Proteomic Analysis of Plasma – Sample Preparation and Multiplexing Workflows for Relative Quantitation

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ABSTRACT

Purpose: Depletion of abundant proteins is required to identify and measure changes in prognostic or diagnostic plasma proteins. We have implemented the use of top14 abundant protein depletion resin, efficient sample preparation using Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit, labeling peptide-level samples with Tandem Mass Tag™ (TMT™) reagents, and high pH reversed-phase fractionation to profile a large set of plasma samples from normal controls and patients with pulmonary hypertension heart failure with preserved ejection fraction (PH-HFpEF) to assess differences in plasma protein abundances.

Methods: Plasma samples were subjected to top14 abundant protein depletion, followed by a quick sample preparation using the EasyPep Mini sample preparation kit. Samples were then grouped into five 11-plex sets and labeled with TMT-reagents. Following the labeling, each grouped set was fractionated by high pH reversed-phase into eight fractions. Fractions were analyzed by LC-MS on Thermo Scientific™ Orbitrap Fusion™ instrument using synchronous precursor selection mode (SPS) for accurate, interference-free quantitative comparison on a 75 cm C_{18} column in a 3 hour gradient. Raw files were processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software.

Results: We have designed a study in which thirty-two plasma samples from healthy donors and individuals diagnosed with pulmonary hypertension heart failure with preserved ejection fraction (PH HFpEF) were prepared and analyzed using the above workflow in five 11-plexed sets, each set consisting of pooled controls and bridging channels. Protein abundances were compared across all individuals as well as between the two pooled cohorts to identify potential biomarker candidates and gain insight into the mechanism of the development and progression of the PH-HFpEF. Overall, 1043 protein groups and 8580 peptide groups were identified in this experiment. Several proteins were shown to have differential abundance in the PH-HFpEF patients samples relative to the normal controls.

INTRODUCTION

The large dynamic range in protein abundance of plasma samples is the main problem associated with plasma/serum-based biomarker discovery experiments, and depletion of abundant proteins is required in order to identify and measure changes in prognostic or diagnostic plasma proteins. Coupled to a simple and efficient protein sample preparation workflow using TMT-reagents for sample multiplexing, it is possible to attain high quality, accurate and reproducible comparative proteomic data in a streamlined and cost-effective manner. We have developed a workflow which combines the above features and assessed it for reproducibility of depletion, protein and peptide yields, number of protein identifications, and quantitative accuracy.

Pulmonary hypertension in the setting of heart failure with preserved ejection fraction (PH-HFpEF) is a growing public health problem that is increasing in prevalence (Figure 1). While PH-HFpEF is defined by a high mean pulmonary artery pressure, high left ventricular end-diastolic pressure and a normal ejection fraction, some HFpEF patients develop PH in the presence of pulmonary vascular remodeling with a high transpulmonary pressure gradient or pulmonary vascular resistance. Ageing, increased left atrial pressure and stiffness, mitral regurgitation, as well as features of metabolic syndrome, which include obesity, diabetes and hypertension, are recognized as risk factors for PH-HFpEF. Qualitative studies have documented that patients with PH-HFpEF develop more severe symptoms than those with HFpEF and are associated with more significant exercise intolerance, frequent hospitalizations, right heart failure and reduced survival. Currently, there are no effective therapies for PH-HFpEF and timely diagnosing of the disease remains a challenge.

We set out to apply our plasma proteomic workflow to a group of plasma samples of patients with PH-HFpEF together with normal control plasma samples for comparative profiling with the aim to find disease-specific biomarkers.

