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RESEARCH ARTICLE

Blood plasma high abundant protein depletion unintentionally carries over 100 proteins

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There is a constant interest in blood-based protein biomarkers, which can help to improve diagnosis and treatment outcomes of multifactorial human pathologies. In this regard, proteomic studies usually employ plasma immunoaffinity fractionation to deplete the most abundant plasma proteins, due to the high dynamic concentration range of proteins. The depletion of high abundant proteins allows to obtain less abundant and, oftentimes, more interesting proteins. However, the removal of the fraction of the high abundant plasma proteins - the depletome - may co-elute many unintended proteins due to protein-protein interactions. Little data is available about the depletome and potential protein biomarkers may be lost during this process. To visualize and characterize these proteins, we analyzed the depletome of 20 plasma samples by shotgun mass spectrometry-based proteomics. Thus, using immunoaffinity depletion followed by 2-D liquid chromatography coupled to an ion mobility-enhanced mass spectrometer, our analysis identified that over 100 proteins are co-eluting with the high abundant fraction. These proteins play roles in several biological processes, such as receptor-mediated endocytosis, complement activation, and regulation of immune response. This study supports that investigating the depletome is important in the quest for biomarkers.

KEYWORDS
Depletome, high-definition mass spectrometry, Plasma proteome

1 | INTRODUCTION

Proteomics are a set of biochemical tools which can elucidate the role of proteins in the molecular complexity of multifactorial pathologies, including, for example, some types of cancer, psychiatric, neurodegenerative and respiratory diseases [1–4].These disorders frequently overlap symptoms with other diseases, challenging physicians to find precise diagnostic or adequate treatment. On this matter, biomarkers - a measurable characteristic from an organism's current state [5,6] - are a current focus to improve outcomes and personalize treatments of diseases of multifactorial etiology.

The discovery of novel protein biomarkers can be enhanced by investigation of blood plasma and/or serum, which is a promising human supply of proteins [7]. However, the

List of abbreviations: 2-D RP/RP, two dimensional reversed phase; DIA, data independent acquisition; $HDMS^E$, high definition MS^E; HSS, high strength silica; IMS, ion mobility separation; MS^E, tandem mass spectrometry alternating low and high collision energy.

