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Second-hand smoke exposure modulates plasma proteins linked to detoxification, inflammation and atherothrombosis

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ABSTRACT

Chronic exposure to second-hand smoke (SHS) contributes to the development of health issues, including cancer and cardiovascular diseases. Molecular mechanisms underlying SHS-related diseases remain poorly understood, highlighting the need for reliable risk assessment biomarkers. Herein, we demonstrate that the plasma proteome of individuals exposed to SHS undergoes significant modulation. Butyrylcholinesterase (BChE) and Vitamin D-binding protein (GC) that are involved in the physiological response to circulating toxic substances, as well as key mediators of systemic inflammation, including Complement C1r subcomponent (C1R), Complement C1q subcomponent subunit C (C1QC), Histidine-rich glycoprotein (HRG), and Vitamin K-dependent protein S (PROS1), were found to be significantly modulated in SHS-exposed individuals. Moreover, strong indicators of a pro-atherothrombotic response such Apolipoprotein A-IV (APOA4) and Alpha-2-antiplasmin (SERPINF2), were also differentially expressed. These findings provide novel insights into the biological pathways linking SHS-exposure to cardiovascular risks, and suggest a panel of candidate proteins with potential utility as SHS-risk assessment biomarkers.

1. Introduction

The World Health Organization (WHO) states that, despite a general decline in smoking habits following the implementation of tobacco control policies, tobacco smoking remains a global public health issue (World Health Organization, 2021). Second-hand smoke (SHS) originates from both the burning end of a cigarette, cigar, or pipe and the smoke exhaled by the smoker. SHS is a hazardous environmental contaminant when it accumulates in indoor spaces. According to the WHO, no level of SHS exposure is risk-free. It is estimated that about 37 % of the global population is exposed to SHS (Flor et al., 2024).

SHS exposure causes approximately 1.2 million deaths among non-smokers each year (Murray et al., 2020). A link has been established between SHS exposure and mortality from diseases such as cancer and cardiovascular conditions in healthy non-smokers (Office on Smoking and Health US, 2006). Since then, studies have shown that non-smokers exposed to SHS have a 5 % - 30 % increased risk of developing coronary artery disease (Digiacomo et al., 2019; Flor et al., 2024; Whincup et al., 2004). Moreover, 20–25 % of lung cancer cases in non-smokers are linked to SHS exposure, with a tendency of increased risk (1 %) in those who lived for $\geq$ 30 years with a smoking partner (Clément-Duchêne et al., 2010; Daylan et al., 2023; Kim et al., 2018; LoPiccolo et al., 2024). Long-term exposure to SHS has also been associated to breast cancer, chronic obstructive pulmonary disease (COPD) and diabetes (Carreras et al., 2019; Flor et al., 2024).

Although research on the molecular mechanisms of tobacco smoking-related diseases is still expanding, the understanding of molecular effects from SHS exposure remains limited.

The harmful effects of SHS begins at the airway level, where inhaled particles and gases trigger local responses. Recently, we reported alterations in the nasal epithelia proteome of a group of healthy non-smoker restaurant employees, exposed to SHS. We found that cigarette smoke induces cellular oxidative damage and modulates the Hypoxia-inducible factor-1α (HIF1α) signaling pathway. Namely, the HIF1α-

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glycolytic targets, such Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Triosephosphate isomerase (TPI1), were overexpressed in SHS-exposed individuals. Proteins related to cell detoxification and differentiation were also seen as modulated due to cigarette smoke. Importantly, we distinguished never and former smokers SHS-exposed proteomic profiles – while in never smokers there was an enrichment in the Glutathione metabolism pathway; in former smokers' proteome, the pathways and biologic processes associated with Warburg effect, Peroxisome pathway (ROS detoxification), DNA biosynthetic process and Leukocyte chemotaxis were enriched. Moreover, a modulation of the anti-apoptotic protein Serpin B3 (SERPINB3) was up-regulated in this subgroup (Neves et al., 2024).

Although our results are significant, a 2022 review highlights that cigarette smoke induces the release of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, as well as the recruitment of immune cells like neutrophils and macrophages, contributing to a persistent inflammatory environment (Dahdah et al., 2022). The SHS-induced local inflammation may extend beyond the lungs, contributing to systemic inflammation, endothelial dysfunction, and atherogenesis (Csordas and Bernhard, 2013). Thus, it becomes imperative to study the SHS-effects in the plasma of SHS-exposed subjects, seeing as this is a matrix that reflects systemic or organ-specific effects of xenobiotic exposure and associated disease progression (Deutsch et al., 2021; Tewari et al., 2011). Moreover, the plasma fraction of blood serves as an excellent sample for studying those alterations and identifying protein biomarkers, offering both accessibility and ease of collection (Deutsch et al., 2021; Tewari et al., 2011).

Indeed, in previous work, we identified several SHS-modulated plasma proteins using two-dimensional difference gel electrophoresis (2D-DIGE), namely ceruloplasmin and Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), both associated with acute-phase inflammation (Pacheco et al., 2013).

Building upon these findings, we evaluated the plasma proteome of the same healthy restaurant workers occupationally exposed to SHS, using a shotgun mass spectrometry approach. Our aim was to explore global protein expression changes in plasma following SHS exposure. We uncovered protein expression profiles linked to SHS exposure related with tissue injury, inflammatory response and atherothrombosis processes. These protein signatures can serve as a foundation for biomarker development to assess and mitigate the health risks associated to SHS exposure, such as cardiovascular diseases.

2. Materials and methods

2.1. Experimental design

The experimental design followed the methodology previously described (Neves et al., 2024). Plasma samples were collected concurrently with nasal epithelium samples analyzed in the earlier study (Neves et al., 2024). The plasma samples were obtained from the same cohort of 48 healthy individuals employed in restaurants or canteens equipped or not equipped with designated smoking areas, as defined by the pre-2017 smoking legislation, consistent with the prior investigation (Neves et al., 2024). The groups were distributed as shown in Fig. 1.

The study was approved by the Ethics Committee of the Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA). Written informed consent was obtained from all volunteers.

2.2. Sample preparation

Plasma was obtained as described in Charro et al. (2011) and stored in a biobank at −80°C. Before analysis, plasma samples were depleted of the 14 most abundant proteins by using a Multiple Affinity Removal Spin (MARS) Human 14 column (MARS, # 5188–6557, Agilent, Santa Clara, CA, USA), according with manufacturer's instructions. Briefly, each sample was diluted 4X with Buffer A (Buffer A, #5185–5987,

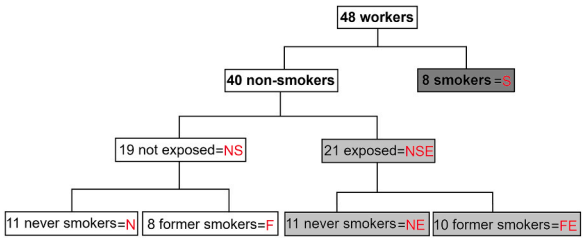

Fig. 1. Cohort distribution in groups and subgroups. 48 healthy workers were included in the proteomics study: 8 were labelled "Smokers" (S) not exposed to SHS at work, and 40 were non-smokers, of which 19 were not exposed (NS) and 21 were exposed to SHS (NSE). Following the established epidemiological criteria, namely the practice of cigarette smoking, NS could be subdivided into 11 "Never Smokers" (N) and 8 "Former Smokers" (F), and the NSE into eleven 11 "Never Smokers Exposed" (NE) and 10 "Former Smokers Exposed" (FE).

Agilent, Santa Clara, CA), filtered with a 0.22 μm spin filter and centrifuged for 1 min at 16,000 xg, then injected in a MARS column. The un-bound fractions were concentrated and buffer-exchanged to 25 mM of ammonium bicarbonate (AmBic - NH_4HCO_3) by centrifugal filtration using molecular weight cut-offs (MWCO) of 3KDa spin concentrators (Amicon® Ultra-4, #UFC800396, Merck KGaA, Darmstadt, Germany). Protein concentrations were established using the bicinchoninic acid (BCA) protein assay (Pierce™ 660 nm Protein Assay, #22662; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Samples were stored at −80°C until further analysis.

Each sample was prepared with 20 μL of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8; 25 % glycerol; 2 % SDS and 0.01 % Bromophenol Blue) with 350 mM Dithiothreitol (DTT). Vortex was applied to constituted samples, followed by centrifugation at 16,100 xg (20 ̊at 4 °C) and heated for 5' at 95°C. Samples were applied in a precast protein gel, with 12 + 2 well (12 % Mini-PROTEAN® TGX™, #4561041; Bio-Rad Laboratories Inc., Hercules, California, USA); using a constant current of 300 V during 3' at room temperature (RT). After, gels were stained (GelCode, #24596; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and the one band/sample coloured zone were cut into nine cubes, each cube with the approximated volume of 1 mm^3. The slices were washed with distain solution (50 % Acetonitrile (ACN) and 25 mM AmBic) 2X, 30´each at RT; and proteins reduced with 10 mM DTT in 100 mM AmBic for 1 h at 57 °C. Protein alkylation was performed by incubating slices in 100 mM AmBic and 55 mM Iodoacetamide (IAM) for 45´at RT, in the dark. Slices were washed twice with 100 μL of 100 mM AmBic followed by ACN 100 % dehydration by freeze speed-vac (T < 0°C) (SpeedVac ™ SRF110, Thermo Fisher Scientific Inc; Waltham, Massachusetts, USA) and digestion with 150 μL of Sequencing grade modified trypsin (Promega Biotech AB, Nacka, Sweden) (12.5 ng/μL in 50 mM AmBic) for 12 h at 37 °C. Peptides were extracted from the gel slices by incubation in 200 μL of 75 % ACN and 5 % trifluoroacetic acid (TFA) during 2 × 30' at RT, under vortex, followed by the incubation with 250 μL of ACN 100 % for 10' and then freeze dehydration by speed-vacuum (T < 0°C) (SpeedVac ™ SRF110, Thermo Fisher Scientific Inc; Waltham, Massachusetts, USA). Samples were stored at −80°C until mass spectrometry (MS) analysis.

