Prof. Roman Fischer
Target Discovery Institute,
University of Oxford

Customer Insights

- High-throughput proteomics advances clinical research with large-scale sample sets

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Researchers at the Target Discovery Institute, University of Oxford, are using the Bruker timsTOF Pro to develop new methodologies for the proteome characterization of clinical cohort samples.

Working with Bruker
The work of Professor Roman Fischer, Associate Professor in Clinical Proteomics at the Target Discovery Institute, University of Oxford, and his research team has led to significant breakthroughs in method development for high-throughput mass spectrometry (MS) on large-scale clinical sample sets, which led to a partnership with Bruker:

"We set out to find an instrument or a platform that can deal with blood-derived, very difficult clinical samples. The Bruker timsTOF Pro is very robust, and that's when we started to work closely together with Bruker. Since then, we have produced really excellent results on the timsTOF Pro and now we're looking to expand with another instrument from Bruker."

High-throughput proteomics at Oxford University

Research at Oxford University's Target Discovery Institute (TDI) focuses on linking recent advances in genetics, genomics, cell and chemical biology, to address the need for accurately defined drug targets to accelerate drug development.

Within the TDI, the Discovery Proteomics Facility provides guidance in experimental design, sample preparation, and sample analysis to researchers from Oxford University, as well as national and international collaborators. Among their achievements, the team has developed sample preparation techniques to access the deep proteome from small sample amounts using instrumentation such as the Bruker timsTOF Pro.

Professor Roman Fischer, PhD, joined Oxford University in 2009 and became the head of Discovery Proteomics Facility in 2016. In that time, he has seen the evolution of clinical proteomics and applications used for the spatial characterization of the proteome in biological structures such as tissues and tumors. His work includes developing methodologies for the proteome characterization of clinical cohort samples at high throughput.

Over the last two decades, significant advances in technology and new methodologies have made proteomics an extremely powerful tool for protein scientists, biologists, and clinical

researchers [1]. The discovery of molecular principles underlying human disease is the driving force of finding new cures for human diseases. Mass spectrometry (MS) can help researchers discover critical 'molecular windows' within complex disease processes. The ongoing developments in MS technology have enabled researchers to start visualizing the proteome – the set of proteins expressed at a particular time – of a cell or organism, with the goal of comprehensively identifying all proteins and their associated biological activities.

Clinical proteomics applies proteomics to patient care, however the analysis of proteomics data from patients requires special strategies. Challenges include how to extract meaningful protein expression signatures from data with high individual variability, how to integrate the genomic background of the patients into the analysis of proteomics data, and how to determine biomarkers and properly estimate their predictive power.

Within the TDI, Roman Fischer and his team of six scientists work in the Discovery Proteomics Facility studying protein function, dynamics, post-translational modifications (PTMs) and their effects on protein turnover, antigen presentation, and metabolic pathways. The group focuses on proteomics, MS, and biochemical approaches to understand disease processes. The expansion of analytical capabilities using integrative approaches have led to a more comprehensive picture of molecular processes in human disease. Prof. Fischer explains:

"Oxford University is a very good location for clinical biomedical science. We have multiple hospital sites and very strong biomedical research. Our Institute is closely connected to the hospitals, and we have many collaborations where proteomics has become part of the major research strategies. So, that translates into increasing demand from clinical researchers to include proteomics analysis of their samples.

At the same time, the average sample numbers per clinical study have gone up quite dramatically. That's where high-throughput proteomics came at the right moment in time."

Challenges for clinical proteomics

When Prof. Fischer began at Oxford University, proteomics was not the driving force of clinical projects as sample sets were typically very small. Additionally, the coverage of complete proteomes was difficult to achieve due to the limited speed, sensitivity, and resolution of mass spectrometers at the time. Clinical samples are typically challenging, with extreme amounts of proteins and a high dynamic range [2]. That presents complications for MS analysis, because the dynamic range of the mass spectrometer is often much smaller than the dynamic range of proteins in clinical samples [3]. Prof. Fischer describes these early days:

"At that time, in clinical proteomics, you would struggle to analyze blood samples. These are usually very difficult samples to analyze with proteomics due to the high dynamic range in protein abundance. Some sample types are 'tricky' because they would contain components that are not really compatible with MS. It was not easy or possible to run a lot of samples using proteomics at that point. At the time, a batch of 200 samples was a lot for a proteomics study. If you can only analyze 10 samples per day, then it's quite easy to calculate how long 200 samples take. Plus, proteomics labs have multiple projects running in parallel and instrument time is at a premium. So, our laboratory needed to dedicate one machine to run for two or three weeks for data acquisition back then, which is a big challenge. High-throughput proteomics in the past has been addressed by just ramping up the number of mass spectrometers in labs analyzing numerous samples at a low throughput.

