforming growth factor beta superfamily, is positively correlated with the clinical analytes triglycerides, small LDL particle number, C-peptide, HOMA-IR, and insulin; it has not been reported to be a marker for cardiovascular

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risk and therefore represents an interesting candidate for follow-up.

We identified several communities containing microbiome taxa, suggesting that there are specific microbiome–analyte relationships. Hydrocinnamate, l-urobilin, and 5-hydroxyhexanoate clustered with the bacterial class Mollicutes and family Christensenellaceae (8 V; 8 E). The Coriobacteriaceae and Mogibacteriaceae families were associated (12 V; 19 E) with phenylacetic acid, eicosadienoic acid, p-cresol glucuronide, taurine, and phenylacetylglutamine. Phenylacetylglutamine, a known microbial metabolite (9), was recently identified as a risk factor for mortality and cardiovascular disease in chronic kidney disease patients (10).

A community formed around microbiome α-diversity (8 V; 7 E), a measure of the number of operational taxonomic units observed and the evenness of their distributions; elevated diversity is generally thought to be associated with better health in part by ameliorating inflammation (11). Microbiome α-diversity was negatively correlated with inflammatory and immune-related proteins, including interleukin-8 (IL-8), FMS-related tyrosine kinase 3 (FLT3LG), and macrophage colony-stimulating factor 1 (CSF1). In contrast, β-nerve growth factor (beta-NGF) was positively correlated with microbiome α-diversity (Figure 2).

**Delta correlation network identifies changes over time**

Thirty-three communities of at least two vertices (mean of 24.9 V and 59.2 E) were identified in the inter-omic delta correlation network. This network contains many interesting relationships not found in the cross-sectional network. For example, changes in HDL cholesterol were positively correlated with changes in galanin, a neuropeptide hormone with many physiological functions, including therapeutic associations with diabetes and Alzheimer’s disease (12).

**Polygenic scores correlate with disease-risk analytes**

Several edges in the cross-sectional network represented correlations between genetic traits and corresponding biomarkers already identified in published studies. For example, blood levels of dihomoy-γ-linolenic acid (DGLA) in our study were positively correlated with a polygenic score computed from genotypes in six variants that were previously associated with DGLA levels (13). Other edges in the network occurred between polygenic disease risk and specific analytes. For example, the genetic risk of inflammatory bowel disease (IBD) in Europeans has been associated with 110 single-nucleotide polymorphisms (SNPs) (14). In our cohort, the polygenic score for IBD calculated from all 110 SNPs was significantly negatively correlated with plasma cystine, the disulfide form of cysteine (Figure 2).

**Coaching and biomarker improvements**

In order to help participants modify their behavior and potentially improve their health throughout the 9-month period of this study, a behavioral coach talked participants through...
actionable possibilities from their data. Each month the coach worked with the participants and made recommendations for lifestyle changes with the aim of altering markers of known clinical significance and/or compensating for genetic predispositions. The most significant improvements for those who began the study out-of-range were observed in vitamin D (+7.2 ng/mL/round), mercury (−0.002 mcg/g/round), and HbA1c (−0.085%/round). We observed consistent improvements in total cholesterol measured by both Quest and Genova (−6.4 mg/dL/round and −5.4 mg/dL/round, respectively). LDL cholesterol, measured only with Genova, significantly decreased (−4.8 mg/dL/round), while HDL cholesterol significantly increased (+4.5 mg/dL/round). Other significant improvements were observed in other diabetes risk factors (fasting insulin and HOMA-IR), and inflammation [IL-8 and tumor necrosis factor-alpha (TNF-alpha)].

**Discussion**

We report here the main findings from the P100 Wellness Project. We computed thousands of statistically significant interomic correlations using personal, dense, dynamic data clouds to identify many associations that could be followed up with perturbation experiments. We partitioned the correlations into data communities, which placed biomarkers in context within biological networks. We identified molecular correlates of polygenic disease risk scores computed from published GWAS data, revealing possible ways in which genetic predisposition is manifested through analyte changes. Finally, the clinical biomarkers of many participants significantly changed in a healthy direction during the course of the study (e.g., type 2 diabetes and cardiovascular risk factors). Together these findings show that personal, dense, dynamic data clouds embody the essence of precision medicine (15) and present possibilities for the discovery of important medical applications. We hope that analyses for a much larger cohort will enable the identification of network perturbations that result in common diseases, the design of diagnostics to detect early disease transitions, and the development of drugs and other interventions to reverse disease at the earliest stages. Personal, dense, dynamic, data clouds are the essence of what precision medicine should be.

**References**

How personal, multi-omic data clouds will usher in precision medicine: A conversation with Leroy “Lee” Hood

Interview by Kendall Powell

Leroy Hood, president of the Institute for Systems Biology in Seattle, has always been a visionary when it comes to the complexity of human biology. His discoveries and inventions helped usher in the ‘omics revolution and earned him the Lasker Award, the Kyoto Prize, and the U.S. National Medal of Science. His vision for medicine in the 21st century is no less sweeping. He sees precision medicine as having four key qualities: predictive, preventive, personal, and participatory.

In a new study, he recruited 108 “pioneers” to have their genes, blood analytes, and microbiomes recorded and tracked for 9 months to create what he calls “personal, dense, dynamic data clouds.” In parsing those clouds, Hood and his colleagues found relationships between genetic risks for disease and circulating factors that could be used to diagnose, and perhaps ultimately treat and reverse, disease processes.

We spoke with Hood about why researchers and physicians should embrace the coming paradigm shift to scientific wellness—using real-time, personal data to monitor health-to-disease transitions and stop disease before it strikes.

Q: You have been called the “father of systems biology.” Do you recall the moment you realized that we’d need a whole systems approach to the problems of human biology?

It really started with my graduate thesis advisor in biology at the California Institute of Technology in the late 1960s, William Dreyer. His advice to me was: If you want to practice biology, then do it at the leading edge—it’s more fun there. Also, if you want to transform the field, you have to invent new technologies that let you make new, faster, or different kinds of measurements.

Q: What are the obstacles to achieving precision medicine?

One of the big barriers to generating personal, dense, dynamic data clouds is the cost of the assays. At $4,000 per patient per year, that’s prohibitively expensive. We need to improve technology to bring costs down by at least 10-fold. A second barrier is the conservative stance that many physicians take to thinking about wellness as an equivalent to disease. They are too caught up in thinking about disease and waiting for it to happen so that they can cure it. But wellness gives you the earliest chance for prevention.

Q: What role do you see protein biomarkers playing in precision medicine?

Any biomarkers that you can think about, including nucleic acids, metabolites, and lipids, will play a role, but proteins are right at the heart of biological action. They are doing the things that make you well or cause disease. So I think protein biomarkers will be the most sought after and most effective.

But the techniques for looking at protein biomarkers still need improvement. We need very simple assays for large numbers of proteins. The Olink Proteomics proximity extension assay (PEA) was among the most reproducible of any of the assays we did in our pioneer study, and that assured us that even small differences could be measured. A PEA also requires very little sample, so it’s easy to do retrospective studies. In addition, the assay has minimal cross-reactivity between the antibodies, which is key for looking across hundreds of proteins at once.

We used both PEA and mass spectrometry together in the pioneer study to capture the most interesting protein biomarker correlations.

Q: Why is there power in running genome-wide association studies (GWAS) alongside protein biomarker studies?

GWAS allowed us to group, or bin, the pioneers by their genetic risks for certain diseases, from lowest to highest. We found that the binned pioneers correlated with a disease phenotype, and identified several molecules that correlated with the participants’ polygenic-disease risk scores. For example, those with the highest risk of heart disease had the highest levels of low-density lipoprotein (LDL) cholesterol and those with the lowest risk had the lowest LDL cholesterol. Even though none of the pioneers had heart disease at the time, the metabolite cholesterol correlated with their genetic risk.

Q: Do those biomarkers reflect the genetic predisposition to a disease or the biology of the disease mechanism playing out?

We have no idea whatsoever—all we can say is that it’s a correlation. But the experiment would be very interesting. We found a negative correlation between the level of circulating cystine (the disulfide form of the amino acid cysteine) and individuals at high risk for inflammatory bowel disease (IBD). If you increased the level of cystine in these people by giving them a supplement, would it lower their risk of getting IBD? That’s a testable hypothesis. And in that sense it would be far more than a biomarker; it would be a drug.

Q: How do we make sense of all the correlations found within these personal, dense, dynamic data clouds?

These clouds are like the Hubble Telescope—they give a resolution we haven’t seen before of the multi-omic statistical correlations that are the determinants of wellness and disease.

But these correlations look like a hairball—with the edges representing the statistical probabilities between two different analytes. We then ask the computer program to cut all the edges that were the lowest probabilities and leave the strongest.

Q: What are the obstacles to achieving precision medicine?

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But these correlations look like a hairball—with the edges representing the statistical probabilities between two different analytes. We then ask the computer program to cut all the edges that were the lowest probabilities and leave the strongest.
When we prune out the things that are less likely … boom, we see relationships that emerge from the hairball and end up with discrete communities, such as the cardiometabolic community of molecules.

Q: You have previously noted that multi-omics from one individual will allow for “N=1 experiments” that will be key to advancing precision medicine. How would those experiments work?

In a sense there are two kinds of N=1 experiments. The first will be studies of how nutrition and complex human genetics play out. How each individual handles nutrients and metabolites is really diverse, and so N=1 experiments done in the context of an individual’s genetics and environment will be critical for our understanding of, for example, nutrition.

Second, there are several reasons why N might only be 20, 30, or 40 for the clinical drug trials of the future. You might begin a trial with an N of 30 to determine any off-target hits by the drug, to stratify the disease into its subtypes, and to stratify the patients into responders and nonresponders. Right now, among the top 10 most-prescribed drugs in the United States—things like Abilify, Nexium, and Crestor—the ratios of responders to nonresponders range from 1 in 4 people to 1 in 25! Most of the people getting drugs are not even responders—it’s a significant waste of health care dollars.

So if you identify really good biomarkers that can indicate who responds to a treatment, then you can do a clinical trial with just 40 responders and get a drug approved because of high levels of responsiveness. If you can stratify your patients correctly, you can cut down all the numbers as well as all the morbidity caused in nonresponders.

Q: Will health care of the future sequence genomes, and create and track personal data clouds for everyone?

Absolutely. The real issue is cost and proving the validity of all the actionable possibilities based on an individual’s data. We’ve been enrolling new participants at Arivale, a consumer scientific wellness company that I cofounded, with the ultimate goal to have data clouds for hundreds of thousands or even millions of people in the next 5-10 years. We will follow them longitudinally to see the wellness-to-illness transitions for all common diseases and figure out how to reverse them.

We could do away with chronic disease by catching people at the very earliest trigger points. For example, since Alzheimer’s costs the U.S. health care system $260 billion a year, we’d eventually be saving that amount by catching most future Alzheimer’s patients at their earliest stage of transition and reversing the disease process. The hospital systems that embrace this enormous paradigm change will put themselves at the forefront of medicine in the 21st century.

Harnessing the potential of plasma biomarkers in precision medicine by efficient control of effects unrelated to disease

Ulf Gyllensten and Stefan Enroth

The high specificity, wide dynamic range, and multiplexing capability of the proximity extension assay (PEA) offer unique opportunities to study the plasma proteome. Promising candidate disease protein biomarkers are often affected by lifestyle and genetic factors that are unrelated to the disease under study. We have examined the determinants of individual variability in the plasma proteome and have developed methods to control for variation that are unrelated to disease. These methods are useful in order to rank biomarker candidates for further study and to increase their diagnostic value in precision medicine.