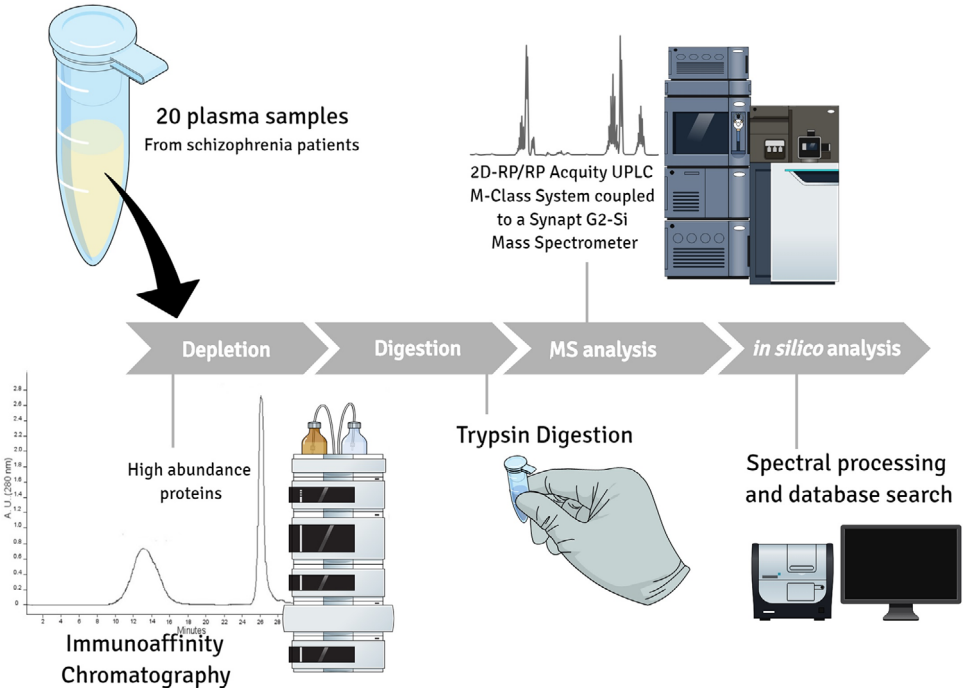

FIGURE 1 Shotgun workflow. High-abundance proteins bound to the column at depletion step and later eluted. Then, protein samples were digested to peptides with trypsin. The resulting peptides were injected in a UHPLC system coupled online to a Synapt G2-Si mass spectrometer with electrospray ionization. Afterwards, proteins were identified and quantified using Progenesis QI against Swiss-Prot Human Proteomic Database

complexity of blood-based protein samples is a challenge because human blood plasma has a high dynamic concentration range of proteins, stretching over 12 orders of magnitude [8,9]. One way to reduce the complexity of blood plasma proteins is by fractionation, discarding the high abundance-fraction after separation by immunoaffinity chromatography. The term "depletome" emerged from the need to investigate the discarded fraction, composed of up to 20 proteins and their isoforms [10,11]. Although depletion technique allows for the identification of many more proteins with low abundance and thusly more potential biomarkers, it can also remove other potential biomarkers due to protein-protein interactions, causing a type of undesired co-immunoprecipitation. Thus, the investigation of depletome requires high sensitivity techniques, such as high-resolution mass spectrometry, that can identify interacting proteins, despite the broad range of concentrations.

Mass spectrometry-based proteomics has potential in dealing with complex protein samples. Furthermore, MS can generate spectra of data from the peptide fragments even with low-abundance proteins [12]. An alternative approach called MS^E employs the alternation of collision energy within the fragmentation cell to promote varied molecular fragmentation of ionized peptides [13]. Ion mobility separation (IMS) adds another dimension in the separation of the ions, reducing interference and improving the detection capacity of the peaks, making this tool especially suitable for complex samples [14,15]. The association of IMS with MS^E is called $HDMS^E$ and increases the detection of precursor ions from complex samples and confidence in the identification of peptides [14].

This study aims to increase the knowledge base about the blood plasma depletome, composed by proteins which interact with high abundant fraction. We identified co-eluted proteins of depletome fraction using $HDMS^E$ shotgun mass spectrometry, and after initial identification, we performed *in silico* analysis to characterize which biological processes and molecular functions are involved in that fraction (Figure 1). Thus, we confirm the relevance of the depletome investigation when the exploration by biomarkers is performed.

2 | MATERIALS AND METHODS

The cohort for this investigation consisted of 20 human plasma samples. Blood samples were collected at the psychiatric clinic of the University of Magdeburg, Germany as previously described [16], and all participants provided written informed consent. This collection has been approved by the ethics committee of the University of Magdeburg, in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Shortly after collection, the blood samples were centrifuged for 10 min at 2000g and the resulting plasma fraction was immediately frozen and stored at -80°C.

