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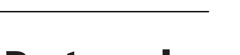

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TECHNICAL BRIEF

Improved breast milk proteome coverage by DIA based LC-MS/MS method

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Abstract

The breast milk composition includes a multitude of bioactive factors such as viable cells, lipids and proteins. Measuring the levels of specific proteins in breast milk plasma can be challenging because of the large dynamic range of protein concentrations and the presence of interfering substances. Therefore, most proteomic studies of breast milk have been able to identify under 1000 proteins. Optimised procedures and the latest separation technologies used in milk proteome research could lead to more precise knowledge of breast milk proteome. This study ($n = 53$) utilizes three different protein quantification methods, including direct DIA, library-based DIA method and a hybrid method combining direct DIA and library-based DIA. On average we identified 2400 proteins by hybrid method. By applying these methods, we quantified body mass index (BMI) associated variation in breast milk proteomes. There were 210 significantly different proteins when comparing the breast milk proteome of obese and overweight mothers. In addition, we analysed a small cohort ($n = 5$, randomly selected from 53 samples) by high field asymmetric waveform ion mobility spectrometry (FAIMS). FAIMS coupled with the Orbitrap Fusion Lumos mass spectrometer, which led to 41.7% higher number of protein identifications compared to Q Exactive HF mass spectrometer.

KEYWORDS
breast milk, colostrum, mass spectrometry, obesity, proteomics

Breast milk is the natural mode of feeding an infant during the first months of life. The composition of breast milk is of vital importance as it provides nutrients for growth and development as well as a multitude of bioactive factors including proteins that contribute to immune function and health even in long-term [1]. Generally, breast milk proteins are grouped into three major classes: caseins, milk serum proteins (whey) and milk fat globule membrane proteins [2] and

Abbreviations: BMI, body mass index; DDT, dithiothreitol,; DIA, data independent acquisition; FAIMS, high-field asymmetric waveform ion mobility spectrometry; HF, high field; IAA, iodoacetamide; GO, gene ontology.

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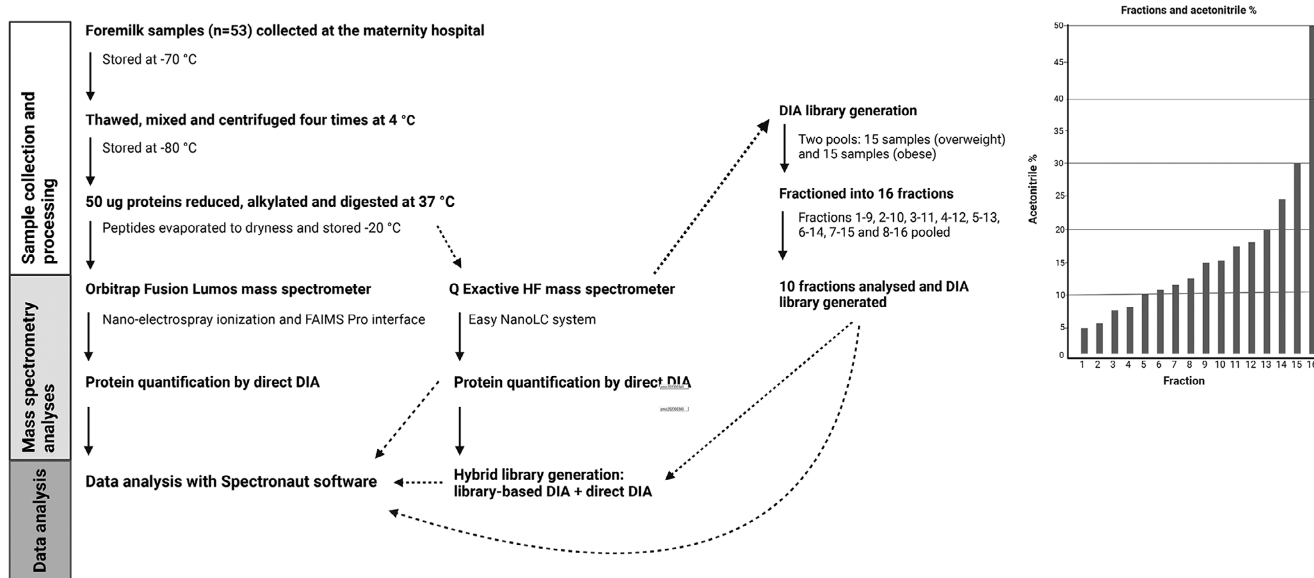