Understanding causes of individual variation in plasma protein levels

Whole blood, serum, and plasma are the dominant sample types collected in routine clinical analyses. Due to leakage and secretion of proteins from organs into the bloodstream, the plasma proteome encompasses proteins originating from a large number of tissues throughout the human body. Ideally, biomarkers for identification of disease should not be affected by non-disease-related factors. However, many biomarkers are not uniquely found in the malignant tissue, and their circulating levels are affected to an unknown degree by an individual’s genetic and physical constitution, lifestyle, and medication. A detailed understanding of such confounding factors and the magnitude of their effect is therefore a necessary prerequisite for evaluating the rapidly growing number of candidate biomarkers.

In order to identify the nature of the determinants and their impact on individual variability in plasma protein levels, we have studied a population-based cohort, the Northern Swedish Population Health Study (NSPHS), for which there exist extensive phenotype and genotype data. The data include information on lifestyle, environmental factors, exposure to medication, and sample handling, making it possible to study a wide range of putative covariates. The cohort has been genotyped using high-resolution genetic single-nucleotide polymorphism (SNP) arrays, and the data has been analyzed to generate a map of the genetic constitution of each individual in the study group, which includes over 10 million genetic markers. Using information on over 150 selected variables, we determined the effect
of common clinical variables and lifestyle factors such as age, sex, blood pressure, blood group, body mass index, medication, and smoking on protein biomarker levels, and we performed genome-wide association studies (GWAS) to determine the heritability of biomarkers. In our initial study of biomarkers for cancer and inflammation, we selected 158 phenotypic covariates available for NSPHS (1) and found significant effects in 52 of the 77 proteins studied. The proportional effect of genetic, clinical, and lifestyle factors on biomarker levels differed dramatically between the 77 proteins studied (Figure 1). While the variability of some biomarkers between individuals was strongly affected by genetic factors (left side of figure), others were predominantly influenced by environmental or clinical factors (right side of figure). Since some of the covariates are not independent, such as blood pressure and medication use (both are related to age), the sum of the explained variance by individual covariates in some cases reached above 100%. Combined
models using the 158 non-genetic variables together with genetic factors explained 20%–56% of the intra-individual variability in biomarker levels (Figure 1B, gray bars). The remaining variability may be due to sample handling, as yet unidentified covariates, or technical viability of the PEA.

More recently, we studied 425 proteins present in 178 pathways in a population-based cohort (n=903), using the Multiplex CVD II and III, the Proseek Multiplex Inflammation I, the Proseek Multiplex Oncology I, and the Proseek Multiplex Neurology I panels from Olink Proteomics to identify determinants of plasma proteome variability (Enroth et al., unpublished data). Anthropometric and lifestyle traits affected about 75% of the proteins, while genetic factors impacted 25%. Together, these factors explained up to 86% of the variation between individuals.

In addition to the lifestyle and genetic constitution of an individual, the handling of clinical samples in the biobank may affect the outcome of the analysis. Using a longitudinal dataset, we studied the effect of sample storage time at -80°C, and the season and month of the year, as well as the patient’s age at sampling, on the abundance of 108 proteins in the blood of 106 Swedish women (Figure 2) (2). Of the 108 proteins that could be detected in more than 80% of samples, the storage time affected 18 proteins (17%) and explained 4.8%–34.9% of the observed variance. Seasonal variation had an effect on 15 proteins (14%), while time of year (number of sun hours) affected 41 proteins (38%), but only explained at most 4.5% of the variance. By comparison, chronological age at sample collection, which is a well-recognized covariate, affected 70 proteins (65%) and explained 1.1%–33.5% of the variance. These results showed that sample storage time and collection date (month and season) had a magnitude of effect on the measured abundance of these proteins in plasma that was similar to the well-recognized covariate of age at sample collection. This implies that detailed data on sample handling—particularly storage time—should be regarded as covariates that are as prominent as age and sex, and need to be included in studies involving protein levels.

**Incorporating covariate information in biomarker identification and clinical use**

Given the prominent effect of certain covariates, as discussed above, we investigated how other biomarker variables might be used to reduce non-disease-related variation (3). Through modeling of the relevant covariates for each biomarker, we developed the concept of *personally normalized plasma protein profiles* (PNPPP).

The methods we developed can be used in biomarker discovery to rank promising biomarkers, or to determine personalized normal threshold levels that can help to increase the diagnostic usefulness of a particular biomarker. These two applications are discussed in more detail below.

The PNPPP method was first used to identify 125 candidate biomarkers for five non-communicable diseases (cataracts, diabetes, hypertension, myocardial infarction, and stroke) in our NSPHS cohort. For each of the diseases studied, we divided the individuals into cases and controls. We then re-examined the significance levels for influencing variables, such as age or genetic markers, for each measured protein in the controls alone by fitting a generalized linear model, including all covariates simultaneously. In this step, the posterior genotype probabilities for the top-ranking marker from the combined protein-specific GWAS were included as possible confounders. The per-protein linear model generated was then applied to each individual in the cases and control groups, and the PNPPPs were calculated for both affected and unaffected individuals. Case-control analyses were subsequently performed using both the unadjusted biomarker measurements and the PNPPP. Using protein abundance values that have not been adjusted for the effects of covariates (denoted “raw”), a large number of proteins showed a significant difference between cases and controls (Figure 3, raw in red). By employing the PNPPP method, the number of covariates for these proteins was reduced dramatically (Figure 3, adjusted in blue), and as a consequence, the number of biomarker candidates was also reduced. In total, for the five diseases we studied, the number of significant differences in abundance levels between cases and controls was reduced from 174, when using unadjusted
abundance levels, to only 64 when the PNPPP method was applied. The most striking effect was for stroke and cataracts, where the number of proteins showing a significant difference between the individuals with a particular disease and the control group number was reduced from 20 to 6 and from 37 to 9, respectively (Figure 3). Several known associations of diseases with previously described biomarkers were replicated, including associations between epidermal growth factor receptor and brain natriuretic peptide (BNP) for cataracts; matrix metalloproteinase-10 and growth differentiation factor 15 (GDF-15) for diabetes; midkine for myocardial infarction and high blood pressure; and spondin 1, which was elevated in individuals with high blood pressure.

The PNPPP method can also be used to improve the clinical utility of protein biomarkers. For example, tissue plasminogen activator (t-PA, Figure 4A) has previously been associated with increased risk of myocardial infarction, but our covariate analysis showed that both weight and systolic blood pressure (SBP) influence circulating levels of t-PA in individuals who have not suffered a myocardial infarction. The association between t-PA abundance and weight in the unadjusted raw values (Figure 4A, dotted line) prohibits the use of a weight-independent cutoff for identifying cases. However, the PNPPP method allowed a constant cutoff to be applied (Figure 4A, solid lines). A second example is the abundance of T-cell immunoglobulin mucin receptor 1 (TIM-1; also known as hepatitis A virus cellular receptor 1, HAVCR1), which differed between cataracts cases and controls. No difference was found after adjusting for weight, SBP, age, waist size, height, usage of insulin and fast-acting analogs, and genetic factors (Figure 4B). TIM-1 is currently used as a biomarker for proximal tubular injury in renal diseases, but has not previously been linked to cataracts, suggesting that the associations we found using the raw values were primarily due to differences in age, SBP, and weight between cataracts cases and controls. Similar patterns were seen for GDF-15 (Figure 4C) in relation to diabetes and SBP, and for human growth hormone with respect to hypertension and weight (Figure 4D). Here, we adjusted the abundance of GDF-15 for sex, height, and weight, and PNPPP allows for linear cutoffs to be applied. This illustrates that proteins that are strongly correlated with anthropometrics or non-disease-related lifestyle factors can increase their clinical usefulness by having their levels normalized. Such normalization improves the utility of the biomarkers and facilitates their clinical interpretation.

These results signal a paradigm shift in biomarker study design. Information on which parameters affect a biomarker can be identified from nonaffected cohorts and used to normalize the values in the affected cohort. This differs conceptually from recruiting, for example, age- and gender-matched controls, and allows for the more efficient use and reuse of control cohorts. Our results also impact how biomarkers are used clinically. The physician will either determine a cutoff depending on a predefined set of prerequisites, such as age, gender, or ethnicity, or recalculate the value based on models generated from nonaffected individuals. The former system quickly becomes unfeasible when several factors need to be taken into account or when noncategorical variables, such as age or blood pressure, are present. The PNPPP method provides ad-
advantages by limiting the number of covariates included in the analysis and providing a set of candidate biomarkers for further validation whose variability is less affected by factors unrelated to disease.

**Lifestyle factor biomarkers and biological aging**

Aging is associated with numerous changes in the body at the molecular level, including telomere length reduction and alterations in metabolic, gene-transcription, and DNA-methylation patterns (4–6). In addition, chronological-time (age) lifestyle factors, such as smoking or stress, can alter DNA methylation patterns and telomere length (7–8). Comparisons of chronological age to that predicted by molecular methods (denoted “biological age”) is an indicator of health status and can provide insight into the effects of lifestyle changes while also aiding in treatment decisions for cancer patients (9).

We studied 77 plasma proteins from the Proseek cardiovascular and cancer panels in 976 individuals from the NSPHS to determine if the protein profile could predict chronological age, weight, height, and hip circumference. Surprisingly, chronological age was predicted within 5.0 years, weight within 6.8 kg, height within 4.7 cm, and hip circumference within 5.1 cm for 50% of the observations (10). The proteins in our analyses are not known to be involved in biological aging, and the changes seen in the protein profile are therefore not likely to be drivers of the aging process per se, but rather a reflection of this process at the metabolic level. Some proteins are expected to be more important for a particular trait. For instance, the strongest effect on height was found to be the growth hormone somatotropin, followed by fatty acid-binding protein 4 (FABP4). For weight, the main predictive proteins were a growth hormone and tissue plasminogen activator (t-PA). For age, the top predictor was osteoprotegerin, then C-X-C motif chemokine ligand 9 (CXCL9), and finally GDF-15.

We subsequently applied our modeling of the plasma protein profile to examine the effect of lifestyle choices on predicted biological age. We first studied smoking by building an age-predicting model from individuals who self-reported as nonsmokers, and then applied these models to smokers. Our model predicted smokers to be on average 2.3 years older (Figure 5) than their chronological age, although the two groups do not differ in chronological age. We then examined the impact of body mass index (BMI) on predicted age by training a model on individuals with normal weight (BMI 18.5 to 25) and applying it to higher BMI intervals. Individuals with a BMI over 40 were predicted to be on average 6.3 years older than their chronological age. We had access to over 1,000 phenotypic traits from our study cohort, including dietary habits, and examined the effect of certain dietary components on predicted age. High soda consumption increased the...
predicted biological age (since soda consumption is known to be age-correlated, we included only individuals between 20 and 50 years of age), while consumption of fatty fish reduced the predicted age. Remarkably, individuals drinking three to six cups of coffee per day were predicted to be on average 5.6 years younger than their chronological age (Figure 3G). Finally, we studied exercise, where participants compared their own level of exercise relative to individuals of the same age in the community (peers). Those that self-reported to “exercise less” had a predicted age 2.3 years higher than their peers, which increased to 5.2 years in those who self-reported to exercise “much less” than their peers.

We envisage that such analyses of the plasma protein profile can be used to motivate lifestyle changes for individuals at risk of developing noncommunicable diseases by monitoring the effect of changes on predicted age. It has been shown that sugar-sweetened beverages cause earlier menarche in girls (11) in the United States, demonstrating that dietary effects are not only confined to specific molecular events but can have a significant impact on the development of the body. Such dietary habits have direct downstream effects on disease risk for an individual. For example, risk of breast cancer is known to increase by 5% for each year of early onset of menarche as a consequence of longer lifetime exposure to estrogen (12). Plasma protein profiles may also be valuable in forensic medicine. Age and gender prediction in forensic medicine have been proposed based on DNA methylation or protein N-glycan levels. The PEA technology described here is applicable to dried whole blood samples (13, 14), and our findings indicate that such an analysis method could provide basic anthropometric data in forensic investigations. A single set of proteins might be sufficient to capture enough variation in these traits to be useful for anthropometric predictions.

