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ORIGINAL RESEARCH

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Proteomic exploration of potential blood biomarkers in haemophilic arthropathy

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Abstract

Background and Aims: The pathophysiology of haemophilic arthropathy (HA) is complex and largely undefined. Proteomic analyses provide insights into the intricate mechanisms of the HA.

Our study aimed to identify differentially expressed proteins in relation to the severity of HA, explore their pathophysiological roles, and evaluate their potential as HA biomarkers.

Methods: Our cross-sectional observational study encompassed 30 HA patients and 15 healthy subjects. Plasma samples were pooled into three groups of 15 samples from those with severe haemophilic arthropathy (sHA), mild haemophilic arthropathy (mHA) and healthy controls. Proteomic analysis was performed using liquid chromatography-mass spectrometry. The severity of HA was assessed using the World Federation of Haemophilia Physical Examination Score and ultrasonography following the Haemophilia Early Arthropathy Detection with Ultrasound (HEAD-US) guidelines.

Results: A total of 788 proteins were identified, with 97% of the uniquely identified proteins being expressed in all analysed groups. We identified several up and downregulated proteins across the groups that were mainly related to inflammatory and immunity-modulating processes, as well as joint degeneration. We highlighted ten proteins relevant for the development of HA: cathepsin G, endoplasmic reticulum aminopeptidase 2, S100-A9, insulin-like growth factor I, apolipoprotein (a), osteopontin, pregnancy zone protein, cartilage oligomeric matrix protein, CD44, and cadherin-related family member 2.

Conclusion: Our analysis identified several proteins that shed further light on the distinctive pathogenesis of HA and could serve for biomarker research. However, these results need to be validated on a larger patient group.

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arthropathy, biomarkers, haemophilia, pathophysiology, proteomics

1 | INTRODUCTION

Haemophilia is a rare genetic condition characterized by a deficiency of coagulation factor VIII (haemophilia A) or factor IX (haemophilia B), with similar clinical presentation. The disorder predominantly affects males who inherit an aberrant X chromosome. The estimated prevalence of haemophilia A is 1 in 5,000 males, whereas haemophilia B is found in 1 in 30,000 males.[1] A hallmark of haemophilia is a tendency of bleeding in muscles and joints, whose severity correlates with the level of clotting factor activity. Patients with severe haemophilia experience spontaneous bleeding and significant joint-related challenges, profoundly affecting their mobility and overall quality of life. Patients with severe haemophilia (F VIII or FIX activity <1%) are treated with prophylaxis therapy to reduce hemarthrosis and haemophilic arthropathy (HA).[2] A notable complication is the development of inhibitors - alloantibodies that some patients produce against the infused clotting factors, reducing treatment efficacy. Occasional spontaneous or prolonged bleeding after minor trauma or surgical procedures is characteristic for moderate haemophilia (FVIII or FIX activity 1 – 5%), while it is rare in mild haemophilia (FVIII or FIX activity 5 – 40%). Bleeding episodes primarily affect large synovial joints such as elbows, knees and ankles leading to painful and debilitating HA.[1,2]

The damage seen in HA [2] has similarities to that in osteoarthritis (OA), and also to the inflammation characteristic of rheumatoid arthritis (RA).[3] However, HA is distinguished by joint haemorrhage, in contrast to the systemic inflammation observed in RA or the age-related degeneration observed in OA.[4] The pathophysiology of HA is derived from direct interactions that involve blood and joint cartilage, as well as indirect interactions driven by inflammatory processes.[1] Although intraarticular bleeding is recognized as a primary risk factor, the precise aetiology of HA remains unresolved.[5] The predominant processes implicated in HA include synovitis, cartilage, and bone degeneration, as well as vascular remodeling.[6] Excessive iron release from erythrocytes into the synovial tissue stimulates synovial proliferation and activates macrophages. This leads to the production of cytokines that inhibit chondrocyte activity and suppress proteoglycan synthesis, initiating cartilage degeneration.[1,6,7] Furthermore, pro-inflammatory milieu promotes the activity of matrix metalloproteinases (MMPs) and aggrecanases, pivotal enzymes involved in the breakdown of collagen and proteoglycans.[8] The resulting inflammation and hypertrophy of the synovium induce a state of hypoxia, leading to increased angiogenesis.[4,9] Direct exposure of cartilage to blood results in deterioration of the extracellular matrix (ECM) and chondrocyte apoptosis.[4,7,8]

The hemarthrosis is the pivotal reason for a disbalance leading to a lowered bone mineral density (BMD) and the onset of osteoporosis in these patients.[8] This is exacerbated by multiple factors including infections such as hepatitis C and the human immunodeficiency virus as well as reduced physical activity that often goes hand in hand with vitamin D deficiency.[8–12] Furthermore, the inflammation-induced bone resorption may be associated to modifications in the RANKL/RANK/OPG pathway, contributing to the pathogenesis of osteoporosis.[8–10,13]

Using proteomics to study haemophilia and HA has the potential to shed light on the complex mechanisms of blood-induced joint damage.[14] So far, this has been addressed by Kriegsman et al., who analysed synovial tissues from six patients with HA. They suggested a predictive value in proteins such as ferritin light and heavy chain, alpha and beta haemoglobin subunits, truncated coagulation factor VIII peptide, beta and gamma fibrinogen peptides and annexin A2, and in peptides derived from cathepsin B and cathepsin D.[5] In our study, we compared the plasma proteomic profiles of patients with severe and mild HA with healthy (non-haemophilia) control group. Our goal was to identify differentially expressed proteins (DEP) in relation to the severity of HA, infer their pathophysiological roles, and evaluate their potential suitability as biomarkers for the detection and classification of HA.