2.3. LC-MS/MS analysis

The 48 samples were individually analysed by liquid chromatography coupled by mass spectrometry in tandem (LC-MS/MS) in a single shotgun proteomic experiment. The samples were re-suspended in 0.1 % Formic Acid (FA) during 30' and subjected to C18 spin column-based cleaning procedure according to the manufacturer's instructions. An ESI-LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc; Waltham, Massachusetts, USA.) interfaced with an Eksigent nanoLC 2DTM plus HPLC system (Eksigent technologies, Dublin, California,

USA) was used. A total of 5 μL, containing a total of 6.67 μg of protein digests, were loaded, trapped and washed with at constant flow rate of 10 μL/minute with 0.1 % FA, for 15' onto a pre-column (PepMap 100, C18, 5 μM, 5 mm×0.3 mm; LC Packings, Amsterdam, Netherlands) - a blank LC-MS/MS run was used between each 5 analysed samples. The peptides were subsequently separated on a 10 μM fused silica emitter, 75 μM x 16 cm (PicoTip™ Emitter; New Objective, Inc. Woburn, Massachusetts, USA), packed in-house with Reprosil-Pur C18-AQ resin (3 μM; Dr Maisch, Ammerbuch-Entringen, Germany). Peptides were eluted with a flow rate of 300 nL/minute with a 60' linear gradient of 3–35 % (v/v) ACN in water, containing 0.1 % (v/v) FA and then increased rapidly to 90 % (until 68 ') and was kept until 78 ´, before re-equilibrating. The LTQ-Orbitrap was operated in data-dependent mode (DDM) to automatically switch between Orbitrap-MS (from *m/z* 400–2000 Da) and LTQ-MS/MS (50–2000 Da) acquisition. Mass spectra of all samples were acquired in the positive ionization mode. Four MS/MS spectra were acquired in the linear ion trap per each FT-MS scan, which was obtained at 60,000 full width at half maximum (FWHM) nominal resolution settings using the lock mass option (*m/z* 445.120025) for internal calibration. The dynamic exclusion list was restricted to $N_{entries} = 500$; using $N_{repeat\ count} = 2$; with a $N_{repeat\ duration} = 20''$ and a $N_{dynamic\ exclusion\ range} = 2$ ´after the second peptide count. Precursor ion "charge state screening" was enabled, to select for ions with at least two charges and rejecting ions with undetermined charge state. The CID energy was set to 35 %, and one micro scan acquired for each spectrum. Data acquired using the Xcalibur (version 2.0.7) (Thermo Fisher Scientific Inc; Waltham, Massachusetts, USA.)

2.4. Mass spectra data analysis

2.4.1. PatternLab software in protein identification and quantification

The collected MS data was studied by Database Dependent analysis (DDA) methodology using the "PatternLab V" bioinformatics platform (http://patternlabforproteomics.org/). (Santos et al., 2022). From the UniProtKB (https://www.uniprot.org), and restricted to the reviewed Swiss-Prot (ID UP000005640), a Homo sapiens (ID 9606) canonical protein's database was retrieved in 06/02/2023 (Consortium, 2023).

The MS raw data were organized in subfolders of groups and subgroups according to the established cohorts (see Fig. 1.). Two independent analyses were done, one using the raw files of NSE, NS and S groups, and the other using the NE, N, FE, F and S subgroups.

In the "PatternLab V" platform, under the "Identify" tab, the "Peptide Spectrum Matching Search and Filtering" options were selected. In "Search and Filtering" menu, the raw files and database were loaded, being the following choices implemented: in "Preconfigured search parameters" sub-menu, the "High-Low" was used; in "Operation Mode" the "Search and Filter" was selected; and in "Processing options" the "Include MS2 in SEPro filtered results" was checked. The post translation modifications (PTM) searched were the "Carbamidomethylation of cysteine", as a fixed PTM, and the following variable PTMs: Deamidation NQ (Delta Mass: 0.984016), Phosphorylation (Delta Mass: 79.9663), Ubiquitination (Delta Mass: 383.2281), Acetylation (Delta Mass: 42.01565), Metlylation (Delta Mass: 14.01565), O-Sulfonation (Delta Mass: 79.95682), Crotonylation (Delta Mass: 68.02622), Glycosylation of serine/threonine with KDO (Delta Mass: 220.0583), Cysteinylation (Delta Mass: 119.1423), Myristoylation (delta Mass: 210.3556), Palmitoylation (Delta Mass: 238.4088), Carboxylation (Delta Mass: 44.0095), SUMOylation by SUMO-1 (Delta Mass: 600.5789) and Oxidation of Methionine (Delta Mass: 15.9949). In the "Advance Parameters" menu the established settings were the default, except for the following: in "Search (Comet)" the "Precursor mass accuracy" was "35", in "Enzyme" option it was chosen the "Trypsin" parameter and in "Enzyme specificity" the "Fully-specific" was selected.

In label-free relative quantification, PatternLab's XIC extraction was used, where the area under the curve of the extracted-ion chromatograms (XIC) of the identified peptides, was taken as a comparative tool for measurement. The quantities were normalized by a distributed "normalized ion abundance factor" approach - dNIAF (Carvalho et al., 2016, 2013). In the "Project Organization" tab, the "Label Free" menu was chosen and the directory of the raw files for each study cohort was specified, while keeping all parameters at their default settings.

The final list of the inferred proteins, proposed for candidate to biomarkers, had to fit two criteria: i) proteins had to present at least one unique peptide and ii) identified proteins had to be present in 80 % of each studied "population group".

2.4.2. Network mapping - ClueGO application

Through ClueGO's functional analysis algorithm application, in Cytoscape, the proteins identified in ≥ 80 % of subjects of a group or subgroup were selected for a single or multiple cluster comparison analysis (Trindade et al., 2019). The cluster analysis allowed to underline the "biological terms" (e.g. "Biological process" of GO terms) and metabolic pathways that showed to be specifically enriched for each group or subgroup's list of proteins (cluster), according with previous protocol (Mlecnik et al., 2018; Trindade et al., 2019).

For the analyses the public database Gene Ontology Knowledgebase (GO) (21/10/2024) (http://geneontology.org/) (Carbon et al., 2021) was used. In this database, the information obtained came from evidences curated, experimental data or inferred from "Reviewed Computational Analysis". ClueGO's selection criteria encompass the statistical Test Enrichment/Depletion (Two-sided hypergeometric test) with a p-value≤ 0.05 and Bonferroni step down correction test. A detailed "Network Specificity" (Min GO Level = 3 and Max GO Level = 8) with "Specific fusion of the "parent-child" GO-terms was employed. Pathways and Terms were grouped by the "Kappa Score" threshold statistic of 0.9.

2.5. Statistical analysis

To address the differences between the independent cohorts studied here, a statistical analysis based on nonparametric tests was employed using the software IBM SPSS Statistics (version 27) (IBM, New York, United States) and Microsoft Excel (version 2016) (Microsoft, Washington, United States). It was assumed that data were not normally distributed and an independent samples Mann-Whitney U Test was applied for group comparisons. The significance level (α) was set at 0.05. The variability in the box plots is captured by the interquartile range (IQR), while the median represents the central value of the distribution. The Venn diagram was accomplished using the software available online at http://www.interactivenn.net/ (Heberle et al., 2015).

3. Results

3.1. Studied cohort

The cohort analysed in this study was previously characterized, as described in our published work (Neves et al., 2024). The same 48 employees were selected for the plasma proteomic study. The subjects were classified in three main groups, "Non-Smokers" (**NS**), "Non--Smokers Exposed" (**NSE**) and current "Smokers" (**S**), or into five subgroups, "Never Smokers" (**N**), "Never Smokers Exposed" (**NE**), "Former Smokers" (**F**), "Former Smokers Exposed" (**FE**) and current Smokers (**S**) (see Fig. 1.).

The epidemiological data is shown in Table 1 (Neves et al., 2024).

3.2. Plasma proteome characterization

Analysis of the groups NS, NSE and S revealed a total of 9262 "peptides ion species" and 1823 proteins identified, whereas the analysis of the subgroups N, NE, F, FE and S, resulted in a total of 8964 "peptides ion species", corresponding to a sum of 1764 proteins (see **Table SI1**).

Through the "ClueGO" application in Cytoscape software, a protein enrichment analysis was established on the plasma proteome, using the

Table 1
Demographic data.

