Bridging discovery and quantification: Complementary insights from ENRICH-iST and Olink in serum proteomics

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Introduction

Biofluids such as serum are invaluable in disease research due to their rich protein content and minimally invasive collection. However, the serum proteome is challenging to analyze due to its high complexity and dynamic range.[1] Two leading technologies, mass spectrometry (MS) and affinity-based platforms, provide distinct but synergistic approaches to address these challenges.[2]

MS offers untargeted, discovery-driven analysis, enabling detection of protein isoforms and post-translational modifications. In contrast, affinity-based methods, such as the Olink® platform, use Proximity Extension Assay (PEA) with NGS readout to deliver high-throughput, targeted quantification of clinically relevant proteins with high sensitivity. Recent advances in MS, including optimized sample preparation workflows and enhanced instrumentation, have significantly increased proteome depth.[3] However, studies consistently report limited overlap between proteins identified by mass spectrometry and those detected by affinity-based methods.[4,5] This highlights the unique strengths of each approach in serum proteomics.

In this study, 132 serum samples from healthy individuals were analyzed using both platforms. Samples were prepared with PreOmics® ENRICH-iST and analyzed on a Bruker timsTOF HT mass spectrometer (ENRICH-iST workflow). In parallel, the Olink® Explore 3072 panel was used for targeted protein quantification. By combining both approaches, proteome coverage and biomarker detection were improved. Although protein overlap between platforms was limited, the quantitative agreement for shared proteins was remarkably high. This demonstrates the precision and reliability of both technologies and underscores the value of integrated serum proteomics.

Keywords

Proteomics, serum analysis, low-abundance protein, dynamic range compression, biomarkers, ENRICH-iST, LC-MS analysis, timsTOF

Key takeaways

Complementary coverage: ENRICH-iST and Olink workflows identified largely distinct protein sets, offering expanded serum proteome coverage when combined.

High quantitative agreement: Shared proteins showed strong quantitative correlation across age groups, confirming the reliability of both platforms.

Deeper biological insight: Integrated analysis improved pathway resolution, revealing more comprehensive insights than either method alone.

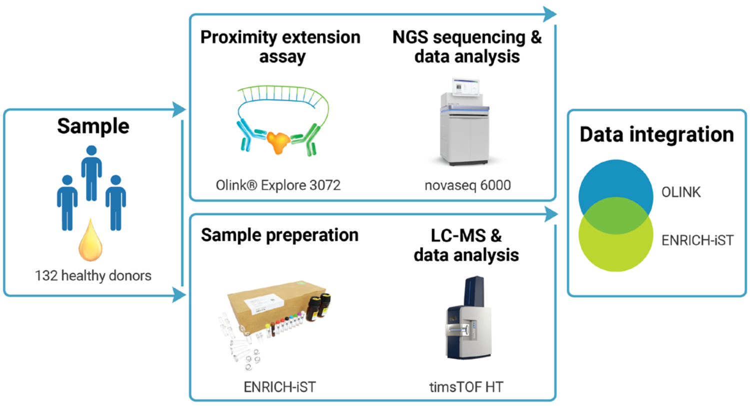

Figure 1 | Overview of the workflow for deep serum proteomics. Serum samples from 132 donors were analyzed using two complementary platforms: ENRICH-iST sample preparation followed by mass spectrometry (MS) analysis, and Olink® Explore 3072 platform for affinity-based profiling. MS data and Olink data were processed, and the resulting datasets were compared both qualitatively and quantitatively to assess proteome coverage, overlap, and platform complementarity.

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Materials and Method

Sample collection

Blood samples were collected from healthy male and female donors of varying ages via venipuncture following standard clinical procedures. Blood samples were allowed to clot at room temperature for 60 minutes. Samples were then centrifuged at 1500 g for 10 minutes at room temperature. Serum was stored at -80°C until analysis. Samples were collected at Sheffield Teaching Hospitals NHS Foundation Trust, UK, under the REC (Research Ethics Committee) approval 15/SC/0132.