2 | METHODS

2.1 | Study participants and study outline

This cross-sectional observational study was approved by the Institutional Ethics Committee of the University Hospital Centre Zagreb (EP- 8.1-20/73-2/02/21). Subjects were enrolled at the University Hospital Centre Zagreb between May 2020 and June 2021, where they also provided a signed informed consent. The inclusion criteria were: haemophilia, male gender, age older than 25 years. Participants who had experienced joint bleeding in the last 30 days, had a bone fracture in the past 3 months, or had hyperparathyroidism, hyper- or hypothyroidism, Paget's disease, inflammatory arthritis, or were currently on glucocorticoid therapy, were excluded from the study. Additionally, patients were screened to rule out potential conditions that could influence the expression of inflammatory markers (Supplementary table 1). Healthy control blood samples were provided by male volunteers without haemophilia (aged 30–53) following the inclusion and exclusion criteria.

To assess global changes in protein expressions between the analysed groups, we decided to pool samples into three groups each consisting of 15 samples from patients with severe HA, patients with mild HA, and healthy controls (Figure 1).[15]

2.2 | Participant stratification

Participants were stratified by the severity of HA using ultrasound and clinical examination. Ultrasonographic evaluation and scoring was performed by two independent board-certified rheumatologists in accordance with the Haemophilia Early Arthropathy Detection with Ultrasound (HEAD-US) guidelines.[16] The clinical assessment was objectivized using the WFH Physical Examination Score (Gilbert Score), a haemophilia-specific physical examination scoring system, which was performed by a physiatrist.[17]

2.3 | Plasma sample collection

Plasma samples from haemophilic patients were pooled according to the severity of HA into severe HA ($n = 15$) and mild HA ($n = 15$), compared with pooled plasma proteomes of healthy individuals ($n = 15$) (Figure 1).

Blood samples (~10 mL per participant) were drawn by venepuncture and stored in two 5 mL vacuette blood collection tubes containing 3.8% sodium citrate (blood to anticoagulant ratio 1:9). Plasma was isolated by centrifugation at 1500 *g* for 15 min and stored at −80°C until further analysis.

2.4 | Liquid Chromatography-Mass spectrometry (LC-MS)

Protein concentrations in the samples were determined by spectrophotometry using the Lowry protein assay (BioRad RC DC assay, Hercules, CA, USA). Pooled protein samples (100 µg in total) in five technical replicates were processed in 10 kDa molecular weight cut-off spin filters. They were first denatured using 8 M urea, then alkylated in 55 mM iodoacetamide in 8 M urea and finally digested with TPCK treated trypsin in a 1:50 ratio (Worthington Industries, Columbus, OH, SAD). The peptides obtained were concentrated, purified using C18 StageTips, and stored at −80°C until analysis.[18]

The eluted peptides were analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) performed using the UltiMate 3000 RSLCnano system online coupled with an Orbitrap Exploris 480 spectrometer (Thermo Fisher Scientific). See the Supplementary methods 1 for full details regarding the analyses. The MS

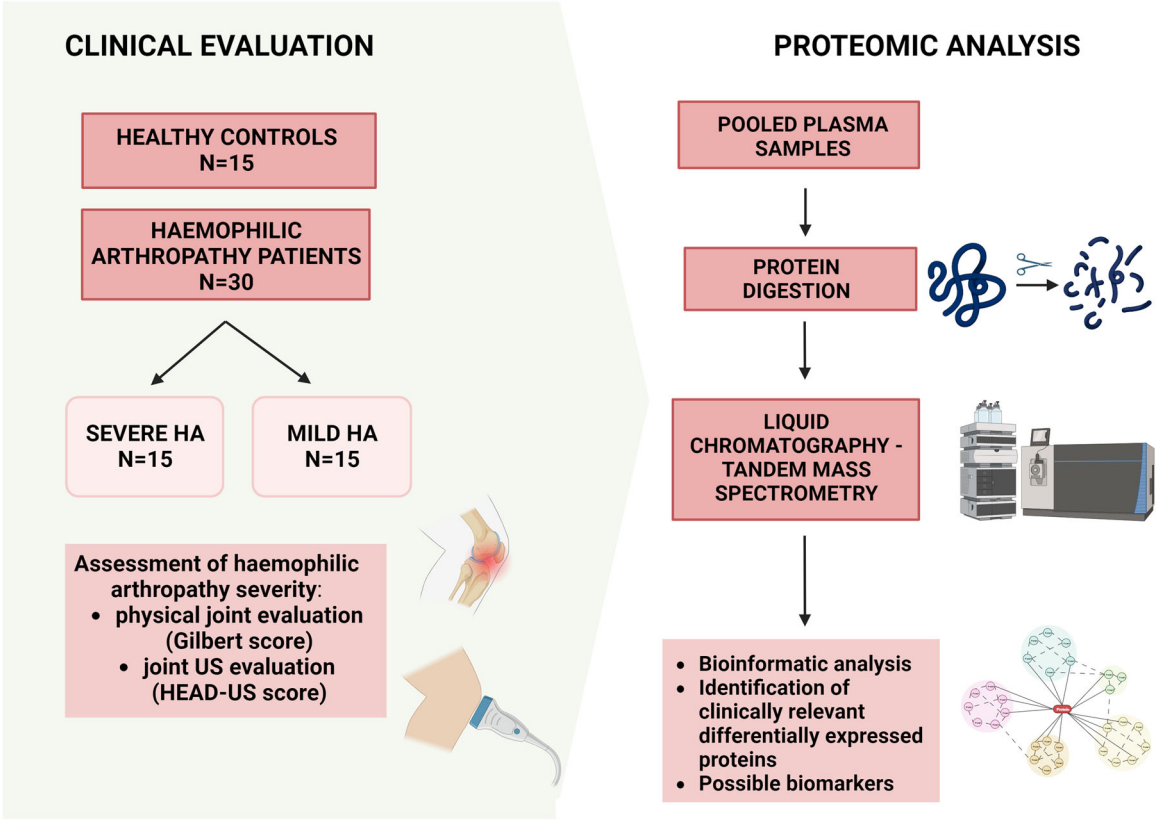


FIGURE 1 Study outline depicting the main steps of clinical evaluation and proteomic analysis methods, as well as the study groups. Image created with Biorender. com.