FIGURE 1 An illustration of the study workflow including sample collection, preparation and processing, LC-MS/MS and data analysis. The 16 DIA library fractions are displayed in the bar chart. Created with BioRender.com.

aqueous proteins in milk, the milk serum proteome, exhibit similarity to blood serum proteome. Several water-soluble components of breast milk with antibacterial and immunomodulatory functions have been identified [3, 4]. Interestingly, it has been suggested that the immunological composition of breast milk could be modulated by the metabolic health of the mother [5]. One such factor may be obesity, which has been linked with systematic low-grade inflammation [6, 7] and further maternal obesity with an increased risk of various complications for both foetus and neonate [8]. A recent study found 15 proteins differently expressed in breast milk of obese women as compared to those with normal weight [9].

A challenge in the extraction and purification of milk and colostrum serum samples is the high concentration of colloidal components, casein-micelles and fat globules [10], which need to be removed without affecting the aqueous phase. Thus, the sample handling, extraction of aqueous proteome and sample preparation for mass spectrometry (Figure 1) requires a specific methodology. Although bioactive components in milk have been recognised for decades, the whole proteome study of breast milk is a relatively new research area [11]. Most proteomic studies of breast milk have identified below 1000 proteins [4, 12, 13]. Recently, one study identified 1296 proteins with DIA library-based quantification [14].

The need for an optimised procedure for milk proteome research has been discussed for some time [15] and it has gained more attention recently [16]. Recent advances in separation technologies and data acquisition methods have the potential to provide much more detailed information about the composition of milk proteome. Here we describe methodologies based on high-field asymmetric waveform ion mobility spectrometry (FAIMS) and data independent acquisition (DIA) methods to detect proteins previously unidentified from colostrum samples: The comparison of data analysis methods included library-based DIA, direct DIA, and hybrid library (combining direct DIA+library-based DIA). We applied these methods to study body mass index (BMI) associated changes in milk proteins.

The breast milk samples (n = 53) used in this study were collected from obese or overweight mothers participating in a mother-infant dietary single-centre intervention trial (clinicaltrials.gov, NCT01922791). The clinical study design has been described previously in detail [17]. The summary of clinical details of the participants has been described in detail [18], Here, the cohort studied is the group receiving a placebo. The Ethics Committee of the Hospital District of Southwest Finland (115/180/2012) has approved the study protocol (decision number T166/2012, 17 December 2012) and the study meets the guidelines of the Declaration of Helsinki 2013 [19]. All participants provided written informed consent for the study and they were recruited in maternal welfare clinics between October 2013 and July 2017.

The foremilk colostrum samples were collected at the maternity hospital by manual expression after lactation had commenced (3.3 ± 1.2 days postpartum). The milk proteins were extracted from the samples using a uniform process (Figure 1): The samples were thawed, mixed and centrifuged four times at 4°C ($2000\,g \times 20$ min $+ 3 \times 10{,}000\,g$ $\times 10$ min) in order to remove fat. To carry out proteome analyses, 50 µg milk proteins were reduced with dithiothreitol (DDT), alkylated with iodoacetamide (IAA) and digested by trypsin o/n at 37°C. Peptides were desalted by Sep-Pak C18 96-well plate. Elution was performed with 0.1% HCOOH/60% acetonitrile. Peptides were evaporated to dryness and kept at −20°C before MS analysis. Digested peptide samples were dissolved in 0.1% formic acid and peptide concentration was determined with NanoDrop (Thermo Fisher Scientific).

LC-MS/MS analyses were performed by Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to Easy NanoLC HPLC system (Thermo Fisher Scientific). All analyses were performed as one uninterrupted series of measurements. The

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DIA method was used for protein quantitation. Samples were spiked with indexed retention time (iRT) peptides in a ratio 1:20 (v/v). A total of 600 ng peptides from each sample were analysed in a randomised order. Wash runs were submitted between each sample to reduce potential carry-over of peptides. Peptides were loaded on 2 cm C18 precolumn and separated by 15 cm analytical column (75 µm × 15 cm, ReproSil-Pur 3 µm 120 Å C18-AQ, Dr Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The mobile phase consisted of water with 0.1% formic acid (solvent A) or acetonitrile/water (80:20 (v/v)) with 0.1% formic acid (solvent B). A 120 min gradient from 7% to 36% solvent B was used to elute peptides. For spectral library generation, peptides from 15 BMI type 1 (overweight) samples and 15 BMI type 2 (obese) samples were pooled together thus forming two pools. These both pooled samples were then fractioned by Thermo's High pH Reversed Phase Peptide Fractionation Kit into 16 fractions by elution with increasing acetonitrile concentrations 5%–50%. Fractions were evaporated to dryness, dissolved in 0.1% TFA (1 mL) and desalted by C18 Sep-Pak 96-well plate. Fractions 1–9, 2–10, 3–11, 4–12, 5–13, 6–14, 7–15 and 8–16 were pooled before LC-MS/MS analysis. Fractions 7 and 9 were analysed without pooling also. Taken together 10 samples were used for DIA library generation. One microgram of peptides was injected and analysed by DIA method.