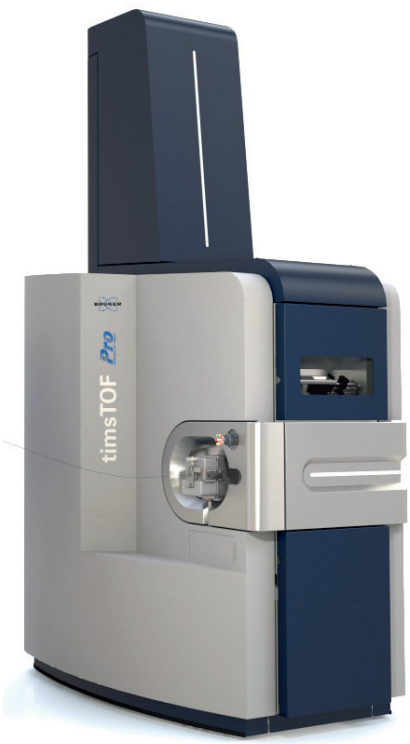
The Bruker timsTOF Pro, in combination with the novel Evosep One HPLC, changed this with its improvements in analysis time and robustness, making high-throughput proteomics possible."

Benefits of the Bruker timsTOF Pro

Researchers at the Discovery Proteomics Facility found that the Bruker timsTOF Pro significantly improved the ability to achieve high-throughput proteomics for clinical studies. One of the biggest benefits of the timsTOF Pro is the speed of analysis. Prof. Fischer describes his team's initial test of the timTOF Pro with the facility's clinical samples:

"We set out to find an instrument or a platform that could deal with blood derived, very difficult clinical samples. So, we created 200 samples for a demonstration to see how different vendors would deal with this kind of request. We made this demo relatively difficult because we had a lot of samples and we needed to analyze them in just two days.

So, we wanted a throughput of 100 samples per day. Bruker returned excellent data to us and had no concerns relating to instrument robustness."



The timsTOF Pro uses trapped ion mobility spectrometry (TIMS), where ions are propelled through the TIMS tunnel by a gas flow. An electrical field controls each ion from moving beyond a position defined by the ion's mobility, where the push it experiences from the gas flow matches the force of the electrical field. Ramping down the electrical field allows selectively releasing ions from the TIMS tunnel according to their mobility.

This process is called parallel accumulation – serial fragmentation, or PASEF. The unique TIMS design allows researchers to reproducibly measure the collisional cross section (CCS) values for all detected ions, and those can be used to further increase the system's selectivity, enabling more and more reliable relative quantitation information from complex samples and short gradient analyses [4]. The novel PASEF design allows for ions to be accumulated in the front section, while ions in the rear section are sequentially released depending on their ion mobility.

The Discovery Proteomics Facility research team found the unique characteristics of the Bruker timsTOF Pro allowed routine measurements of ion mobility information and enabled high-quality 4D feature alignment in retention time, *m/z* and