TABLE 1 Participants' clinical characteristics.

	Groups			
	Healthy control $n = 15$	Mild HA $n = 15$	Severe HA $n = 15$	*p*-value
Age (years): median (IQR)	39.0 (33.0 – 46.0)	40.0 (31.0 – 45.0)	52.0 (46.0 – 57.0)	<0.001[b]
BMI (kg/m^2): median (IQR)	27.8 (23.5 – 30.4)	26.1 (24.8 – 27.5)	26.6 (22.2 – 31.9)	0.631[b]
Haemophilia type A: n (%)		13 (86.7)	13 (86.7)	>0.999[c]
Patients with inhibitors: n (%)		0 (0.0)	6 (40.0)	0.017[c]
HEAD US total score: median (IQR)		7.0 (3.0 – 11.8)	33.0 (30.8 – 39.3)	<0.001[b]
Pain: median (IQR)[a]		0.0 (0.0 – 1.0)	1.0 (1.0 – 2.0)	0.003[b]
Bleeding: median (IQR)[a]		0.0 (0.0 – 0.0)	1.0 (0.0 – 1.0)	<0.001[b]
Joint physical examination: median (IQR)[a]		2.0 (1.8 – 8.3)	46.5 (41.0 – 53.7)	<0.001[b]

Abbreviations: BMI, body mass index; HA, haemophilic arthropathy; IQR, interquartile range (25th – 75th percentile); US, ultrasound.

[a]Joint evaluation according to the World Federation of Haemophilia Physical Examination Score (Gilbert Score).

[b]Evaluated by Mann-Whitney *U* test.

[c]Evaluated by Fishers' exact test.

raw data were deposited at the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD045469.

2.5 | Data analysis

Participants' characteristics, categorical data (haemophilia type, number of patients with detected inhibitors) are presented as frequencies with corresponding percentages, while continuous data are presented as medians with corresponding interquartile ranges. Differences in categorical variables were analysed by Fisher's exact test while the Mann-Whitney U test was used to assess differences in continuous data. Differences between HA groups and healthy control group in age and BMI were assessed with Kruskal-Wallis test. Statistical calculations were performed with MedCalc® Statistical Software version 22.013.[19] Type I error (alpha) was set at 0.05.

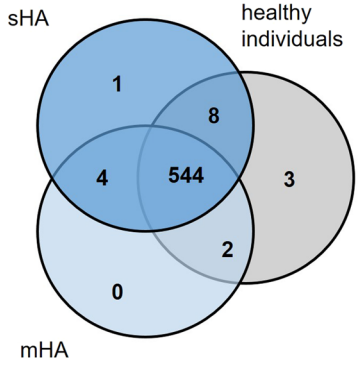
MS data were evaluated using protein maximum label free quantification (MaxLFQ) of intensities reported in the DIA-NN main report file, and further processed using the software container environment (https://github.com/OmicsWorkflows), version 4.6.3a. The processing workflow is available on request. Briefly, it covered: (a) removal of low-quality precursors and contaminant protein groups, (b) protein group MaxLFQ intensities log2 transformation, (c) elimination of protein groups not quantified in more than half of the replicates of at least one sample type, (d) imputation of missing values from the random distribution around the global minimal value, (e) differential expression analysis using the LIMMA statistical test. Proteins with an adjusted p-value < 0.05 and a fold change >2 were considered significantly changed.

The interactions between detected proteins, as well as their gene ontology categories and functional enrichment, were evaluated using String 11.5 software and the SR plot.[20,21] An extensive literature search of selected biomarker candidates was performed in the MEDLINE database, and their pathophysiological significance was estimated by manual curation.

3 | RESULTS

This study included $N = 15$ patients with severe HA, $N = 15$ patients with mild HA and $N = 15$ healthy controls. The descriptive data on the clinical characteristics of the patient are summarized in Table 1. All patients with severe HA had severe haemophilia, and all patients with mild HA had mild haemophilia. All patients with severe haemophilia received prophylactic therapy, while those with mild haemophilia received on-demand therapy. Patients with mild HA had a median HEAD US score of 7.0 (IQR: 3.0 to 11.8), while those with severe haemophilia displayed a markedly elevated median score of 33.0 (IQR: 30.8 to 39.3), underscoring a more pronounced degree of joint impairment in this cohort (Table 1). For patients with mild HA, the median joint physical examination score was a low 2.0 (IQR: 1.8 to 8.3); those with severe HA had a significantly higher median of 46.5 (IQR: 41.0 to 53.7), demonstrating clinically significant joint impairment (Table 1). The joint physical examination score and the HEAD-US score were influenced by the fact that five patients with severe haemophilia each had a total knee replacement, while one patient with mild haemophilia had a single total knee replacement. We note that joints with endoprostheses were not scored clinically or by ultrasound (Table 1).

(A)



(B)

Group(s)	Proteins specifically detected only in experimental groups
sHA (compared to mHA and healthy individuals)	Tubulin beta chain
sHA and mHA (compared to healthy individuals)	Adenylate kinase isoenzyme 1 Extracellular serine/threonine protein kinase FAM20C Arylacetamide deacetylase-like 3 60 kDa heat shock protein, mitochondrial
sHA and healthy individuals (compared to mHA)	Leukocyte immunoglobulin-like receptor subfamily A member 3 Glucosidase 2 subunit beta Interferon-induced protein with tetratricopeptide repeats 2 N-fatty-acyl-amino acid synthase/hydrolase PM20D1 Contactin-4 Phosphatidylethanolamine-binding protein 4 Coronin-1A Bone morphogenetic protein 1
mHA and healthy individuals (compared to sHA)	Myosin light chain 1/3, skeletal muscle isoform PDZ and LIM domain protein 1
healthy individuals (compared to sHA and mHA)	Tubulin alpha-4A chain Platelet-derived growth factor receptor beta Probable non-functional immunoglobulin heavy variable 3-35

FIGURE 2 (A) Venn diagram depicting the overlapped, and group-specific proteins identified in the plasma of analysed patient groups. (B) Proteins specifically detected in sHA and healthy individuals, as well as combinations of mutually expressed proteins in the analysed experimental groups. mHA, mild haemophilic arthropathy; sHA - severe haemophilic arthropathy.