References

pQTL studies and examples: Understanding biological mechanisms and creating a foundation for precision medicine

Anders Mälarstig

Precision medicine is an approach to drug development that requires a deep understanding of the molecular pathways involved in human disease, the right molecular tools to modulate and monitor implicated disease pathways, and the incorporation of such tools in clinical trial design and execution. Precision medicine should bring more efficacious and safer medicines to patients across multiple therapeutic areas and decrease the proportion of drug-target candidates stopped after phase 2 testing due to lack of efficacy (1). Here I discuss how genomics, in combination with large-scale proteomics, builds a solid foundation for precision medicine by maximizing a priori information on a drug target, the biomarkers associated with that target, and that target’s relevance in human disease.

A priori precision medicine drug development to reduce clinical-stage attrition

Our understanding of human disease biology and the druggable target space has advanced significantly in the past decade, but clinical-stage attrition remains relatively high, in large part because drugs are failing to meet efficacy benchmarks (2, 3). Multiple reasons exist as to why a candidate drug fails to show clinical efficacy, including incomplete understanding of the drug’s mechanism of action in disease pathogenesis, incomplete data on target engagement and occupancy, and incomplete matching of the mechanism under study to the clinical features of patients in the trial (4). Failed clinical trials may benefit from deep profiling of the trial population using new proteomics and genomics technologies. Plasma/serum proteomics, in particular, has taken a significant leap forward in the past few years, with sensitive, specific, and robust multiplex assays now available to rapidly analyze thousands of proteins (5). Post hoc ‘omics profiling may reveal subgroups of patients that responded better to treatment or were at heightened risk of suffering adverse events. If such subgroups are found, better-designed trials that incorporate the new information can be planned. While postmortem profiling in a trial with partial responders can be informative, perhaps a more effective approach would be to apply strong a priori information in the initial stages.
of drug development and avoid the need for post hoc profiling.

**Connecting genomics to proteomics**

Genome-wide association studies (GWAS) have provided thousands of robust connections between genetic variants and susceptibility to multiple, common, complex human diseases and traits, so-called genetic loci (6). The biological mechanisms underpinning the regulation of different genetic loci are often difficult to understand, and additional molecular annotation and functional studies are needed. If combined with other data such as RNA and protein expression, genetic loci can provide a map of those biological pathways most relevant to the development, progression, and outcomes of a particular disease. In addition to explaining complex disease traits, GWAS can be combined with proteomics to identify so-called protein quantitative trait loci (pQTLs). A pQTL denotes a genetic variant robustly linked to protein expression data. A cis-pQTL is located near the gene encoding the protein being investigated, while a trans-pQTL is located elsewhere in the genome. pQTLs have multiple applications in drug development. First, cis-pQTLs can be used to establish whether a disease biomarker is likely to be causal or observational using Mendelian randomization, and thus inform drug-target discovery and validation (7). Second, trans-pQTLs can be used to map effects downstream of a gene and identify mechanistically relevant biomarkers that can in turn be evaluated as defining markers for the trial-patient population or as surrogates for efficacy (8). Third, since many pQTLs explain a significant proportion of interindividual variation in protein levels, the endpoint prediction performance of a protein biomarker can theoretically be enhanced by taking such information into account (9). Likewise, safety biomarkers with significant variability explained by pQTLs can be avoided, or at least better understood.

**Discovery and follow-up of pQTLs: The IMPROVE study**

IMPROVE is a multicenter, observational study that recruited 3,711 men and women from five European countries, aged 55–79 years, with at least three cardiovascular disease (CVD) risk factors, but without symptoms. To identify novel pQTLs, a total of 5 million imputed single-nucleotide polymorphisms (SNPs) were tested as determinants for 83 plasma proteins on the Olink Proteomics CVD I panel in all IMPROVE baseline samples. A total of 79 genome-wide significant (p < 5 x 10^-8) pQTLs for 56 proteins (67%) were discovered (10). It could be concluded that most proteins are significantly influenced

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**FIGURE 1.** The 29 proteins investigated in the IMPROVE study. The bars represent the percentage of explained variance (R^2) based on one or more single nucleotide polymorphisms for each of these proteins.
by genetic variation. Of the 56 proteins, 30 were influenced by SNPs acting in cis- only, 14 by SNPs acting both in cis- and in trans-, and 12 by SNPs acting in trans- only. In line with other studies, pQTLs explained a large proportion of protein-biomarker variance ($R^2$), as illustrated in Figure 1 (10). In 29 of the 56 proteins associated with pQTLs, SNPs explained 2% or more of the total trait variability. A practical consequence of the large percentage of variability associated with cis-pQTLs is that the statistical power for Mendelian randomization studies to establish biomarker causality is enhanced in comparison to traits with low $R^2$.

The cis-pQTLs identified in the IMPROVE study were investigated for association with coronary heart disease (CHD), which resulted in replication of the risk locus in the interleukin-6 receptor gene and provided some new potential CHD risk genes, including matrix metalloproteinase 12 ($MMP12$), placental growth factor ($PGF$), and macrophage colony-stimulating factor 1 ($M-CSF1$). Interestingly, the proteins encoded by the risk gene candidates $MMP12$, $PGF$, and $M-CSF1$ have all been previously associated with prognosis in patients with CVD (11–13). Using data on the relationship between (1) protein biomarker and disease risk, (2) SNP and protein biomarker level, and (3) SNP and disease risk, a quantitative Mendelian randomization framework allows for formal testing of whether certain biomarkers play a causal role in disease. The importance of Mendelian randomization in drug-target validation has been highlighted by studies comparing genetics with phase 3 outcome studies (14).

Several of the pQTLs identified in the IMPROVE study were trans-acting (Table 1). The trans-pQTLs were characterized using data from analysis of linkage disequilibrium, gene expression, protein–protein interactions, literature mining, and pleiotropy analysis (9). The analysis revealed known receptor–ligand pairs such as macrophage inflammatory protein 1-beta/C-C chemokine receptor 5, and protein–transcription factors pairs such as interleukin-18/NLR family CARD domain-containing protein 4, as well as connections that seemed to be regulated by postprocessing of circulating proteins. For example, a trans-pQTL near $MMP9$ was found to be associated with plasma levels of stem cell factor (SCF), and it has also been shown that MMP9 enzymatic activity cleaves membrane-bound SCF, providing orthogonal evidence of this connection (15). Another trans-pQTL near the $IFI30$ gene, which encodes γ-interferon-inducible lysosomal thiol reductase (GILT), was associated with cathepsin-L levels. Separate evidence indicates that GILT is the only enzyme identified in lysosomes with the potential to catalyze reduction of cysteine cathepsins (16). Other trans-pQTL associations were statistically convincing but without apparent biological explanation. Using GWAS, a locus near thetribbles homolog 1 ($TRIB1$) gene has repeatedly been associated with lipoprotein levels, liver enzyme levels, CHD risk, inflammatory bowel disease, and, to some extent, nonalcoholic fatty liver disease (NAFLD) (17).

**TABLE 1.** Selected trans-pQTLs from the IMPROVE study of 83 plasma proteins on the Olink Proteomics CVD I panel. Protein names are commonly used short forms, whereas genes are annotated according to Entrez records. The candidate genes for driving the trans-pQTL are given under the gene column. Likely causal candidates after analysis are marked with an asterisk. Further details are provided in (9). SNP, single-nucleotide polymorphism; MIP-1β, macrophage inflammatory protein 1 beta; ST2 (IL1RL1), interleukin 1 receptor-like 1; SCF, stem cell factor; CA-125, cancer antigen 125; PAPPA, pregnancy-associated plasma protein A.

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<th>Protein</th>
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<tr>
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<td>Follistatin</td>
<td>rs1260326</td>
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| Interleukin-18 | rs7599125 | LTBP1
|             |                | NLR4*  |
| ST2 (IL1RL1)| rs35166255     | TIRAP |
| ST3GAL4*    | rs4810479      | TRAP |
| SCF         | rs12469459     | PLTP |
| CA-125      | rs14000161     | GAL3ST2* |
| PAPPA       | rs2954029      | PRG2 |
| MMP9*       |                | D2HGDH |
| TRIB1       |                |      |
IMPROVE study showed that SNPs at the TRIB1 locus are also significantly linked to plasma levels of YKL-40, encoded by the chitinase-3-like protein gene. YKL-40 is upregulated at sites of inflammation, and high levels are associated with a wide range of diseases linked to inflammation, including CHD and NAFLD (18). Taken together, one may speculate that TRIB1 links lipid metabolism and liver biology to peripheral inflammation.

At a higher level, the IMPROVE pQTL study of Olink CVD I panel proteins showed that this approach could identify both causal pathways for disease and associated disease-related biomarkers. To better understand novel trans-pQTLs, perturbation experiments that validate the pQTLs on a functional level will be necessary.

**Systematic application of pQTLs in precision medicine drug discovery**

Studying the genetics of a disease can provide causal evidence for connections between specific molecular pathways and that disease, and also offer clues as to whether therapeutic benefit requires inhibition or stimulation of that pathway. Naturally occurring gene variants with a clear phenotype can be regarded as models for the possibility of pharmacological intervention (19). Proteins found in blood and other tissues are the most common target for small molecules and antibody biotherapeutics that aim to modulate disease development and progression. Proteins can now be more systematically assessed thanks to developments in mass spectrometry and immune-based multiplex assays. In combination, human genomics and proteomics can be effectively harnessed to inform target discovery, target validation, and the identification of mechanistic biomarkers that are capable of discriminating patients with complex disease into subgroups based on the underlying pathophysiology of the disease. An alternative model for precision medicine drug discovery would therefore be to focus on studying the expression of disease-related phenotypes, including case/control comparisons, patients with slow/rapid progression of disease, or responders/nonresponders to established treatments. Broad proteomics profiling brings the promise of comprehensively mapping differences among such groups with the purpose of finding discriminative biomarkers. As a second step, pQTLs could be mapped for the protein biomarkers that discriminate between these groups. The cis-pQTLs for such proteins can then be used in a Mendelian randomization framework to establish whether the observation is likely to cause disease or be caused by the disease, whereas trans-pQTLs provide alternative pathways to target the protein upstream of the protein biomarker. Moreover, genetic variants that act in cis- and that are robustly linked to the disease phenotype are amenable to reverse-genetics approaches—such as phenome-wide screens—whereby the accessible proteome is compared between carriers and noncarriers of the genetic variant. This approach promises to further our understanding of the consequences of genetic perturbation at the proteomic level and yield high-value protein biomarkers that could act as surrogate endpoints. Its success depends on several factors, including the availability of genetic data from carefully phenotyped contemporary patient collections across multiple diseases, and access to the proteomic data from relevant tissues. These dependencies notwithstanding, the approach enables target and biomarker discovery to follow a similar path, which by design can connect mechanism to disease.

In conclusion, the integration of genomics and proteomics provides new tools at the intersection of precision medicine and drug development. If effectively applied at the right stage of the drug discovery process, these tools could reduce patient attrition in clinical studies as well as abrogate the need for post hoc identification of patient subgroups, leading to improved treatment outcomes.