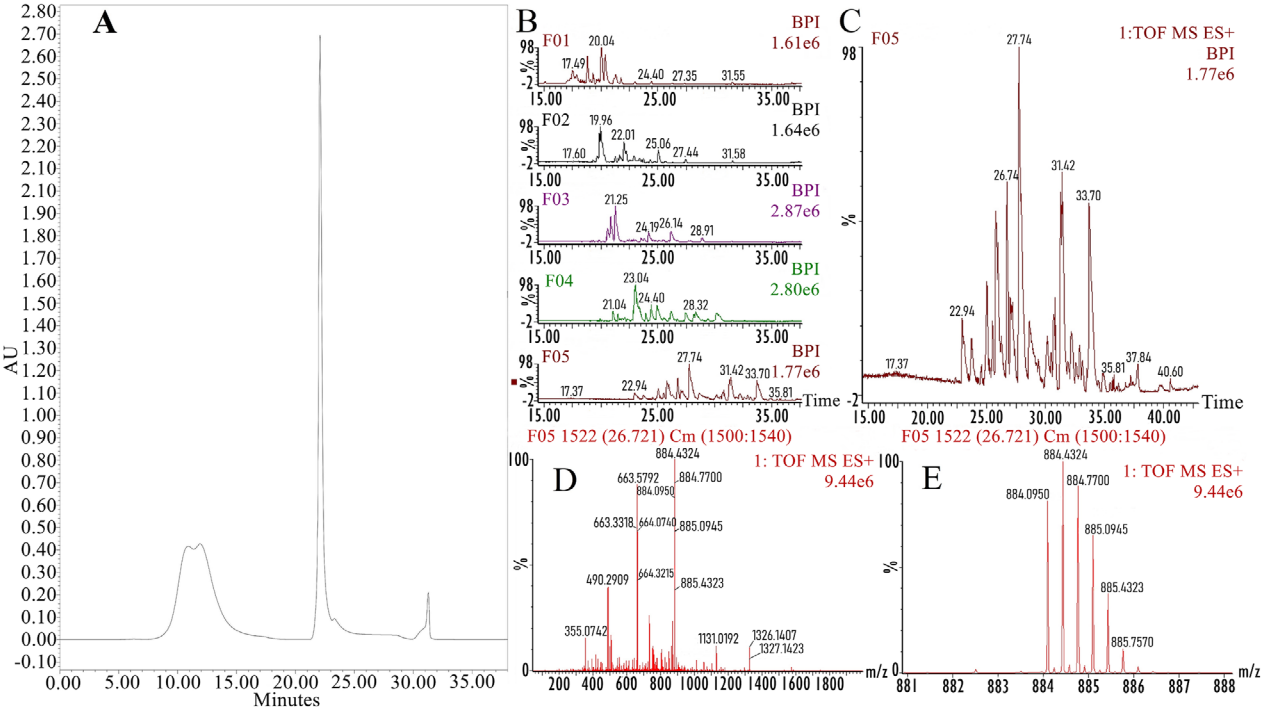

FIGURE 2 (A) Representative chromatogram of one depletion run. The first peak represents the low abundance proteins and the second peak represents the high abundance proteins or the depletome. (B) Five representative chromatograms of 2-D LC fractionation before MSE analysis. (C) Zoom in on the last chromatogram of figure 2B, related to fraction 5 from that run. (D) Representative spectrum corresponding to data acquisition at the peak at 26.74 of fraction 5. (E) Zoom in on spectrum shown in D

In the depletion step, 30μL of plasma was diluted in 90 μL phosphate buffer (pH 7.4) prepared with 2.5% (v/v) phosphate buffer solution 1M (Sigma-Aldrich), 10.0% (v/v) NaCl 5M and 0.02% (m/v) sodium azide in water. The phosphate buffer was also used to carry the sample into the MARS Hu14 Immunodepletion System column (Agilent Technologies), according the manufacturer's protocol. Then, urea acidic buffer - 2.0M urea, 0.5M glycine in water, pH 2.25, adjusted with HCl - was used to elute the high abundance proteins bound to the column, releasing the depletome and thus allowing the loading of the next sample. A representative chromatogram of depletion process is represented in Figure 2A. Depletome buffer was exchanged with 50 mM ammonium bicarbonate using Vivaspin 6 (Sartorius) cartridges. After buffer exchange, proteins were reduced with dithiothreitol (100 mM, 60°C, 30 min) and alkylated with iodoacetamide (300 mM, 30 min, room temperature, in the dark). Then, we proceeded with protein samples digestion into peptides with trypsin (Promega) at a ratio of 1:100 (w/w trypsin/protein) for 16 h at 37°C. Digestion was quenched with 5% trifluoroacetic acid for 15 minutes at room temperature. The samples were centrifuged at 20,817g at 6°C for 30 min. The supernatant was recovered and then pH was adjusted using 0.5 μL of 1 N ammonium hydroxide prior to analysis.