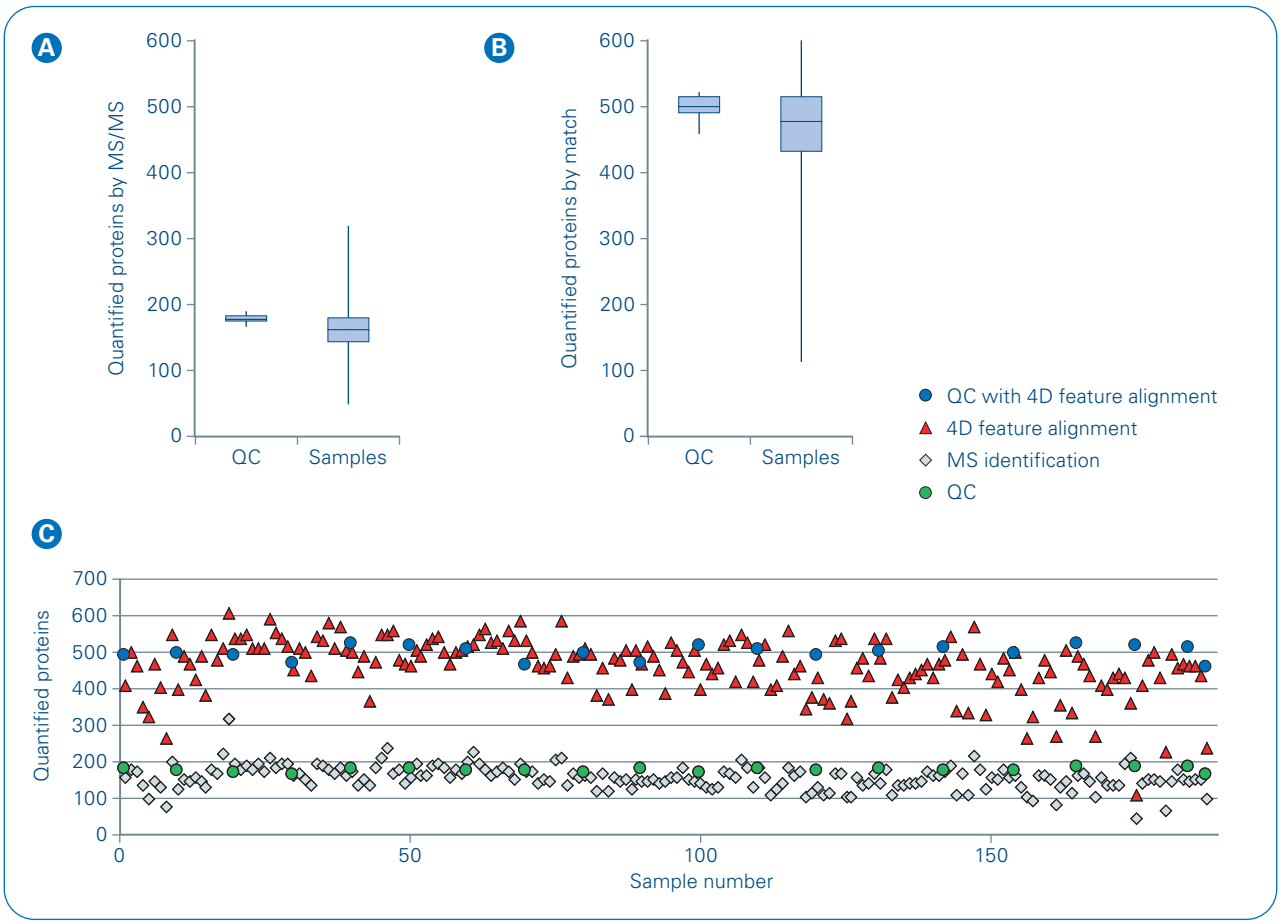


Figure 1: Number of quantified proteins by MS/MS identification and 4D feature alignment. **A** *Number of quantified proteins by MS/MS in pooled samples (QC) and samples.* **B** *Number of quantified proteins per sample if MS/MS identifications are transferred between runs with a 4D feature alignment in retention time, m/z, ion mobility (1/K_0) and intensity.* **C** *Time course of LC-MS/MS measurements with and without 4D feature alignment.*

ion mobility ($1/K_0$) and MS1 intensity (Figure 1). The alignment boosted the number of quantified plasma proteins up to more than 500 proteins in a single 11.5 min LC-MS/MS run and 772 collectively quantified if all runs are combined (Figure 2) [5]. Achieving this depth offers completely new possibilities to analyze large sample cohorts of hundreds to thousands of samples for biomarker discovery in blood plasma.

PASEF technology can achieve >100 Hz sequencing speed without losing sensitivity or resolution. This is achieved by synchronizing the quadrupole isolation mass window with the elution time of the specific peptide packages from the TIMS tunnel. The MS/MS spectra quality of the low abundant peptides can be increased by selecting them several times. Superior results can be obtained from less than 200 ng sample load, therefore reducing both sample preparation costs and MS maintenance frequency. Using a 90 min gradient length, more than 5400 protein groups can be identified from a typical human cell line lysate.