PARAMETERS		OCCUPATIONAL SECONDHAND SMOKE EXPOSURE							
	Never Smokers (N)	**Never Smokers Exposed (NE)**	**Mann-Whitney U Test**	**Former Smokers (F)**	**Former Smokers Exposed (FE)**	**Mann-Whitney U Test**	**Smokers (S)**	**Kruskal-Wallis Test**	
Subjects (n)	11	11	na	8	10	na	8	na	
Age (years)	42.0 [19;66]	33.0 [23;48]	retain null hypothesis	48.0 [26;63]	33.5 [25;57]	retain null hypothesis	44.0 [34;62]	retain null hypothesis	
Gender (F|M)	4|7	1|10	na	3|5	2|8	na	3|5	na	
Body mass index (kg/m^2)	25.8 [22.2;31.5]	24.5 [18.9;34.8]	retain null hypothesis	24.6 [21.2;28.7]	25.4 [20.3;31.3]	retain null hypothesis	24.7 [18.0;29.7]	retain null hypothesis	
Pulmonary function									
FVC (%)[a]	87.0 [75;98]	93.0 [80;105]	retain null hypothesis	100.0 [79;112]	96.5 [75;123]	retain null hypothesis	102.5 [78;149]	$p < 0.05$	N *vs* F $p < 0.05$ N *vs* S $p < 0.05$
FEV_1 (%)[b]	91.0 [72;102]	96.0 [79; 104]	retain null hypothesis	98.5 [74;120]	94.5 [67,124]	retain null hypothesis	97.0 [74;140]	retain null hypothesis	
FEV_1 /FVC (%)[c]	81 [73;90]	83 [73;94]	retain null hypothesis	81.5 [71;88]	79.5 [69;93]	retain null hypothesis	76.5 [70;84]	retain null hypothesis	
Tobacco smoking									
Smoking habit duration (years)	na	na	na	11.0 [1;30]	6.0 [1;22]	retain null hypothesis	30.5 [18;48]	$p < 0.05$	F *vs* FE $p < 0.001$ S *vs* FE $p < 0.001$
Cigarette number per day	na	na	na	7.8 [1;20]	6.6 [1;80]	retain null hypothesis	12.5 [5;20]	retain null hypothesis	
Smoking withdrawal duration (years)	na	na	na	14.0 [2;23]	13.0 [1;36]	retain null hypothesis	na	na	
Urine Cotinine (ng/mL)	5.0 [5.0;6.0]	8.4 [5.0;27.9]	$p < 0.05$	5.0 [5;10]	5.7 [5.0;7.1]	retain null hypothesis	1358.9 [513.0;2324,6]	$p < 0.001$	N *vs* NE $p < 0.05$ F *vs* NE $p < 0.05$ N *vs* S $p < 0.001$ F *vs* S $p < 0.001$ NE *vs* S $p < 0.05$ FE *vs* S $p = 0.001$
Time in workplace (years)	2.0 [0.3;34]	5.0 [0.3;11]	retain null hypothesis	10.5 [3;21]	2.3 [1;34]	retain null hypothesis	9 [0.3;41]	retain null hypothesis	
Working time (hours/week)	40.0 [5;60]	40.0 [20;50]	retain null hypothesis	50.0 [40;60]	40.0 [14;40]	$p < 0.001$	40.0 [40;66]	$p < 0.05$	NE *vs* F $p < 0.05$ FE *vs* F $p < 0.001$
[PM2.5] at work place ($\mu g/m^3$) Maximum	38.0 [25;153]	220.0 [131;309]	$p < 0.001$	48.0[28;119]	247.0 [164;693]	$p < 0.001$	100.50 [47;153]	$p < 0.001$	N *vs* FE $p < 0.001$ F *vs* NE $p < 0.001$ S *vs* NE $p < 0.05$ S *vs* FE $p < 0.05$ N *vs* NE $p < 0.001$ F *vs* FE $p < 0.001$

na - not applicable
[a] Forced volume vital capacity (the determination of the vital capacity from a maximally forced expiratory effort)
[b] Volume that has been exhaled at the end of the first second of forced expiration
[c] Tiffeneau-pinelli index (expressed as FEV1 %)

"Biological processes" term in GO's database. The Fig. 2 shows the main "Biological processes" enriched in the plasma samples, with the three top processes: "fibrinolysis" (GO:0042730), "complement activation" (GO:0006956) and "regulation of coagulation" (GO:0050818). Other major biological processes worth mentioning are: "protein activation cascade" (GO:0072376), "regulation of fibrinolysis" (GO:0051917), "regulation of microtubule depolymerization" (GO:0031114), "plasminogen activation" (GO:0031639), "cytolysis by host of symbiont cells" (GO:0051838) and "complement activation, classical pathway" (GO:0006958).

3.3. Plasma proteome – exposure to SHS

3.3.1. Identification and proteins distribution between cohorts

To robustly identify differentially expressed proteins (DEPs) associated with the biological condition under study, additional criteria were

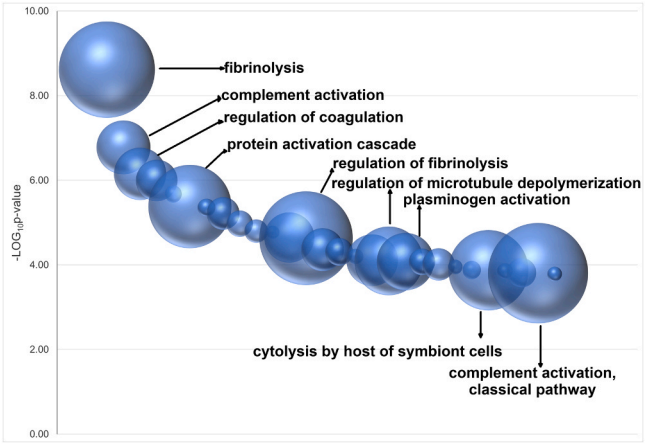

Fig. 2. Bubble chart representing the main biological processes identified in plasma proteome. Each circumference (bubble) represents a biological process. Larger bubbles indicate a higher percentage of identified proteins that belong to the biological process. The variable on the y-axis is given by the $-Log_{10}$ of the p-value and describes the probability of the biological process being linked to the analysed proteome. The probability is obtained from a two-sided hypergeometric test with Bonferroni step down correction (analysis in Cytoscape with application ClueGO using the Gene Ontology (GO) terms (Biological process); with 10799 total unique genes tested as described in (Mlecnik et al., 2018)).

applied in the protein identification algorithm: in addition to requirement of at least "one unique peptide", proteins must be detected in at least 80 % of samples within each group or subgroup.

The application of these criteria resulted in a more restricted yet reliable set of identified proteins: 117 for the groups and 128 for the subgroups. Fig. 3 displays the Venn diagrams showing the identified protein distribution among NS, NSE and S groups (Fig. 3**A**) and N, NE, F, FE and S subgroups (Fig. 3**B**). The list of proteins identified in each group/subgroup is shown in **Table SI2**.

Table 2 lists the proteins exclusively detected in over 80 % of subjects within NSE group and, NE and FE subgroups. In NSE group, the Immunoglobulin heavy constant gamma 4 (IGHG4) protein is linked with humoral immunity (Bateman et al., 2025); the Zinc finger protein 235 (ZNF235) likely plays a role in gene transcription (Bateman et al., 2025); and the Ras-related protein Rab-24 protein (RAB24) is associated with autophagy-related processes (Bateman et al., 2025). In the NE subgroup, the Alpha-1-acid glycoprotein 1 (ORM1) functions as blood transport protein and modulates the immune system during an acute phase reaction (Bateman et al., 2025), whereas, KICSTOR complex protein (SZT2) is involved in mTORC1 signalling inhibition under amino

Table 2
Proteins identified exclusively in the SHS exposed groups and subgroups.

Specific Proteins of "Non-Smokers Exposed" to Second-hand Smoke		
Uniprot Acession Number	**Gene Name**	**Protein Name**
P01861	**IGHG4**	Immunoglobulin heavy constant gamma 4
Q14590	**ZNF235**	Zinc finger protein 235
Q969Q5	**RAB24**	Ras-related protein Rab−24
Specific Proteins of "Never Smokers Exposed" to Second-hand Smoke		
Uniprot Acession Number	**Gene Name**	**Protein Name**
P02763	**ORM1**	Alpha−1-acid glycoprotein 1
Q5T011	**SZT2**	KICSTOR complex protein SZT2
Specific Proteins of "Former Smokers Exposed" to Second-hand Smoke		
Uniprot Acession Number	**Gene Name**	**Protein Name**
P19652	**ORM2**	Alpha−1-acid glycoprotein 2
P05154	**SERPINA5**	Plasma serine protease inhibitor
Q969Q5	**RAB24**	Ras-related protein Rab−24

acid deprivation and may contribute to oxidative stress responses (Bateman et al., 2025). In the FE subgroup, the Alpha-1-acid glycoprotein 2 (ORM2) shares similar functions with ORM1 (Bateman et al., 2025), and the Plasma serine protease inhibitor (SERPINA5) regulates proteolytic activities, exhibiting both procoagulant and anticoagulant functions in plasma (Bateman et al., 2025).

3.3.2. *Functional enrichment by cluster-based analysis*

The results of the network enrichment by cluster-based analysis and the associated proteins resulting from the comparison of NSE *versus* NS groups are summarized in Table 3 and **SI3**. Similarly, the results from the comparison NE *versus* N and FE *versus* F are represented in Table 4, **SI4** and **SI5**.