**References**

6. European Bioinformatics Institute, GWAS Catalog (2017); available at [https://www.ebi.ac.uk/gwas](https://www.ebi.ac.uk/gwas).
The SCALLOP consortium

Anders Mälarstig

The genetic contribution to the variability of plasma-protein biomarkers can be explored in genome-wide association studies using single-nucleotide polymorphisms (SNPs). These associations are also known as protein quantitative trait loci (pQTLs) (1, 2). We and others have previously shown that pQTLs are important for validating and discovering novel biological pathways and for establishing causality when correlating protein biomarkers with disease endpoints (3). Evidence suggests that pQTLs that act in cis- often explain a significant proportion of the total protein variability, whereas pQTLs acting in trans- are more likely to have a smaller effect (4–6). To enable the discovery of novel trans-pQTLs, the SCAled-up coLLaboration for Olink Proteins (SCALLOP) consortium was initiated by institutions that analyze large patient populations through genome-wide genotyping and plasma proteomics (using the Olink proximity extension assay).

The SCALLOP consortium is set up in a similar way to other genetics consortia, such as the Psychiatric Genomics Consortium (PGC) and the Genetic Investigation of ANthropometric Traits (GIANT) consortium, which both pool data in order to more rapidly advance genomic discoveries. Members are invited to contribute, lead, or co-lead subprojects within the SCALLOP framework by providing data and expertise. Authorship of reports and presentations is based on the effort applied by members to specific projects and is discussed during the monthly SCALLOP teleconference calls. All members have agreed to a memorandum of understanding that outlines the basic principles for conduct. The current subprojects within SCALLOP are aimed at pQTL discovery, with follow-up work focused on identifying causal pathways for human disease and the relevance of any novel findings. The information technology infrastructure for SCALLOP is supported by the European Union ELIXIR (European Life-Science Infrastructure for Biological Information) Tryggve project, a secure server that enables the sharing of sensitive data (7). If the need arises, SCALLOP has the technical infrastructure to drill down to patient-level data to address questions that cannot be handled using summary-level statistics of SNP-protein associations. To date, a total of 17 European cohorts with approximately 25,000 patients and controls have joined the effort. Several of the cohorts have been involved in plasma or serum proteomics studies using Olink panels.

References
**FIGURE 1.** Preliminary data from Random Forest analyses of the relative strength of biomarker associations with resilience.

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Patients and study cohorts

The inclusion of patients in this resilience multimarker substudy was based on a random sample of 2,933 patients derived from the 14,124 patients for whom plasma samples were available. The predefined event for this substudy was survival without any of the following events: death of any cause; myocardial infarction; stroke or transient ischemic attack; coronary revascularization; hospitalization for unstable angina; hospitalization for heart failure; any new cancer; new onset of diabetes mellitus; any cardiovascular procedure; any other vascular endpoint (e.g., limb amputation); noncoronary revascularization; or hospitalization for any noncoronary ischemic event.

Biochemical analyses

Venous blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) or citrate tubes at randomization, frozen in aliquots, and stored at -70°C or colder until analysis. The plasma levels of high-sensitivity cTnT protein (hs-cTnT), NT-proBNP, GDF-15, cystatin C, and IL-6 were determined as previously reported (3–5). Blinded samples without accompanying clinical data were provided to the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University for the proteomic analyses. The determinations were performed with the Olink Proseek Multiplex CVD kit and the Olink Proseek Multiplex Inflammation kit, which together simultaneously measured 169 selected CVD- and inflammation-related proteins in plasma samples (6).

Statistics

Baseline characteristics, established biomarkers, and PEA biomarkers were evaluated by conventional descriptive statistics. Random Forest analyses were performed to provide an unbiased grading of the prognostic importance of all variables including all clinical characteristics, PEA protein levels, and levels of other biomarkers. The Boruta algorithm was used to confirm which of these variables in the Random Forest analyses had a larger-than-random association to outcomes. Cox regression analysis was performed for the established and PEA biomarkers, both unadjusted and after adjustment for baseline characteristics [i.e., age, sex, body mass index, current smoking, hypertension, diabetes, previous myocardial infarction, previous coronary revascularization, previous stroke, previous peripheral arterial disease (PAD), and randomized treatment]. If several measurements of the same biomarker were available, only one result for each biomarker was included in these analyses. The significance levels for the Cox regression analyses were determined using 10,000 random permutations simulating the effect under the null hypothesis. Based on the effective number of tests of significance, the level of significance was 2.96 x 10^-4 after applying the Bonferroni correction.

Preliminary results

In our initial analysis, a comparison between the group without (n=1,977) and the group with (n=956) any event, the former was observed to be younger, less likely to be obese, and had less-frequent history of hypertension, diabetes, or polyvascular disease. The levels of the established cardiovascular, inflammatory, and renal biomarkers were significantly lower in the group without any event. The crude bivariate comparison between cases and noncases concerning all 155 PEA biomarkers was significant (p < 0.001) for 39 of the biomarkers.

An unbiased selection of the variables with linear or nonlinear associations with the absence of events was performed by Random Forest analysis (Figure 1). According to the Boruta function, 39 biomarkers were of confirmed importance for the identification of patients with no events (Figure 2). The independent linear association of these 39 biomarkers with the absence of events was also investigated by Cox regression analyses, including

FIGURE 2. Boruta analysis of the relative strength of statistical significances, showing biomarkers with confirmed (green) and tentative (yellow) association with resilience. Red indicates no association with resilience. Data is unpublished and preliminary.
adjusting for clinical baseline characteristics. Based on multiple statistical testing, only biomarkers with a $p$-value of $<2.96 \times 10^4$ were considered to have significant associations with absence of events in these analyses. When requiring confirmed importance in the Boruta analyses and statistical significance in the Cox regression, there remained 22 of the original 39 biomarkers showing prognostic importance for the absence of events. This approach identified low levels of NT-proBNP, BNP, GDF-15, IL-6, and hs-cTnT as the most important variables for the identification of patients with no events. These were followed by matrix metalloproteinase 12 (MMP-12), tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), cystatin C, and fibroblast growth factor 23 (FGF-23). Thereafter followed 13 additional biomarkers of a lower level of significance. After also adjusting for collinearity with clinical baseline characteristics, there remained eight biomarkers with significant and confirmed independent association with the absence of both cardiovascular and cancer events, namely low levels of NT-proBNP, BNP, hs-cTnT, GDF-15, IL-6, MMP-12, TRAIL-R2, and FGF-23.

### Identifying disease risks

The biomarker profiling capacity provided by the Olink protein panels has been previously used to demonstrate associations of 77 plasma proteins with chronological age and lifestyle factors related to biological aging (7, 8). Together, these studies support the contention that this type of protein profiling might be helpful for identification of different risks for major noncommunicable diseases, and provide a promising new avenue for the characterization of biomarkers related to biological age and resilience against disease.

The most important prognostic biomarkers indicating absence of events in the current study were low levels of the cardiac biomarkers NT-proBNP, BNP, and hs-cTnT. Similar findings had previously established a lower risk of almost all types of cardiovascular events in elderly individuals with decreased levels of these proteins (9). The identification of these proteins in this study suggests that CVD was the most common event during follow-up of the patients tested. Low levels of the inflammation markers GDF-15 and IL-6 also showed strong correlation with the absence of events. These two markers have previously been found to correlate with a raised risk of both cancer and CVD (9). The identification of these biomarkers using the PEA method as well as by conventional assays (such as the Elecsys GDF-15 kit from Roche Diagnostics) supports the validity of the PEA platform for the accurate identification of new biomarkers.

The three additional prognostic markers—FGF-23, MMP-12, and TRAIL-R2—are less well established as risk indicators in CVD and cancer. FGF-23 is a hormone produced by osteocytes and osteoblasts that increases phosphate excretion by the kidney and acts as a negative feedback regulator for activated vitamin D synthesis. In recent studies, elevated FGF-23 levels have been associated with cardiovascular diseases, i.e., hypertension, left-ventricular hypertrophy, endothelial dysfunction, cardiovascular events, and mortality (10). FGF-23 has also been found associated with nonvascular diseases and cancer (11). MMP-12 is associated with atherosclerosis and diabetes (12), but thus far there are no data associating circulating MMP-12 levels with cancer. TRAIL-R2 is a cell-surface receptor of the TNF-receptor superfamily that binds TRAIL and mediates apoptosis. To date, only one report connecting circulating levels of TRAIL-R2 to CVD has been published (13).

The associations between resilience and lower levels of FGF-23, MMP-12, and TRAIL-R2 are novel findings and require further exploration and confirmation. However, even if not fully understood, a biomarker profile to identify patients with low risks of cardiovascular or cancer events might still be very useful both in clinical management and for the design of clinical trials. Although they may not contribute to risk stratification in multivariable analyses, biomarkers of this type might nonetheless be of pathophysiological importance for resilience. The pathophysiological importance of biomarkers associated with cardiovascular or cancer events might be further characterized by Mendelian randomization analyses of outcomes in relation to the corresponding regulatory genes and/or by functional studies in animals or cell systems. In order to identify associations between biomarker levels and specific cardiovascular or cancer events, we are now expanding the sample set to include material from all available patients with events from the entire STABILITY trial. Fuller biomarker profiles associated with the risk for specific cardiovascular or cancer events will be provided in a more complete report of this pilot study.

### Conclusions

The Olink PEA technology, providing profiles of biomarker levels in very small volumes of plasma, was tested for identification of proteins in patients with stable CAD and resilience against cardiovascular and cancer events over 3-5 years. The usefulness of the technology was verified, as low levels of two well-established cardiac markers (NT-proBNP and BNP) and low levels of two well-established inflammation markers (IL-6 and GDF-15) were independently and similarly associated with resilience with the PEA method and conventional assays. In addition, PEA screening identified three additional novel, independent resilience markers, namely low levels of FGF-23, MMP-12, and TRAIL-R2. Although the importance of the new prognostics biomarkers need to be verified in other materials, the usefulness of the Olink PEA technology for screening of biomarkers for different purposes is strongly supported by these initial findings.

### Disclaimer

The manufacturer of the protein assay, Olink Proteomics, had no input on the study design and analysis, or in the manuscript preparation.

### References

A comparison of the proximity extension assay with established immunoassays

Agneta Siegbahn, Niclas Eriksson, Johan Lindbäck, and Lars Wallentin

The last few decades have seen a large number of potential biomarkers described in the scientific literature, but only a small percentage of these have been translated into clinical practice. At the same time, it has also been clearly demonstrated that expression patterns of multiple biomarkers can provide a more complete picture of pathophysiologic pathways and disease mechanisms than single biomarkers alone. In this context, multiplex protein biomarker assays are proving to be promising tools for developing diagnostic, prognostic, and treatment strategies for a variety of complex diseases, including cardiovascular diseases (CVDs) and cancer.

To enable the discovery of large sets of candidate biomarkers in sample collections, multiplexed and sensitive detection assays with low sample consumption are required. One new technology that meets these criteria is the high-throughput proximity extension assay (PEA) (1). Just 1 µL of sample is needed for the measurement of 92 biomarkers in each sample and in 90 samples simultaneously. Using this technology, we performed screening studies using plasma samples from three large international trials testing novel pharmaceutical treatments for cardiovascular diseases. In these trials several protein biomarkers were previously measured by conventional immunoassays, detecting single analytes in samples. The aim of the present foundational substudy was to perform a comparison between PEA and conventional assays. Two questions were addressed: (1) Is PEA technology reliably reflecting the levels of all measured biomarkers simultaneously? (2) Do the biomarker levels measured by PEA provide the same associations to outcome as conventional immunoassays?

Study design and participants

In the PLATElet inhibition and patient Outcomes (PLATO) trial, 18,624 patients were randomly assigned treatment with the platelet adenosine diphosphate–receptor blockers ticagrelor or clopidogrel, following acute coronary syndrome (2). In the present substudy, plasma samples were collected from 400 PLATO study patients with myocardial infarction—200 with and 200 without a new event—plus 100 age- and sex-matched apparently healthy elderly individuals included in the population-based Swisch study (3). These samples were analyzed together as a single set.