For the analyses including FAIMS separation, equal amounts of samples were analysed using the same liquid chromatography setup coupled to the Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) equipped with a FAIMS Pro interface. A slightly modified 120 min gradient was used to elute peptides (62 min from 5% to 21% solvent B and 48 min from 21% to 36% solvent B followed by the wash stage with 100% Solvent B).

MS data was acquired automatically by using Thermo Xcalibur 4.4 software (Thermo Fisher Scientific). Instrument performance was monitored by repeated analyses of Pierce™ HeLa Protein Digest Standard (ThermoFisher Scientific). In a DIA method, a duty cycle contained one full scan (400–1000 m/z) and 40 DIA MS/MS scans covering the mass range 400–1000 with an isolation width 15 m/z. In a DIA method, FAIMS compensation voltages –50 V and –70 V were used and a duty cycle contained one full scan (resolution 120,000, AGC target 7E5, maximum injection time 50 ms, 400–1000 m/z) and 30 DIA MS/MS scans (resolution 30,000, AGC target 1e6, maximum injection time 52 ms) covering the mass range 400–1000 with variable width isolation windows.

Data was analysed by Spectronaut software (Biognosys; version 16.0.220606.53000). Direct DIA approach was used to identify proteins and label-free quantifications were performed with MaxLFQ. Main data analysis parameters in Spectronaut were: (i) Enzyme: Trypsin/P; (ii) Missed Cleavages: 2; (iii) Fixed modifications: Carbamidomethyl (cysteine); (iv) Variable modifications: Acetyl (protein N-terminus) and oxidation (methionine); (v) Protein database: Homo Sapiens Swiss-Prot reference proteome (Uniprot release 2021_04); and (vi) Normalization: Local normalization. We applied a false discovery rate cutoff of less than 0.01 for protein identifications. On average, protein groups exhibited 8.6 peptides, with 41 proteins being detected in a one sample. Differential abundance analysis was performed using in-built functionality of Spectronaut software with unpaired Student's *t*-test on log2 transformed protein quantity values and multiple-testing correction of the *p*-values with Benjamini–Hochberg method. Statistical analysis was performed using SPSS software (version 27). The functional enrichment analysis was performed using g:Profiler (version e107_eg54_p17_bf42210) with g:SCS multiple testing correction method applying significance threshold of 0.05 [20]. Figure 2A was created using GraphPad Prism version 8.0 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com). Overrepresentation analysis of the downregulated proteins associated with gene ontology (GO) biological pathway terms was performed using R Statistical Software (4.3.1; R Core Team, enrichGO function of the clusterProfiler R package [21, 22]). The gene ontology annotation was made for proteins that were significantly different between overweight and obese mothers. The entire list of expressed proteins was included to serve as a background list. No upregulated pathways have been statistically significantly enriched.

In the comparison of different data analysis methods ($n = 53$), the results of the protein and peptide identifications from the hybrid library (library-based DIA+direct DIA), direct DIA, and library-based DIA are summarised in Figure 2A. The average number of identified for the hybrid was 2400 ± SD 221 proteins and 19,760 ± SD 2710 peptides, for direct DIA 1850 ± SD 72 proteins and 15,440 ± SD 1070 peptides, for library-based DIA 1990 ± SD 140 proteins and 15,960 ± SD 1430 peptides. The list of identified proteins are presented in Supplement 1. We found that all samples included epithelial proteins of both palmar and simple epithelia. However, their relative abundance was low compared to secreted milk proteins (Supplement 2) suggesting that there were no major contaminations and the proportion of exfoliated mammary gland epithelial cells was relatively low as well [23, 24]. From a biological and nutritional perspective, the origin of breast milk proteins—whether derived from milk or skin—may not significantly impact the overall nutritional value. Notwithstanding, it is crucial to acknowledge that skin and other forms of contamination are inevitable during the manual expression of milk into tubes.