Affinity-based platform (Olink workflow)

Sample preparation

Proteomic profiling was conducted using the Olink® Explore 3072 platform, which features eight specialized panels: Cardiometabolic, Cardiometabolic II, Inflammation, Inflammation II, Neurology, Neurology II, Oncology, and Oncology II. For each sample, 1 µL of serum was used per panel, resulting in a total input of 8 µL.

PEA sample analysis and data processing

Olink technology utilizes a Proximity Extension Assay (PEA), in which pairs of antibodies specific to a target protein are each labeled with unique, complementary oligonucleotide probes. Upon binding to the target, the probes come into proximity and hybridize, enabling DNA polymerization and amplification. The resulting DNA signal is then quantified using next-generation sequencing (NGS). Sequencing data were processed using Olink® NPX Explore software, which provides quality control data and performs normalization. Protein expression levels were reported as Normalized Protein eXpression (NPX) values on a $\log_2$ scale. For valid value filtering, only proteins with values above the assay-specific limit of detection (LOD) were included in downstream analysis. Each assay plate included internal controls and pooled plasma reference samples to ensure data quality and consistency. Inter-plate normalization and quality control were performed according to Olink's standard procedures.

MS-based platform (ENRICH-iST workflow)

Sample preparation

Serum samples were processed using the ENRICH-iST 96x kit (PreOmics) according to the manufacturer's protocol. For protein enrichment, 20 µL of serum was used and enrichment, lysis, and digestion were performed in a batch. Purification was carried out using the provided 96-well plates. Samples were eluted in 100 µL of ELUTE buffer and then dried in a vacuum concentrator. Peptides were reconstituted in 50 µL of LC-LOAD buffer, and 4 µL was injected for each LC-MS/MS analysis.

LC-MS/MS and data processing

MS data were acquired on a timsTOF-HT mass spectrometer (Bruker Daltonics) operated in DIA-PASEF mode and coupled to a nano-RSLC system (Ultimate 3000 RSLC; Thermo Fisher Scientific). Tryptic peptides were automatically loaded onto a C18 trap column (300 µm inner diameter × 5 mm, Acclaim PepMap100 C18, 5 µm, 100 Å; LC Packings) at a flow rate of 30 µL/min. For chromatographic separation, an Aurora Ultimate column (25 cm × 75 µm, C18, 1.7 µm; AUR3-25075C18-CSI, IonOpticks, Australia) was used at a flow rate of 250 nL/min, employing a 70-minute non-linear acetonitrile gradient from 3% to 40% in 0.1% formic acid.

Data were acquired in dia-PASEF® mode using a dia-PASEF isolation scheme with a precursor range of 300–1500 m/z, using 32 PASEF windows of variable m/z width tailored to a similar number of eluting precursor ions in each window.[6] The ion mobility range ($1/K_0$) was set from 0.60 to 1.50 V·s/cm^2 with a ramp time of 100 ms (i.e., TIMS mode is active), thus resulting in a cycle time of 1.8 s. Rolling average was enabled (10×). Ion polarity was set to be positive with an ionization voltage of 1500V. The collision energy was ion mobility dependent and determined by a linear function.

DIA files were analyzed in DIA-NN (version 1.9). An in-house spectral library was generated from the high-pH fractionation of various matrices (plasma, serum, CSF, and extracellular vesicles isolated from plasma) and sample preparation methods (ENRICH-iST, iST-BCT, ProteoMiner beads, and perchloric acid). Fractions were acquired in data-dependent acquisition PASEF mode under identical chromatographic separation and ionization conditions, and the spectral library (46,070 precursor spectra, 3,897 proteins) was generated using Proteoscape (version 2024b). For protein annotation, the canonical SwissProt human database (Release 2020_02, 20,432 sequences) was used. The default DIA-NN settings were applied, except that cross-run normalization was set to global normalization, match-between-runs was enabled, and mass accuracy was fixed at 10 ppm for MS1 and 20 ppm for MS2. Quantification was based on summed MS2-level fragment ion intensities.