A total of 788 proteins (655 protein groups) were identified by LC-MS, 86% (562) of which were sucessfully annotated to their respective genes by the used gene ontology software. The five replicates were consistent, i.e. had a comparable number of protein identifications and intensity ranges (Supplementary Figure 1). A high level of homogeneity in the proteins identified based on the unique (proteotypic) peptides was identified, as 97% (544) of them were shared. Three proteins were specific to healthy individuals, and only one to severe HA. Healthy individuals shared two proteins with mild HA that were not expressed in sHA, and eight proteins with severe HA patients that were not expressed in mHA. Groups with HA shared four common plasma proteins that were not expressed in healthy individuals (Figure 2).

Proteins expressed in patients with severe HA and healthy individuals that had markedly different expression levels were plotted on a volcano plot. Predetermined thresholds were established with a statistical significance p-value < 0.05 and a minimum of two times the difference in under-expression/overexpression between the two groups, determined by their ratios of log2 transformed LFQ intensities (Figure 3). A total of 16 proteins were significantly upregulated and 26 proteins were significantly downregulated in severe HA compared to healthy individuals (Table 2).

A clustering of up- and downregulated proteins was observed in severe HA group in comparison to the healthy control group (Figures 4A and 4B)). The downregulated proteins roughly follow three main categories: cell-cell interaction (ECM receptor interaction, focal adhesion), infection, and immune response (HPV infection, phagosomes, malaria) and signalling and cellular regulation (TGF-β signalling pathway, PI3K-Akt pathway) (Figure 4C). Proteins upregulated in severe HA are related to cancer and different cellular signalling pathways (glioma, HIF-1 and Rap-1 signalling), metabolic processes (thiamine and histidine metabolism) and others (Figure 4D).

There are 18 proteins that are upregulated in severe HA when compared to mild HA. These proteins are involved in inflammatory process (CD44, RBP4, PBP, CNTN4, PF4, SPARC, THBS1), immunity process (IGF-I, HLA-A, SPARC, BMPG, THBS1, immunoglobulins), cell adhesion (CD44, CNTN4), angiogenesis (PBP, CD44, ROBO4, THBS1), cancer (CD44, ROBO4, multimerin-1), transport (RBP4), degenerative joint disease (RBP4, SPARC, THBS1), reactive oxygen species signalling and endoplasmatic reticulum stress (THBS1), cytokine regulation (LTBP-1), cytokine and chemotaxis (PBP, PF4), cell growth (SPARC), coagulation (multimerin-1).

There are 12 proteins that are downregulated in severe HA compare to mild HA. This proteins are involved in inflammatory process (ENPP2, SAA-1, SAA-2, CRP), immunity process (immunoglobulin, HLA-C), cell proliferation and migration (ENPP2) cell adhesion (Cadherin 2), cancer (ENPP2), degenerative joint disease (CKM), cytokine and chemotaxis (ENPP2), lipid metabolism (ENPP2), carbohydrate metabolism (Pancreatic alpha-amylase), energy (CKM), oxido-redox process (Protein disulphide isomerase), metabolic enzyme (CES1P1).

4 | DISCUSSION

We compared pooled plasma proteomes from participants with severe HA, mild HA and healthy controls to identify DEPs associated with the disease severity, infer their pathophysiological roles and potential utility as biomarkers for distinguishing and grading HA. Notably, due to the nature of disease progression, there was a significant age difference between the HA patient groups, with the severe HA group being older. Changes in protein expression between the groups were associated to inflammation and immune modulation, implying their roles in HA pathophysiology. Among the 16 proteins