As shown in Figure 2A, the library generation delivered a modest improvement compared to direct DIA, and it needs to be weighted against the additional cost and labour. There were no specific highlighted pathways when compared to direct DIA to library-based DIA-expressed proteins in enrichment analysis. The enrichment was calculated against the background of proteins identified in this study. In addition, as shown in Figure 2C, the individual variation is greater than the variation between mean values of mothers who had overweight or obesity. Almost all proteins which differed significantly between mothers with obesity and overweight, were downregulated (Figure 2B). Proteins, for which expression varies significantly between research groups (log2 fold change $\leq$–0.75 or $\geq$0.75 with $p < 0.05$, red and blue dots in Figure 3A) consist 7% of total LC-MS/MS hybrid library identifications.

Individual variation is greater than the variation between mean values of mothers who had overweight or obesity, as shown in Figure 2B. Interestingly, the majority of proteins that differed significantly between mothers with obesity and overweight, were downreg-

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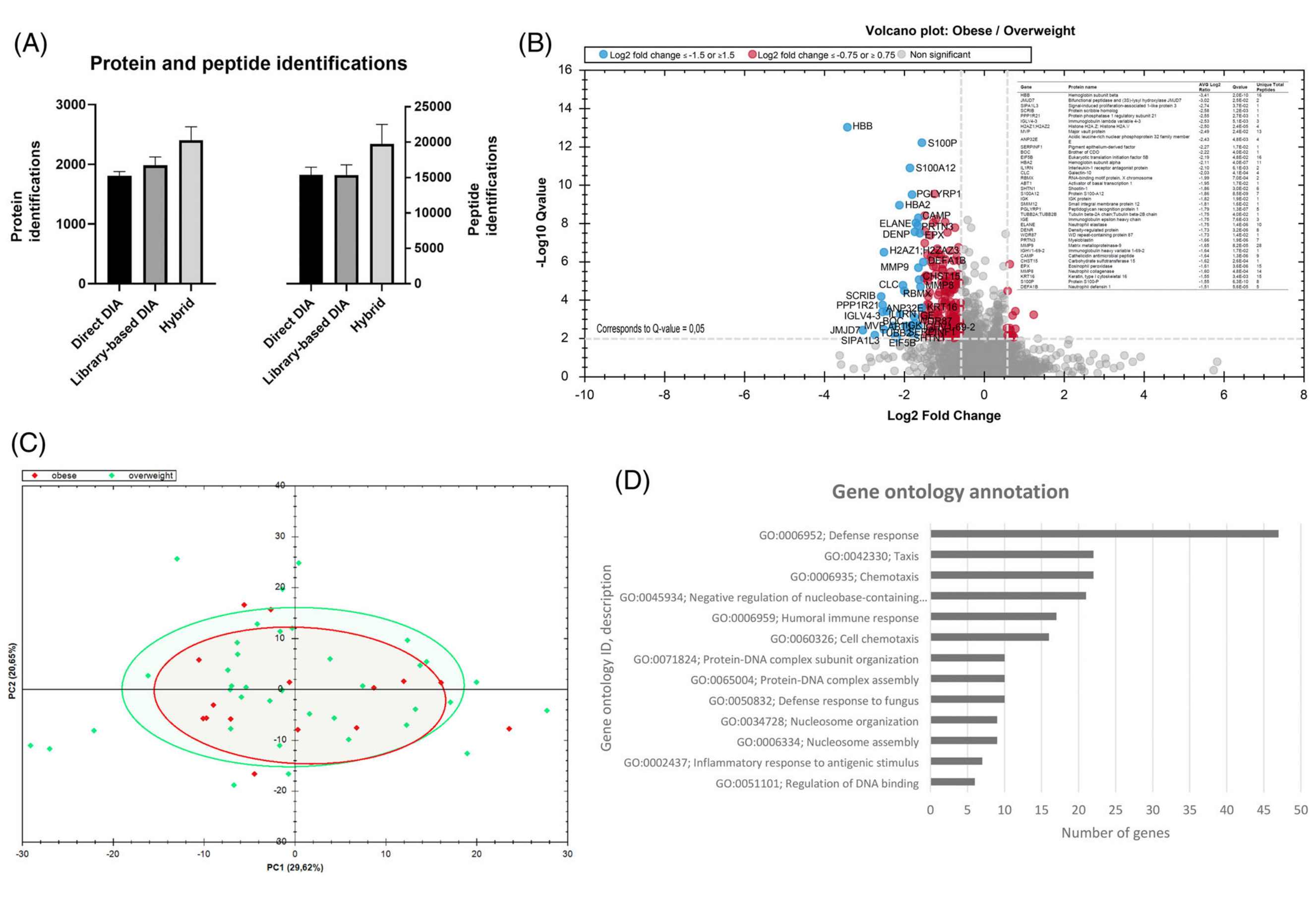