We randomized the samples before mass spectrometry analysis. The peptides were subjected to 2-D UHPLC HDMS^E analyses by injection into a 2-D RP/RP Acquity UPLC M-Class System (Waters) coupled online to a Synapt G2-Si Mass Spectrometer (Waters). Discontinuous steps of ACN (11, 14, 17, 20 and 50%) were used to perform the first-dimension chromatography using an ACQUITY UPLC M-Class Peptide BEH C18 Trap Column (Waters). The second-dimension separation column (ACQUITY UPLC M-Class HSS T3, Waters) was set to acetonitrile gradient from 7 to 85% (v/v) for 36 min at a flow rate of 0.4 μL/min directly into the Synapt G2-Si HDMS. The mass spectrometer was operated in resolution mode with an m/z ratio resolving power of 40,000 FWHM, using ion mobility separation with cross-sectional resolving power of 40 Ω/ΔΩ and data independent acquisition method (DIA). Fragmentation spectra were obtained by MS/MS analysis, performed with a NanoLock Spray (Waters) ionization source in positive ion mode [17]. Representative chromatograms of five fractions related to five steps of acetonitrile and the corresponding spectrum of last fraction is represented in Figures 2B,C,D and 2E.

Afterwards, spectra were processed and proteins were identified and quantified with Progenesis QI for Proteomics® (Nonlinear Dynamics; Waters Corporation; version 4.0) employing Apex3D (Waters) for peak detection and searching against the Swiss-Prot Human Proteomic Database. Quantitation was performed using Hi-N (Hi3). To obtain the preliminary dataset of proteins, the following parameters

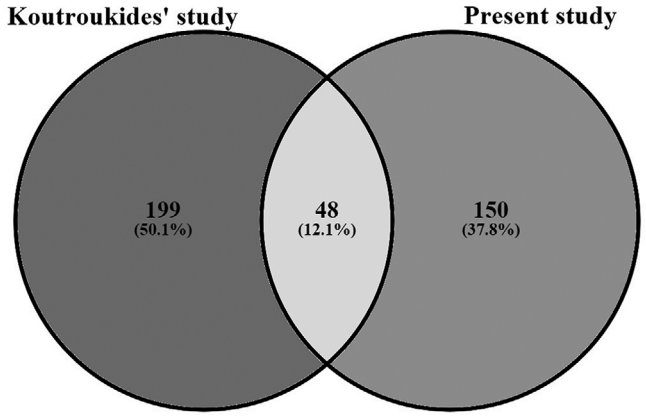

FIGURE 3 Venn diagram comparing the proteins identified in the Koutroukides' study and the present characterization

were considered: trypsin digestion with the maximum of one missed cleavage; variable modification by oxidation (M) and fixed modification by carbamidomethyl (C); false discovery rate (FDR) less than 1%; and mass error less than 20 ppm. In addition, the ion matching requirements were set to select proteins with at least two ions per peptide, five ions per protein, and one peptide per protein. Then, protein grouping was applied, hiding proteins whose peptides are subset of another protein's peptides. Mass spectrometry data were deposited in the ProteomeXchange database and are available under the identifier PXD010273.

Then, the final list of proteins was narrowed down to select proteins identified by at least two unique peptides, and proteins whose presence was detected in at least 70% of samples. Keratin and identifications which do not attend these parameters were excluded. Aside from the 20 most abundant proteins and their isoforms [10,11], bioinformatics analyses were performed on the 81 remaining proteins using DAVID functional annotation tool and the PANTHER Classification System.

3 | RESULTS AND DISCUSSIONS

We detected here 12,714 peptides, corresponding to 281 proteins. After protein grouping, 198 proteins remained. Thus, using the Supplementary Table 1 available at "supporting information" of Koutroukides' paper [10], the comparison between the depletome dataset obtained by Koutroukides and his group and data obtained here revealed 150 non-redundant proteins unique to our study. Although the sum of proteins identified in the two studies were 397 proteins, only 48 are common to both studies (Figure 3).