Post-processing for both platforms: Data analysis and integration

To process Olink and MS data similarly, Perseus 2.0.11 was used.[7] For data processing, Olink values above LOD were included, and MS data were filtered for 0.01% FDR at the precursor and protein group levels. In Perseus, both platforms underwent the following steps: categorical annotation of age groups: 30–39 years (18 individuals), 40–49 years (13 individuals), 50–59 years (24 individuals), 60–69 years (61 individuals), and 70 years and older (16 individuals); averaging groups based on mean values; applying valid value filters of 50% in each group for CV calculation and 20% in each group for quantification; combining main columns to calculate $\log_2$ fold changes ($\log_2$FC); and using a two-sample t-test to assess the significance of the $\log_2$FC. Additionally, a limma batch correction was applied for the MS data.

ClueGo analysis

Gene ontology enrichment analysis was performed using ClueGO v2.5.10 in Cytoscape, using UniProt Accession IDs for Homo sapiens (taxon ID 9606) across Biological Process, Cellular Component, and Molecular Function ontologies. Functional enrichment was assessed using a two-sided hypergeometric test with Bonferroni step-down correction, considering GO levels 3–5 and applying GO term fusion and Kappa score-based grouping (threshold 0.4). The analysis included two gene clusters (39 and 14 recognized genes, respectively), and results were visualized with seven final functionally grouped GO terms.

Results and Discussion

Evaluation of protein identification overlap and reproducibility of MS- and affinity-based platforms

Because serum proteins contain important information about biomarkers and diseases, and the matrix presents challenges due to its high dynamic range, maximizing data collection is essential. To address this, two leading proteomics platforms, MS and PEA, were compared to potentially increase the number of identified and quantified proteins and to integrate the data and analyze their similarities.

Using the ENRICH-iST workflow, a total of 1,815 protein groups (filtered at 1% FDR, using global q-values for protein groups and both global and run-specific q-values for precursors) were identified from 132 serum samples. In comparison, 2,923 proteins above the LOD were detected with the Olink workflow from the same set of samples. The overlap between proteins identified by both platforms was 18.2% (Fig. 1A), which is relatively low given the total number of proteins identified, a finding commonly reported in the literature.[5] Notably, 2,193 proteins (54.7%) were uniquely covered by Olink, while 1,085 proteins (27.1%) were uniquely detected with ENRICH-iST.

To assess the robustness and comparability of the two platforms and the measured datasets, the coefficient of variation (CV) was calculated across different age groups. The 132 samples were divided into five age groups: 30–39, 40–49, 50–59, 60–69, and 70 years and older. For MS data, CVs were calculated using the mean of raw abundance values, applying a 50% valid value filter within each group. For Olink data, CVs were calculated using linearized NPX values above LOD, also with a 50% valid value filter per group. Both platforms exhibited similar trends in CVs, reflecting biological variability within age groups (Fig. 1B, C). Additionally, technical reproducibility was evaluated by calculating CVs from control samples. For MS, 23 HeLa samples were measured throughout the sample queue, resulting in a technical CV of 15.35%. For Olink, 10 pooled plasma control samples (2 per plate) yielded a CV of 12.4%. These results indicate that both platforms exhibit strong technical reproducibility, and that the higher CVs observed in serum samples are primarily due to biological variation.

Correlation assessment of commonly identified proteins

Since Olink and ENRICH-iST are relative quantification methods, absolute values are not directly comparable. To enable a meaningful comparison between the two platforms, the ratios of the age differences were used as a basis for analysis. Subsequently, $\log_2$ fold changes ($\log_2$FC) were calculated across different age groups, and Pearson correlation was applied to assess the quantitative relationship between the platforms. For the quantitative analysis, a 20% valid value filter was applied within each group. The following comparisons were created: $\log_2$FC (30–39 vs. ≥70, 40–49 vs. ≥70, 50–59 vs. ≥70, and 60–69 vs. ≥70).

$\log_2$FC values from the Olink data were plotted against the corresponding $\log_2$FC values from the MS data, considering either all commonly identified proteins (Fig. 3, panels A–D) or only those with a p-value below 0.05, indicating a significant difference between age groups (Fig. 3, panels G–H).

The Pearson correlation coefficient was calculated to quantify the linear relationship between the Olink and ENRICH-iST datasets. Notably, the correlation was particularly strong when considering only proteins with statistically significant p-values, indicating that both platforms provide reliable quantitative measurements for significant $\log_2$FC comparisons.