In the Stabilization of Atherosclerotic plaque By Initiation of darapLadib TherapY (STABILITY) trial, 15,828 patients with stable coronary heart disease were randomly assigned to receive either a once-daily dose of darapladib, a selective oral inhibitor of lipoprotein-associated phospholipase A2, or a placebo (4). Samples from a randomly selected group of 4,373 patients were included in our substudy.

The Apixaban for Reduction In StROKE and Other ThromboemboLic Events in Atrial Fibrillation (ARISTOTLE) trial was a double-blind, double-dummy, randomized clinical trial that enrolled patients with atrial fibrillation and increased risk of stroke. 18,201 patients were randomized to receive apixaban, an oral, direct factor Xa inhibitor, or warfarin (5). In our substudy, samples from 5,697 ARISTOLE patients were used.

Procedures

Blood samples were collected in ethylenediaminetetraacetic acid tubes at randomization and centrifuged immediately. The plasma samples were frozen in aliquots and stored at -70°C until analyzed centrally at the Uppsala Clinical Research (UCR) Laboratory, Uppsala, Sweden. PEA analysis was performed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University.

Gold standard immunoassays

Growth-differentiation factor 15 (GDF-15) concentrations were determined with the Elecsys GDF-15 commercial kit from Roche Diagnostics, Basel, Switzerland, and N-terminal pro B-type natriuretic peptide (NT-proBNP) concentrations with the Roche immunoassay on a cobas e 601 analyzer (Roche Diagnostics).

Interleukin-6 was analyzed using the Quantikine HS Human IL-6 immunoassay (R&D Systems, Minneapolis, Minnesota) on a Tecan Freedom EVOlyzer. All analyses were done according to the instructions of the manufacturer and have been described in detail previously (6-10).

Multiplex proteomic analysis

Analyses were performed with the PEA technique using the Olink Proseek Multiplex CVD I (PLATO and STABILITY), CVD II, CVD III, and inflammation (ARISTOTLE) panels. With the PEA technology, 92 oligonucleotide (PEA probes)–labeled antibody pairs bind their respective target present in the sample. Upon binding their specific antigens, they give rise to new DNA amplicons, and each individual DNA sequence is then detected and quantified using specific primers by microfluidic quantitative PCR using a Fluidigm Biomark HD system. The reproducibility and repeatability for the different panels had mean intra-assay and interassay coefficients of variation of 8% and 12%, respectively. The average intersite variation was 15% (1). The concentrations are expressed as Normalized Protein eXpression (NPX), where a high protein value corresponds to a high protein concentration, on a log2 scale. The lower level of detection (LOD) is defined as the negative control plus three standard deviations.

Statistical analysis

Comparisons between established immunoassays (gold standard methods) and PEA measurements of NT-proBNP,
GDF-15, and IL-6 were performed using scatter plots, Bland–Altman diagrams, and Pearson correlations. The association between each biomarker and time to cardiovascular death, adjusted for baseline characteristics (age, sex, body mass index, current smoking, hypertension, diabetes, previous myocardial infarction, previous revascularization, previous stroke, previous peripheral arterial disease, and randomized treatment) was evaluated using Cox regression analyses. The results were presented as hazard ratios, with corresponding 95% confidence intervals, for an increase of one standard deviation of the biomarker, and were illustrated as a forest plot. The C-statistic was used as a measure of discriminative ability of each model. It ranges from 0.5 to 1, where a value of 1 means that we have perfect discrimination between cases and controls, and 0.5 means that the model is no better than chance.

**The ARISTOTLE study**

To investigate if the measurements of different proteins included in both the CVD II and inflammation panels were comparable, we used samples from the ARISTOTLE substudy. Using Bland–Altman diagrams, seven biomarkers included in both of these panels were compared (Figure 1). The measurements from the two different panels indicated highly comparable results for four of the markers. Three of the markers, fibroblast growth factor 21 (FGF-21), stem cell factor (SCF), and interleukin-18 (IL-18) had higher NPX values in the CVD II panel than the inflammation panel. However, the correlation between the measurements in the two panels appeared to be very good in initial tests (Figure 1). A possible explanation for the difference in NPX values for these biomarkers is that different PEA probes, generated with the same FGF-21, SCF, or IL-18 antibodies, were used to probe the two panels. Moreover, NPX is an arbitrary unit and not an exact quantification. Our data, however, clearly demonstrate that biomarkers measured in different PEA panels should be treated in each PEA panel as a unique assay.

**PEA versus gold standard assays**

In further work to validate the PEA assay, comparisons between gold standard methods and PEA were performed. GDF-15 protein levels in samples from the PLATO, STABILITY, and ARISTOTLE studies were measured using a Roche immunoassay. This immunoassay is based on the specific Elecsys electrochemiluminescence (ECL) detection system (Roche Diagnostics). The analytical range is 400 ng/L–20,000 ng/L with a total coefficient of variation (CV) of 2%. We observed excellent agreement between GDF-15 concentrations obtained by ECL technology and the PEA NPX values measured in the three studies using both CVD I and CVD III panels (Figure 2). We found good correlation.
for IL-6 concentrations obtained using high-sensitivity (hs) ELISA (enzyme-linked immunosorbent assay) and PEA (Figure 3). The hs-IL-6 ELISA has an analytical range of 0.04 ng/L–10 ng/L and a total coefficient of variation (CV) of 6%. The largest deviations were found when measurements were close to the upper limit of detection.

When NT-proBNP was measured in samples from the PLATO and STABILITY studies using the CVD I panel, and compared to results obtained from the ECL immunoassay (analytical range 5 ng/L–35,000 ng/L and total CV 2%), a good correlation was found. However, at higher concentrations of NT-proBNP, the so-called hook effect was observed, caused by an excess of antigen relative to the amount of antibody reagent, and resulting in artificially lower levels (Figure 4A, B). This effect was seen at approximately 2,000 ng/L in the CVD I panel. To overcome this problem, NT-proBNP detection was moved to the Olink CVD III panel, in which all samples were diluted 1:100 before analysis; this panel was used to compare PEA data from the ARISTOTLE patients with results obtained by the standard method. Previous data from the ARISTOTLE study showed a wide range of NT-proBNP concentrations (5 ng/L–30,000 ng/L) (9). Correlations between signals obtained by the ECL immunoassay and PEA were high, but 448 (8.2%) samples had concentrations below LOD (~200 ng/L) (Figure 4C). After finalization of the above-mentioned substudy, Olink Proteomics has carried out further optimization of the NT-proBNP assay in CVD III, improving its sensitivity to approximately 60 ng/L (Olink Proteomics, unpublished observations). The revised NT-proBNP assay is currently being implemented. This indicates that NT-proBNP detection using the CVD III panel is best suited for samples in which NT-proBNP protein levels above 60 ng/L are expected.

To measure NT-proBNP using PEA in samples where the concentration is low, NT-proBNP has also recently been included in the new Olink Metabolism panel, in which all
samples are diluted 1:10. To test this new panel and compare the results to ECL, we used 27 samples from the ARISTOTLE study with NT-proBNP concentrations varying from below 5 ng/L to 23,000 ng/L, and 56 samples from healthy individuals (Figure 4D). The protein could be reliably detected using the PEA panel at 5 ng/L–25,000 ng/L, an analytical range comparable with that of the standard method.

The association between baseline levels of three biomarkers, NT-proBNP, GDF-15, and IL-6, was determined using ECL and PEA analysis in samples from the STABILITY study, and time to cardiovascular death was investigated using Cox regression analyses (Figure 5). Adjusting for baseline characteristics resulted in almost similar C-statistics for all three biomarkers, independent of the assay used.

**Summary**

The multiplex PEA is a high-throughput technique for detection of protein biomarkers that encompasses low sample volume, high specificity, high sensitivity, and no cross-reactivity during multiplexing—all desirable properties of a multiplex immunoassay. The data from the foundational studies described above indicate that the PEA technology reliably reflects the levels of all measured biomarkers simultaneously. Moreover, PEA results provided the same associations to outcome—cardiovascular death—as conventional assays and can, we believe, be used as a substitute. The PEA technique offers a novel tool that is suitable for screening studies of complex diseases. For implementation of the PEA technique in a clinical setting, we would, however, recommend a panel/chip that tests a smaller number of biomarkers, allowing for more robust quantification of the protein concentrations.

**References**

Searching for sentinels: The need for more informative fluid-based biomarkers in neurodegenerative disease

Kaj Blennow¹ and Henrik Zetterberg¹,²

Neurodegenerative diseases such as Alzheimer’s disease (AD), multiple sclerosis (MS), Parkinson’s disease, and amyotrophic lateral sclerosis (ALS) affect hundreds of millions of patients worldwide and are expected to increase in prevalence as the generation born after World War II lives longer than ever before. And so far, medicine has few effective treatments for slowing or stopping neurodegeneration.

The inaccessibility of the brain and the many confounding factors that arise during clinical evaluation of neuronal loss represent major challenges. For a 70-year-old who presents in the clinic with mild cognitive impairment, it’s nearly impossible to say whether it’s due to normal aging, AD, vascular damage, or depression without a biological marker that signals a particular pathology.

Today, the field of neurodegeneration has a handful of blood- and cerebrospinal fluid-based biomarkers that give physicians a useful picture of disease pathology and serve as effective tools for diagnosis. Nevertheless, it’s imperative to keep searching for better fluid-based biomarkers that reflect different brain pathologies and can warn of the earliest points at which brain cells encounter trouble. In addition, the field needs easily accessible biomarkers that can stratify patients into subtypes of neurodegenerative disorders and signal when patients respond to treatments.

Beyond classical biomarkers

The cerebrospinal fluid (CSF) surrounds the brain, flows deep into the brain’s ventricles, and can be sampled from a lumbar puncture to the lower back (the procedure is more aptly called “CSF sampling” or “CSF collection”). CSF biomarkers for MS and AD (1) have been used for decades.

As amyloid beta 42 (AB42) is deposited into AD amyloid plaques, its levels in CSF drop. Phosphorylated tau (P-tau) is a marker that increases with tangle pathology found in AD. Total tau (T-tau) protein is a marker of axonal injury, and increases in CSF with increasing severity of neurodegeneration. We have shown that T-tau levels in blood can also be used to detect concussion and track rehabilitation in professional hockey players (2).

Numerous studies have shown that an increase in T-tau alongside a decrease in AB42 in a patient’s CSF sample indicates AD pathology throughout the clinical stages, from preclinical to dementia (3). For MS, the presence of CSF-selective oligoclonal bands of immunoglobulin G (IgG) in CSF, which reflect immunoglobulins produced by autoreactive plasma cells within the CNS, indicates CNS inflammation, and the CSF biomarker neurofilament light subunit denotes injury to the myelinated, large-caliber neurons affected in MS (4).

To date, these biomarkers have proved most useful for diagnostic purposes and give limited information on disease severity and progression (5). In addition, there are a host of brain diseases for which we have no fluid biomarkers of any kind—including frontotemporal dementia, Lewy body dementia, dystrophic myotonia, Parkinson’s disease, and ALS. Misdiagnoses are unacceptably common due to broad overlaps in disease symptoms and pathologies. In the dementia stage of AD, the accuracy of a clinical diagnosis is around 80%, and is much lower in earlier disease stages (6).

Although advances in brain imaging offer improved diagnostics, fluid-based biomarkers could reflect the broad range of biological activities ongoing in the brain and point to which pathways are most active in disease. Tracking a dynamic biomarker could signal changes in pathology in response to treatment, the correct dose for treatment, or potential side effects. In addition, pathology-specific, dynamic biomarkers would be incredibly useful in clinical research to follow a cohort of patients over time to monitor exactly when pathologies appear. Finally, better biomarkers should also allow more precise matching of patients to clinical trials.