After stringent filtering, of the 81 low-abundance proteins identified in the depletome, two have experimental evidence of the existence of a transcript, although the existence of protein has not been strictly proven, according to the Swiss-Prot Database: haptoglobin-related protein with Uniprot accession number P00739 and testis- and ovary-specific PAZ domain-containing protein with Uniprot accession number Q8N9V7. Comparing the concentrations of identified proteins with the highest and lowest abundances, the dynamic range spanned nearly 7 orders of magnitude (Figure 4).

Results from the DAVID functional annotation chart using the GOTERM Direct category (p-value < 0.01) reported 35 main biological processes (Figure 5). Receptor-mediated endocytosis was the biological process with higher number of associated proteins - 19 proteins of 81 low abundance proteins

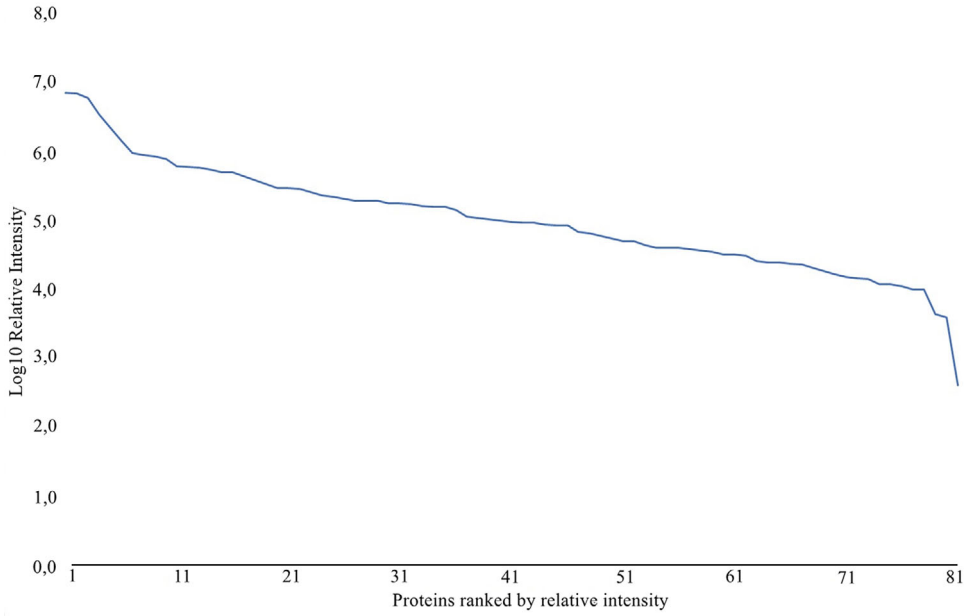