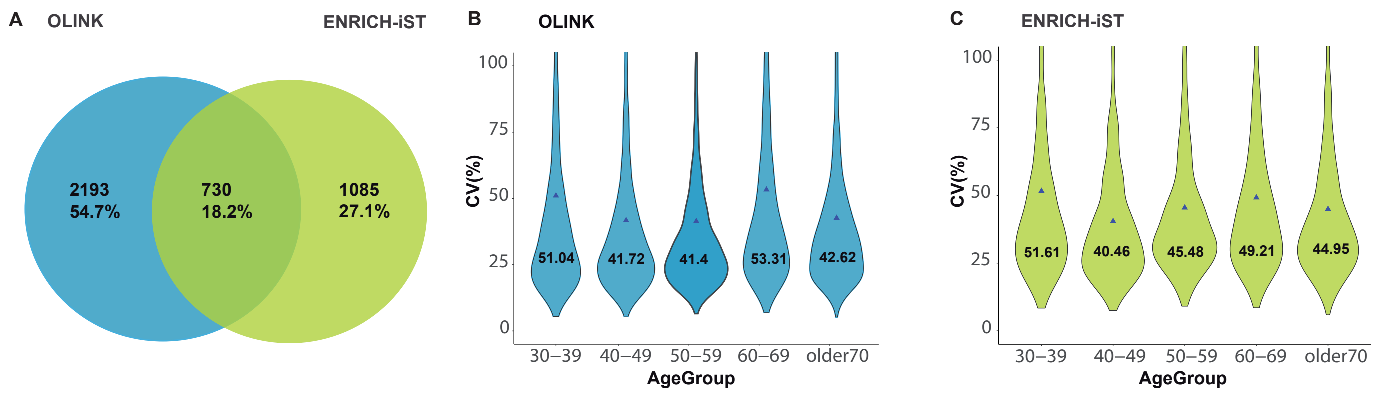

Figure 2 | Number of protein groups identified and technical variability. (A) Comparison of total identified protein IDs, showing a low overlap between Olink and ENRICH-iST. Coefficient of variation (CV) across age groups for (B) Olink and (C) ENRICH-iST. Only proteins detected in at least 50% of the samples per group were retained for the CV calculation (blue, Olink measurements; green, ENRICH-iST measurements). Technical CV (not shown in the figure) was assessed using HeLa samples for ENRICH-iST and plasma for Olink, resulting in CVs of 15.35% (7314 protein groups) and 12.4% (2680 protein groups), respectively.

In contrast, the correlation across all overlapping proteins was significantly lower. This can be attributed to the smaller biological variance among non-significant proteins, which results in a less pronounced data spread. Since Pearson correlation relies on variability to detect linear relationships, a narrow spread in the data limits its ability to capture meaningful associations, thereby weakening the overall correlation.

To further demonstrate the correlation between the two platforms, three proteins that were significantly detected and regulated within all different age groups were selected (Fig. 3I). FBP1 is a central regulator of gluconeogenesis whose altered levels are linked to metabolic diseases and cancer progression, making it both biologically insightful and clinically relevant as a potential biomarker.[8] ELN degradation products, indicative of both aging and inflammatory processes, contribute to the regulation of key biological pathways, and finally, CHI3L1, a recognized biomarker of aging, progressively increases in blood-derived samples with advancing age.[9,10] The longitudinal log_2FC patterns were very similar between both technologies showing that, especially for differentially abundant proteins, both platforms perform consistently.

