

Poor Clearance of Free Hemoglobin Due to Lower Active Haptoglobin Availability is Associated with Osteoarthritis Inflammation

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Ashish Sarkar^{1,2}
Monu^{1,2}
Vijay Kumar³
Rajesh Malhotra³
Hemant Pandit⁴
Elena Jones⁴
Frederique Ponchel⁴
Sagarika Biswas¹

¹Department of Integrative and Functional Biology, CSIR-Institute of Genomics and Integrative Biology, New Delhi, 110007, India; ²Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, 201002, India; ³All India Institute of Medical Sciences, New Delhi, 110029, India; ⁴Leeds Institute of Rheumatic and Musculoskeletal Medicine, School of Medicine, University of Leeds, Leeds, UK

Introduction: Circulating plasma proteins play an important role in various diseases, and analysis of the plasma proteome has led to the discovery of various disease biomarkers. Osteoarthritis (OA) is the most common chronic joint disease, mostly affecting people of older age. OA typically starts as a focal disease (in a single compartment, typically treated with unicompartmental knee replacement), and then progresses to the other compartments (if not treated in time, typically treated with total knee replacement). For this, identification of differential proteins was carried out in plasma samples of OA cases and compared with healthy controls. The aim of this study was to identify circulatory differentially expressed proteins (DEPs) in knee-OA patients undergoing total knee replacement or unicompartmental knee replacement compared to healthy controls and assess their role, in order to have better understanding of the etiology behind OA pathophysiology.

Methods: DEPs were identified with two-dimensional gel electrophoresis (2DE) and isobaric tags for relative and absolute quantification (iTRAQ), followed by liquid chromatography with tandem mass spectrometry. Validation of DEPs was carried out using Western blot and ELISA. Posttranslational modifications were checked after running native gel using purified protein from patients, followed by detection of autoantibodies.

Results: In total, 52 DEPs were identified, among which 45 were distinct DEPs. Haptoglobin (Hp) was identified as one of the most significantly upregulated proteins in OA ($P=0.005$) identified by both 2DE and iTRAQ. Decreased levels of Hp tetramers and increased levels of autoantibodies against Hp β were observed in OA plasma.

Conclusion: Our data suggest that poor clearance of free hemoglobin and low levels of Hp tetramers may be associated with OA pathogenesis and inflammation.

Keywords: osteoarthritis, blood, haptoglobin, hemoglobin, biomarker, inflammation

Introduction

Osteoarthritis (OA) is the most common chronic joint disease associated with aging.¹ Currently, OA accounts for 25% of joint problems² and is the major cause of disability, leading to poor quality of life in the adult population. The etiology of OA is not yet well understood. OA pathogenesis is multifactorial, and its contributing factors are broadly linked to genetics, diet, lifestyle, injury, and obesity.^{3,4}

There is currently a lack of knowledge regarding the cellular and molecular mechanisms initiating the disease and factors leading to bone and cartilage degradation and progression.⁵ Due to this lack of knowledge, OA treatment is largely

Correspondence: Sagarika Biswas
Department of Integrative and Functional Biology, CSIR-Institute of Genomics and Integrative Biology, Near Jubilee Hall, Mall Road, New Delhi, 110007, India
Tel +91 11-2766-2581
Email sagarika.biswas@igib.res.in

symptomatic (ie, controlling pain or modifying lifestyle), allowing the disease to progress. Uncontrolled progression of OA ultimately leads to the need for a joint replacement, which is associated with considerable socioeconomic burden and medical complications.⁶ Currently, various imaging techniques, such as X-rays and magnetic resonance imaging, are used for diagnosis and grading of severity of OA, but these can only detect cartilage and bone degradation once they have already taken place.⁷

For joint replacement, OA patients are recommended either unicompartmental knee replacement (UKR) or total KR (TKR) surgery. In UKR, both cruciate ligaments and the two knee compartments are preserved, leading to faster recovery, more normal gait, better knee flexion, and lower cost than TKR.^{8,9} Normally, TKR is preferred, because of perception that it is more durable,^{10–12} despite the fact that up to 50% patients requiring KRs are suitable for a UKR.^{13–15} It would be useful to identify any specific biomarkers that are more commonly found in patients needing a UKR or those needing a TKR. There is significant interest and an absolute need to identify biomarkers, in order to understand the etiology behind OA pathogenesis and inform early clinical diagnosis, prognosis, and surgical choices for OA. Recently, strategies for biomarker discovery have included genomics, proteomics, metabolomics, and transcriptomics to identify candidate markers.^{16,17}

The aim of the present study was to identify differentially expressed proteins (DEPs) between UKR and TKR OA cases and healthy controls (HCs). For this, we applied proteomic techniques (isobaric tags for relative and absolute quantification [iTRAQ] and two-dimensional gel electrophoresis [2DE]) followed by liquid chromatography with tandem mass-spectrometry (LC-MS/MS) using blood as a source sample. Blood is widely used for screening of potential protein biomarkers, because it is easy to access and offers the possibility of using large cohorts and repeated sampling.^{18,19}

Proteomics has emerged as a highly promising technique for rapid detection of differentially expressed proteome profiles and biomarker discovery in biological samples. This method is widely used for the discovery of disease-associated biomarkers and understanding the pathogenesis of a number of diseases,²⁰ where the most common method applied is a combination of 2DE and mass spectrometry.²¹ However significant improvements can be made by applying more advanced technique — iTRAQ.²²

In this study, we identified 52 DEPs in OA patients' plasma using both 2DE and iTRAQ. Haptoglobin (Hp), a free hemoglobin-scavenging protein,²³ was identified as the most

significant DEP, validated by Western blot and ELISA. Further, pure protein was purified by Immunoprecipitation (IP) and checked for posttranslational modifications (PTMs). Free hemoglobin detection in OA plasma was also carried out to validate our findings and demonstrate its utility and potential specificity for clinical diagnosis.

Methods

Patients

All OA patients (TKR and UKR, n=58) recruited fulfilled the American College of Rheumatology criteria for diagnosis of knee OA,²⁴ with detailed disease history. All patients included in this study had a Kellgren–Lawrence (KL) score of 4 and had been referred to undergo either TKR or UKR. The KL grading system is a common method of classifying²⁵ the severity of knee OA (using grades of 0–4, 4 being most severe).