MATERIALS AND METHODS

All plasma samples were obtained from ClinicalTrials.gov (NCT01431313). 10µL of each plasma sample were individually depleted using Thermo Scientific™ High Select™ Top14 Abundant Protein Depletion columns and depleted samples were processed using Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit. Pooled plasma samples: normal pooled, PH-HFpEF pooled, and combined pooled were prepared by combining equal volumes of the group- appropriate plasma samples, and 10µL of each were processed as described above. For the multiplexed sets, bridging samples were constructed by combining equal amounts of the individual samples. After labeling with the TMT-reagents (Thermo Scientific™ TMT10plex™ Isobaric Mass Tag Labeling Reagent Set plus TMT11-131C; product #A34808), the labeled samples were combined to create five sample sets as shown in Figure 3. All samples were analyzed on Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer and processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software. TMT-reagent labeled samples were analyzed using synchronous precursor selection mode (SPS).

Figure 1. Graphical definition of pulmonary hypertension heart failure with preserved ejection fraction (PH-HFpEF).

PH-HFpEF = PAH + elevated pressure in the pulmonary veins coming from left-sided heart diastolic dysfunction

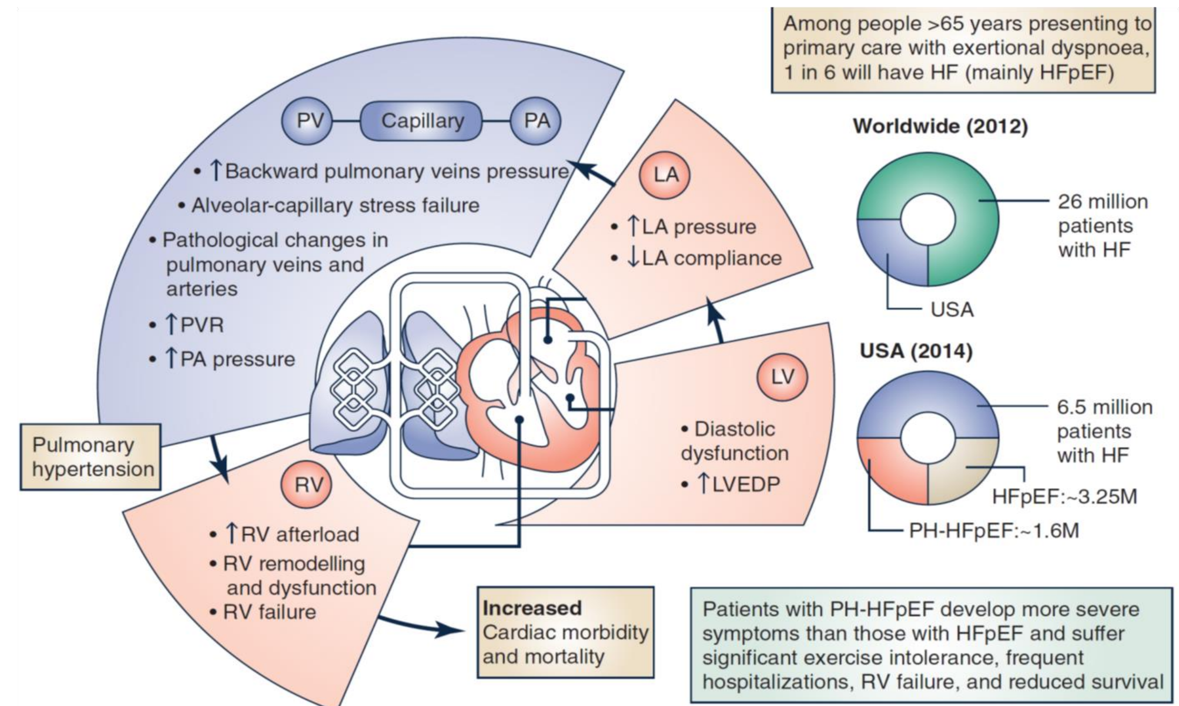


Figure 2. EasyPep sample preparation workflow compared to the traditional method.