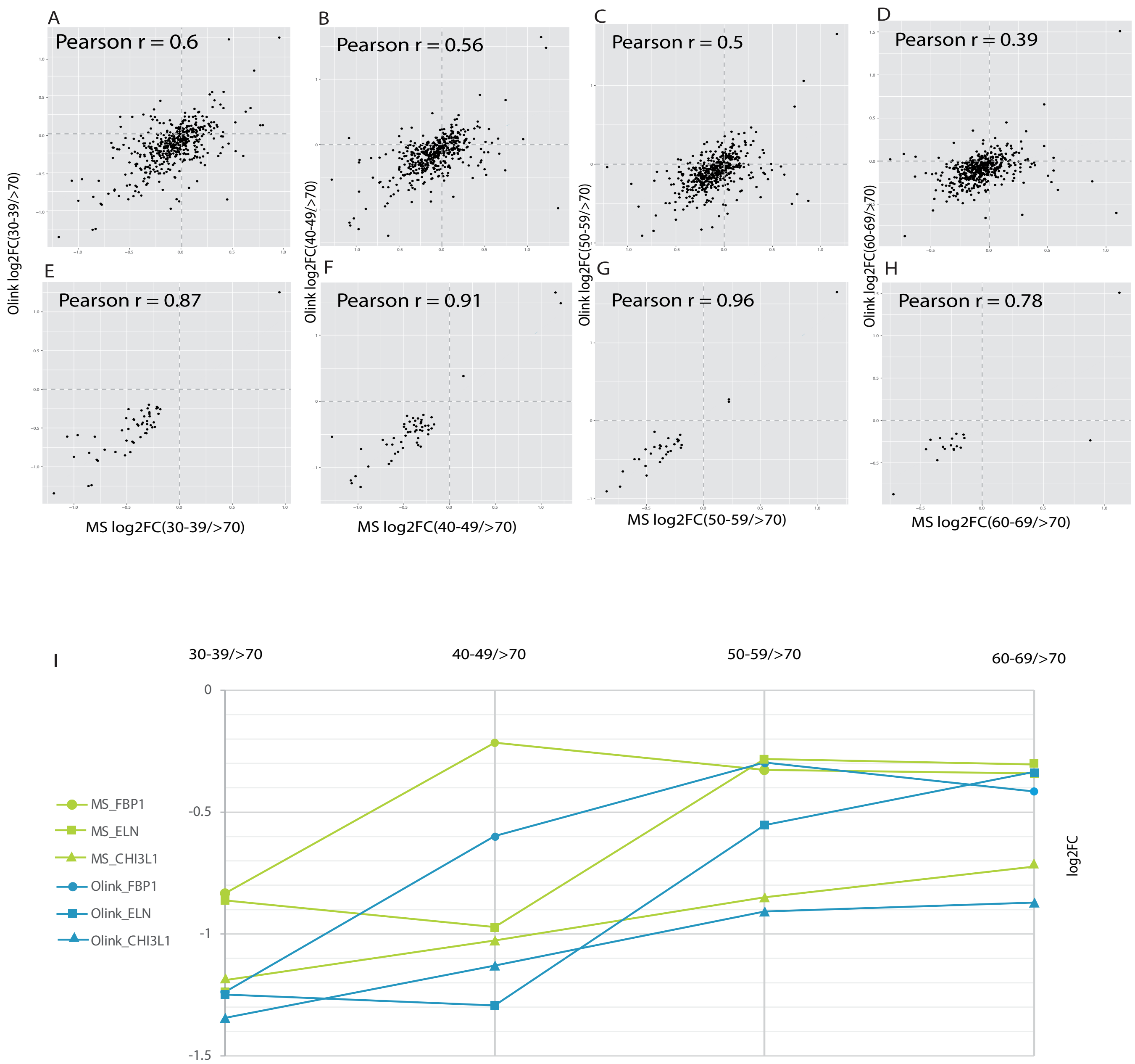

Figure 3 | Pearson correlation overlapping proteins. Correlation of different age groups comparisons plotting log_2FC of Olink measurement vs. MS measurements. The upper panel (A-D) shows all proteins in common between technologies with a valid value filter of 20%. The lower panel (E-H) shows proteins that are significantly differentially abundant between respective age groups, with a p-value of less than 0.05. (I) Variation of log_2FC for the different age comparisons for three differentially abundant proteins (FBP1, ELN, CHI3L1).

Cross-platform integration of biological pathways and functions

To demonstrate the complementarity of the MS- and affinity-based platforms, a pathway integration analysis was performed using ClueGO (v2.5.10) within the Cytoscape environment (Fig. 4). Significantly downregulated proteins identified in the comparison of 30–39 years vs. ≥70 years, independently by ENRICH-iST and Olink, were used as input lists. The integrated analysis showed that many of the significantly regulated proteins are involved in shared biological pathways, while each platform also contributed additional proteins to these pathways individually. This provided a more comprehensive and biologically meaningful view of the affected processes. The results emphasize the added value of combining both platforms, improving pathway resolution and aiding in the interpretation of proteomic changes.