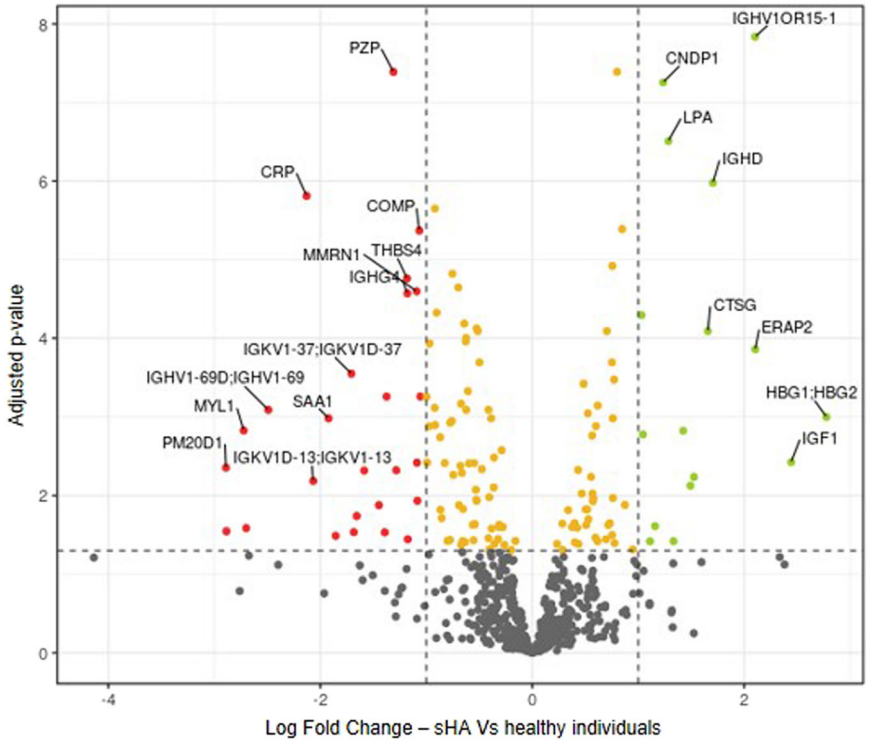


FIGURE 3 Volcano plot comparing protein expression levels in the plasma of patients with severe haemophilic arthropathy (sHA) and healthy control subjects. The dashed lines on the graph indicate thresholds: first, *p*-value ≤ 0.05 (values adjustment on multiple hypothesis testing using the Benjamini & Hochberg method, -log10-transformed) and second, log2-transformed ratio of severe HA vs control samples higher than 1, or lower than −1. Up- and downregulated proteins that meet both thresholds are shown in green and red dots, respectively. The yellow dots represent proteins that did not meet the second threshold. For clarity, full protein names are omitted and are available as supplementary data through the PRIDE database.

upregulated in severe HA (compared to healthy subjects), an interesting dichotomy was observed, as we saw an increase in proteins associated to both disease worsening and alleviation. Several proteins indicated the activation of inflammatory processes and cartilage degradation, namely cathepsin G (CTSG). Its role in the synovial fluid of knee OA patients was suggested to be the degradation of lubricin, an important factor in degenerative joint disease.[22] Increased CTSG activity was also found in the synovial fluid of RA patients, and its chemotactic role was suggested in the pathogenesis of synovial inflammation in RA.[23] Furthermore, elastase and CTSG affect the level of active MMPs, governing ECM degradation, which is critical for the loss of integrity of articular cartilage in RA. We found an upregulated CTSG in the plasma of patients with mild and severe HA compared to healthy subjects. Secondly, we found an upregulation of endoplasmic reticulum aminopeptidase 2 (ERAP2) in both severe and mild HA, compared to healthy subjects. While direct links between ERAP2 and HA have yet to be made, it has however been linked to inflammatory arthritis. A study by Zhang et al. showed that that gene and protein expression of ERAP2 is significantly higher in CD4 + T cells from RA patients, which triggered inflammasome assembly, activated Caspase-1, and induced pyroptosis in CD4 + T cells. Pyroptosis is a type of programmed cell death that contributes to inflammation and is therefore an important pathogenetic factor in RA.[24] While RA has a distinct and different pathogenesis from HA, this data taken together with our results point to possible associations of ERAP2 in multiple arthritides.

Finally, in severe HA, the inflammation-inducing S100-A9 protein was upregulated, highlighting its possible role in leukocyte recruitment and stimulation of cytokine production. Lee et al. identified it as one of the proteins that have the potential to distinguish RA patients from healthy controls, but its exact role in HA is to be further studied.[25]

In contrast to those that likely worsen disease outcome, we identified several proteins with seemingly protective roles in inflammatory joint diseases, like the upregulated IGF-1 in severe HA. IGF-I enhances the growth and differentiation of chondrocytes and osteoblasts *in vitro*, and in the serum of RA patients, it affects T cell apoptosis.[26,27] In severe and mild HA, we also saw the upregulation of apolipoprotein (a) (Apo(a)), a protein that suppresses the production of pro-inflammatory cytokines TNF-α and IL-1β in synovial tissue. Furthermore, it is absent in patients in remission, suggesting its specific role during the active disease phase and therefore it is important in RA development.[28] IL-1β and TNF-α were shown to contribute to cartilage degradation and synovial inflammation in HA.[6] Taken together, the upregulation of mediators that could potentially improve HA can reflect a systemic 'defence mechanism' to counter disease progression.

We also identified numerous downregulated immunity/inflammation mediators like osteopontin (OPN), which promotes synovitis and bone/cartilage deterioration, thus contributing to RA and OA.[29] This protein could be a potential biomarker for early-stage blood-induced joint disease. Elevated synovial fluid OPN was previously associated to OA progression and knee damage, suggesting its role as a biomarker of OA severity.[30,31] In severe haemophilia, a significant increase in plasma OPN was observed in paediatric patients with synovitis, as was a positive correlation to MRI scores in haemophiliacs.[32] Additionally, Czajkowska et al showed a correlation between the concentrations of selected markers of bone turnover and

TABLE 2 Significantly differentially expressed proteins in severe haemophilic arthropathy (sHA) compared to healthy individuals.