Sample Collection and Processing

Whole-blood samples (4 mL) were collected from OA patients (TKR 45, UKR 13) in EDTA-coated vacutainers (Ptech, India) prior to surgery. Similarly, blood samples from HCs (n=40) with no clinical history of any joint-related disease, joint pain, or joint discomfort were collected and included.²⁵ Plasma samples were separated by centrifugation at 1,300 g for 10 minutes at 4°C and stored in multiple 20 µL aliquots in an Eppendorf tube at –80°C deep freezer until use. Demographic and clinical information about the OA patients and HCs is presented in [Supplementary Table 1](#).

iTRAQ Labeling and Protein Identification

Three sets of pooled plasma samples were prepared from each group (TKR, UKR, and HCs, each n=12) and processed for protein digestion by trypsin (Promega, USA) followed by iTRAQ labeling using different isobaric tags (TKR tag115, UKR tag116, HCs tag117) and incubated for 2 hours at room temperature. Labeled peptides were then pooled, lyophilized, and analyzed with iTRAQ using standard protocols. For quantitative analysis, proteins with two or more unique peptides in at least one experiment with an unused score ≥ 2 only were considered²⁶ (see details in [Supplementary material](#)).

Two-Dimensional Gel Electrophoresis

Plasma samples were also pooled and proteins (80µg) were subjected to 2DE:^{27,28} the first dimension with isoelectric focusing and the second with SDS-PAGE. Gels were then silver-stained using standard protocols.^{28,29}

Experiments were repeated at least thrice using different pooled samples. Based on densitometry values, protein spots with fold change ≥ 2 in TKR/UKR compared to HCs were screened for protein identification (see details in [Supplementary material](#)).

In-Gel Digestion of Proteins and Preparation of LC MS/MS Analysis

Protein spots were excised from 2DE gels (minimum three gels), processed, and run on the LC MS/MS platform (Applied Biosystems, USA). MS/MS spectra were procured in reflector-positive mode over a mass scope of 10–20 kDa, and proteins having more than two unique peptides were considered⁴ (see details in [Supplementary material](#)).

Relationship with Major Pathways

DEPs were analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery; version 6.8), an online tool for alignment of DEPs, with major pathways described. It provides functional interpretation of large numbers of genes/proteins derived from experimental studies.³⁰ Proteins identified were first converted to their NCBI gene ID, then searched for associated pathways with maximum DEP hits.

Western Blot

Pooled plasma protein (20 μ g) from each group (TKR, UKR and HC) was separated by 12% SDS-PAGE and Western blot performed with an anti-Hp antibody (1:3,000), using standard procedures³¹ (see details in [Supplementary material](#)). Densitometry values of Western blot analysis were normalized with total protein, as well as a housekeeping protein (actin) as loading control.^{32,33} Experiments were repeated at least thrice.

Enzyme-Linked Immunosorbent Assays

ELISAs were performed using a commercial ELISA kit (CloudClone, USA) as per the manufacturer's guideline using TKR (n=40), UKR (n=11), and HCs (n=17). We also performed indirect ELISAs manually, wherein plates were coated with diluted plasma samples (dilution 1:200) from individuals of each group (TKR 45, UKR 13, HCs 40) using standard protocols (see details in [Supplementary material](#)). The anti-Hp antibody (1:2,000) was used to detect Hp.²⁷

Immunoprecipitation and Glycoprotein Staining

IP was performed to isolate pure Hp protein from OA and healthy plasma samples for detection of alteration in its native form using IP columns (Catch and Release version 2.0, Millipore, USA) and the anti-Hp antibody (2 μ g). Concentration of protein in the plasma was measured by the Bradford method. From each group, 500 μ g total protein was added to the IP column as per the manufacturer's guidelines. Immunoprecipitated protein samples (20 μ L) from each group (TKR, UKR and HC) were then run on native (nondenaturing conditions) gel and transferred onto nitrocellulose (NC) membranes. The membranes were then subjected to glycoprotein detection using a GelCode glycoprotein-staining kit (Thermo Fisher Scientific, USA). Protein bands thus obtained were scanned using a ChemiDoc MP imaging system (Bio-Rad, USA).³⁴ The IP-eluted samples (20 μ L) were also run on SDS-PAGE, followed by silver staining. All experiments were repeated for at least thrice (see details in [Supplementary material](#)).

ProQ Diamond Staining

Phosphoprotein staining was performed for phosphoprotein detection using a ProQ gel-staining kit (Invitrogen, USA). Native gel was run as mentioned earlier, followed by phosphoprotein staining of gels and scanning using the ChemiDoc (see details in [Supplementary material](#)).

Autoantibody Detection

Proteins (2 μ g) from immunoprecipitated samples of each group (TKR, UKR, and HCs) and controls (IgG control and plasma control) were separated by 12% SDS-PAGE, transferred to NC membranes, and incubated with diluted plasma (1:500 in PBS) as a source of primary autoantibodies, and incubated for 3 hours at room temperature followed by washing, incubation with goat antihuman horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 5,000, Jackson, USA) for 1 hour and developed with an enhanced chemiluminescence agent (G Bioscience, USA). Densitometry analysis was carried out using Image Lab 3.00 (Bio-Rad). Experiments were repeated at least thrice (as described in [Supplementary material](#)).

Ubiquitination

Proteins (10 μ g) from immunoprecipitated samples of each group (TKR, UKR, and HCs) were separated with 12%

SDS-PAGE, subsequently transferred to NC membranes, and blocked with 5% BSA. Blots were then incubated with antiubiquitin antibody followed by incubation with HRP-conjugated secondary antibody after washing. The membranes were then developed as mentioned earlier (see details in [Supplementary material](#)).

Free-Hemoglobin Detection

Free hemoglobin detection was carried out in plasma samples using a human free-hemoglobin ELISA kit (G Bioscience) as per the manufacturer's guidelines (details in [Supplementary material](#)).

Statistical Analysis

Data were normalized using suitable controls according to the platform used. Graph was plotted using optical density and densitometry values using Microsoft Excel and GraphPad Prism (version 5). To compare data from different groups, we used nonparametric tests, as data were not normally distributed. Mann-Whitney *U* tests and ANOVA were used to compare data from two or more groups and χ^2 for categorical data. $P < .05$ was considered significant, and Bonferroni correction for multiple testing was applied.

Results

Identification and Quantification of Differentially Expressed Proteins in OA-Patient Plasma

In total 52 DEPs were identified using proteomic techniques (2DE and iTRAQ, [Table 1](#)): 15 with 2DE ([Figure 1](#)) and 37 with iTRAQ ([Table 1](#)). However, with the 2DE method, several spots with the same accession number were identified per protein (probably reflecting isoforms), resulting in eight distinct DEPs. With iTRAQ, all 37 identified DEPs were distinct proteins. Therefore, 45 DEPs were identified in KL4-grade OA-patient plasma samples compared to HCs ($P < 0.05$, [Table 1](#)). Among these 45 distinct DEPs, 30 were upregulated in TKR and 23 in UKR, while 21 were found to be common to both TKR and UKR patients compared to HCs ([Figure 2A](#)). Similarly, 13 proteins were downregulated in TKR and 20 in UKR, while ten were downregulated in both TKR and UKR patients compared to HCs ([Figure 2B](#)). Among these proteins, ten were highly upregulated or downregulated (samples 3–7, 29, 30, 32, and 34 upregulated, sample 8 downregulated; [Table 1](#)) with fold change ≥ 2 in OA-patient plasma compared to HCs. Among all DEPs, Hp

and serum albumin were found to be up regulated on both techniques (2DE and iTRAQ, [Table 1](#)). Hp was one of the most significantly upregulated proteins (2.39-fold, $P = 0.05$) in OA patients compared to HCs. As such, the mechanism of its overexpression in OA was investigated further.