FIGURE 2 Hydrid library data analysis results the most peptide identifications. A) Average of protein and peptide identifications (semi-quantified) by direct DIA, library-based DIA and hybrid library data analysis methods (n = 53). The bar chart includes standard deviations. B) A volcano plot comparing differently expressed breast milk proteins from mothers with obesity or overweight using Spectronaut software. Protein identifications obtained by LC-MS/MS with hybrid library data analysis. Significantly differently (Q-value ≤ 0.05) expressed proteins (log2 fold change ≤–0.75 or ≥0.75 with $p < 0.05$) between groups are represented by red dots and most different (log2 fold change ≤–1.5 or ≥1.5 with $p < 0.05$) are presented by blue dots, name labels and listed in the table. C) PCA of participants proteome comparing mothers with overweight and obesity using Spectronaut software. One diamond represents one breast milk sample, whereas the red diamonds are samples from mothers with obesity and green are samples from mothers with overweight. D) Bar plot of the 10 most abundant biological pathways of the downregulated proteins in obese mothers (adjusted p-value < 0.05). There were no statistically significant upregulated pathways.

ulated (Figure 2C). Proteins, for which expression varies significantly between research groups (log2 fold change ≤–0.75 or ≥0.75 with $P < 0.05$, red and blue dots in Figure 2C) consist 8.8% of total LC-MS/MS hybrid identifications. These relevant proteins are listed in Supplement 3. According to enrichment analysis, 32 of these proteins may contribute to neutrophilic activity (REAC:R-HSA-6798695). This finding warrants future studies focusing on the neutrophilic activity in breast milk. In gene ontology annotation (Figure 2D) between mothers with overweight and obesity the defence response was most abundant.

In order to compare the difference between FAIMS and conventional LC-MS/MS mass spectrometry methods five samples were analysed with both methods. These five samples were selected randomly from the original 53 samples. As shown in Figure 3, the FAIMS method led to 41.7% higher number of protein identifications. It should be noted that the increase in identifications could be partly attributable to the difference between Q Exactive HF and Lumos, which has the high-capacity ion transfer tube. However, the addition of FAIMS most likely explains the majority of the increase in the number of protein identifications compared to the conventional method, and the fractional increase in the number of protein identifications was larger than what is typically observed with tissue or whole-cell samples [25]. It is prudent to recognize that additional identifications may be attainable through alternative experimental parameters or methodologies. However, it is noteworthy that our approach was designed with considerations for both time and financial constraints. This approach not only optimised resource utilisation but also holds relevance for researchers planning proteomic analyses of breast milk. A limitation of this study is that we analysed only five samples using a combination of hybrid DIA method with FAIMS. Analysing a larger sample panel using the combination would likely result in an even higher number of identifications. This is an important issue for future research.

In conclusion, breast milk proteome analysis benefits greatly from the addition of ion mobility separation and modern AI-augmented DIA data analysis. Our comparison of different protein quantification methods suggests that the hybrid (direct DIA+ library-based DIA) led to the highest number of protein and peptide identifications. Based on our

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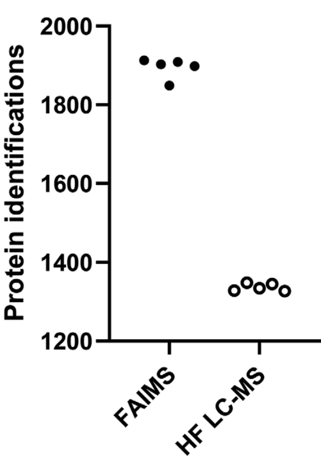

FIGURE 3 Protein identifications by DIA method comparing FAIMS coupled to the Orbitrap Fusion Lumos mass spectrometer (Mean 1890 ± SD 26) and Q Exactive HF mass spectrometer (Mean 1340 ± SD 9.7) (n = 5, randomly selected from n = 53).

comparison of breast milk proteome from obese and overweight mothers, maternal BMI is associated with differences in the proteome but is also subjected to significant individual variation.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available through the University of Turku systems in the context where access to data would not be considered a transfer, the use of data is approved by the researcher as well as the signatory of the University of Turku and by signing a co-operation agreement.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202300340 in the Supporting Information section at the end of the article.

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