Proteins upregulated in sHA versus healthy individuals		
Accession	**Description**	**log fold change**
P69891	Hemoglobin subunit gamma-1 (Gamma-1-globin) (Hb F Agamma) (Hemoglobin gamma-1 chain) (Hemoglobin gamma-A chain)	2.78
P05019	Insulin-like growth factor I (IGF-I) (Mechano growth factor) (MGF) (Somatomedin-C)	2.44
Q6P179	Endoplasmic reticulum aminopeptidase 2 (EC 3.4.11.-) (Leukocyte-derived arginine aminopeptidase) (L-RAP)	2.10
A0A075B7D0	Immunoglobulin heavy variable 1/OR15-1 (nonfunctional)	2.10
P01880	Immunoglobulin heavy constant delta (Ig delta chain C region) (Ig delta chain C region NIG-65) (Ig delta chain C region WAH)	1.71
P08311	Cathepsin G (CG) (EC 3.4.21.20) [Cleaved into: Cathepsin G, C-terminal truncated form]	1.66
P13727	Bone marrow proteoglycan (BMPG) (Proteoglycan 2) [Cleaved into: Eosinophil granule major basic protein (EMBP) (MBP) (Pregnancy-associated major basic protein)]	1.53
P00568	Adenylate kinase isoenzyme 1 (AK 1) (EC 2.7.4.10) (EC 2.7.4.3) (EC 2.7.4.6) (ATP-AMP transphosphorylase 1) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase) (Myokinase)	1.49
Q9UKU6	Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) (TRH-degrading ectoenzyme) (EC 3.4.19.6) (Pyroglutamyl-peptidase II) (PAP-II) (TRH-specific aminopeptidase) (Thyroliberinase)	1.42
P0DP23	Calmodulin-1	1.33
P08519	Apolipoprotein(a) (Apo(a)) (Lp(a)) (EC 3.4.21.-)	1.29
Q96KN2	Beta-Ala-His dipeptidase (EC 3.4.13.20) (CNDP dipeptidase 1) (Carnosine dipeptidase 1) (Glutamate carboxypeptidase-like protein 2) (Serum carnosinase)	1.23
Q4KWH8	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-1 (EC 3.1.4.11) (Phosphoinositide phospholipase C-eta-1) (Phospholipase C-eta-1) (PLC-eta-1) (Phospholipase C-like protein 3) (PLC-L3)	1.16
P35579	Myosin-9 (Cellular myosin heavy chain, type A) (Myosin heavy chain 9) (Myosin heavy chain, non-muscle IIa) (Non-muscle myosin heavy chain A) (NMMHC-A) (Non-muscle myosin heavy chain IIa) (NMMHC II-a) (NMMHC-IIA)	1.11
P05062	Fructose-bisphosphate aldolase B (EC 4.1.2.13) (Liver-type aldolase)	1.04
P06702	Protein S100-A9 (Calgranulin-B) (Calprotectin L1H subunit) (Leukocyte L1 complex heavy chain) (Migration inhibitory factor-related protein 14) (MRP-14) (p14) (S100 calcium-binding protein A9)	1.03
Proteins downregulated in sHA versus healthy individuals		
Accession	**Description**	**log fold change**
Q6GTS8	N-fatty-acyl-amino acid synthase/hydrolase PM20D1 (EC 3.5.1.114) (EC 3.5.1.14) (Peptidase M20 domain-containing protein 1)	−2.89
Q9NP78	ABC-type oligopeptide transporter ABCB9 (EC 7.4.2.6) (ATP-binding cassette sub-family B member 9) (ATP-binding cassette transporter 9) (ABC transporter 9 protein) (hABCB9) (TAP-like protein) (TAPL)	−2.89
P05976	Myosin light chain 1/3, skeletal muscle isoform (MLC1/MLC3) (MLC1F/MLC3F) (Myosin light chain alkali 1/2) (Myosin light chain A1/A2)	−2.72
P58166	Inhibin beta E chain (Activin beta-E chain)	−2.70
A0A0B4J2H0	Immunoglobulin heavy variable 1-69D	−2.49
P02741	C-reactive protein [Cleaved into: C-reactive protein(1-205)]	−2.13

(Continues)

TABLE 2 (Continued)

Proteins downregulated in sHA versus healthy individuals		
Accession	**Description**	**log fold change**
A0A0B4J2D9	Immunoglobulin kappa variable 1D-13	−2.07
P0DJI8	Serum amyloid A-1 protein (SAA) [Cleaved into: Amyloid protein A (Amyloid fibril protein AA)	−1.92
P10321	HLA class I histocompatibility antigen, C alpha chain (HLA-C) (HLA-Cw) (Human leukocyte antigen C)	−1.85
A0A075B6S9	Probable nonfunctional immunoglobulinn kappa variable 1-37	−1.71
A0A075B6K2	Immunoglobulin lambda variable 3-12	−1.68
P62701	40S ribosomal protein S4, X isoform (SCR10) (Single copy abundant mRNA protein) (Small ribosomal subunit protein eS4)	−1.66
P10451	Osteopontin (Bone sialoprotein 1) (Nephropontin) (Secreted phosphoprotein 1) (SPP-1) (Urinary stone protein) (Uropontin)	−1.59
Q9Y279	V-set and immunoglobulin domain-containing protein 4 (Protein Z39Ig)	−1.45
P35590	Tyrosine-protein kinase receptor Tie-1 (EC 2.7.10.1)	−1.39
P01718	Immunoglobulin lambda variable 3-27 (Ig lambda chain V-IV region Kern)	−1.38
P20742	Pregnancy zone protein (C3 and PZP-like alpha-2-macroglobulin domain-containing protein 6)	−1.31
P04746	Pancreatic alpha-amylase (PA) (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)	−1.28
P35443	Thrombospondin-4	−1.18
P01861	Immunoglobulin heavy constant gamma 4 (Ig gamma-4 chain C region)	−1.18
A0A0C4D-H43	Immunoglobulin heavy variable 2-70D	−1.18
Q13201	Multimerin-1 (EMILIN-4) (Elastin microfibril interface located protein 4) (Elastin microfibril interfacer 4) (Endothelial cell multimerin) [Cleaved into: Platelet glycoprotein Ia*	−1.09
Q0VF96	Cingulin-like protein 1 (Junction-associated coiled-coil protein) (Paracingulin)	−1.09
Q14766	Latent-transforming growth factor beta-binding protein 1 (LTBP-1) (Transforming growth factor beta-1-binding protein 1) (TGF-beta1-BP-1)	−1.08
P49747	Cartilage oligomeric matrix protein (COMP) (Thrombospondin-5) (TSP5)	−1.07
P15814	Immunoglobulin lambda-like polypeptide 1 (CD179 antigen-like family member B) (Ig lambda-5) (Immunoglobulin omega polypeptide) (Immunoglobulin-related protein 14.1) (CD antigen CD179b)	−1.06

interleukin 6 to the development of HA.[33] Another study also identified abnormal markers of bone metabolism in patients with haemophilia.[34] This further affirms the possible link between bone metabolism and HA which needs to be studied further.