DAVID Pathway Analysis of DEPs

DAVID pathway analysis of all the identified DEPs provided the maximum hits for the “complement and coagulation pathway”. Seven proteins — A2M, F13A1, C3, FGA, SERPINA1, SERPINC1, and SERPINF2 — were found to be associated with this pathway ([Supplementary Figure 1](#)). Pathway enrichment indicated not only involvement of immunoresponse and inflammation but also homeostasis or related problems among OA patients.³⁵

Validation with Western Blot and ELISA

For initial confirmation and validation, OA (TKR 12, UKR 12) and HC (12) plasma samples were pooled from same-age and -sex participants. The DEP level of Hp was then validated in pooled samples with Western blotting ([Figure 3A](#)). Densitometry values were normalized by total protein ([Figure 3A](#), lower panel) and actin ([Supplementary Figure 2](#)) as loading control. Densitometry analysis followed by normalization revealed 1.5-fold and 1.4-fold increased Hp expression in TKR and UKR, respectively, compared to HCs ([Figure 3B](#)). ELISAs on with individual plasma samples of OA (TKR 40, UKR 11) and HCs (17), revealed 0.8-fold ($P = 0.0003$) and 0.95-fold ($P = 0.05$) lower Hp levels in TKR and UKR than HCs, respectively. Moreover, there was 0.89-fold ($P = 0.05$) lower expression in TKR than UKR. The corresponding concentration of Hp was 1,417 ng/ μ L in TKR and 1,584 ng/ μ L in UKR compared to 1,655 ng/ μ L in HC plasma samples ([Figure 3C](#)). Further, the results of indirect ELISAs performed manually with increased sample sizes also revealed similar results ([Supplementary Figure 3](#)). The ELISA results were thus observed to be contradictory to our findings on 2DE gel, iTRAQ, and Western blot analysis. The main difference between Western blot and ELISA is that Western blot measures denatured protein with linearized epitope antibodies and is more likely to be able to quantify total protein. In contrast, ELISA measures native protein and may be sensitive to PTMs, protein complexes, and structural epitopes.

Table 1 Differentially expressed proteins (DEPs) identified by iTRAQ and 2DE in TKR/UKR blood plasma samples compared to HCs

	% Cov (95)	Accession	Name	Peptides (95%)	List of DEPs identified by 2DE			List of DEPs identified by both techniques
					Spots, n	Name/accession	Peptides (95%)	
1	56	sp P02768 ALBU_HUMAN	Serum albumin OS= <i>Homo sapiens</i> OX=9606 GN=ALB PE=1 SV=2	181	1	Haptoglobin/ sp P00738 HPT_HUMAN	24	Serum albumin/ sp P02768 ALBU_HUMAN
2	46.7	sp P02787 TRFE_HUMAN	Serotransferrin OS= <i>Homo sapiens</i> OX=9606 GN=TF PE=1 SV=3	147	2	Transthyretin/ tr A6XGLI A6XGLI_HUMAN	209	Haptoglobin/ tr J3QR68 J3QR68_HUMAN
3	47.4	sp P01023 A2MG_HUMAN	α_2 macroglobulin OS= <i>Homo sapiens</i> OX=9606 GN=A2M PE=1 SV=3	140	3	Transthyretin/ tr A6XGLI A6XGLI_HUMAN	27	
4	59.8	sp P01009 A1AT_HUMAN	α_1 antitrypsin OS= <i>Homo sapiens</i> OX=9606 GN=SERPINA1 PE=1 SV=3	138	4	Haptoglobin/ sp P00738 HPT_HUMAN	72	
5	58.7	tr J3QR68 J3QR68_HUMAN	Hba (fragment) OS= <i>Homo sapiens</i> OX=9606 GN=HP PE=1 SV=1	87	5	Haptoglobin/ sp P00738 HPT_HUMAN	42	
6	81.7	sp P69905 HBA_HUMAN	Hba OS= <i>Homo sapiens</i> OX=9606 GN=HBA1 PE=1 SV=2	69	6	Epididymis secretory sperm binding protein/ tr E9KL23 E9KL23_HUMAN	3	
7	37.6	sp P00450 CERU_HUMAN	Ceruloplasmin OS= <i>Homo sapiens</i> OX=9606 GN=CP PE=1 SV=1	51	7	cDNA FLJ78413/ tr A8K9P0 A8K9P0_HUMAN	50	
8	19.2	sp P01024 CO3_HUMAN	Complement C3 OS= <i>Homo sapiens</i> OX=9606 GN=C3 PE=1 SV=2	32	8	sp P02768 ALBU_HUMAN	100	
9	65.3	sp P68871 HBB_HUMAN	Hb β OS= <i>Homo sapiens</i> OX=9606 GN=HBB PE=1 SV=2	32	9	cDNA FLJ78413/ tr A8K9P0 A8K9P0_HUMAN	131	

(Continued)

Table 1 (Continued).

					List of DEPs identified by 2DE			List of DEPs identified by both techniques
	% Cov (95)	Accession	Name	Peptides (95%)	Spots, n	Name/accession	Peptides (95%)	Name/accession
10	43.1	sp P02647 APOA1_HUMAN	ApoA1 OS= <i>Homo sapiens</i> OX=9606 GN=APOA1 PE=1 SV=1	30	10	Epididymis secretory sperm binding protein/ tr E9KL23 E9KL23_HUMAN	3	
11	33.8	sp P02790 HEMO_HUMAN	Hemopexin OS= <i>Homo sapiens</i> OX=9606 GN=HPX PE=1 SV=2	28	11	Haptoglobin/ sp P00738 HPT_HUMAN	178	
12	26.9	sp P04217 A1BG_HUMAN	α_{1B} glycoprotein OS= <i>Homo sapiens</i> OX=9606 GN=A1BG PE=1 SV=4	27	12	HP Protein/ tr Q6NSB4 Q6NSB4_HUMAN	212	
13	58.5	sp P02042 HBD_HUMAN	Hb δ OS= <i>Homo sapiens</i> OX=9606 GN=HBD PE=1 SV=2	26	13	Haptoglobin/ tr J3QR68 J3QR68_HUMAN	196	
14	57.8	sp P02766 TTHY_HUMAN	Transthyretin OS= <i>Homo sapiens</i> OX=9606 GN=TTR PE=1 SV=1	18	14	HP Protein/ tr Q0VAC5 Q0VAC5_HUMAN	92	
15	32.8	sp P00739 HPTR_HUMAN	Haptoglobin-related protein OS= <i>Homo sapiens</i> OX=9606 GN=HPR PE=2 SV=2	17	15	Haptoglobin/ tr J3QR68 J3QR68_HUMAN	75	
16	25.4	sp P02763 A1AG1_HUMAN	α_1 -acid glycoprotein I OS= <i>Homo sapiens</i> OX=9606 GN=ORM1 PE=1 SV=1	15				
17	14.7	tr C9J V77 C9J V77_HUMAN	α_2 -HS glycoprotein OS= <i>Homo sapiens</i> OX=9606 GN=AHSG PE=1 SV=1	14				
18	20.4	tr D6RF35 D6RF35_HUMAN	Vitamin D-binding protein OS= <i>Homo sapiens</i> OX=9606 GN=GC PE=1 SV=1	12				