Finding fluid-based clues

For elderly patients with motor or cognitive impairments, it would be ideal to access specific biomarkers from a simple blood-draw. Of course, blood brings its own challenges. CSF contains relatively few circulating factors, and nearly all are derived from one organ, the brain. Blood, on the other hand, requires another layer of analytical sensitivity to pull out biomarkers from a source that is diluted into 4-5 liters and contains proteins, metabolites, lipids, and other molecules from every organ system. In addition, blood biomarkers are likely to vary with a patient’s liver and kidney function, sleep patterns, diet, and other environmental influences.

Researchers have three main options for pulling novel biomarkers out of complex biological fluids. The first is the candidate biomarker approach—a targeted search for one or two critical molecules involved in known pathogenesis pathways that would act as indicators for those pathways. This approach requires deep knowledge of disease mechanisms and available enzyme-linked immunosorbent assay (ELISA)-based tests for detecting and measuring key molecules.

A second approach takes the form of completely blind, non-hypothesis-driven exploration to separate and measure all components in a sample that might be sentinels of disease. This requires using mass spectrometry-based techniques, which hold some limitations in terms of throughput and ability to detect low abundance proteins (7).

The third approach meets somewhere in the middle of these first two. Multiplexed protein panels, grouped by disease families or processes, can be used to survey patient fluid samples for biomarkers. The Olink proximity extension assay (PEA), which amplifies and translates a dual-antibody binding signal into a quantitative nucleic acid signal, is one such system. The assay’s high level of specificity avoids cross-reactivity problems often seen with multiplexed antibody-based assays, and its signal amplification aspect allows reproducible measurements of protein markers across a broad dynamic range using just a few microliters of sample.
We recently used Olink Proteomics PEA panels to search for novel biomarkers in half a dozen neurodegenerative diseases using paired CSF and plasma samples from 500 patients and healthy controls. A subset of this project explored which pathways were active in AD beyond tau and β-amyloid. And finally, we also wanted to probe whether the neuroinflammatory component that has been reported for AD is upstream, downstream, or concurrent to neuronal injury.

Using the Olink panels for proteins implicated in neurodegeneration and inflammation, we would like to assess how other proteins—including astrocyte- and microglia-derived inflammatory markers—might correlate with the classical tau and AB42 biomarkers as well as with severity of patient symptoms. We have also stratified AD patients into three groups for comparison: preclinical AD (patients who have the classical biomarkers but no symptoms), prodromal AD (patients with biomarkers and mild cognitive impairment), and pure AD (patients with biomarkers and a clinical AD diagnosis).

Future early warning and response systems

One goal for our AD research is to find biomarkers to track the other pathophysiology that occur alongside amyloid deposition and tau tangles such as inflammation, synaptic degeneration, and oxidative stress. Specific biomarkers could be used during clinical trials to detect whether treatments ameliorate these destructive processes and improve prognosis. Another important research goal for the field of neurodegenerative biomarkers will be to determine whether useful correlations exist between CSF and blood samples.

Because fluid-based biomarkers are limited in their ability to report on anatomical information, we see the need to cross-validate fluid biomarkers with improved-resolution molecular imaging techniques that are also in development. Of course, the best biomarkers might be protein fragments, peptides, or even posttranslational modifications to proteins, so the field will need ultrasensitive technologies to dig deeper into the proteome moving forward.

Ultimately, the field aims to discover neurological biomarkers that point us to the very beginnings and the most dominant pathology of a disease. Such a marker would delineate, for example, the tipping point between a person developing AD or not.

Fluid biomarkers will help doctors treating neurodegeneration move toward personalized medicine. Ideally, a fluid biomarker will also rapidly reflect a patient's response to a treatment. Imagine in the not-too-distant future a finger-prick device, much like those used for blood glucose monitoring, that could tell a patient how many neurons were recently lost and how to correct the course of treatment for maximum neuronal preservation.

References


Making it personal: Better biomarkers will bring personalized medicine to heart failure

Faiez Zannad1,2 and Anne Pizard1,2

Heart failure, which affects 1-2% of the population in developed countries and more than 10% of people over age 70, will continue to be a growing epidemic for an aging global population. A debilitating condition that still needs more satisfactory treatments, heart failure (HF) causes significant loss of productivity, quality of life, and economic burden for society.

HF is a complex syndrome that involves structural and functional abnormalities of the heart, blood vessels, and kidneys. It can be triggered by an acute injury such as myocardial infarction, or it can develop as a result of a long list of risk factors that coexist in many patients: long-term hypertension, coronary artery disease, sustained arrhythmias, diabetes, systemic inflammation, hyperlipidemia, obesity, and aging.

All of these conditions can result in strain on or injury to the heart muscle tissue, or myocardium, which can lead to the activation of cardiac fibroblasts and a downstream cascade of inflammation and fibrosis. These processes can also result in the loss of the heart muscle cells, called cardiomyocytes, or changes in these cells’ structure and function. As fibroblasts lay down collagen, heart tissue can stiffen, strangling coronary bloodflow and altering the mechanical properties of the heart. To compensate and maintain cardiac output, the heart’s chambers undergo significant remodeling.

Eventually, the heart can no longer pump blood efficiently to all areas of the body. Half of all patients hospitalized for HF will either die of the condition or be back in the hospital with complications within one year after an admission for worsening heart failure.

Stiffening of the large arteries, progressive deterioration of the vasculature endothelium function, chronic inflammation, redistribution of blood flow, compensatory stimulation of neurohormonal systems, loss of oxidative function in the skeletal muscle, and decline in renal function work in concert to make the disease become systemic, resulting in inexorable deterioration.

Failing patients

Current HF therapies include an array of drugs, initially developed for high blood pressure or as diuretics. These treatments have proven somewhat effective for only one subtype of HF, HF with reduced ejection fraction (also called systolic heart failure). Moreover, there is strong evidence that many of these life-saving medications are not properly applied to a diverse patient population, due to contraindicating comorbidities and physicians’ excessive caution surrounding drug safety (1).

In rare cases, a few single-gene mutations have been linked to the occurrence of HF. Most commonly, the syndrome develops as the result of multifactorial interactions and complex physiologies

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that are triggered by precipitating conditions and aggravated by multiple comorbidities (2). This complexity makes HF a perfect testing ground for developing personalized medicine. Indeed, it is tempting to segment the HF patient population into different phenotypic categories. Most current phenotypic stratifications are based on underlying risk factors, etiologies, and clinical predictors of severity and do not provide actionable instruments for personalized medicine.

In addition, an overwhelming number of clinical trials for HF therapies have failed, most likely because trials have been conducted for “all-comers.” That is, the patients in the trials were not stratified by disease profiles or underlying discrete disease mechanisms that matched the specific mechanisms of action for the trialed drugs (3).

To tackle this looming global health epidemic, researchers must find better HF biomarkers. Physicians must get ahead of the HF downward spiral by identifying at-risk patients early, before myocardial injury even begins, with the goal of preventing HF. Clinical research must be aimed at finding treatments that focus on blocking or delaying those first steps in the cascade. Discovering predictive biomarkers and disease-subtype mechanistic biomarkers will be crucial to finding patients and stratifying them for the most appropriate corrective courses of treatments. Finally, identifying biomarkers that signal a positive response or an adverse event in reaction to novel therapies will also be important to personalizing HF treatment.

Data mining for markers

There are only two established biomarkers used in HF patients: high sensitivity troponin T (hsTnT) can be used to diagnose myocardial infarction and myocyte injury, and B-type natriuretic peptides [BNP/N-terminal proBNP (NTproBNP)], released by changes in cardiac stress level, can be used to diagnose acute HF and to establish disease severity in chronic HF.

The literature also holds a handful of promising emerging biomarkers for HF, such as galactin-3, soluble ST2 (sST2), growth-differentiation factor 15 (GDF-15), and insulin-like growth factor (IGF)-binding protein 7 (IGFBP-7) (4). Some of these biomarkers and others are associated with disease severity and have been occasionally associated, in combination with other clinical measures, with reverse remodeling or improvement in cardiac function in response to treatment (5). To date, no single biomarker or multiplex of biomarkers is used routinely to select, initiate, optimize or guide therapy.

Two large, Europe-based, multi-omics studies will sift through thousands of patients’ data to find biomarkers to advance personalized medicine for HF. The 11 academic and industry partners of the FIBROTARGETS project (6) aim to discover and validate drug targets key to the cardiac fibrosis mechanism, one major culprit in HF. The project will eventually test novel drugs against those targets in the hopes of preventing or slowing the fibrosis process. In addition, researchers will identify biomarkers that can be used to track the biological activity of these targets to help select and screen effective candidate drugs.

The Heart OMics in AGEing (HOMAGE) study (7), which will include patients from eight European countries and the United States, will probe for biomarkers that can identify patients at high-risk of developing HF before they have symptoms. The collaborators will also seek biomarkers that tag those HF patients most likely to respond to specific therapies, and test these prospectively in a dedicated trial.

In a retrospective arm of the HOMAGE study, my collaborators and I will survey samples from thousands of patients who did or did not develop HF, to reveal predictive biomarkers. Using the Olink Proteomics proximity extension assay (PEA) panels for cardiovascular disease (CVD II and III) and inflammation, we have already identified potential predictive biomarkers that are activated in patients who developed HF years later. These markers will be validated using an independent cohort of HF cases and control patients. Other ‘omics biomarkers, such as microRNAs (miRNAs) and metabolomic and transcriptomic biomarkers, are being explored; and the value of ‘omics multiplexes is being examined using sophisticated bioinformatics and complex network analyses.

The prospective HOMAGE trial will look at the efficacy of an antifibrotic drug, the mineralocorticoid receptor antagonist spironolactone, and ask whether there are biomarkers that can predict drug response among patients. To date, 350 out of an expected 800 patients in Europe who are at increased risk of HF have been enrolled in the study. The trial will examine the interaction between biomarker bioprofiles and the antifibrotic response to spironolactone.

Using customized PEA panels with the biomarkers of interest, patients in this trial will have a baseline bioprofile established and will then be followed for nine months during treatment to build a picture of their fibrosis readout. By tracking multiple biomarkers in these at-risk patients, we hope to discover a relationship between their starting bioprofiles and their response to the antifibrotic treatment.

A future without failure?

Both the FIBROTARGETS and HOMAGE projects include components that will search for nonprotein biomarkers in HF, such as miRNAs, metabolites, and single-nucleotide polymorphisms that might also be informative. But it is likely that scouring the proteome will bear more fruit for HF biomarker research in the near-term, both because the technology is more mature and poses fewer research challenges, and because proteins remain more stable in the circulation. It will be crucial for researchers to keep an open mind to what a successful HF biomarker might look like: HF is a systemic condition affecting multiple organ systems, and therefore a specific warning signal could come from almost anywhere in the body.

The HF field must eventually move beyond the merely predictive, diagnostic, and prognostic biomarkers to find “highly actionable” biomarkers that will guide physicians to the appropriate therapy for an individual patient’s particular suite of pathologies. Actionable biomarkers would also flag patients who are likely drug responders and those who are not, as well as patients who are most likely to tolerate the specific drug, so that the right drug is given at the right dose to the right patient. Then, and only then, will HF treatment move from the realm of today’s imprecise medicine toward the precision medicine of the future.