We also detected pregnancy zone protein (PZP) which inhibits or antagonizes the synthesis or activity of proteases. Elevated PZP was confirmed in synovial fluid-derived exosome proteomes in patients with RA compared to OA.[35] Additionally, PZP was also elevated in the sera of RA patients compared to control subjects.[36] Interestingly, our findings of downregulated OPN and PZP in severe and mild HA are in contrast to the aforementioned studies in other arthropathies and even in HA.[32] These differences underline the need for future confirmation studies and also imply differences in the underlying pathophysiological mechanisms that distinguish HA from other arthropathies. Furthermore, the discrepancy in OPN expression could be attributed to the fact that our study involved adult patients, stressing the influence of patient age and clinical condition in studying the role of OPN in HA.

Several studies on haemophiliac patients have addressed the expression of cartilage ((oligomeric matrix protein (COMP), a cartilage turnover marker that stabilizes type II collagen fibres, however, the results were inconsistent.[37–39] COMP is correlated to joint space narrowing in adult patients with HA, but not with overall radiological score, while biomarker combinations of uCTX-II, COMP, and CS846

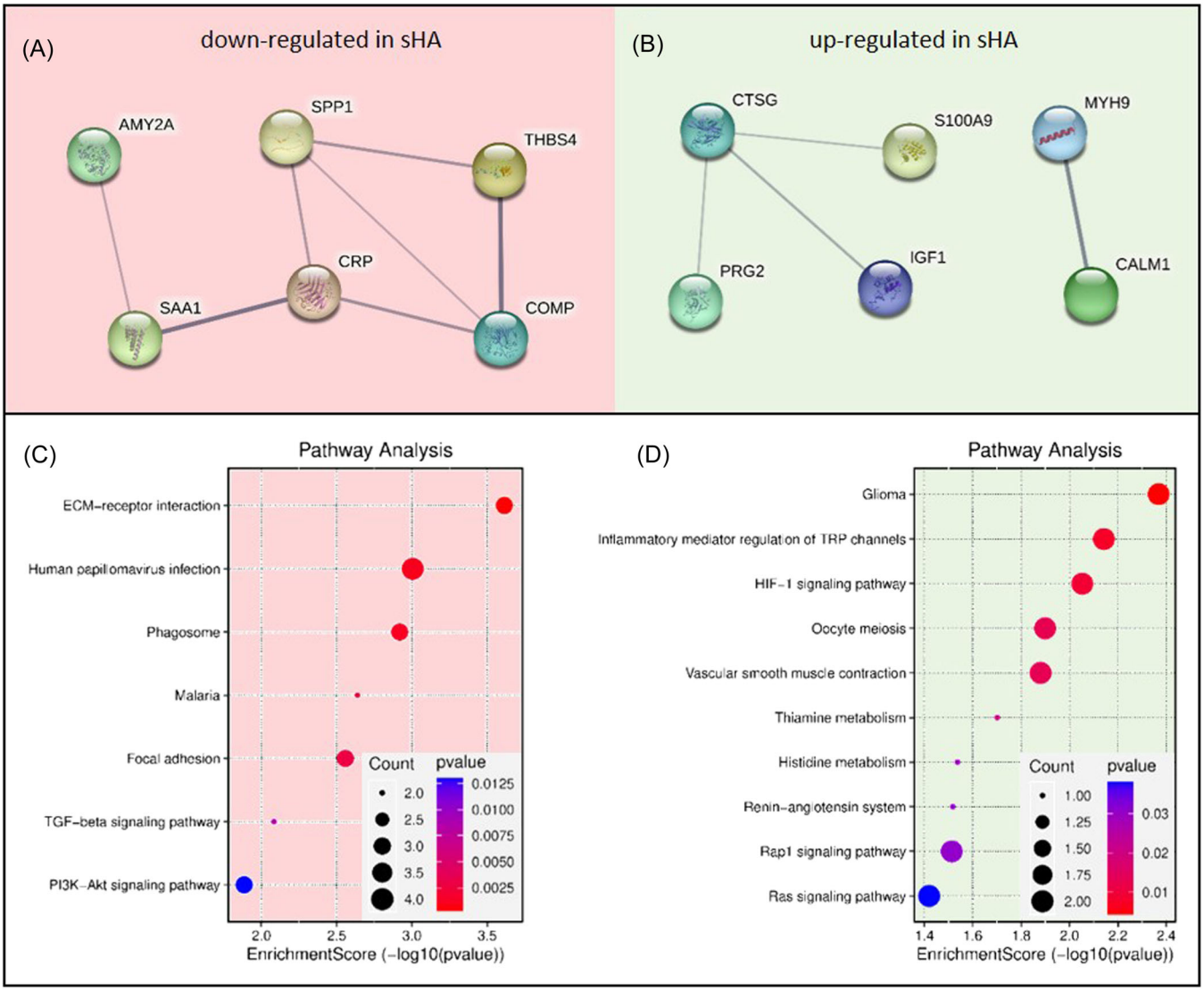


FIGURE 4 Interaction network showing connections between proteins that are statistically significantly (p-value < 0.05) downregulated (A) and up- regulated (B) in the plasma of patients with severe haemophilia (sHA) compared to healthy individuals. The thickness of the grey line depicts the confidence of interaction as predicted by the String 11.5 software. For clarity, full protein names are omitted and are available as supplementary data through the PRIDE database. The lower panels display the analysis of the pathway, that is, the cellular pathways linked to downregulated (panel C; red background) and upregulated (panel D; green background) in sHA compared to healthy individuals. The size of the dot corresponds to the number of proteins ascribed to a certain pathway within the group. The presented molecular pathways are ranked from left to right (lowest to highest) and colour coded according to their EnrichmentScore, i.e. statistical significance. ECM, extracellular matrix; HIF, hypoxia-inducible factor; PI3K, phosphatidylinositol 3-kinase; TGF, transforming growth factor; TRP, transient receptor potential.

show the best correlation to HA severity.[40] Higher levels of COMP were reported in patients with severe adult haemophilia, but no correlation was found to the radiological and physical joint examinations.[41] On the contrary, similar to our results, Sun et al. found lower levels of COMP in adult patients with severe haemophilia.[37] Finally, a number of studies failed to find changes in COMP expression in severe and moderate adult haemophilia.[42–44] These opposing results warrant caution when interpreting the significance of COMP, as additional research is obviously needed.