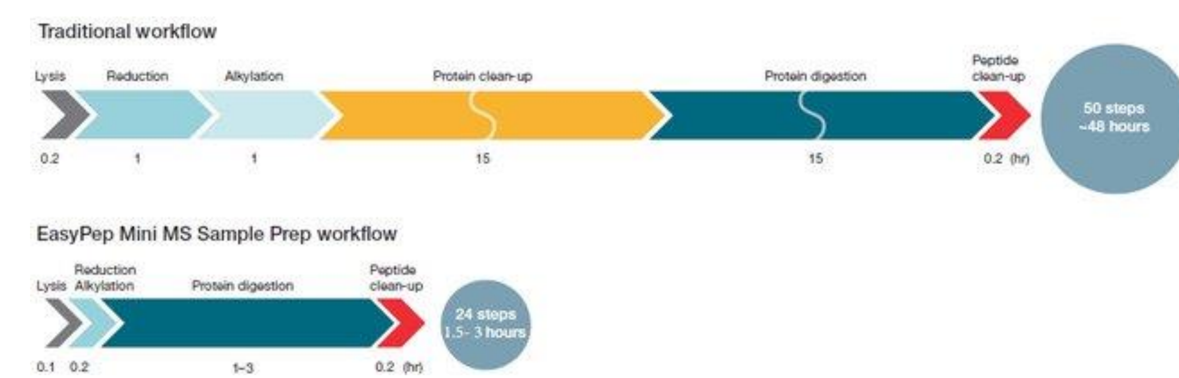
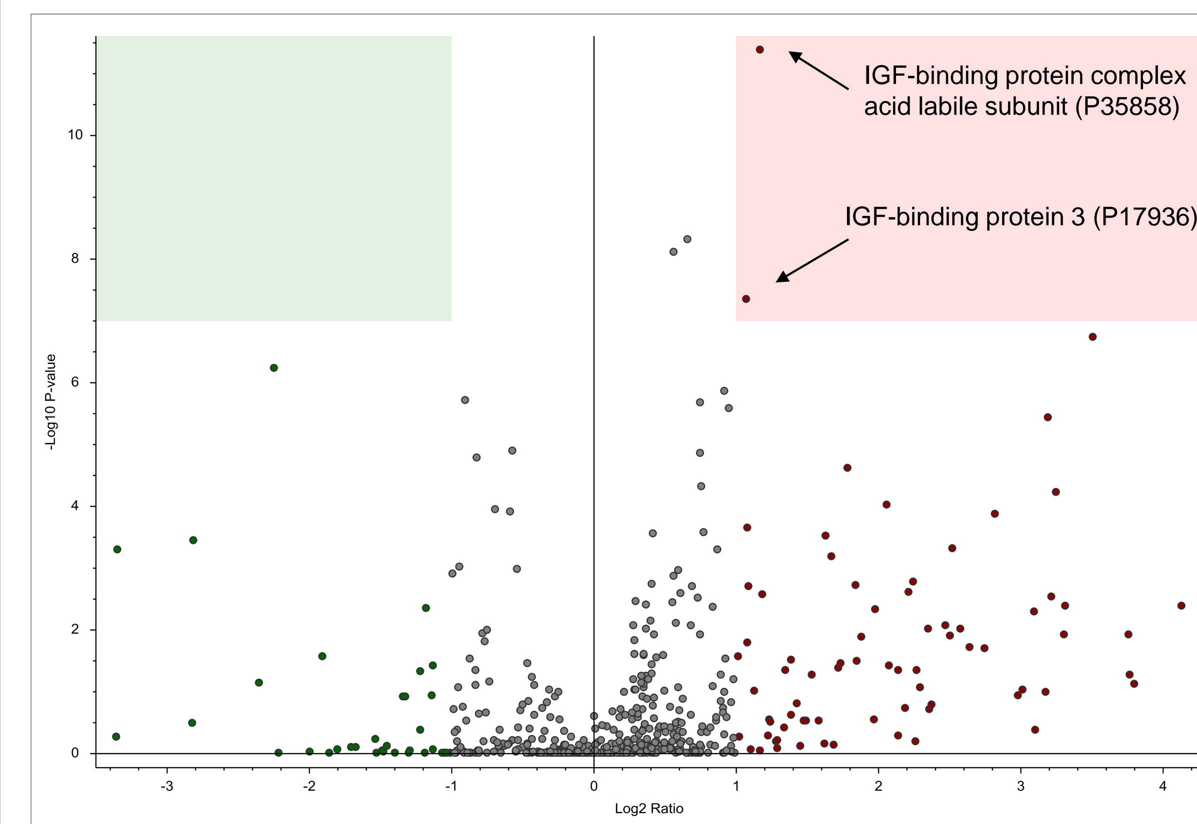


Table 1. Experimental design sample map.