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The human plasma proteome: Exploring new frontiers in precision medicine

Mathias Uhlén1,2*, Åsa Sivertsson1, and Linn Fagerberg1

Human blood plasma consists of molecular targets of great interest for clinical diagnostics and precision medicine. The human plasma proteome comprises proteins that are either actively secreted from various human tissues or leaked by the millions of cells undergoing cell death in our bodies at any given moment. The actively secreted proteins, also called the “secretome,” constitute the vast majority of human plasma proteins—as few as 10 of these proteins make up more than 90% of the total protein mass in human blood. The dynamic range of the protein concentrations is more than 10 orders in magnitude, from albumin at 45 mg/mL of blood to low-abundance proteins, such as the cytokine IL-1, at less than 5 pg per mL. The dynamic range is even greater if leakage products are included, since it is likely that all human intracellular proteins will be leaked into the blood, although in many cases at minute concentrations.

The analytical challenge of determining the concentration of human plasma proteins is therefore a mammoth task, particularly in light of the huge dynamic range between various blood proteins and the vast number of protein targets, likely in the tens of thousands.

**Achieving precision**

One objective of precision medicine is to develop better diagnostics to allow for more individualized therapeutic regimens. A major focus today is the use of genomics to better understand the genetic influence on health and disease, particularly the involvement of various genetic variants and epigenetic changes in disease susceptibility. However, it is also important to analyze other constituents of the human body of relevance to diseases. This could be done using noninvasive methods such as medical imaging, or through in vitro analysis of tissue or blood samples. Millions of blood tests are performed every year, but the total number of protein targets available for clinical analysis is restricted to just a few hundred. This situation emphasizes the need for multiplex methods to facilitate the analysis of a larger portion of the human plasma proteome, and also the need to generate validated antibodies for such analysis (1).

**Defining the secretome**

As pointed out above, an important fraction of the plasma proteome is made up of actively secreted proteins.

The number of putative secreted proteins, defined as having a signal sequence and no transmembrane regions, was previously predicted using various algorithms. The analysis revealed 2,918 genes, corresponding to 15% of all human genes (2). Here, we performed an updated analysis using the new genome assembly and also included annotations by the Universal Protein Resource (UniProt), which resulted in an estimation of the number of human putative secreted proteins to be 3,395 (Figure 1A). If genes with little relevance as targets for plasma proteome efforts are excluded, a shorter priority list of 2,193 genes is obtained. The excluded gene products include proteins with an intracellular location, such as mitochondria and lysosomes; gastric enzymes; immunoglobulins; and proteins with low evidence of existence.

An analysis of the tissue-specificity based on the Human Protein Atlas data showed that a majority of these 2,193 proteins show a tissue-restricted expression (Figure 1B). Interestingly, the fraction of “housekeeping” proteins expressed in all tissues is only 13%, which is much lower than the fraction for all genes (43%) (2). In summary, we are focusing our secretome efforts on this priority list, and an important objective now is to develop resources of reagents and multiplex assays to allow the quantitative exploration of these proteins in human plasma.

**The Human Secretome Project**

To enable the analysis of the human secretome, we have initiated the Human Secretome Project (HSP), a collaboration between the Wallenberg Center for Protein Research in Stockholm, Sweden, the Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark, and the Science for Life Laboratory, KTH Royal Institute of Technology, Stockholm, Sweden, the Novo Nordisk Foundation Center for Protein Research in Stockholm, Sweden, the Wallenberg Center for Protein Research in Stockholm, Sweden, and the Science for Life Laboratory, KTH Royal Institute of Technology, Stockholm, Sweden.

The number of putative secreted proteins, defined as having a signal sequence and no transmembrane regions, was previously predicted using various algorithms. The analysis revealed 2,918 genes, corresponding to 15% of all human genes (2). Here, we performed an updated analysis using the new genome assembly and also included annotations by the Universal Protein Resource (UniProt), which resulted in an estimation of the number of human putative secreted proteins to be 3,395 (Figure 1A). If genes with little relevance as targets for plasma proteome efforts are excluded, a shorter priority list of 2,193 genes is obtained. The excluded gene products include proteins with an intracellular location, such as mitochondria and lysosomes; gastric enzymes; immunoglobulins; and proteins with low evidence of existence. An analysis of the tissue-specificity based on the Human Protein Atlas data showed that a majority of these 2,193 proteins show a tissue-restricted expression (Figure 1B). Interestingly, the fraction of “housekeeping” proteins expressed in all tissues is only 13%, which is much lower than the fraction for all genes (43%) (2). In summary, we are focusing our secretome efforts on this priority list, and an important objective now is to develop resources of reagents and multiplex assays to allow the quantitative exploration of these proteins in human plasma.

**FIGURE 1. The human secretome.** (A) Pie chart showing the number of genes coding for putative secreted proteins and thus being potential targets for the Human Secretome Project (HSP). In total, 3,395 genes have been identified coding for putative secreted proteins, based on the prediction algorithms as described earlier (2) combined with UniProt annotations. The analysis shows that 2,193 of these remain as priority targets after excluding some gene products, such as proteins with an intracellular location (e.g., mitochondria and lysosomes), gastric enzymes, immunoglobulins, and proteins with low evidence of existence. (B) Pie chart showing the tissue-specific expression of 1,999 of the 2,193 priority secretome targets with available RNA-seq expression data in the Tissue Atlas (www.proteinatlas.org/humanproteome).
for Biosustainability in Copenhagen, Denmark, and the pharmaceutical company AstraZeneca in Cambridge, United Kingdom. The ultimate objective is to generate a resource consisting of all secreted human proteins, expressed in cultured mammalian cells, and to use this resource of purified recombinant proteins for various studies, including exploring the human secretome in phenotypic screens. The project, started in 2016, currently generates an average of two new validated proteins per working day using a pipeline involving synthetic biology, cell production, affinity tag purification, and quality assessment, using mass spectrometry and standard protein analytics. The HSP sits well into the framework of the international Human Protein Atlas program (3), which aims to explore all the protein-coding human genes using a combination of genomics, transcriptomics, proteomics, and antibody-based profiling, and includes the Tissue Atlas (3), the Cell Atlas (4), and the Pathology Atlas (5). The Human Protein Atlas, which was recently selected as a European core resource in life science by ELIXIR (European Life-Science Infrastructure for Biological Information, www.elixir-europe.org), is available through an open-access knowledge portal (www.proteinatlas.org) that provides a framework with which to untangle and model the plethora of dynamic, interacting components that produce life, and thus creates a resource for applied research aimed at developing new therapeutic regimens and diagnostic methods for personalized and precision medicine.

Wellness profiling
Within this framework, a collaboration was recently started with the Swedish biobank program Swedish CArdioPulmonary biomImage Study (SCAPIS) (6) to analyze the clinical profiles of 100 apparently healthy individuals between 50 and 65 years of age. Samples from these individuals are taken every three months, and the health profiles at the time of each visit are monitored by classical clinical chemistry, proteomics, metabolomics, immune-cell profiling, microbiome analysis, and transcriptomics of blood cells, and complemented with whole-genome sequencing and an initial advanced medical imaging of each subject. An important part of the health profiling is to enable the collection of multiplex plasma protein profiles. In this project, we have explored the application of the Olink Proteomics platform based on the “padlock” principle originally developed by Ulf Landegren and coworkers (7), which allows for high sensitivity and specificity even for low-abundance proteins. Using 11 panels, it was possible to quantitatively analyze 750 protein targets with high technical reproducibility, as exemplified in Figure 2A. Analysis of protein levels in samples taken at three-month intervals showed large variations (Figure 2B). It was evident that for some targets, there were distinct differences in the range of protein levels between individuals (Figure 2C), and that these differences were stable over the study period (Figure 2D). More than 400,000 data points were generated for these individuals over the first four visits, covering a full year of proteome profiling for a large fraction of the human secretome (unpublished data).

Conclusions
The human plasma proteome is a highly interesting set of proteins in the field of precision medicine and is important for the development of new drugs and diagnostics. Our preliminary results suggest that the Olink platform can be used to explore the plasma proteome using a multiplex method sufficiently sensitive to analyze low-abundance proteins in the blood. In addition, we have reported on the newly initiated HSP, which aims to generate a resource of all actively secreted human proteins to allow for in-depth studies of the human plasma proteome.

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Acknowledgments
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PEA: An enabling technology for high-multiplex protein biomarker discovery

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In the wake of the genomics era, the study of proteins is now emerging as the new frontier for understanding complex biological systems. Protein biomarker discovery enables identification of signatures with pathophysiological importance, bridging the gap between genomes and phenotypes. This type of data may have a profound impact on improving future health care, particularly with respect to precision medicine, but progress has been hampered by the lack of technologies that can provide high throughput, good precision, and high sensitivity.

The proximity extension assay (PEA) is a molecular technique optimized to meet these demands (1) and was commercialized by Olink Proteomics AB to develop its range of Olink biomarker panels. PEA successfully merges an antibody-based immunoassay with the powerful properties of PCR and quantitative real-time PCR (qPCR), resulting in a multiplexable and highly specific method where up to 92 protein biomarkers can be quantified simultaneously.

The basis of PEA is a dual-recognition immunoassay, where two matched antibodies labeled with unique DNA oligonucleotides simultaneously bind to a target protein in solution (Figure 1A). This brings the two antibodies into proximity, allowing their DNA oligonucleotides to hybridize, serving as the template for a DNA polymerase–dependent extension step (Figure 1B). This creates a double-stranded DNA “barcode” which is unique for the specific antigen and quantitatively proportional to the initial concentration of target protein. The hybridization and extension are immediately followed by PCR amplification (Figure 1C), and the amplicon is then finally quantified by microfluidic qPCR (Figure 1D).

Traditional immunoassays do not lend themselves well to multiplexing, since cross-reactive binding of antibodies contribute to the signal readout. This problem escalates exponentially with the degree of multiplexing (Figure 2A). In contrast, the DNA-based readout of PEA circumvents this by requiring both dual recognition of correctly matched PEA probes, and DNA sequence-specific protein-to-DNA conversion to generate a signal (Figure 2B). This provides a highly scalable method with an exceptional readout specificity.

The exponential amplification properties of PCR are utilized in PEA to achieve a strong readout signal, providing assay sensitivity on par with traditional enzyme-linked immunosorbent assays (ELISAs). Importantly, this also means that only 1 μL of sample is needed to measure 92 different proteins simultaneously, which is greatly beneficial when precious samples are in limited supply, such as in studies using human samples from clinical cohorts or biobank material. An additional benefit of the low sample volume is that concentrations of potentially interfering substances are minimized, which in conjunction with specifically tailored blocking reagents in the PEA protocol reduces sample matrix interference to a minimum. While the assay has been most vigorously validated on plasma and serum samples, it has also been shown to work very well with an array of sample types, including cerebrospinal fluid (2), dried blood spots (3), and tissue lysates, such as tumor biopsies (4).

The final detection of the 92 unique DNA barcodes in the PEA protocol is performed using microfluidic qPCR, enabling very high throughput. Typically, 90 samples are assayed against 92 different proteins per run, generating over 8,000 data points in under 24 hours. The use of automated microfluidics also reduces the number of manual pipetting steps needed, contributing to the exceptional repeatability and reproducibility obtained using the PEA technology.

In summary, PEA is a method well suited for large-scale studies of precision proteomics, offering good sensitivity, rapid, high-throughput analysis; and exceptional specificity at high-multiplex levels. Commercialized as a broad range of 92-plex Olink biomarker panels, it enables scientists to cast a wide net in the search for new protein signatures, which can be applied to improve disease detection, make available more personalized health care, and allow a better understanding of biology.

References
Development and validation of customized PEA biomarker panels with clinical utility

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INTRODUCTION

Several studies have demonstrated the value of proteomics in identifying relevant biomarkers for noncongenital diseases, and to monitor disease progression, treatment responses, and efficacy. While the genome can largely be considered as static within an individual (at least in terms of DNA sequence), the proteome varies considerably in response to a wide range of physiological and pathological processes, making it a more immediate barometer of the state of the body. Proteomics is important because proteins represent the functional effector molecules in the cell. Drugs elicit their effects most often through interactions with proteins. Gene expression analyses have discovered numerous genes that are differentially expressed between malignant and benign tissues (1), but few have proven to be suitable as biomarkers, mainly because the messenger RNA levels do not always correlate well with protein abundance (2).