When comparing severe to mild HA cases, we also detected several DEP involved in inflammatory processes. Bone marrow proteoglycan (BMPG) and IGF-1 were upregulated in severe HA Vs healthy, and even more so in severe Vs mild HA patients, suggesting an enhanced inflammatory response relative to disease severity.[45] Our research revealed that LTBP-1, which is involved in regulating transforming growth factor beta 1 (TGF-β 1), is downregulated in both severe and mild HA compared to healthy subjects.[46] However, it is upregulated in severe versus mild HA. Given the role of TGF-β 1 in immune function, the differential expression of LTBP-1 suggests a potential modulation of immune function as the disease progresses and becomes more severe. We found that, CD44, a surface receptor implicated in pathological angiogenesis, was upregulated in severe

HA compared to mild, highlighting its potential significance in differentiating between levels of HA severity.[47] Furthermore, our findings indicate that Cadherin-related family member 2 (CDHR2) is under-expressed in severe versus mild HA and more in mild HA compared to healthy individuals.[48] Given its role, this suggests a possible disruption of the endothelial barrier in severe cases, leading to increased vascular permeability and thus contributing to bleeding episodes. Finally, Multimerin 1 (MMRN1) which regulates thrombus formation, is downregulated in both severe and mild HA, potentially affecting coagulation.[49] Interestingly, MMRN1 levels are higher in severe HA than in mild, suggesting a possible compensatory response to more severe coagulation challenges.

Factor VIII plays a crucial role in the coagulation process, and its status can affect inflammatory processes. Activated protein C (APC), generated through the thrombin–thrombomodulin–endothelial protein C receptor (TM-EPCR) complex, can inactivate the coagulation cofactors VIII/VIIIa and V/Va, thereby reducing the generation of thrombin and factor Xa. This mechanism is essential for maintaining the balance between coagulation and inflammation.[50] This system also plays a role in modulating inflammation, innate immunity, and tissue repair. When APC is not bound to EPCR, it can inactivate factors Va/VIIIa and promote fibrinolysis, thereby increasing plasmin, which regulates fibrin clot size, clears cross-linked fibrin, and recruits inflammatory cells to the site of injury for healing. These mechanisms highlight the importance of factor VIII in the crosstalk between coagulation and inflammation, which may be relevant to the pathophysiology of haemophilic arthropathy.

There are several weaknesses of our study. Firstly, as this was a single-centre study, one of its inherent limitations is cohort size, that is, the number of subjects, which is additionally limited by the rarity of the disease studied. The pooling of samples can be acknowledged both as a limitation and as a strength, as it provides a biological "average" of the analysed samples, but also possibly hinders the identification of certain proteins that might be observed in the single sample analysis. When interpreting the results of our study, it is important to recognize that there was a statistically significant age difference between the HA groups, with patients in the severe HA group being older. This is expected as severe HA is more prevalent in older patients compared to those with mild HA, but could be a potential source of bias. Namely, it is possible that some of our findings could be a reflection of age difference, and not of HA severity. Patients with severe HA showed a statistically significant difference in recovery rates from hepatitis C infections compared to those with mild HA, which could be a potential source of bias. It's also important to note that inhibitors were only present in patients with severe HA. Finally, the expression changes driving the pathophysiological mechanisms in HA that are predominantly happening locally in the joint could be masked, and "diluted" when analysing the full plasma proteome of HA patients. Therefore, a superior approach would be to directly sample and analyse the proteome of synovial fluid. On the other hand, plasma analysis would be a less invasive option for patients.

In this study we profiled for the first time the plasma proteome of HA patients with different disease severity, finding several potential biomarkers that require further investigation. Our findings underscore the complex molecular dynamics of HA, however, a gene ontology analysis failed to detect significant differences between disease stages. Although there is no large-scale shift of the plasma proteome over sample groups, the expression patterns of individual proteins indeed revealed observable differences. The subtlety of these differences is further underlined by the lack of exclusively expressed proteins among the different groups. This indicates that refined changes in the amounts of key molecular players could be driving the progression of HA.

AUTHOR CONTRIBUTIONS

Nataša Kalebota: Conceptualization; Visualization; Writing—original draft; Writing—review and editing. **Ruđer Novak**: Conceptualization; Methodology; Visualization; Writing—original draft; Writing—review and editing. **Stela Hrkač**: Visualization; Writing—original draft; Writing—review and editing. **Porin Perić**: Conceptualization; Visualization; Writing—original draft; Writing—review and editing. **Grgur Salai**: Visualization; Writing—original draft; Writing—review and editing. **Marko Močibob**: Validation; Writing—review and editing. **Marija Pranjić**: Methodology; Visualization; Writing—original draft; Writing—review and editing. **Zbyněk Zdráhal**: Methodology; Writing—original draft; Writing—review and editing. **Václav Pustka**: Methodology; Writing—original draft; Writing—review and editing. **Nadica Laktašić Žerjavić**: Visualization; Writing—original draft; Writing—review and editing. **Milan Milošević**: Methodology; Writing—original draft; Writing—review and editing. **Marijo Vodanović**: Validation; Writing—original draft; Writing—review and editing. **Silva Zupančić Šalek**: Writing—original draft; Writing—review and editing. **Lovorka Grgurević**: Conceptualization; Validation; Writing—original draft; Writing—review and editing.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The MS raw data were deposited at the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD045469.

TRANSPARENCY STATEMENT

The lead author Lovorka Grgurević affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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