Robustness was another major advantage of the timsTOF Pro for the Discovery Proteomics Facility team. Many MS instruments used for shotgun proteomics require biweekly or monthly cleaning when run 24 hours a day on large sample cohorts, and performance degradation is noticeable over even shorter time periods. Prof. Fischer describes the impact of this capability on proteomics research:

"The timsTOF Pro remains remarkably clean inside, even after a large number of samples are injected. Typically, an instrument would need to be cleaned after just 200 blood derived samples. In a high-throughput setting, that would mean we need to clean the instrument every two days, which includes venting the instrument and taking apart the insides for cleaning. It's a two- or three-day operation to get back to normal, which is not feasible with a hundred blood samples per day. The timsTOF Pro has really changed things for us because of the way the instrument is constructed. The ion source remains clean for a long time.

The largest sample batch that we have run on the timsTOF Pro instrument was 4500 injections of non-depleted blood – which is one of the most challenging samples you can throw at it.

During the preventive maintenance service after this big batch, the engineer said the machine was like new inside. It changed the game for us in terms of how we can run these samples."

The timsTOF Pro can also deliver reproducible quantitative information from smaller amounts of highly complex mixtures. Prof. Fischer explains:

"The Bruker timsTOF Pro can achieve 100+ Hz, which means it has the potential to identify 100 or more peptides per second.

When compared to other instruments, we can really shorten the gradient times, even for samples like total cell lysates, without compromising the depths of the data that we get. Plus, the instrument is very sensitive as well. So, we don't compromise sensitivity for the increased speed and robustness. That's usually not the case for any lab instrument – there's always a tradeoff. But not with the timsTOF Pro."