The proteome of the NS group showed group-specific enrichment (>60 % of associated proteins) for the GO term "substrate adhesion-dependent cell spreading" (GO:0034446), a process linked to cell flattening upon substrate adhesion. The protein specifically associated with this process is Fibulin-1 (FBLN1), which is involved in cell adhesion, extracellular matrix interactions, and possibly in haemostasis and thrombosis via fibrinogen binding. No specific GO term was found for NSE group.

In subgroup analysis, the NE subgroup proteome was enriched in GO terms related to immune and inflammatory responses: "homotypic cell-cell adhesion" (GO:0034109), "positive regulation of tumour necrosis factor production" (GO:0032760), and" interleukin-1 beta production" (GO:0032611). These processes were specifically associated with the

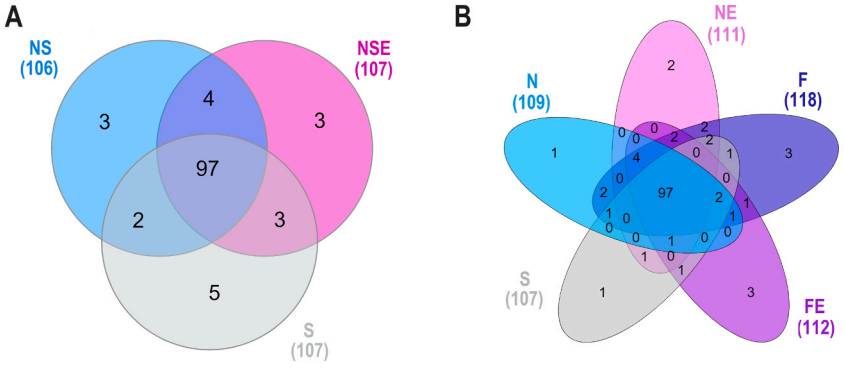

Fig. 3. Venn diagram of proteins detected in ≥ 80 % in each group or subgroup. (**A**) Protein distribution among the three main groups: "Non-Smokers" (NS) not exposed and "Non-smokers Exposed" (NSE) to SHS and "Smokers" (S). (**B**) Protein distribution among the five subgroups: "Never Smokers" not exposed (N), "Never Smokers Exposed" (NE), "Former Smokers" not exposed (F) and "Former Smokers Exposed" to SHS (FE) and Smokers (S). Venn diagram was obtained by the online software http://www.interactivenn.net/.

Table 3
Biological processes specifically associated to the proteome of "Non-Smokers" SHS exposed and not exposed.

SPECIFIC FOR CLUSTER "NON-SMOKERS EXPOSED" TO SECOND-HAND SMOKE							
ID	Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Genes Cluster NSE	Genes Cluster NS
–	–	–	–	–	–	–	–
SPECIFIC FOR CLUSTER "NON-SMOKERS"							
ID	**Term**	**Term PValue Corrected with Bonferroni step down**	**Group PValue Corrected with Bonferroni step down**	**% Associated Genes**	**Nr. Genes**	**Genes Cluster NSE**	**Genes Cluster NS**
GO:0034446	**substrate adhesion-dependent cell spreading**	1.08E−02	3.25E−02	4.41	3.00	**[APOA1, FN1]**	**[APOA1, FBLN1, FN1]**

Table 4
Biological processes specifically associated to the proteome of "Never" and "Former" smokers SHS exposed and not exposed.

SPECIFIC FOR CLUSTER "NEVER SMOKERS EXPOSED" TO SECOND-HAND SMOKE							
ID	**Term**	**Term PValue Corrected with Bonferroni step down**	**Group PValue Corrected with Bonferroni step down**	**% Associated Genes**	**Nr. Genes**	**Genes Cluster NE**	**Genes Cluster N**
GO:0034109	**homotypic cell-cell adhesion**	9.95E−04	7.35E−04	10.87	5.00	**[FGA, FGB, FGG, IL6, MEGF10]**	**[FGA, FGB, FGG]**
GO:0032760	**positive regulation of tumor necrosis factor production**	2.05E−02	1.93E−02	6.25	4.00	**[CLU, IL6, LBP, ORM1]**	**[CLU, LBP]**
GO:0032611	**interleukin−1 beta production**	3.14E−02	3.14E−02	4.92	3.00	**[APOA1, IL6, ORM1]**	**[APOA1]**
GO:0032651	**regulation of interleukin−1 beta production**	3.14E−02	3.14E−02	4.92	3.00	**[APOA1, IL6, ORM1]**	**[APOA1]**
SPECIFIC FOR CLUSTER "NEVER SMOKERS"							
ID	**Term**	**Term PValue Corrected with Bonferroni step down**	**Group PValue Corrected with Bonferroni step down**	**% Associated Genes**	**Nr. Genes**	**Genes Cluster NE**	**Genes Cluster N**
GO:0034446	**substrate adhesion-dependent cell spreading**	2.81E−02	2.81E−02	4.41	3.00	**[APOA1, FN1]**	**[APOA1, FBLN1, FN1]**
SPECIFIC FOR CLUSTER "FORMER SMOKERS EXPOSED" TO SECOND-HAND SMOKE							
ID	**Term**	**Term PValue Corrected with Bonferroni step down**	**Group PValue Corrected with Bonferroni step down**	**% Associated Genes**	**Nr. Genes**	**Genes Cluster NE**	**Genes Cluster N**
–	–	–	–	–	–	–	–
SPECIFIC FOR CLUSTER "FORMER SMOKERS"							
ID	**Term**	**Term PValue Corrected with Bonferroni step down**	**Group PValue Corrected with Bonferroni step down**	**% Associated Genes**	**Nr. Genes**	**Genes Cluster NE**	**Genes Cluster N**
GO:0034109	**homotypic cell-cell adhesion**	1.03E−03	7.82E−04	10.87	5.00	**[FGA, FGB, FGG]**	**[FGA, FGB, FGG, IL6, MEGF10]**

proteins: Interleukin-6 (IL6), which plays various roles in immunity and vascular permeability; Multiple epidermal growth factor-like domains protein 10 (MEGF10), a receptor involved in phagocytosis of apoptotic cells; and ORM1, an acute-phase protein with immunomodulatory and transport functions. On the other hand, the N subgroup presented an enrichment for" substrate adhesion-dependent cell spreading", consistent with the NS group. The F subgroup was enriched for "homotypic cell-cell adhesion", similarly to NE, whereas the FE subgroup did not display a distinct enrichment.

3.3.3. Relative quantification analysis - differentially expressed proteins

A label free strategy was used for relative quantification of the identified proteins by using the NIAF values obtained from the extracted-ion chromatogram (XIC). The relative abundance of proteins simultaneously present in all three groups ($n_{proteins} = 97$ in NS, NSE and S), or in the five subgroups ($n_{proteins} = 97$ in N, NE, F, FE and S), were compared. Twelve proteins were found to be modulated across the three main groups, while eleven were identified as differentially abundant among the five subgroups, as represented in Fig. 4 and Fig. 5.

Table 5 lists the DEPs identified in the comparisons between groups (NSE *versus* NS) and subgroups (NE *versus* N; and FE *versus* F). It is important to note that, despite the statistical difference displayed by Albumin (ALB) in group comparisons (NSE *versus* NS; NE *versus* N), this protein was not considered a true DEP, as it was depleted from plasma prior to analysis, and the observed variations in expression likely reflect technical or experimental bias.

In the NSE *versus* NS comparison, Vitamin D-binding protein (GC) presents the smallest p-value (p-value<0.004; Log_2fold change of −0.24), followed by Histidine-rich glycoprotein (HRG) (p-value=0.002; Log_2fold change of +0.45). The proteins with the most extreme difference in terms of fold change were Leucine-rich alpha-2-glycoprotein (LRG1) (p-value 0.014; Log_2fold change of −0.65) and Immunoglobulin heavy constant gamma 3 (IGHG3) (P-value 0.044; Log_2fold change of −0.56). Other DEPs showed less pronounced statistical significance (higher p-values) and smaller differences in abundance (lower fold changes).

In the NE *versus* N and FE *versus* F subgroup comparisons, several DEPs identified in the NSE *versus* NS group analysis - such as Apolipoprotein A-IV (APOA4), Cholinesterase (BCHE), Complement C1r subcomponent (C1R), and Thyroxine-binding globulin (SERPINA7) - did not