FIGURE 4 Dynamic range of quantified proteins

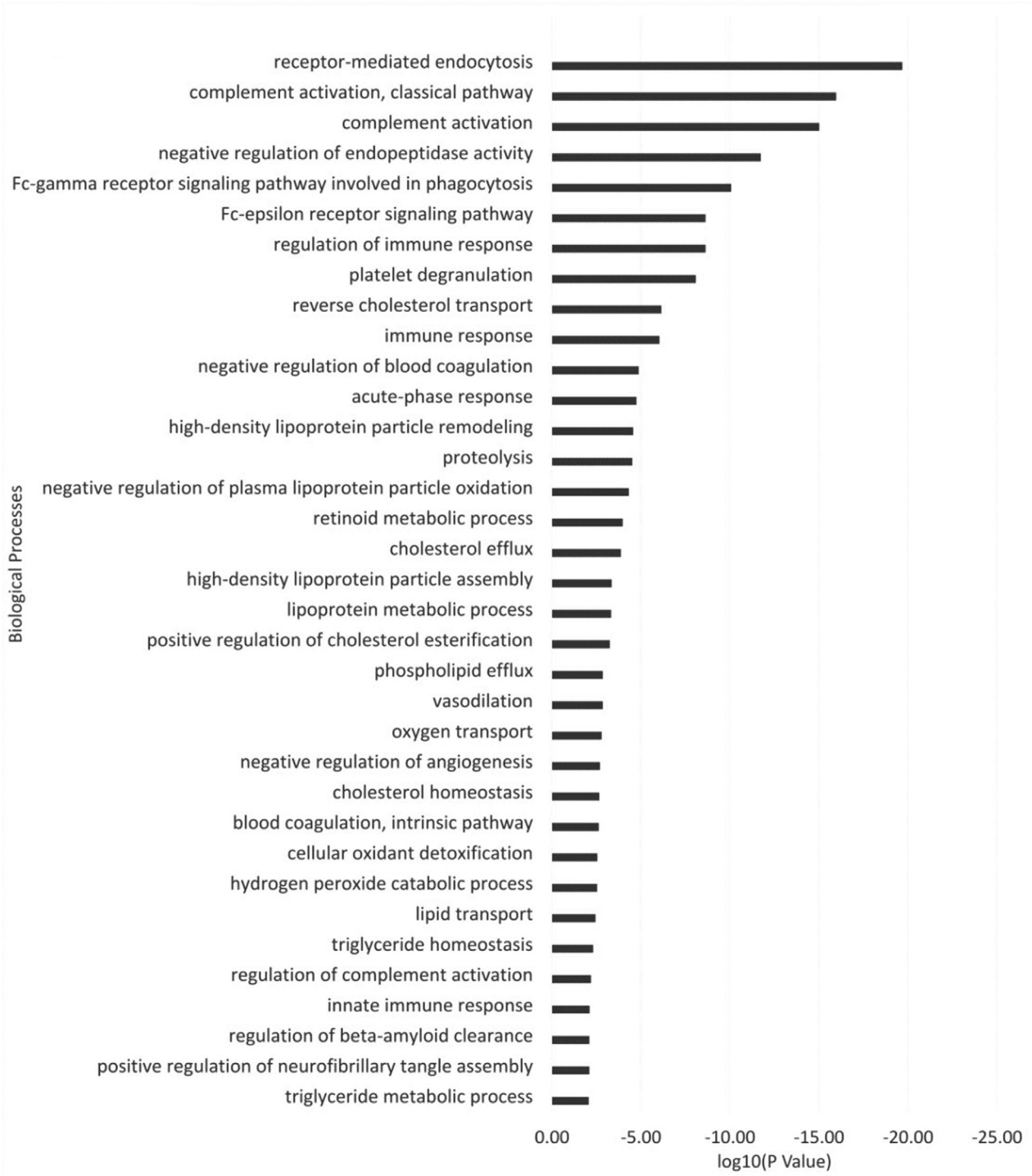

FIGURE 5 Enrichment analysis expressed in p-value from the DAVID functional annotation chart using GOTERM Direct category reported 35 main biological processes

(23%) were associated with this biological process. Fourteen different molecular functions (Figure 6) were associated with low abundance proteins found in the depletome. Some identified proteins were not able to be classified in terms of biological processes (7%) and molecular functions (11%), according to the PANTHER Classification System.

Thus, this study complements the dataset obtained by Koutroukides et al. (2011), by using a similar methodological approach. There is a minor overlap of identified proteins between both studies, which may be related to the differences of depletome acquirement. Although the two studies present limited comparability of results, the different identifications