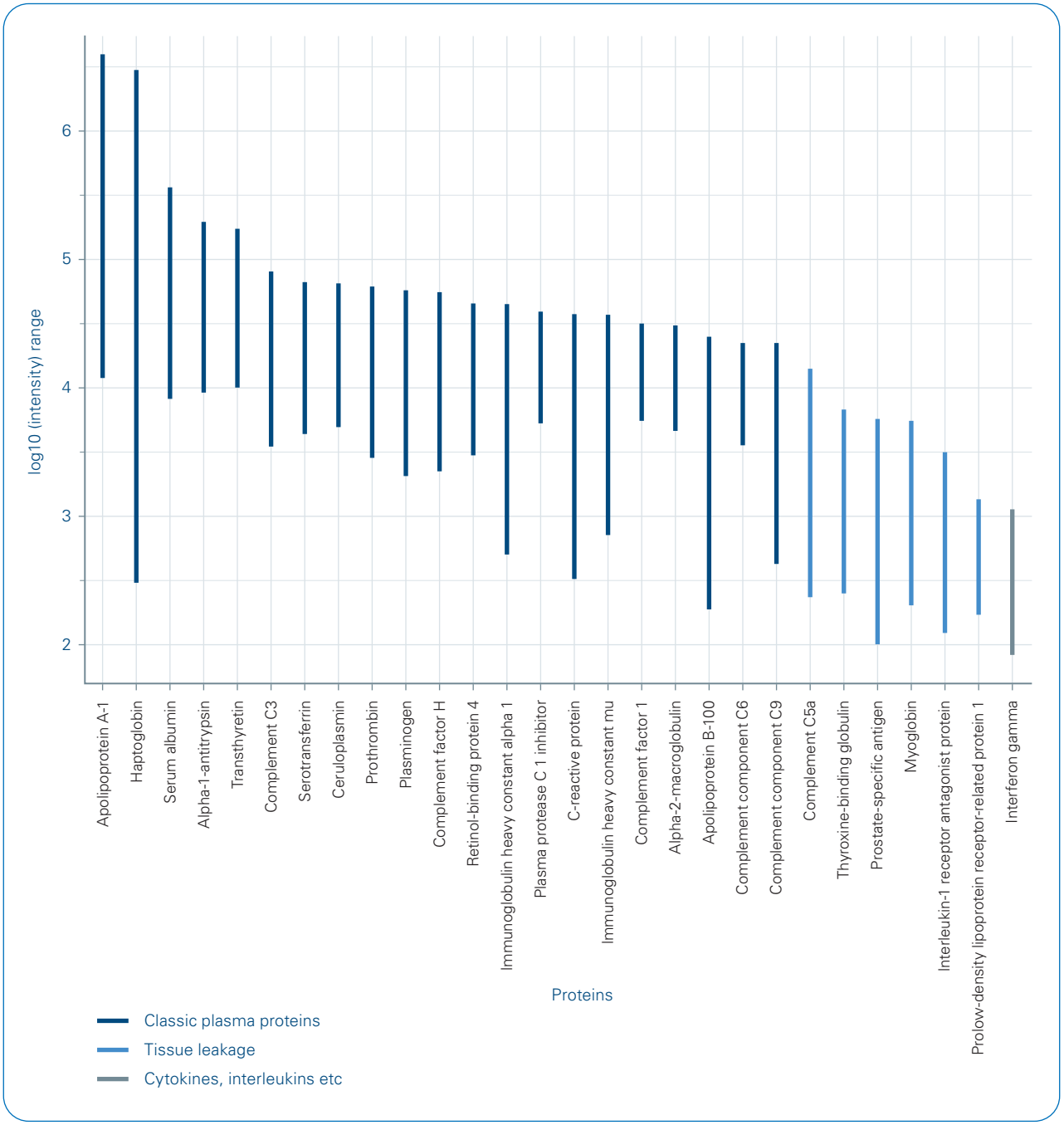


Figure 2: Selected proteins from plasma samples covering close to five orders of magnitude dynamic range. The analytical depth is sufficient to quantify classical plasma proteins (e.g. CRP), tissue leakage proteins (e.g. PSA) and cytokines (e.g. IFN-γ). Together with the high throughput and sensitivity demonstrated, the Bruker timsTOF Pro is a powerful tool for biomarker discovery in large sample cohorts.

Growing demand for high-throughput proteomics

As clinical proteomics has evolved, Prof. Fischer and his team have seen a large increase in the number of samples included in projects, which has doubled in the last two years alone. That enables researchers to include more conditions and better controls, as well as time courses instead of single time points. Prof. Fischer explains why:

"Proteomics is much more accessible now. There's software that can analyze the data relatively easily, and you can visualize it nicely. The demand has risen considerably since more and more people implement proteomics aspects into their projects, and it is complimentary to the other -omics disciplines that are often applied in clinical research such as genomics or classical biochemistry methods."

One such study involves tumor research, where Prof. Fischer and his team use proteomics to determine how a tumor functions in a spatial context. He explains:

"It's very timely because now we can combine proteomics with other technologies such as laser capture microdissection (LMD). It's a really interesting approach because it allows us to maintain the spatial context of a proteome.

At the moment, we are still really trying to understand the spatially resolved molecular interactions in a large structure like a tumor or organ. For example, we see inflammation markers around a blood vessel in a tumor, but nowhere else, so we're trying to understand what's going on at the molecular level in these tumors. This will impact which proteins are to be targeted and how drugs can be delivered to a location where they would be most effective. By combining MS with LMD we can redefine the phenotype. Essentially the proteome becomes part of the phenotype of a cell as it is now observable with spatial resolution. I like to call this the "Pheno-proteome". Obviously, this approach can also be combined with a genomic or other -omic analysis in a spatial corresponding fashion."