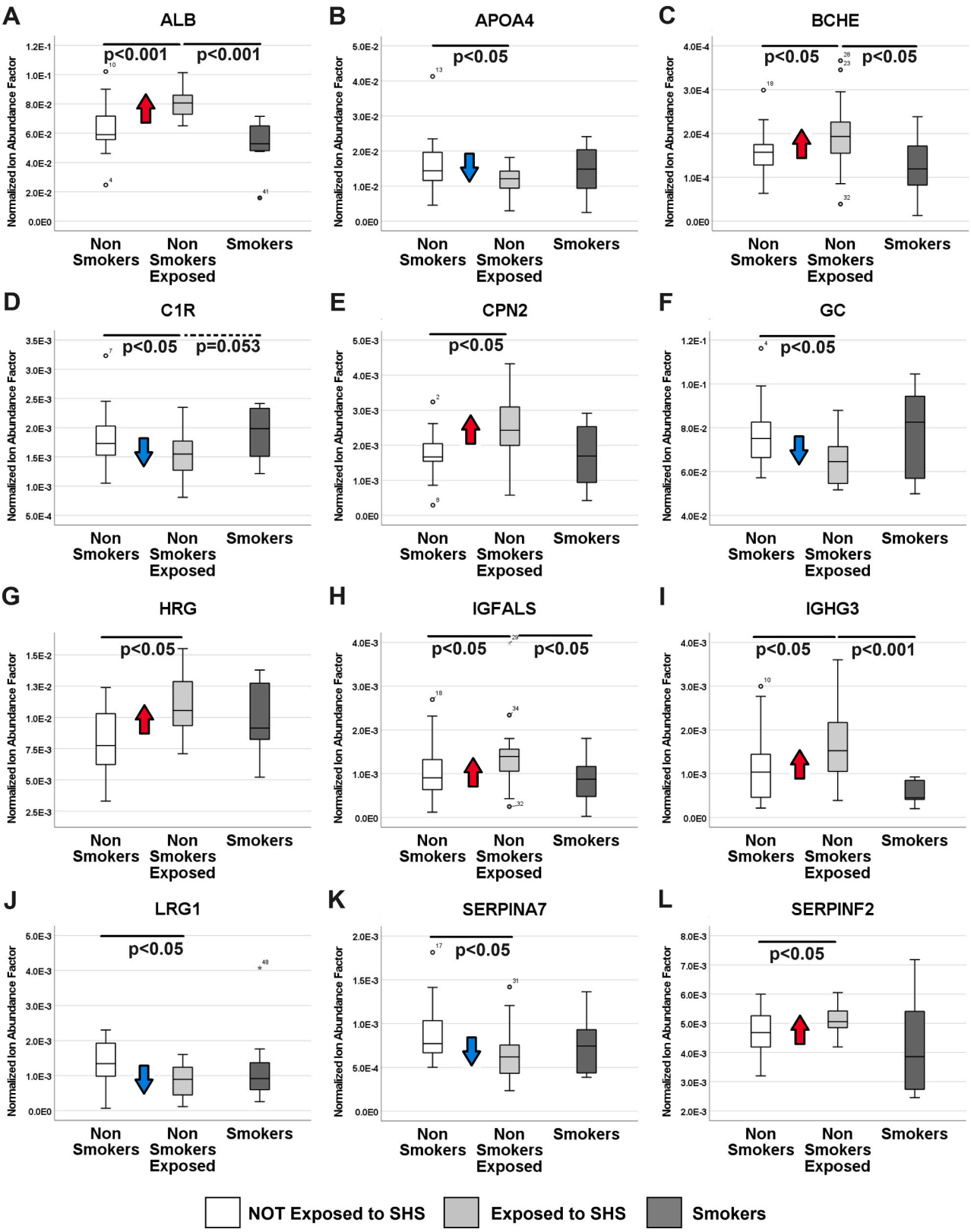

Fig. 4. Differentially expressed proteins between groups: Non-Smokers Exposed, Non-Smokers not exposed to SHS and Smokers. (**A**) Albumin (ALB), (**B**) Apolipoprotein A-IV (APOA4), (**C**) Cholinesterase (BCHE), (**D**) Complement C1r subcomponent (C1R), (**E**) Carboxypeptidase N subunit 2 (CPN2), (**F**) Vitamin D-binding protein (GC), (**G**) Histidine-rich glycoprotein (HRG), (**H**) Insulin-like growth factor-binding protein complex acid labile subunit (IGFALS), (**I**) Immunoglobulin heavy constant gamma 3 (IGHG3), (**J**) Leucine-rich alpha-2-glycoprotein (LRG1), (**K**) Thyroxine-binding globulin (SERPINA7) and (**L**) Alpha-2-antiplasmin (SERPINF2). A nonparametric independent-samples Mann-Whitney *U* test (α=0.05) was applied. Box plots show the median and interquartile values. Red or blue arrows indicate an increase or decrease in quantity, respectively. Statistically (p-value <0.05) or almost statistically (p-value < 0.1) significant differences are represented by a full or dashed line, respectively.

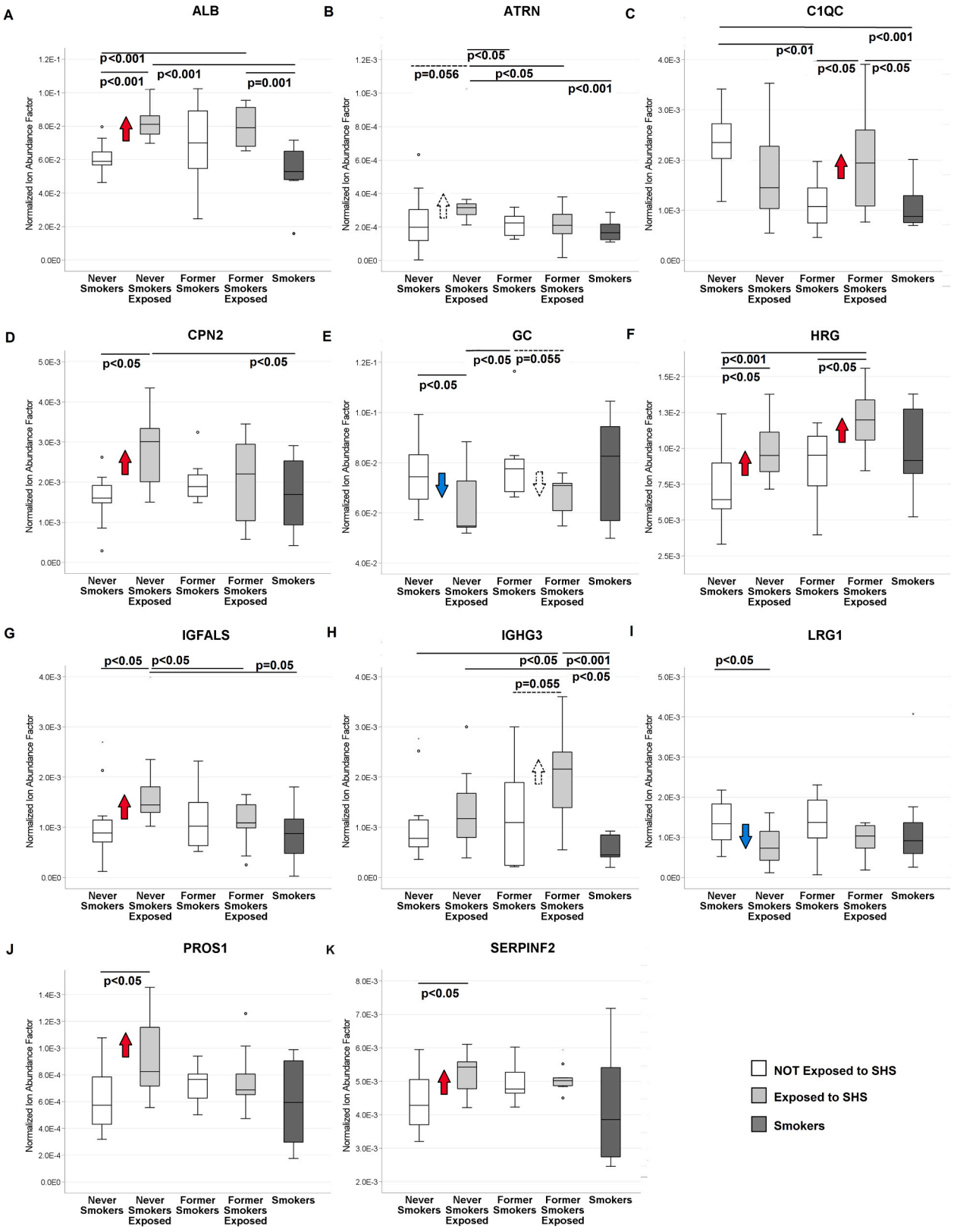

Fig. 5. Differentially expressed proteins between groups: Never Smokers Exposed, Never Smokers not exposed, Former Smokers Exposed, Former smokers not exposed and Smokers. (**A**) Albumin (ALB), (**B**) Attractin (ATRN), (**C**) Complement C1q subcomponent subunit C (C1QC), (**D**) Carboxypeptidase N subunit 2 (CPN2), (**E**) Vitamin D-binding protein (GC), (**F**) Histidine-rich glycoprotein (HRG), (**G**) Insulin-like growth factor-binding protein complex acid labile subunit (IGFALS), (**H**) Immunoglobulin heavy constant gamma 3 (IGHG3), (**I**) Leucine-rich alpha-2-glycoprotein (LRG1), (**J**) Vitamin K-dependent protein S (PROS1) and (**K**) Alpha-2-antiplasmin (SERPINF2). A nonparametric independent-samples Mann-Whitney *U* test (α=0.05) was applied. Box plots show the median and interquartile values. Red or blue arrows indicate an increase or decrease in quantity, respectively. Statistically (p-value <0.05) or almost statistically (p-value < 0.1) significant differences are represented by a full or dashed line, respectively.

Table 5
Differentially expressed proteins (DEPs).