(Continued)

Table 1 (Continued).

					List of DEPs identified by 2DE			List of DEPs identified by both techniques
	% Cov (95)	Accession	Name	Peptides (95%)	Spots, n	Name/accession	Peptides (95%)	Name/accession
19	17.6	tr A0A286YEY1 A0A286YEY1_HUMAN	IgG ₁ (fragment) OS= <i>Homo sapiens</i> OX=9606 GN=IGHA1 PE=1 SV=1	12				
20	22	sp P06727 APOA4_HUMAN	ApoA4 OS= <i>Homo sapiens</i> OX=9606 GN=APOA4 PE=1 SV=3	9				
21	20.1	tr A0A0G2JPA8 A0A0G2JPA8_HUMAN	α_2 antiplasmin OS= <i>Homo sapiens</i> OX=9606 GN=SERPINF2 PE=1 SV=1	9				
22	11	sp P43652 AFAM_HUMAN	Afamin OS= <i>Homo sapiens</i> OX=9606 GN=AFM PE=1 SV=1	9				
23	22.9	sp P19652 A1AG2_HUMAN	α_1 -acid glycoprotein 2 OS= <i>Homo sapiens</i> OX=9606 GN=ORM2 PE=1 SV=2	9				
24	36.8	tr V9GYM3 V9GYM3_HUMAN	ApoA2 OS= <i>Homo sapiens</i> OX=9606 GN=APOA2 PE=1 SV=1	8				
25	6.7	tr B7ZKJ8 B7ZKJ8_HUMAN	ITIH4 protein OS= <i>Homo sapiens</i> OX=9606 GN=ITIH4 PE=1 SV=1	8				
26	11.8	sp P01019 ANGT_HUMAN	Angiotensinogen OS= <i>Homo sapiens</i> OX=9606 GN=AGT PE=1 SV=1	6				

(Continued)

Table I (Continued).

					List of DEPs identified by 2DE			List of DEPs identified by both techniques
	% Cov (95)	Accession	Name	Peptides (95%)	Spots, n	Name/accession	Peptides (95%)	Name/accession
27	9.9	tr A0A286YFY4 A0A286YFY4_HUMAN	Ig heavy constant γ_2 (fragment) OS= <i>Homo sapiens</i> OX=9606 GN=IGHG2 PE=I SV=1	6				
28	13.3	tr A0A1B0GUU9 A0A1B0GUU9_HUMAN	Ig heavy constant μ (fragment) OS= <i>Homo sapiens</i> OX=9606 GN=IGHM PE=I SV=1	5				
29	3.8	sp P02671 FIBA_HUMAN	Fibrinogen α chain OS= <i>Homo sapiens</i> OX=9606 GN=FGA PE=I SV=2	4				
30	7.8	sp P02750 A2GL_HUMAN	Leucine-rich α_2 glycoprotein OS= <i>Homo sapiens</i> OX=9606 GN=LRG1 PE=I SV=2	4				
31	4.6	sp P00488 F13A_HUMAN	Coagulation factor XIII A chain OS= <i>Homo sapiens</i> OX=9606 GN=F13A1 PE=I SV=4	4				
32	14.7	sp P27169 PONI_HUMAN	Serum paraoxonase/ arylesterase I OS= <i>Homo sapiens</i> OX=9606 GN=PONI PE=I SV=3	4				
33	8.2	sp P01008 ANT3_HUMAN	Antithrombin III OS= <i>Homo sapiens</i> OX=9606 GN=SERPINC1 PE=I SV=1	3				

(Continued)

Table 1 (Continued).

	% Cov (95)	Accession	Name	Peptides (95%)	List of DEPs identified by 2DE		List of DEPs identified by both techniques
					Spots, n	Name/accession	
34	21.5	sp P00915 CAHI_HUMAN	Carbonic anhydrase I OS= <i>Homo sapiens</i> OX=9606 GN=CA1 PE=1 SV=2	3			
35	0.6	sp P04114 APOB_HUMAN	ApoB ₁₀₀ OS= <i>Homo sapiens</i> OX=9606 GN=APOB PE=1 SV=2	2			
36	5	sp P22792 CPN2_HUMAN	Carboxypeptidase N2 OS= <i>Homo sapiens</i> OX=9606 GN=CPN2 PE=1 SV=3	2			
37	7.1	sp P02760 AMBP_HUMAN	Protein AMBP OS= <i>Homo sapiens</i> OX=9606 GN=AMBP PE=1 SV=1	2			

Posttranslational Modification and Level of Hp Tetramer Protein in Plasma

In order to decipher these contradictory results, Hp PTM was checked. Native protein was purified using IP from OA and HC plasma (pooled from 12 patients in each group of TKR, UKR, and HCs), followed by protein separation with SDS-PAGE and native gels. The gels were then glycostained or phosphostained to detect any change in PTM of Hp tetrameric structure. Glycostaining analysis of Western blot membranes showed (Figure 4A) reductions in glycosylated Hp tetramers to 0.69-fold and 0.83-fold, respectively, in TKR and UKR plasma samples compared to HCs (Figure 4B), suggesting that the decreased levels of glycosylated Hp tetramer in OA plasma may have contributed to the discrepancy between Western blot and ELISA data. However, phosphostaining of native gels showed no significant difference in band intensities of TKR, UKR, or HCs, indicating that

there was no change in the phosphorylation status of Hp (Supplementary Figure 4). To further strengthen our data, the presence of autoantibodies in OA plasma was checked.