11-plex sample sets	reporter ion channels										
	126	127N	127C	128N	128C	129N	129C	130N	130C	131N	131C
Sample Set 1	normal_1	PH-HFpEF_1	normal_2	PH-HFpEF_2	normal_3	PH-HFpEF_3	normal_4	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 2	PH-HFpEF_4	normal_5	PH-HFpEF_5	normal_6	PH-HFpEF_6	normal_7	PH-HFpEF_7	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 3	normal_8	PH-HFpEF_8	normal_9	PH-HFpEF_9	normal_10	PH-HFpEF_10	normal_11	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 4	PH-HFpEF_11	normal_12	PH-HFpEF_12	normal_13	PH-HFpEF_13	normal_14	PH-HFpEF_14	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 5	normal_15	PH-HFpEF_15	mixed_pooled	normal_16	PH-HFpEF_16	mixed_pooled	normal_17	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge

Figure 3. Volcano plot comparing differences in protein abundance between the normal controls and the PH-HFpEF patients cohorts.



RESULTS

In our previous work, we optimized the conjugation chemistries and blending protocols of immobilized antibody resins to achieve >95% depletion efficiency of target high abundance proteins in human plasma samples. Post-depletion, we have evaluated several sample preparation workflows including those with protein-level desalting by solvent precipitation and peptide-level only desalting for ease of use, reproducibility, and yield. We have also assessed compatibility of the newly developed and commercialized protein sample preparation kits, which were designed to minimize the sample handling steps and processing times, with the post-depletion plasma samples, TMT-reagent labeling and high pH reversed-phase peptide sample fractionation. All solution and reagent concentrations were optimized for processing of 10µL plasma samples, and the clean-up procedure of the workflow enables high yields of recovered peptides free of undesired salts, detergents, and excess TMT-reagents.

Using this workflow, we designed a study in which thirty-two plasma samples from healthy donors and individuals diagnosed with pulmonary hypertension heart failure with preserved ejection fraction (PH-HFpEF) were prepared and analyzed using the above workflow in five 11-plexed sets, each set consisting of pooled controls and bridging channels. Each set was further fractionated into eight high pH reversed-phase fractions. Protein abundances were compared across all individuals as well as between the two pooled cohorts to identify potential biomarker candidates and to gain insight into the mechanism of the development and progression of the PH-HFpEF. The use of the EasyPep sample preparation workflow afforded easy, convenient and quick sample preparation of the plasma samples.

Overall, 1043 protein groups and 8580 peptide groups were identified in this experiment, with digestion efficiency of >90% as measured by the percentage of total peptides identified with 0 missed cleavage sites. Analysis of the searched data revealed the presence of several proteins exhibiting a significant difference in abundance in the normal controls and the PH-HFpEF cohorts (Figure 3). IGF-binding proteins showed the most significant difference, with the abundance changes of IGF-binding protein complex acid labile subunit and the IGF-binding protein 3 having the largest fold change (Figure 4). These were also shown to be lower in the PH-HFpEF cohort. IGF-II was also detected at lower abundance levels, consistent with the relative abundance level of its binding proteins.