"Non-Smokers" Exposed to Second-hand Smoke *versus* "Non-Smokers" not Exposed			
ID	**Name**	**Independent samples Mann-Whitney U Test p-value**	**Log_2Fold change**
P02768	Albumin (**ALB**)	< 0.001***	0.31
P06727	Apolipoprotein A-IV (**APOA4**)	0.027*	−0.50
P06276	Cholinesterase (**BCHE**)	0.038*	0.35
P00736	Complement C1r subcomponent (**C1R**)	0.041*	−0.27
P22792	Carboxypeptidase N subunit 2 (**CPN2**)	0.020*	0.46
P02774	Vitamin D-binding protein (**GC**)	< 0.004**	−0.24
P04196	Histidine-rich glycoprotein (**HRG**)	0.002**	0.45
P35858	Insulin-like growth factor-binding protein complex acid labile subunit (**IGFALS**)	0.044*	0.39
P01860	Immunoglobulin heavy constant gamma 3 (**IGHG3**)	0.044*	0.56
P02750	Leucine-rich alpha−2-glycoprotein (**LRG1**)	0.014*	−0.65
P05543	Thyroxine-binding globulin (**SERPINA7**)	0.027*	−0.40
P08697	Alpha−2-antiplasmin (**SERPINF2**)	0.023*	0.15
"Never Smokers" Exposed to Second-hand Smoke *versus* "Never Smokers" not Exposed			
ID	**Name**	**Independent samples Mann-Whitney U Test p-value**	**Log_2Fold change**
P02768	Albumin (**ALB**)	< 0.001***	0.41
O75882	Attractin (**ATRN**)	*0.056*	0.62
P22792	Carboxypeptidase N subunit 2 (**CPN2**)	0.010*	0.80
P02774	Vitamin D-binding protein (**GC**)	0.034*	−0.23
P04196	Histidine-rich glycoprotein (**HRG**)	0.028*	0.44
P35858	Insulin-like growth factor-binding protein complex acid labile subunit (**IGFALS**)	0.013*	0.73
P02750	Leucine-rich alpha−2-glycoprotein (**LRG1**)	0.047*	−0.73
P07225	Vitamin K-dependent protein S (**PROS1**)	0.040*	0.56
P08697	Alpha−2-antiplasmin (**SERPINF2**)	0.028*	0.25
"Former Smokers" Exposed to Second-hand Smoke *versus* "Former Smokers" not Exposed			
ID	**Name**	**Independent samples Mann-Whitney U Test p-value**	**Log_2Fold change**
P02747	Complement C1q subcomponent subunit C (**C1QC**)	0.043*	0.84
P02774	Vitamin D-binding protein (**GC**)	*0.055*	−0.25
P04196	Histidine-rich glycoprotein (**HRG**)	0.021*	0.42
P01860	Immunoglobulin heavy constant gamma 3 (**IGHG3**)	*0.055*	0.78

Independent Samples Mann-Whitney U Test was used for pairwise analysis. The significance level (α) was set at 0.05. * Statistically significant at the 5 % level, ** Statistically significant at the 1 % level and *** Statistically significant at the 0.1 % level. Fold change tells how much the relative quantity of the protein changes between two biological conditions ("Non-Smokers" Second-hand Smoke Exposed (NSE) *versus* "Non-Smokers not Exposed" (NS), "Never Smokers" Second-hand Smoke Exposed (NE) *versus* "Never Smokers" not exposed (N) and "Former Smokers" Second-hand Smoke Exposed (FE) *versus* "Former Smokers" not exposed (F)) and it was calculated by the rate between groups (NSE/NS, NE/N and FE/F). In the Log_2(Fold Change) the value of + 1 corresponds to 2-fold upregulation, the −1 corresponds to 2-fold down-regulation and 0 to "no change".

reach statistical significance. However, they retained the same expression trends (increase or decrease) observed in the NSE *versus* NS group comparison (see Fig. 4 and Fig. 6). Notably, additional DEPs emerged in these subgroup contrasts, including the Vitamin K-dependent protein S (PROS1) in NE *versus* N comparison and the Complement C1q subcomponent subunit C (C1QC) in FE *versus* F.

4. Discussion

Previously, we demonstrated that non-smoking healthy employees from a group of Lisbon restaurants with smoke-free dining rooms (under pre-2015 legislation) exhibited elevated urinary cotinine levels and 2DIGE-based proteomics alterations in their plasma linked to workplace SHS exposure (Pacheco et al., 2013). In the present study, we employed a shotgun proteomic analysis to achieve in-depth characterization of the plasma proteome of these same individuals. The main goal was to identify plasma proteins whose expression changes with SHS exposure, with the potential to be developed as biomarkers for early detection or risk assessment of SHS-related diseases.

The functional study of all plasma samples analysed in this work reveals an enrichment in GO terms typically associated with the most abundant plasma proteins, like "fibrinolysis" (GO:0042730), "complement activation" (GO:0006956) and "regulation of coagulation" (GO:0050818). However, in individuals exposed to SHS, the modulated proteins showed mainly associated with biological processes, including response to toxic elements in the blood, systemic inflammation/autoimmunity, and blood vessel diseases.

4.1. Response to toxic elements in the blood

Butyrylcholinesterase (BChE), one of the modulated proteins identified as associated with SHS exposure, is an enzyme involved in neutralizing toxic compounds, including organophosphates and nerve agents, primarily through sequestration and irreversible binding (Gok et al., 2024; Lockridge, 2015). In healthy individuals, BChE activity is relatively stable, but its plasma levels tend to decrease following exposure to cholinesterase inhibitors, likely due to increased enzyme turnover or downregulated expression in response to sustained toxic insult (Johnson and Moore, 2012; Lockridge, 2015; Sun et al., 2024). Consistent with this, reduced BChE levels have been reported in individuals occupationally exposed to organophosphorus pesticides (Mishra, 2006; Sutoluk et al., 2011). Similarly, we observed a significant downregulation of BChE in smokers (S), suggesting a comparable response to mainstream tobacco smoke exposure. Moreover, there are works that mention the presence of pesticides in the cigarette smoke that persist pyrolysis (Dane et al., 2006; López Dávila et al., 2020). Interestingly, BChE was upregulated in non-smokers exposed to SHS, possibly reflecting a distinct biological response. This divergence may be attributed to differences in the chemical composition and toxicity burden of SHS, potentially triggering induction of BChE as part of a compensatory detoxification response.

The Vitamin D-binding protein (GC), a plasma glycoprotein, was downregulated in the plasma of Never (NE) and Former Exposed (FE) subgroups. In addition to transporting vitamin D, GC plays a key role in actin clearance, working in tandem with gelsolin: while gelsolin depolymerizes F-actin into G-actin, GC binds G-actin to prevent repolymerization and facilitate hepatic clearance (Lisowska-Myjak et al., 2020; Speeckaert et al., 2014). We propose that SHS exposure may lead to epithelial injury and actin release into circulation, increasing the demand for these scavenger proteins. The observed downregulation of GC,

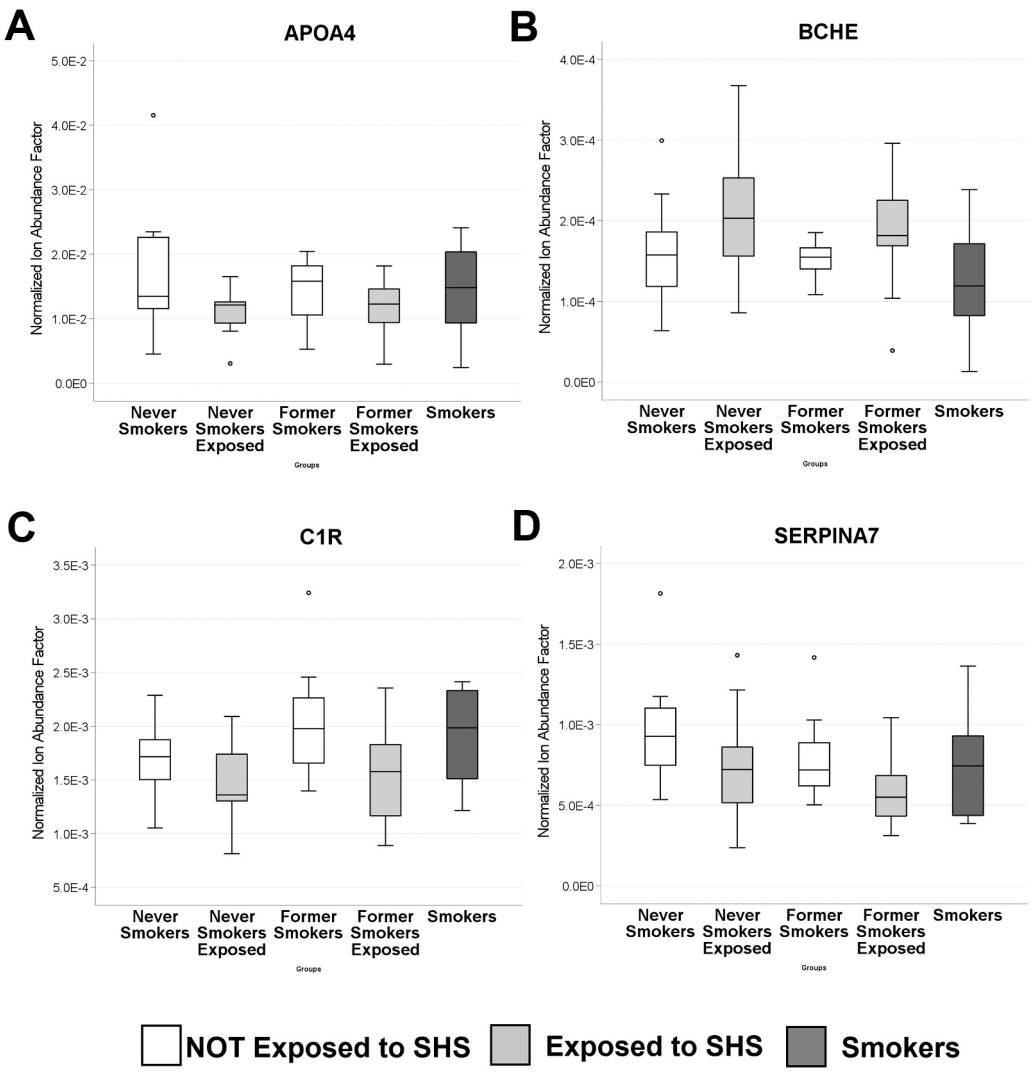

Fig. 6. Proteins differentially expressed between groups but not within subgroups. (**A**) Apolipoprotein A-IV (APOA4), (**B**) Cholinesterase (BCHE), (**C**) Complement C1r subcomponent (C1R) and (**D**) Thyroxine-binding globulin (SERPINA7). Box plots show the median and interquartile values.