Presence of Autoantibodies Against Hp in Plasma

Although OA has not been considered an autoimmune disease,³⁶ many autoantibodies have been reported in OA against such proteins as MAT2 β -AAb,³⁷ CHST14,³⁸ and extracellular matrix proteins,³⁹ but there have been no reports of the presence of autoantibodies against Hp. Interestingly, we identified Hp autoantibodies in OA samples. A band at 52 kDa was observed, suggesting recognition of Hp β (Figure 4C). Densitometry analysis suggested 1.57- and 1.31-fold increase in expression of autoantibodies against Hp β in TKR and UKR patients, respectively, compared to HCs (Figure 4D).

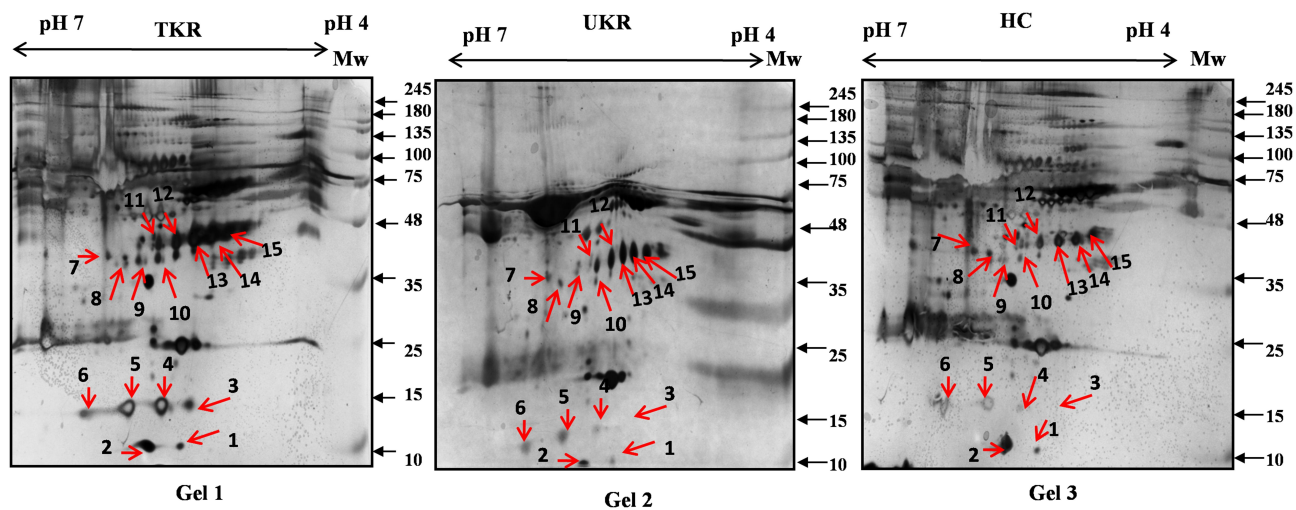


Figure 1 Two-dimensional gel electrophoresis (2DE) of plasma proteins. Representative 2DE gel images of plasma proteins showing differentially expressed protein spots in TKR (gel 1), UKR (gel 2), and HCs (gel 3), marked with arrows and numbered 1–15. Protein spots were excised from the protein gel and identified by liquid chromatography with tandem mass spectrometry.

Abbreviations: Mw, molecular weight; TKR, total knee replacement; UKR, unicompartmental knee replacement; HCs, healthy controls.

DIFFERENTIALLY EXPRESSED PROTEINS (DEPs)

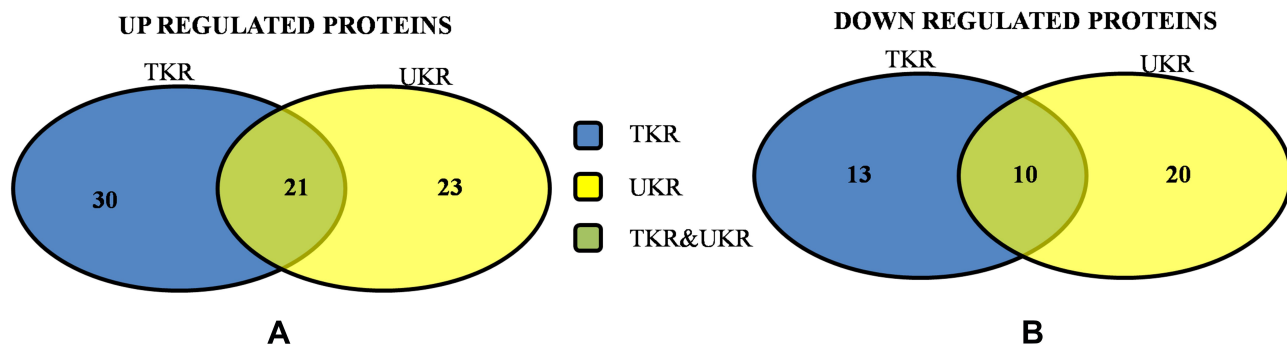


Figure 2 Venn diagrams of distribution of differentially expressed proteins identified in TKR and UKR. **(A)** Upregulated DEPs and their distribution among TKR and UKR: 30 proteins were upregulated in TKR and 23 in UKR patients, while 21 were common to both TKR and UKR patients. **(B)** Similarly, 13 proteins in TKR and 20 proteins in UKR patients were downregulated and ten common to both TKR and UKR patients compared to healthy controls.

Abbreviations: TKR, total knee replacement; UKR, unicompartmental knee replacement.

Lower Levels of Hp β and Its Ubiquitination in Disease

The ubiquitination status of Hp β was checked to find the reason for the presence of autoantibodies against Hp. Densitometry analysis of Western blots of ubiquitinated Hp from TKR/UKR and HCs was performed (Figure 5A). The results showed 0.22-fold and 0.37-fold lower ubiquitination in TKR and UKR samples, respectively, compared to HCs (Figure 5B). We further checked Hp β expression in disease using IP-eluted samples. SDS-PAGE of these followed by silver staining (Figure 5C) suggested that there was significantly less Hp β in TKR (0.47-fold) and UKR (0.41-fold) patients than HCs (Figure 5D). We further

evaluated changes in ubiquitination of Hp β , and found only 45% and 86% ubiquitination in TKR and UKR patients, respectively, compared to 96% ubiquitination of Hp β in HCs. The change in ubiquitination levels among TKR/UKR patients compared to HCs suggests that ubiquitination may possibly be PTMs involved in the production of autoantibodies against Hp in OA patients.