Broader biological coverage for deeper insights

A key finding of the study was the relatively low overlap in identified proteins, highlighting the complementary nature of the two technologies. Proteins uniquely identified by each platform contributed to distinct biological functions: Olink-exclusive proteins were enriched in environmental information processing, including signal transduction and signaling molecule interactions, while ENRICH-iST-unique proteins were more involved in cellular processes, particularly vesicle transport (Fig. 5). The low overlap between the MS- and affinity-based platforms can be explained by Olink's higher sensitivity for low-abundance proteins within its targeted panels, which is an advantage driven by its predefined assays but also inherently limits its ability to identify proteins outside these panels. In contrast, MS, especially when combined with ENRICH-iST enrichment, can detect a broader range of proteins, including many from extracellular vesicles, parts of which are missed by Olink Explore 3072 since they are not covered by its assays. These results emphasize the value of combining both platforms to achieve broader biological coverage and a more comprehensive understanding of the serum proteome.

Furthermore, quantitative trends for shared proteins remained consistent across age comparisons, especially for significantly differentially abundant proteins, highlighting the robustness and reliability of both methods. Integrating datasets provided a more complete view of biological pathways, with each platform offering unique protein-level information. This combined approach deepens proteome analysis, supporting a more detailed understanding of age-related changes in serum protein composition. While this study focused on protein abundance and pathway integration, it is important to note that MS offers additional benefits not explored here, including the distinction of protein isoforms and the ability to detect post-translational modifications.[11]

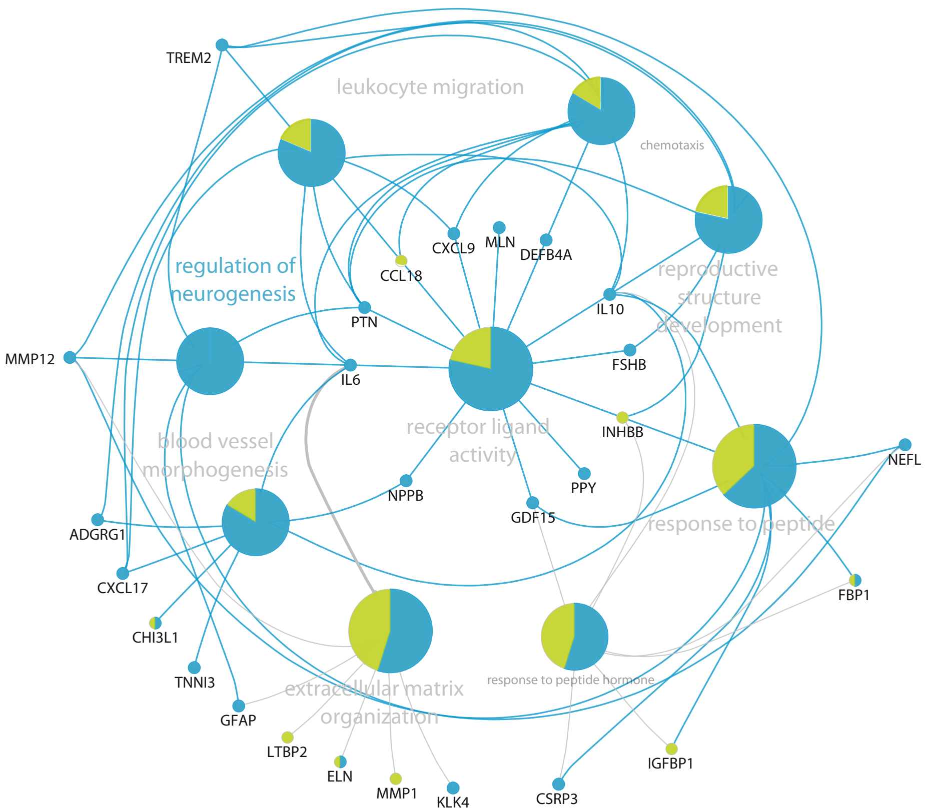

Figure 4 | Pathway integration. A functionally grouped network based on Gene Ontology (GO) terms. Nodes are colored according to data source: blue indicates proteins or pathways identified by Olink, and green shows those identified by ENRICH-iST. The colored segments within each pathway node represent the proportion of proteins contributed by each platform, highlighting their individual and overlapping contributions to the enriched biological processes.