together with previously reported reduced plasma gelsolin in SHS-exposed non-smokers (Pacheco et al., 2013), may reflect their consumption during actin clearance. This mechanism is consistent with reports linking lower GC levels to tissue injury and poor clinical outcomes (Lisowska-Myjak et al., 2020; Speeckaert et al., 2014). Our findings also align with studies reporting reduced GC in smokers (Bortner et al., 2011), and with our own previous work showing evidence of airway tissue disruption in SHS-exposed individuals, including reduced Tubulin beta-4B (TUBB4B) expression in nasal epithelium (Neves et al., 2024).

Besides acting as an actin scavenger, GC also plays an immunomodulatory role in inflammation (see discussion below).

4.2. *Inflammation/autoimmunity*

One of the detrimental molecular effects that cigarette smoke has in the body is its alteration of the immune system including the inflammatory response (reviewed in (Gonçalves et al., 2011). Several epidemiologic works have demonstrated the association between SHS exposure and elevated systemic inflammatory markers (Dahdah et al., 2022; Digiacomo et al., 2019). Using 2D-DIGE, we identified nine plasma proteins modulated by SHS exposure - particularly the acute-phase proteins ceruloplasmin (CP) and Inter-α-trypsin inhibitor heavy chain H4 (ITIH4) (Pacheco et al., 2013).

GC is regarded as an acute-phase protein and is involved in the innate immune inflammatory response. In neutrophils, it binds to the cell surface and associates with the protein annexin A2 to the CD44 molecule, thereby enhancing complement C5a-dependent chemotaxis (Lisowska-Myjak et al., 2020; Speeckaert et al., 2014). GC can also be converted into a deglycosylated proteoform, denominated macrophage-activating factor (MAF), which is antiangiogenic and boosts macrophage phagocytic activity. In chronic inflammation, reduced GC may reflect its conversion to MAF, later internalized by activated B and T cells (Lisowska-Myjak et al., 2020; Speeckaert et al., 2014). As so, the observed downregulation of plasma GC in the NE and FE subgroups may be indicative of an inflammatory process occurring in the endothelium.

Additional evidence of a systemic proinflammatory state comes from several differentially expressed plasma proteins, including Histidine-rich glycoprotein (HRG), Complement C1q subcomponent subunit C (C1QC), Complement C1r subcomponent (C1R), Leucine-rich alpha-2-glycoprotein 1 (LRG1) and Vitamin K-dependent protein S (PROS1).

HRG was significantly upregulated in non-smokers (never and former) exposed to SHS. It interacts with various blood cells like endothelial cells, erythrocytes, neutrophils, T cells and platelets, modulating its immune responses and limiting their excessive activation under pathological conditions (Gao and Nishibori, 2021; Pan et al., 2022). Particularly, HRG enhances apoptotic cell clearance and promotes a

balanced proinflammatory/anti-tumor macrophage response in inflammatory environments by acting as an intermediary between Fc-gamma receptors (FcγR) and apoptotic cell-derived DNA, thereby upregulating phagocytic activity (Pan et al., 2022). Its upregulation in SHS-exposed individuals suggests a role in modulating immune response in the presence of cellular debris.

The subunit of C1q, C1QC, was found to be downregulated in S and NE subgroups (though not significantly in NE), and upregulated in FE. C1q is central to the classical complement pathway, playing a key role in recognition of antigen-antibody complexes and contributing to the prevention of autoimmunity through enhanced phagocytosis, regulation of cytokine production by the Antigen-Presenting Cells (APCs) and T cell maturation favouring Treg proliferation (Thielens et al., 2017). The circulating levels of C1q has been proposed as biomarker in several diseases where the decrease levels are associated with autoimmune diseases, inflammatory diseases (e.g., pneumonia and emphysema), and cancer, including lung adenocarcinoma (van de Bovenkamp et al., 2021). Smokers with emphysema presented lower C1QC mRNA expression in lung APCs and reduced plasma levels of C1q inversely correlated with disease severity (Yuan et al., 2019). Based on these findings, the reduced C1QC levels in SHS-exposed individuals may indicate an impaired capacity for immune regulation and clearance of cellular debris, potentially contributing to chronic inflammation or autoimmune-like responses.

In line with this, we also observed a downregulation of C1R in the NSE group. C1R, together with C1q and C1s, constitutes the C1 complex, the initial component of the classical complement system, and as a serine protease, it is responsible for the activation of C1s. Deficiencies in C1 components such as C1R and C1q are associated to poor clearance of immune complexes and the development of autoimmune and inflammatory conditions (McMurray et al., 2024). Accordingly, reduced C1R levels in SHS-exposed individuals may compromise cascade activation, weakening the system's ability to eliminate autoantigens and regulate immune function. Moreover, a recent work reported that a decrease in C1R expression resulted in heightened malignant characteristics of hepatocellular carcinoma cells through the activation of HIF-1α-mediated glycolysis (Ma et al., 2024). Interestingly, our previous work revealed an upregulation of HIF-1α glycolytic targets, GAPDH, TPI, in the nasal epithelium of SHS-exposed individuals (Neves et al., 2024). The downregulation of C1R in plasma and upregulation of glycolysis-associated targets in epithelial tissue raise the possibility of a link between complement dysregulation, metabolic reprogramming, and inflammatory signalling in SHS exposure, which warrants further investigation.

LRG1 was downregulated in NE *versus* N, with a similar trend in FE *versus* F, though not statistically significant. LRG1 has been found to be upregulated in inflammatory and autoimmune conditions via IL-6/IL-1β-induced STAT3 activation. Through this pathway, LRG1 promotes pro-inflammatory Th17 cells differentiation by upregulating IL-6R in naïve CD4 T cells, contributing to autoimmune diseases like rheumatoid arthritis (Camilli et al., 2022; Nasuno et al., 2023; Yoshimura et al., 2021; Zou et al., 2022). Therefore, its decreased expression in SHS-exposed individuals contrasts with the expected upregulation in a proinflammatory context. This finding is particularly relevant when considered alongside the reduced levels of C1QC and CR1, proteins involved in immune complex clearance and regulation of inflammation.

However, LRG1 also plays a critical role in tissue repair and preservation of physiological tissue integrity, limiting fibrosis through modulation of TGFβ signalling (Camilli et al., 2022). Thus, its downregulation may reflect an impaired reparative response rather than an acute inflammatory state. This pattern is consistent with clinical evidence showing that cigarette smoking is associated with delayed wound healing and a higher incidence of postoperative complications (Matharu et al., 2019; Vu and Lussiez, 2023), supporting the notion that tobacco exposure may impair tissue repair mechanisms. Although evidence in humans is still limited, exposure to SHS may also interfere with tissue repair mechanisms, as suggested by experimental studies (Santiago et al., 2017; Wong et al., 2004). In our study, the expression profile of LRG1 suggestive of an increased propensity for a reduced capacity for tissue repair was mainly identified in non-smokers exposed to SHS, indicating that the biological response of these individuals may differ from that of active smokers.

PROS1 is an anticoagulant factor that binds to negatively charged phospholipid membranes of apoptotic cells while simultaneously acting as an agonist for TAM family tyrosine kinase receptors on immune cells, thereby promoting apoptotic cell clearance and suppressing inflammatory responses (Dahlbäck, 2018). PROS1 was upregulated in NE individuals and remained elevated in F and FE compared to N control, supporting the hypothesis of increased apoptotic cell burden and an adaptive mechanism aimed at modulating the immune response.

Further support for a SHS-induced proinflammatory response comes from unique proteins identified in SHS-exposed individuals. IGHG4 (an immunoglobulin component) and RAB24 (involved in autophagy) (Bateman et al., 2025) were exclusively found in the Non-smokers exposed to SHS (NSE) group. Both proteins are closely related to immune function and cell death pathways, and their presence may be linked to autoantibody production secondary to tissue damage. Additionally, functional enrichment analysis of the SHS-exposed plasma proteome revealed Gene Ontology (GO) terms linked to immune regulation and epithelial integrity. These pathways reflect endothelial alterations and increased proinflammatory signalling, consistent with prior studies on cytokine upregulation following SHS exposure (Bhat et al., 2018; Mahabee-Gittens et al., 2019; Schamberger et al., 2014; Tatsuta et al., 2019; Zhang et al., 2002).