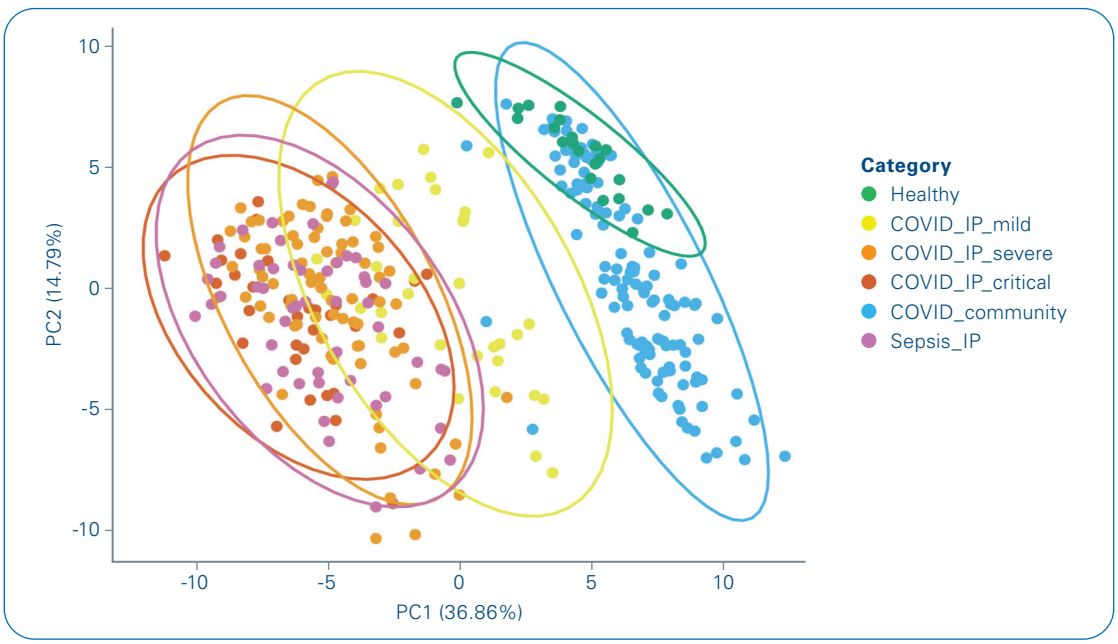


Figure 3: Principal Component analysis of COVID-19 disease severity groups using Shotgun high throughput proteomics data acquired by a Evosep 1/TimsTOF Pro platform. Adapted from reference [6] in accordance with Creative Commons Attribution 4.0 International License.

Categorizing COVID-19 patients

One of the most recent projects where the researchers at the Discovery Proteomics Facility found the capabilities of the Bruker timsTOF Pro particularly valuable was a study to enable deep phenotyping of the host immune response in COVID-19 infections [6].

The COVID-19 Multi-omics Blood Atlas (COMBAT) consortium is part of a large multi-department collaboration at the University of Oxford that is characterizing the immunological response to SARS-CoV-2 infections. Specifically, COMBAT aims to perform deep phenotyping of the peripheral blood response in SARS-CoV-2 infection to understand why some patients with COVID-19 develop severe disease, with the goal of identifying such patients early and treating them in a targeted manner. To do this, large-scale datasets derived from a core set of patient samples taken at different time points of infection are being generated using a wide array of molecular techniques (multi-omic and immunological) at different labs in Oxford. The project analyzed roughly 500 samples and looked at proteins that correlated with disease severity. Prof. Fischer explains:

"We've got a really clear picture of a relatively small number of proteins that correlate with the patient's condition at that point in time in the clinic. These proteins then would clearly point to the acute phase response in the body. And from that point of view, many already available drugs can address this acute response. So, in that context, we could contribute to supporting these findings with insights into underlying biology."

This study involved 200-300 patients, some with samples taken at multiple time points, and analyzed using high-throughput proteomics on the Bruker timsTOF Pro. The study took a new approach by using subgroups of these samples to analyze with other -omics techniques, such as site analysis of certain blood cells. The plasma proteome enabled sub-phenotyping into patient clusters, which were predictive of severity and outcome (Figures 3 and 4) [6]. Prof. Fischer explains:

"Because some technologies are less capable of high throughput, we're in a new position with proteomics. Usually, it's the other way around and you'd use a genomics approach on the whole sample set, and then you'd further analyze a subset with proteomics. This project was the first time it was flipped around so we could use