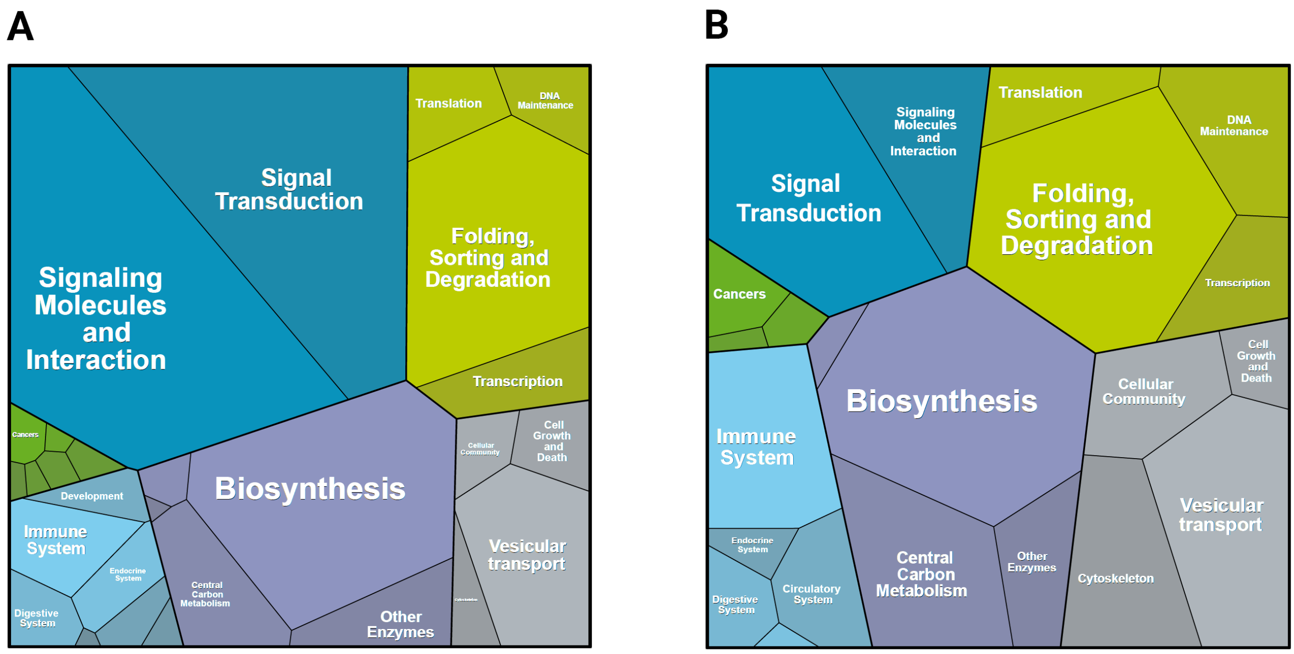

Figure 5 | Protein composition. Proteomaps pathway analysis of (A) proteins identified only by Olink and (B) proteins identified only by ENRICH-iST.

Conclusion

In this study, we assessed the performance and complementarity of the ENRICH-iST sample preparation method for LC–MS/MS in comparison with the Olink Explore 3072 platform, analyzing serum samples from 132 individuals across different age groups. By focusing on protein identification, quantitative correlation, and pathway-level integration, the study offers valuable insights into the strengths of each platform and their combined potential in clinical research.

By combining ENRICH-iST with affinity-based platforms like Olink, researchers can have access to a more comprehensive and detailed proteomic landscape. This is particularly useful for biomarker discovery, pathway analysis, and translational research. This dual-platform approach holds strong potential to advance clinical proteomics and improve our understanding of complex biological processes in health and disease.

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Products

Product	Manufacturer	Product Code
ENRICH-iST 96x	PreOmics GmbH	P.O.00164

Ordering information

http://www.preomics.com/quote
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