In summary, individuals exposed to SHS exhibit a modulation in plasma proteins indicative of an inflammatory and potentially autoimmune-prone environment, due to the presence of autoantibodies from tissue injury. These changes include the upregulation of HRG and PROS1 associated with apoptotic cell clearance and the downregulation of C1QC and C1R, which may impair immune complex clearance and favour autoimmunity. These results suggest that SHS exposure can lead to tissue damage, increased apoptotic burden, and dysregulated immune responses with potential long-term health consequences.

4.3. *Blood vessels diseases - atherosclerosis and thrombosis*

Atherosclerosis has long been recognized as a major contributor to cardiovascular diseases. Mild systemic inflammation coupled with cellular debris from cell death and its accumulation within the endothelium, is also linked to cardiovascular disease (Ząbczyk et al., 2023). SHS is associated with chronic cardiovascular diseases and contributes to acute atherothrombotic events, including stroke and myocardial infarction. The biological mechanisms underlying the ability of SHS to induce acute atherothrombosis include direct damage to endothelial cells, alteration of atherosclerotic plaque composition, and disruption of the coagulation system promoting the thrombus formation (reviewed in (Csordas and Bernhard, 2013; Digiacomo et al., 2019)).

In this study, we identified a set of proteins that suggest that SHS-exposed individuals exhibit an imbalance between a pro-atherogenic/pro-thrombotic microenvironment and an anti-thrombotic one, shifting the balance toward an atherothrombotic state. Elevated levels of alpha-2-antiplasmin (SERPINF2), which plays a crucial role in blood clotting regulation, were detected in the NE, F, and FE groups. An upregulation of SERPINF2 expression is associated with atherosclerosis and thrombosis risk and, as so, with ischemic stroke and myocardial infarction (Humphreys et al., 2023). SERPINF2's anti-fibrinolytic function contributes to thrombosis by interfering with plasmin-fibrin binding through plasmin inhibition or by disrupting plasminogen-fibrin complexes (Alsayejh et al., 2022; Carpenter and Mathew, 2008). This expression profile, suggests that SHS-exposed individuals are more prone to blood clotting.

APOA4 was another protein whose profile was found to be modulated by SHS. This molecule is involved in a broad range of biologic

processes, like lipid absorption and metabolism, anti-atherosclerosis, platelet aggregation and thrombosis (Kronenberg, 2017; Peng and Li, 2018; Qu et al., 2019). In this work, APOA4 is downregulated in the NSE group (a result also seen in NE and FE, but without statistical significance). Studies have shown that overexpression of APOA4 leads to the prevention of atherosclerosis (Qu et al., 2019), with the reduction of inflammation (Peng and Li, 2018). APOA4 may attenuate atherosclerosis by promoting HDL-mediated reverse cholesterol transport and by reducing LDL oxidation (Qu et al., 2019). This protein also inhibits platelet aggregation, and so when an unbalance in APO4A activity occurs thrombosis unfolds, rising the risk of heart attack or stroke (Peng and Li, 2018; Qu et al., 2019). Low levels of APOA4 in plasma were associated with a poor outcome in cardio vascular diseases, being APOA4 a risk factor for coronary heart disease and its plasma concentration a diagnostic parameter (Peng and Li, 2018). Thus, our results suggest that exposure to SHS predisposes individuals to a pro-atherogenic and pro-thrombotic state.

These findings contrast with the upregulation of PROS1 (encoding Protein S), an anti-coagulant molecule and cofactor for activated Protein C (APC) (Dahlbäck, 2018). Indeed, PROS1 is essential for APC activity in the degradation of coagulation factors Va and VIIIa. PROS1 also interacts directly with the coagulation factors Xa, Va, and VIIIa to inhibit their function (Dahlbäck, 2018). Contrary to this result is the specific detection in SHS-exposed individuals (FE subgroup) of Plasma serine protease inhibitor (SERPINA5), a protein with pro-coagulant and pro-inflammatory properties. SERPINA5 inhibits APC, and its dysregulation is linked to venous thromboembolism (Alnor et al., 2024). Furthermore, the modulation of HRG in the pro-atherothrombotic balance can result in different outcomes according to the protein's function: in the presence of blood vessel injury HRG can protect the vascular endothelial cells from strong activation and apoptosis, being a negative regulator of the platelet function, inhibiting the erythrocytes aggregation and adhesion to the damaged endothelium (Gao and Nishibori, 2021), the typical atherogenesis process; on the other hand, this protein also contributes to a pro-thrombotic state, by inhibiting heparin's anti-coagulant function, thereby promoting blood coagulation and thrombotic events(Pan et al., 2022). Altogether, these findings point to a modulation of atherothrombotic process upon to SHS exposure.

4.4. Other proteins differentially expressed

Several additional proteins showed altered expression in SHS-exposed individuals, potentially reflecting systemic responses to exposure.

The Carboxypeptidase N subunit 2 (CPN2) was significantly upregulated in the NE group. CPN2 stabilizes the Carboxypeptidase N (CPN), a metalloprotease complex, protecting it from degradation and clearance (Matthews et al., 2004). CPN degrades inflammatory peptides like bradykinin, which increase vascular permeability and cause bronchoconstriction and hypotension (Matthews et al., 2004). These pathways possibly link CPN2 expression to inflammatory responses.

The insulin-like growth factor-binding protein complex acid labile subunit (ALS/IGFALS), which forms part of a ternary complex with Insulin-like growth factor 1 and 2 (IGF-1/2) and Insulin-like growth factor binding protein 3 and 5 (IGFBP-3/5), prolongs the half-life of the IGF/IGFBP circulating binary complexes and regulates the bioavailability of circulating IGFs (Baxter, 2024; Macvanin et al., 2023). Its levels were significantly increased in NE individuals. Altered ALS levels have been associated with cardiovascular risk, although findings in the literature are mixed, with both elevated and reduced levels reported in coronary heart disease patients (Baxter, 2024).

The Thyroxine-binding globulin (SERPINA7), a transporter of T3 and T4 thyroid hormones, was downregulated in SHS-exposed groups, namely in NSE. Although no direct link with tobacco smoke has been reported, increased SERPINA7 expression has been associated with the severity of lung emphysema (Carolan et al., 2014).

Attractin (ATRN), primarily secreted by activated T cells, was increased the NE subgroup. Its soluble isoform is involved in immune cell clustering and may regulate chemokine activity during inflammation (Duke-Cohan et al., 2000).

The Immunoglobulin heavy constant gamma 3 (IGHG3) was upregulated in SHS-exposed individuals (NSE, NE, FE), though not significantly in all cases, and downregulated in smokers. This may reflect increased antigen-binding activity upon SHS exposure and apparent humoral immunosuppression in active smoking.

5. Study limitations

While a longitudinal study with multi-year follow-up would be more appropriate to fully evaluate long-term risks and disease development, the proteomic signatures identified here already reveal early biological responses compatible with inflammation, immune dysregulation, and a pro-atherothrombotic state. These findings suggest that even within a cross-sectional framework, it is possible to detect molecular changes indicative of elevated susceptibility to SHS-related conditions, reinforcing the potential of these proteins as early biomarkers of exposure and risk.

6. Conclusions

Exposure to SHS seems to modulate the plasma proteome through two main mechanisms: a direct response to circulating toxic compounds and tissue injury, and an indirect response that promotes a proinflammatory, autoimmune, and pro-atherothrombotic state.

We observed altered expression of BChE, a key enzyme in xenobiotic metabolism, and notably, a downregulation of vitamin D binding protein, GC, which, along with gelsolin, is involved in the clearance of F-actin filaments released during cell damage. RAB24, a protein related to autophagy-related processes, was exclusively detected in SHS-exposed individuals, suggesting enhanced cellular stress responses.

Additionally, classical complement proteins C1QC and C1R were downregulated, supporting the presence of an inflammatory and potentially autoimmune environment induced by toxic exposure. Proteins such as HRG and PROS1 were upregulated and are known to modulate immune responses, towards a proinflammatory context, and facilitate the clearance of apoptotic cells. These findings further reinforce a proinflammatory systemic effect of SHS.

Consistent with previous reports linking SHS exposure to a pro-atherothrombotic state, our data show downregulation of anti-atherogenic protein like APOA4 and upregulation of SERPINF2, a plasmin inhibitor involved in clot stabilization. Moreover, SERPINA5, a pro-atherosclerotic protein, was specifically identified in former smokers exposed to SHS, further highlighting the cardiovascular risk associated with this exposure.

In summary, the observed proteomic alterations highlighted several proteins as promising biomarkers to assess the biological response and risk associated with SHS exposure.

Ethics

The study was approved by the National Institute of Health Doctor Ricardo Jorge's Ethics Committee.

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CRediT authorship contribution statement

Cristina Valentim-Coelho: Methodology, Investigation. **Joana Saraiva:** Methodology, Investigation. **Solange A. Pacheco:** Methodology, Investigation, Data curation. **Fátima Vaz:** Project administration, Methodology, Investigation, Data curation. **Sofia Neves:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Deborah Penque:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Peter James:** Supervision, Resources. **Tânia Simões:** Investigation, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT /OpenAI in order to support the revision of grammar and sentence structure in the preparation of this manuscript, as the main authors are not native English speakers. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Consent to participate

Informed consent was obtained from all the individuals/workers recruited for this work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104864.

Data availability

Data will be made available on request.

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