Confirmation of Increased Levels of Free Hemoglobin in OA Patients

Evaluation of free-hemoglobin levels in plasma was performed using ELISAs. Average levels of free hemoglobin in TKR (n=45) and UKR (n=13) patients were

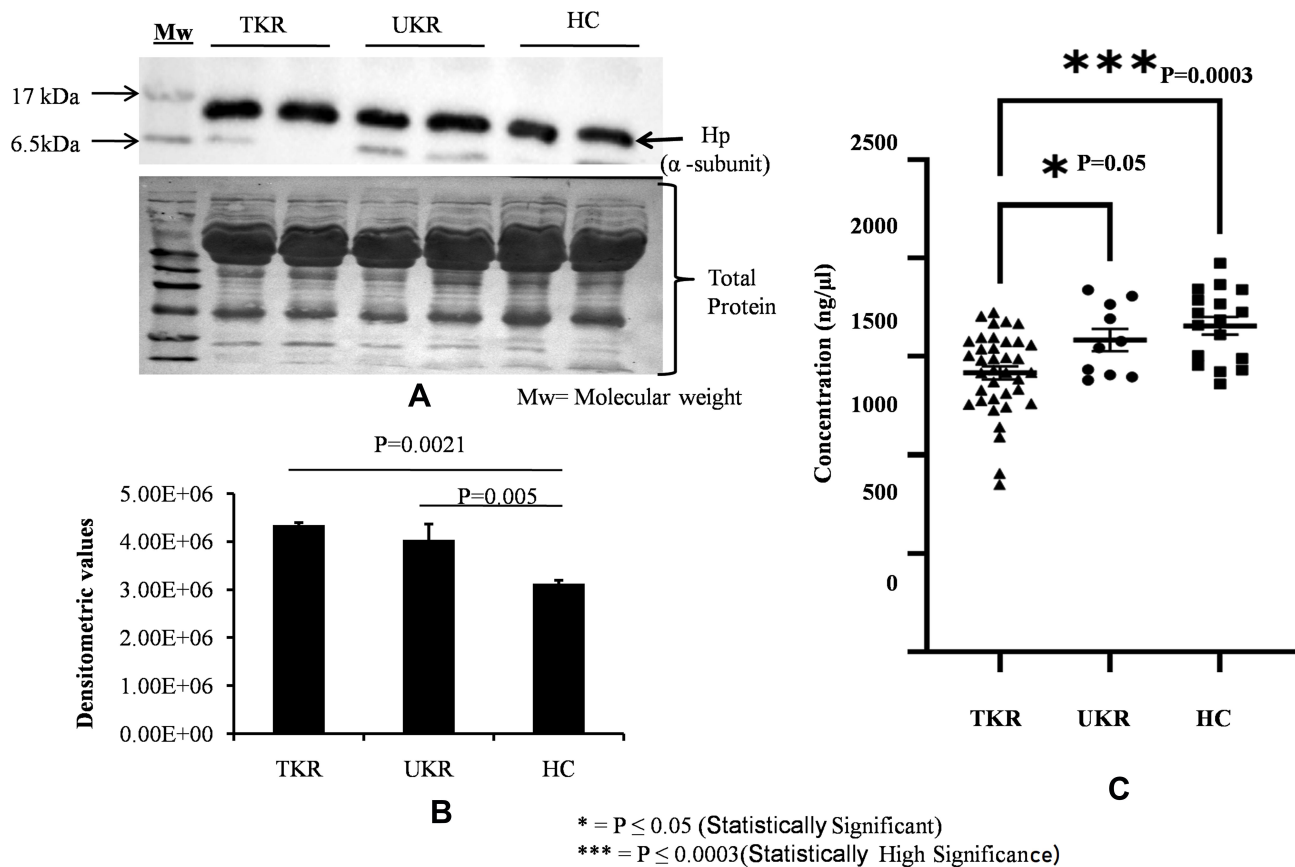


Figure 3 Validation of haptoglobin (Hp) expression in plasma. **(A)** Representative Western blot image of pooled plasma samples (TKR = 12, UKR = 12, HCs = 12) developed using anti-Hp antibody to detect Hp α . Hp α protein expression was normalized with total protein (lower panel) with Ponceau staining of membrane. **(B)** Densitometric analysis showing 1.5 fold upregulated Hp α in TKR ($p=0.0021$) and 1.4-fold in UKR ($p=0.005$) compared to HCs. Analysis was done using mean values of three independent experiments. **(C)** ELISA results showing Hp concentrations in blood plasma of TKR ($n=40$), UKR ($n=11$) and HCs ($n=17$). Average Hp concentration was found lowered by 1,417 ng/ μ L in TKR and 1,584 ng/ μ L compared to 1,655 ng/ μ L in HC plasma samples. The average fold change was found to be 0.8 ($P=0.0003$) and 0.95 ($P=0.05$) in TKR and UKR compared to HCs, respectively. TKR also showed 0.89-fold ($P=0.05$) lower levels of Hp than UKR.

Abbreviations: TKR, total knee replacement; UKR, unicompartmental knee replacement; HCs, healthy controls.

significantly increased to 71 μ g/ml and 66 μ g/mL, respectively, compared to 50 μ g/mL (basal level in human plasma sample) in HC samples ($n=40$, $P=0.003$; Figure 6A). Specificity (95%) and sensitivity (95%) evaluated by a receiver-operating characteristic (ROC) curve (Figure 6B) confirmed true-positive results against false-positive ones.

Discussion

The current study was designed to identify novel OA-associated protein markers in severely affected OA patients (TKR and UKR cases, KL4 grade). The inconclusive clinical difference in deciding the type of joint replacement needed (TKR/UKR) for patients led us to perform a differential protein-profile study, with a belief that DEPs among TKR and UKR patients and HCs may additionally develop into biomarkers for surgeon's choices.⁴⁰ Using a proteomic

approach, Hp was observed to have a better peptide score (209, Table 1), and was hence chosen for detailed study. The Hp protein is well known for its free hemoglobin-clearance activity, linked with the "complement and coagulation pathway"⁴¹ on DAVID pathway analysis. Free hemoglobin has been found to be associated with inflammation,^{42–44} and triggers complement activation via intravascular hemolysis.⁴⁵ Hp consists of 2 α and 2 β chains: Hp α typically has 14–18 kDa molecular mass, while the molecular mass of Hp β is 45 kDa.⁴⁶ The *HP* gene harbors three types of Hp (1-1, 2-1, and 2-2).⁴⁷ Hp1 and Hp2 are susceptible to various diseases (eg, cancer, cardiovascular disease, diabetes, rheumatoid arthritis),^{44,48} but their role in OA is not known. Although some reports have shown that Hp is upregulated in human OA serum samples^{49,50} and in synovial fluid of knee OA, further validation has not been carried out.⁵¹ We found significantly upregulated expression of Hp α in OA