A higher power of discrimination can be obtained by combining more than one biomarker (3–6) to create a protein signature. However, large-scale studies of protein levels have been hampered by a lack of high-throughput methods. Using protein signatures could transform the future of disease diagnosis, treatment, and our understanding of health, but only if these technical limitations can be overcome using improved proteomics technologies.

Recent advances such as Olink Proteomics’ proximity extension assay (PEA) technology (7) have enabled highly multiplexed analysis using many protein biomarker assays. This has allowed scientists to cast a wider net for new protein signatures that can be used to stratify patients, predict disease and treatment outcomes, and understand pathophysiology or discover new drug targets (8).

Standard Olink panels enable the simultaneous relative quantification of 92 proteins, using only 1 µL of sample. The assays have been thoroughly validated; and panel composition is designed to focus on specific diseases or biological processes, and is optimized for the expected dynamic range of the target protein concentrations in clinical samples. To tailor health care to individual patients, the next step is to transpose protein signatures from discovery into the clinic using low-plex custom panels. To meet these needs and to support flexible and efficient customization, we have developed a new PEA protocol using an optimized molecular design and new conjugation chemistry, creating an assay in which high- and low-abundance analytes could be combined into a single panel. In the proof-of-principle study described below, calibrators were used for normalization and to enable absolute quantification of the measured proteins.
To verify the results from the screening phase, a set of 80 samples were analyzed both with the 19-plex panel and the corresponding screening panels. Relative quantification, NPX (Normalized Protein eXpression) values were compared among the different analyses, plotted, and correlated. Figure 1B-D shows example plots for FABP4, IL-8, and PVRL4. Note the high correlation obtained for FABP4 even after applying a shift in dynamic range on top of other protocol changes. The average coefficient of determination ($R^2$) across the 19 assays was 0.90. The lowest correlation ($R^2 = 0.73$) was observed for the SOD2 assay, reflecting that the samples tested were within a narrow range (<2 NPX). These data demonstrate that PEA assays can be combined in different configurations and panel formats while maintaining the same performance. This supports previous findings demonstrating the high scalability of the PEA technique (7). Together, these findings demonstrate the suitability of the technique for developing more focused panels for verification of screening data.

SENSITIVITY

Owing to the real-time PCR (qPCR) readout utilized in PEA, high sensitivity and wide dynamic range can be obtained. Standard curves were generated in multiplex for all assays included in the panel, using recombinant antigens for all assays. All assays were analyzed as triplicate measurements in two consecutive experiments. Data were normalized and a four-parameter logistic regression (4-PL) curve fitting was applied. The EN-RAGE standard curve had a slight plateau in the middle, possibly due to variable timerization levels (10), and was therefore unsuitable for absolute quantification and excluded from further analyses. A 4PL nonlinear curve fitting was applied to all data points (2 runs, 2 replicates, and 30 concentrations at 2-fold dilutions) and used to determine limit of detection (LOD), lower, and upper limit of quantification (LLOQ, and ULOQ) for each assay (Figure 2; data not shown).

BACKGROUND

In an ongoing project, more than 400 proteins were screened as potential markers to identify different diseases or disease stages, using standard Olink 92-plex panels. From the candidate biomarkers identified during this screening phase, 19 were selected to build a custom panel using the new PEA protocol. These customized panels were then used to verify the initial findings in additional patient samples. In the study presented here, we have scrutinized the technical performance of the 19-plex panel with the aim of guiding a decision on whether the technology is fit-for-purpose for future clinical utility, such as in early-phase clinical trials. The study design was inspired by a white paper generated by the AAPS Biomarker Discussion group (9).

TRANSITIONING FROM SCREENING TO VERIFICATION

SCALABILITY

A clinically useful biomarker signature may include both high- and low-abundant proteins. If assays for high-abundance proteins that normally require prediluted samples are combined with those for lower abundance proteins that require undiluted samples, the dynamic range must be shifted to cover the endogenous concentrations. In the current panel, an assay for FABP4 that normally uses samples diluted 100-fold was included, and therefore required dynamic range optimization. Figure 1A demonstrates the ~10-fold shift of the dynamic range for the FABP4 assay from the original 92-plex screening panel to the 19-plex panel. The other assays targeted were: CDH3, CPE, Dkk-4, EN-RAGE, FABP4, FGF-23, FR-alpha, Gal-1, IL-10, IL-17C, IL-8, KLK11, MK, MMP-7, NTRK3, PARP-1, PRSS8, PVRL4, and SOD2.

FIGURE 2. Antigen standard curves were generated for all 18 assays and analyzed in multiplex. Data were normalized against an internal control assay and calibrators, and curve-fitting was performed using 4-PL. Circles show the average of all data points from two runs, and lines show the fitted curve.
Each run also included triplicate measurements of four different calibrators (Hi, Mid, Low, and Blank) for normalization and absolute quantification, as well as four control sample pools (healthy individuals or patients with different disease types) for precision calculations. Overall, the assays gave precise measurements and high sensitivity. The most sensitive assay in this panel was IL-8 with an LOD and LLQ of 30 fg/mL. Another four assays (IL-10, FR-alpha, PARP-1, and PVRL4) exhibited very high sensitivity with LLQ ≤ 1 pg/mL. The median LOD and LLQ were 7.6 pg/mL and 11.4 pg/mL, respectively. Figure 2 displays the antigen standard curves for all assays.

**DYNAMIC RANGE**

To visualize if the dynamic range was sufficient to accurately quantify proteins in a clinical context, a set of 36 relevant samples was analyzed, quantified, and plotted in the context of LOQ. Figure 3 shows the distribution of the three different groups of plasma samples, with the upper and lower LOQ indicated. The dynamic range obtained covered samples from both healthy and diseased subjects very well, with only a few exceptions. The mean log10 range was 3.7 (2.1–5.4). IL-8 had the widest dynamic range of all assays with a log10 range of 5.4. Altogether, the assays spanned 6.4 logs in concentration from the lowest to the highest control sample. The shifting of the dynamic range for FABP4 proved to be successful, resulting in samples being distributed around the middle of the dynamic range (second assay from the left).

**ASSAY RELATED PROTEIN COVERAGE IDENTITY CROSS-REACTIVITY**

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**FURTHER ASSESSMENT OF SUITABILITY FOR CLINICAL UTILITY**

**MATRIX INTERFERENCE**

Several plasma components are known to potentially interfere with immunoassays. In a second study, the potential impacts of bilirubin, lipids, and hemolysate were evaluated at different spiked concentrations of analytes. These additions represent different patient health conditions and/or sample collection irregularities. For all assays, bilirubin and lipids could be added to concentrations corresponding to at least 8 (630 g/mL) or 10 times (20 mg/mL) normal values (11, 12), respectively, without disturbing assay performance (data not shown). In two out of 20 assays (PARP-1 and IL-8), a slight signal increase was observed by the addition of high concentration (15 and 7.5 mg/mL, respectively) of hemolysate (calculated from the hemoglobin level in the original blood sample). A concentration of 15 mg/mL of hemolysate represents 10% hemolysis of a sample. The reason for this is most likely due to analyte-specific leakage from disrupted erythrocytes rather than technical interference.

These data were in good agreement with the results obtained for corresponding assays using Olink’s screening panels (13).
CROSS-REACTIVITY

Cross-reactive events are a common problem for multiplex immunoassays, such as the sandwich ELISA (enzyme-linked immunosorbent assay). The dual-recognition, DNA-coupled readout provided by PEA, however, can overcome this and provides exceptional specificity even at high multiplexing levels (7): Multiple blocking reagents are also included in the immunoassay step to avoid nonspecific binding. PEA probes are designed for pairwise hybridization, and detection requires double recognition of two specific primers to give a signal. This degree of specificity is a hallmark of PEA. However, since the current panel is built on a new PEA design, including entirely new DNA sequences, crosstalk was reassessed by testing each assay for recognition of the other 18 antigens, all at endogenous concentrations. Only one assay (SOD2) gave rise to a weak signal with nonsignificant contribution to the specific signal (0.5%; data not shown). This study confirmed that the new PEA protocol did not bring about any unexpected crosstalk events.

Previous data have demonstrated that PEA could distinguish between human and corresponding chicken proteins (14). However, homologous human proteins have not been studied in the context of cross-reactivity. In a second approach, a set of highly homologous proteins were used to search for cross-reactive recognition of related proteins and to further challenge specificity. Homologous proteins from Olink’s antigen library (n=1,500) were included if they had an amino-acid sequence coverage ≥90% and/or identity ≥50% according to Protein BLAST (Table 1). Endogenous levels (15; and data not shown) were used for both specific and homologous proteins as indicated in the table. Despite testing the most related proteins, cross-reactivity was not observed. FR-alpha showed some recognition of its highly homologous relative FR-beta (77% identity and 87% coverage), although at a nonsignificant level in plasma (0.1%). This systematic approach demonstrated that the assays can distinguish between very similar human proteins, and yet again highlights the high specificity of PEA.

LINEARITY

Linearity of dilution is often assessed by diluting a native sample with assay buffer, and thereby determining the minimum required dilution (MRD). However, in the current 21-plex protocol, crude samples were analyzed so that linearity was studied under true matrix conditions. This was done by mixing a sample containing a relatively high endogenous level of the protein analyte with a sample containing a low level at different ratios, to give 5 equally spaced concentrations (16). Native samples were chosen to give as wide a range as possible, requiring several different sample combinations to be included in the test, all depending on the endogenous concentrations. Analysis was performed...
by calculating the expected concentrations of the three intermediate points (based on the highest and lowest samples) and plotting the measured concentration against the expected (theoretical) concentrations. Figure 4A-D shows the results for four assays, and Figure 4E presents the $R^2$, range (fold-change: high/low) and maximum accuracy for all assays. Assays were linear in general (both at high and low ranges), with somewhat poorer accuracy observed for the MK assay, where the highest sample concentration was close to the estimated ULOQ.

**STABILITY**

A robust diagnostic test requires that both biomarkers and reagents are stable throughout the protocol and are preferably also resistant to suboptimal handling. In a stability study, both whole-kit and reference samples were exposed to either three or five cycles of freeze-thawing, or to a 24-hour room temperature (RT) storage to assess stability compared to untreated references. Samples and kits were found to be insensitive (criteria: +/- 30% deviation) to both freeze-thawing and RT storage. The exceptions were MMP-7, which was sensitive to RT storage both in plasma and in buffer (calibrator), and MK, which showed a decrease (38%) in calibrator signal after 24 hours at RT (data not shown). As a follow-up, MMP-7 and MK will be studied along with the other biomarkers in both short-term and long-term storage studies to better define their utility as potential biomarkers.

**CONCLUSIONS**

The technical verification studies described here, performed on a custom 19-plex PEA panel, demonstrate that multiplex PEA is a highly scalable technique that is compatible for both screening and verification studies.

Several key immunoassay parameters, including sensitivity, dynamic range, specificity, linearity, precision, and stability, have been studied, and the results presented here will serve as a guide in determining whether PEA is fit for your research or clinical purposes.

To move further along the path toward clinical decision-making and in vitro diagnostics, long-term reagent supply and readout platforms are also factors under active investigation. To those ends, PEA assays are being developed using antibodies developed in-house by Olink, and agnostic readout using standard qPCR machines is also being explored. Both these important developments show great promise, and early results indicate that compatibility with PEA methodology is excellent.

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