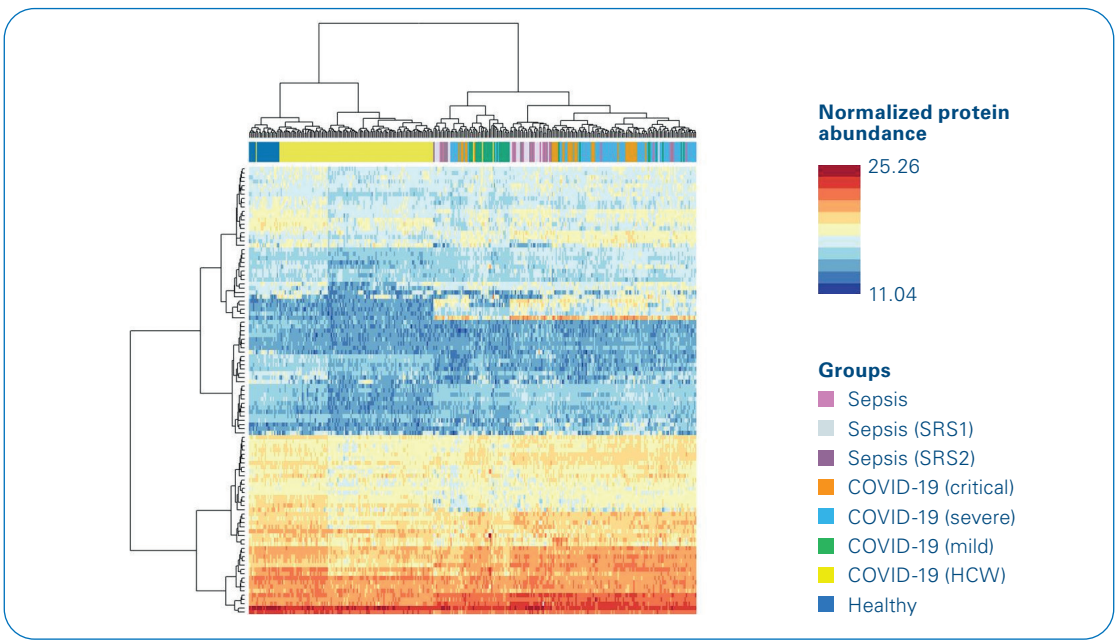


Figure 4: Unsupervised hierarchical clustering of quantified proteins reveals plasma protein clusters correlating with disease severity. Adapted from reference [6] in accordance with Creative Commons Attribution 4.0 International License.

proteomics to cover the whole sample set, and other technologies would be used to cover a subgroup.

Among the 8 types of -omics that we used in this large study on the same set of samples, proteomics was the best suited to stratify the COVID-19 patients into different disease severity groups, while other technologies provided data to help understand the underlying biology.

Additionally, we could identify indicators or markers that would help us see how the disease progresses and even allow prediction of outcomes, with the eventual aim of intervention and tailored treatment for these patients."

This proteomic analysis identified specific plasma acute phase protein levels as indicators of severe disease, with evidence for hallmarks of acute phase inflammation, complement activation/attack, fibrin clots, proteases, serum amyloid, tissue necrosis, receptor mediated endocytosis and cholesterol transport. The Consortium discovered plasma protein signatures that can be used to stratify acute hospitalized COVID-19 cases into disease sub-phenotypes, with cluster membership informative for response state and associated with differential 28-day mortality.

Studies such as this multi-omics blood atlas will provide essential insights to inform future drug development, clinical trial design and personalized medicine approaches for COVID-19.

Future steps

Prof. Fischer sees a future where high-throughput proteomics is used in a clinical setting – where the instrumentation is installed in a hospital and being used to conduct day-to-day screening of patient samples. Because MS enables the measurement of multiple analytes at the same time, proteomics could potentially analyze a whole pathway or a whole assembly of proteins from a patient sample, which could lead to improved diagnoses. Another benefit is, instead of being tested for one disease, most patients have comorbidities that could be identified with MS in a systems biology-based approach.

However, clinical proteomics comes with challenges that require special strategies to cope with obtaining this type of data from patients in a hospital setting. Additionally, logistical issues such as certification and ease of use for non-MS specialists means this possibility is still several years away. Prof. Fischer explains:

"Among other factors, we must work out challenges like making sure that analyzing the same sample across different locations gives you the same result.

But later on, I suspect proteomics in a clinical setting can be used to develop better targeted drugs, and even patient specific or tailored treatment regimens."

Since that original trial with the timsTOF Pro, the Discovery Proteomics Facility researchers also have continued to expand their collaboration with Bruker on other instrumentation and equipment. Prof. Fischer explains how this partnership has helped with the facility's other projects:

"Bruker is hugely supportive in helping us with our lipidomics analyses, which we just started. Bruker has a group of experts who are experienced in that field, and they are very helpful.

They're also assisting our work where we're combining proteomics with LMD, and now want to supplement with MALDI Imaging using Bruker's timsTOF fleX. Bruker is working on completely new ways of data analysis too, with the goal of real-time data processing.

These new developments are vital for the progression of clinical proteomics research, and I'm looking forward to seeing how our research continues to evolve with these new technological advancements."

For more information about the timsTOF Pro, please visit https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/o-tof/timstof-pro.html.

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About the Target Discovery Institute

The Target Discovery Institute (TDI) is a major new collaborative research initiative led by 2019 Nobel Laureate Professor Sir Peter Ratcliffe, FRS. Strategic investment through the Department and collaborative use of existing research resources from the Department of Cardiovascular Medicine, the Department of Oncology, specifically the Radiation and Oncology Unit, the Oxford Branch of the Ludwig Institute for Cancer Research and the Centre for Medicines Discovery (CMD)established the Target Discovery Institute in the new NDM Research Building. The TDI now encompasses several groups including Chemical Biology, High Throughput, Phenotypic Screening, Proteomics and Mass Spectrometry and Medicinal Chemistry.

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Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com - www.bruker.com

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