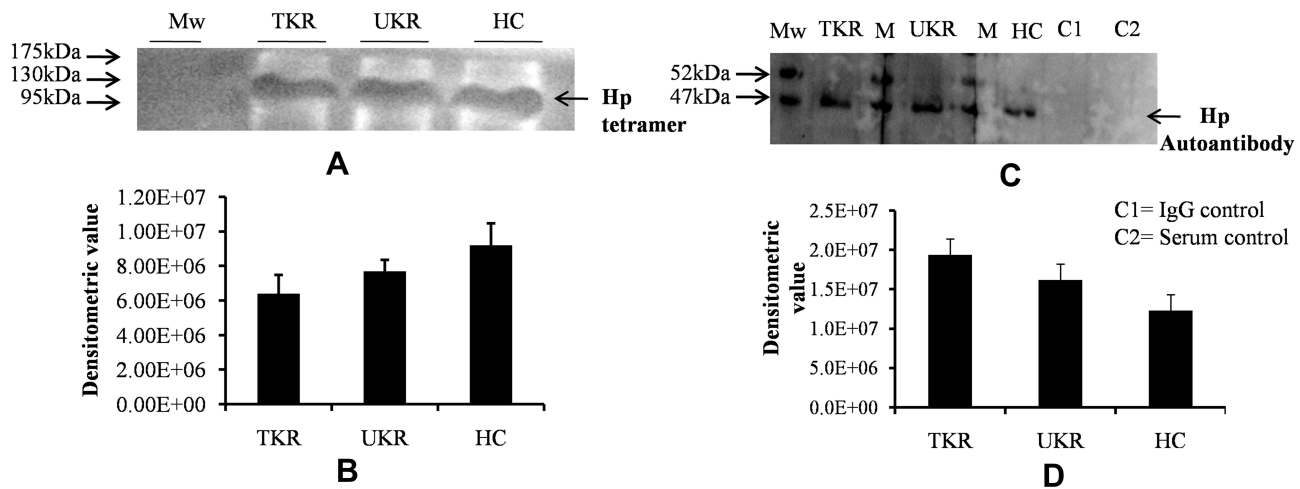


Figure 4 Native-gel analysis and autoantibody detection against haptoglobin (Hp). **(A)** Representative glycoprotein-stained image of Western blot after running native PAGE using purified native Hp protein (tetramer) by immunoprecipitation from plasma samples indicated low levels of Hp tetramer (120 kDa) in TKR and UKR compared to HCs. **(B)** Densitometric analysis of glycosylated Hp native band shows lower levels of Hp tetramer in TKR (0.69-fold) and UKR (0.83-fold) than HCs. **(C)** Western blot was carried out after running SDS-PAGE using IP-eluted purified Hp protein and using blood plasma as source of primary antibodies followed by incubation with antihuman secondary antibody. The image represents increased levels of autoantibodies against Hpβ in TKR and UKR plasma compared to HCs. **(D)** Densitometric analysis of Western blot indicating subsequent increased level of autoantibodies (1.57-fold in TKR and 1.31-fold in UKR) against Hpβ compared to HC plasma samples.

Abbreviations: Mw, molecular weight; TKR, total knee replacement; UKR, unicompartmental knee replacement; HCs, healthy controls; Hp, haptoglobin.

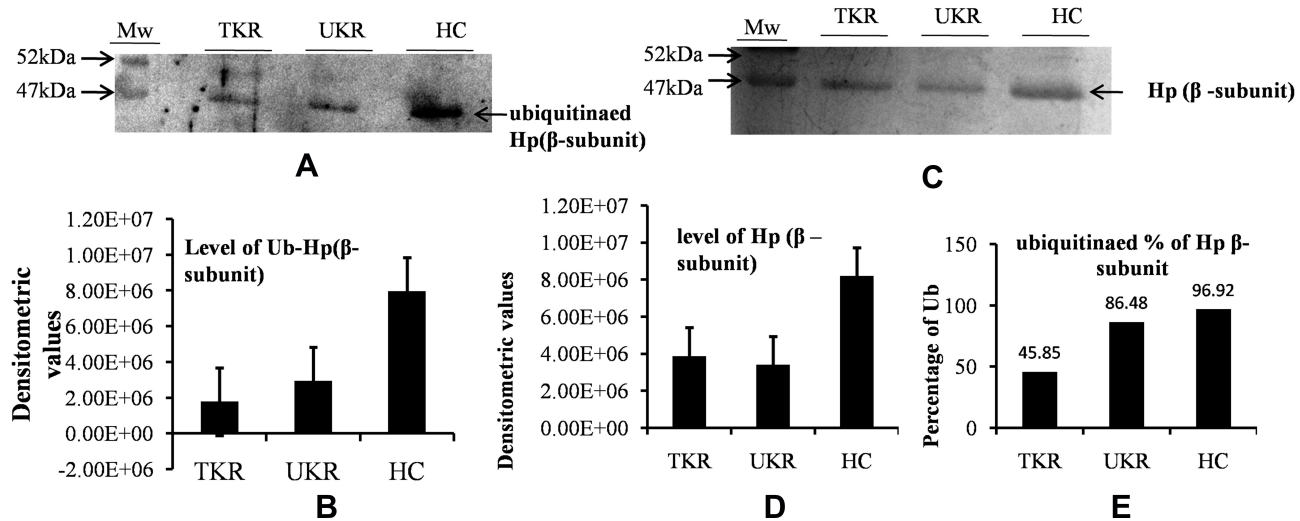


Figure 5 Ubiquitination of haptoglobin (Hp) by Western blot analysis. **(A)** Representative Western blot image showing that there were decreased levels of ubiquitinated Hpβ in TKR/UKR compared to HCs. Ubiquitination of Hpβ was detected using antiubiquitin antibody after immunoprecipitated Hp had been separated on SDS-PAGE followed by Western blot. **(B)** Densitometry analysis showing decreased ubiquitination of Hp in TKR (0.22-fold) and UKR (0.37-fold) compared to HCs. **(C)** Silver-stained image of gel after running SDS-PAGE using purified Hp, indicating lower levels of Hpβ in TKR and UKR patients than HCs. **(D)** Densitometry analysis of gel image for Hpβ showing lower levels of Hpβ in TKR (0.47-fold) and UKR (0.41-fold) than HCs. **(E)** Ubiquitination percentages of Hpβ in TKR, UKR, and HCs, showing 45.85% Hpβ ubiquitinated in TKR and 86.48% in UKR compared to 96.92% in HCs.