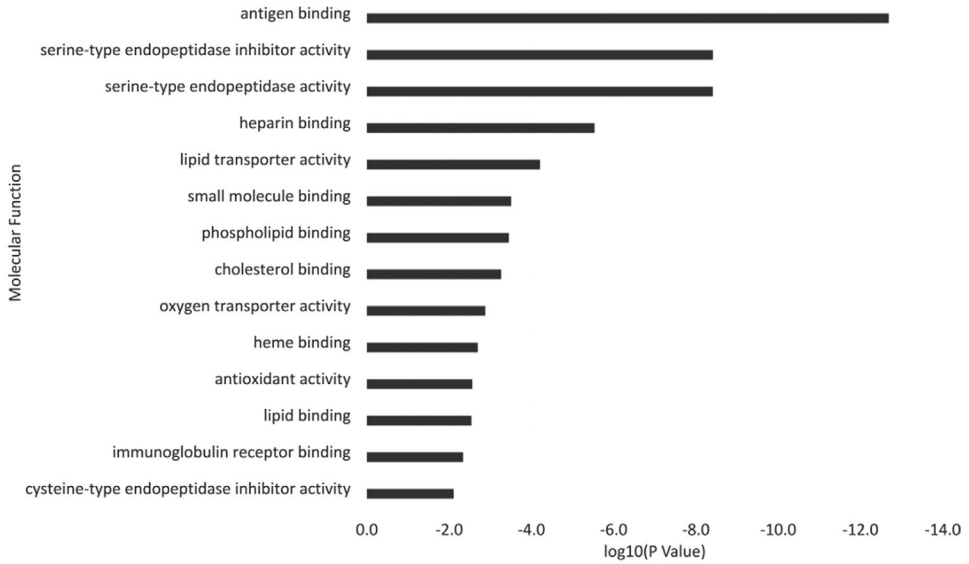

FIGURE 6 Enrichment analysis expressed in p-value from the DAVID functional annotation chart using GOTERM Direct category reported 14 different molecular functions

between both studies may also be associated to depletome complexity.

Proteins which participate in receptor-mediated endocytosis, and negative regulation of endopeptidase activity biological processes were found in the depletome (Figure 5). Alterations in endocytic mechanisms controlling traffic of lipids and proteins may be involved in the onset of several diseases, such as psychiatric and immune-related disorders, and some types of cancer [18–20]. Moreover, endocytic mechanisms can be explored with the purpose of personalized delivery of drugs, improving outcomes of leukemic and mesenchymal cancers and other disorders [21–23]. However, endocytosis pathways regulation remain to be fully understood even after 40 years since its discovery [24,25]. Therefore, more in-depth investigation into the role of the depletome proteins in several disturbances can bring important insights into the biochemical pathways related to diseases and patient response to medication.

Another important feature of the depletome is that it includes proteins related to immune processes and the complement system. Thus, investigation of the depletome can bring insights about autoimmune, metabolic, neurodegenerative and psychiatric diseases [26–30]. Several studies suggest that certain changes in the central nervous system may be caused by an imbalance of the peripheral immune system [31], possibly due to a blood-brain barrier rupture and consequent disturbance of the hypothalamic-pituitary-adrenal axis [32]. Approximately 20 proteins involved with immune response and activation of the complement system were found in the depletome, making this fraction a source of possible biomarkers related to the neurodegenerative and psychiatric disorders.

4 | CONCLUDING REMARKS

In future studies, it would be necessary to obtain a better resolution of the depletome fraction, since very large quantities of some proteins such as albumin interfere with the detection of the proteins with which they interact. One potential solution to the problem of low-abundance proteins co-eluting with the depletome could be to create different pH steps or a gradient instead of releasing the depletome all at once. Standardization with mass spectrometry analyses would determine if some co-eluting proteins can dissociate from the column at intermediary pH values without releasing the most abundant proteins from the column.

Considering the intrinsic potential of plasma to participate in and exhibit changes in response to endogenous and exogenous stimuli, plasma fractions are excellent samples to search for biomarkers related to several disorders. Outlook regarding diagnosis-dependent differential expression of proteins in the depletome could be interesting for future research, when looking for potential diagnostic or therapeutic potential biomarkers.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DATA AVAILABILITY STATEMENT

The mass spectrometry proteomic datasets for this study can be found in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010273.

AUTHOR CREDIT STATEMENT

Licia C. Silva-Costa: Formal Analysis, Investigation, Writing – Original Draft. Sheila Garcia-Rosa: Methodology, Investigation. Bradley J. Smith: Writing – Review & Editing. Paulo A. Baldasso: Methodology. Johann Steiner: Resources, Writing – Review & Editing. Daniel Martins-de-Souza: Conceptualization, Supervision, Writing – Review & Editing.

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