Interestingly, even after depletion, we were able to see the differences in the albumin abundance between the two cohorts (Figure 5A) Albumin levels were significantly lower in the PH-HFpEF patient samples, suggesting possible loss of albumin in urine due to kidney dysfunction, which is uncommon for patients with a metabolic syndrome. Another protein, Von Willebrand factor. was detected in the higher abundance in the PH-HFpEF patients which has been reported to be higher than normal levels in plasma of patients with cardiovascular diseases. In contrast, the relative abundance levels of complement C-4B and complement C-4B binding protein do not appear to change between the two cohorts.

Figure 4. Abundances of IGF-related proteins identified in the plasma samples.

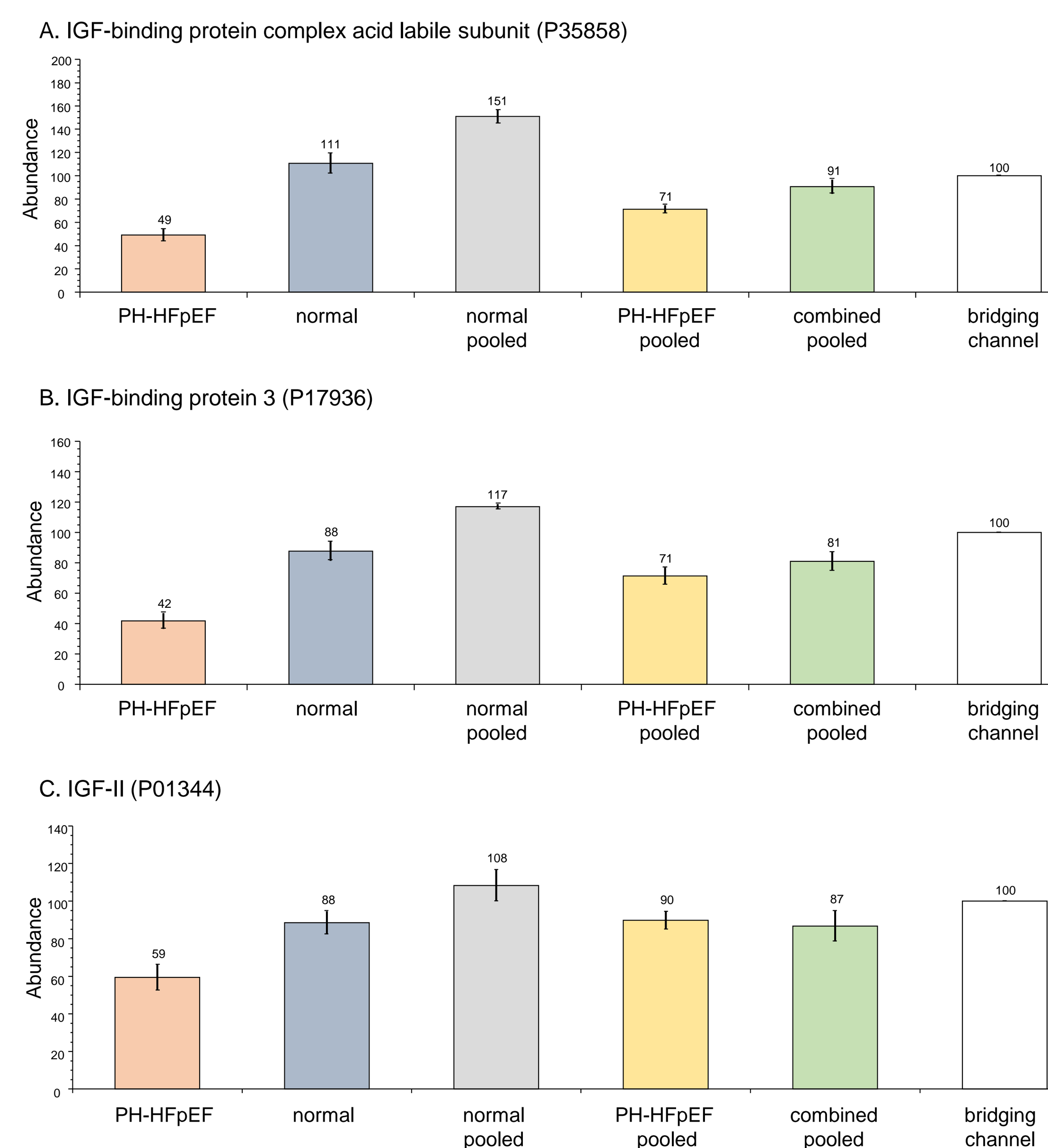
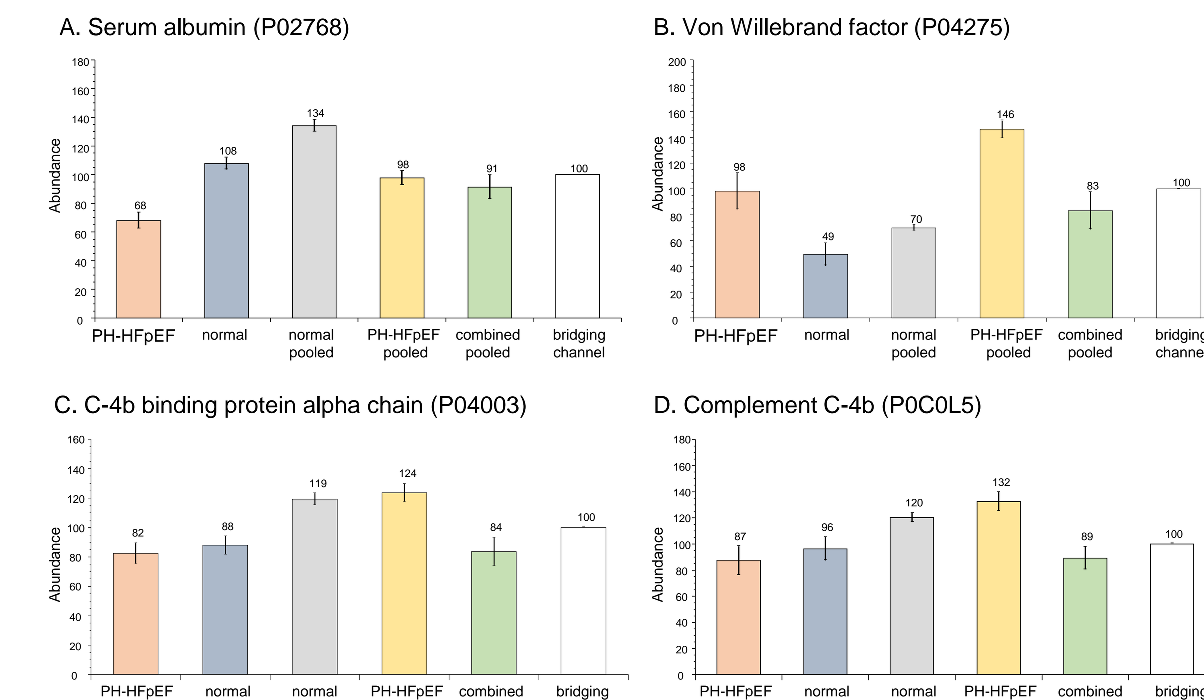


Figure 5. Abundances of selected plasma proteins found in the samples.



CONCLUSIONS

- Reproducible depletion of abundant proteins is obtained by using Pierce Top14 abundant protein depletion spin columns.
- Abundant protein depletion from plasma allows for detection of more proteins in the sample enabling better detection and quantitation of relevant biomarkers.
- While reproducible depletion of the abundant proteins can be attained, MS-based label-free quantitation ultimately depends on the quality of sample preparation after the depletion, in terms of reproducibility of reduction/alkylation, digestion efficiency and peptide recovery.
- Abundant plasma protein depletion can be used effectively for quantitative comparison proteomics studies of plasma samples.

REFERECES

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2. Lai YC; Wang L; Gladwin MT; Insights into the pulmonary vascular complications of heart failure with preserved ejection fraction; *J Physiol.;* **2018**; 597(4)

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