Abbreviations: TKR, total knee replacement; UKR, unicompartmental knee replacement; HCs, healthy controls; Ub, ubiquitination.

cases (TKR and UKR) compared to HCs (Figure 3A and B), but this result was not replicated on ELISA. Three Hp proteins have been identified but a reason for their up- or down-regulation has not been put forward.^{48,49} These seemingly contradictory results supported our findings, and we reasoned that this may be possible if there is a difference in native

tetramer form and its denatured form due to the possibility of degradation of protein monomers.⁴⁶ Decreased levels of glycosylated Hp tetramer by native gel in OA plasma (Figure 4A) indicated that there was possibly degradation of proteins (Hp) and that may have triggered autoantibody production.^{52,53} Similar results were also noted while using

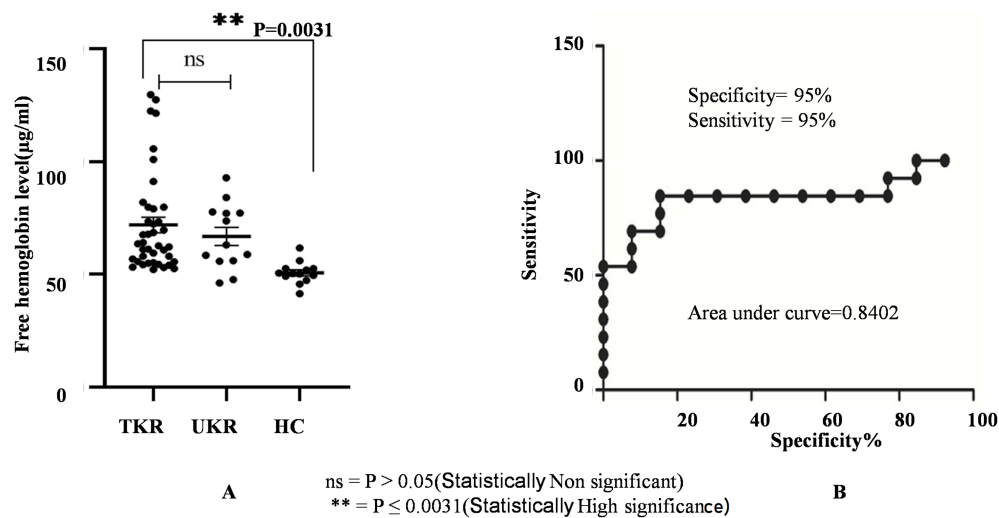


Figure 6 Free hemoglobin plasma-level analysis. (A) Circulating free hemoglobin in plasma was quantified in TKR (n=39), UKR (n=13), and HCs (n=13) using ELISA. The dot plot shows increased circulating free hemoglobin in TKR and UKR blood plasma compared to HCs. Free hemoglobin in TKR was found 71 µg/mL and 66 µg/mL in UKR compared to 50 µg/mL basal level in HCs. (B) ROC plot indicating individual protein abundance in each group with area under curve (AUC) of 0.8402 with 95% sensitivity and 95% specificity between the groups.

Abbreviations: TKR, total knee replacement; UKR, unicompartmental knee replacement; HCs, healthy controls; ROC, receiver-operating characteristic; NS not significant.

individual plasma samples from OA patients (Supplementary Figure 5). However, the inaccurate protein folding in old age may also be an added reason for reduction in glycosylated active Hp.⁵⁴ To confirm our hypothesis, autoantibodies against Hpβ subunit were identified and found to be increased in TKR and UKR (Figure 4, panel C). The involvement of other PTMs (phosphorylation, ubiquitination) status of Hp tetramer was also checked. Phosphorylation status revealed no change in Hp phosphorylation, indicating that autoantibody production was not due to phosphorylation (Supplementary Figure 4).

In ubiquitination, the E3 ubiquitin ligase depletes MARCH2 by targeting substrate-protein degradation, which may be responsible for reduced secretion of Hp.^{54,55} Highly decreased levels (Figure 5B) of ubiquitination in OA cases (TKR 45% and UKR 86%) compared to HCs (97%; Figure 5E) revealed decreased levels of E3 ubiquitin ligase and altered degradation pathway for Hp.⁵⁶ Deregulated ubiquitination events are associated with immunological disorders and autoimmune and inflammatory diseases,⁵⁷ indicating that ubiquitination may be responsible for triggering autoantibody formation in OA. For more confirmation, protein subunits were separated and detected with SDS-PAGE⁵⁸ using purified patient protein (Hp), revealing lower Hpβ levels at 45 kDa (Figure 5C and D) and upregulation in Hpα (Supplementary Figure 6) in UKR/TKR patients compared to HCs. The autoantibody against the Hpβ may lead to its degradation,

and hence lower levels of Hpβ may be responsible for lowering of Hp tetramer formation. However, the key factor responsible for autoantibody formation leading to the degradation of Hpβ remains unclear.

It has reported that the Hp precursor is an N-linked glycoprotein⁵² cleaved into α and β chains,⁴⁶ and it is also known that active Hp binds with hemoglobin at specific sites on Hpβ and β-globin chain of hemoglobin⁵⁹ and alters Hp's biochemical profile,⁴³ and thus the level of Hp increases for the clearance of circulating free hemoglobin in blood,²³ playing a role in the maintenance of cellular iron homeostasis. The increase in free-hemoglobin levels in UKR and TKR patients in our study suggested that Hp has strong potential to neutralize the adverse physiological effects of free Hb.⁴¹

Some reports have concluded that obesity is a low-grade systemic inflammation condition⁶⁰ positively correlated with body-mass index (BMI), progressively increasing the risk of knee OA.^{61,62} As per our observations (supplementary Table 1) the difference in BMI between the OA groups was not found to be significant (TKR and UKR). This may have been due to the small sample of UKR patients. Obese mice deficient in TNFα or TNFα receptors do not exhibit the typical increase in Hp mRNA.⁶³ This signifies that upregulation of Hp is specifically associated with inflammation,⁶⁴ neither due to the obesity nor increased BMI of OA patients. However, future studies looking at a larger cohort may be helpful to understand the chain of events more precisely.

Inappropriate functioning of Hp leads to wear and tear of the joints.^{65,66} Our study provides new evidence that lower levels of Hp tetramer may be one of the factors associated with initiation of inflammation in OA.

Conclusion

Hp was found to be an important factor in regulating hemoglobin homeostasis and inflammation in severely affected knee-OA patients (TKR and UKR surgical cases). Downregulation of active Hp tetramer accompanied by increased levels of free hemoglobin indicated poor clearance of free hemoglobin in patients with severe OA, and may be responsible for disease severity. This study thus contributes to understanding of the inflammation cascade in severe OA patients, and should be helpful in understanding its etiology and developing new treatment strategies.

Data Sharing Statement

For all original data and detail protocol, please contact Dr Sagarika Biswas(sagarika.biswas@igib.res.in).

Ethics Statement

The study protocol was approved by the Medical Ethics Committee of the All India Institute of Medical Sciences, Department of Orthopaedics, New Delhi, India (CSIR-IGIB/IHEC2017-18 Dt.08.02.2018) and the Council of Scientific and Industrial Research, Institute of Genomics and Integrative Biology, Delhi, India (CSIR-IGIB/IHEC/2017-18, February 8, 2018). All study protocols complied with the Declaration of Helsinki.

Consent for Publication

Written signed consent was obtained from all participating patients and healthy volunteers.

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those of the NIHR or the Department of Health and Social Care.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, took part in drafting the article or revising it critically for important intellectual content, agreed to submit to the current journal, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

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