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The alarmins high mobility group box protein 1 and S100A8/A9 display different inflammatory profiles after acute knee injury

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SUMMARY

Objective: To compare the concentrations of high mobility group box 1 protein (HMGB1) and S100A8/A9 in synovial fluid between patients with knee injuries and osteoarthritis (OA), and knee healthy subjects. To investigate associations of alarmin levels with different joint injuries and with biomarkers of inflammation, Wnt signaling, complement system, bone and cartilage degradation.
Methods: HMGB1 and S100A8/A9 were measured in synovial fluid by immunoassays in patients with knee injuries, with OA and from knee healthy subjects, and were related to time from injury and with biomarkers obtained from previous studies. Hierarchical cluster and enrichment analyses of biomarkers associated to HMGB1 and S100A8/A9 were performed.
Results: The synovial fluid HMGB1 and S100A8/A9 concentrations were increased early after knee injury; S100A8/A9 levels were negatively associated to time after injury and was lower in the old compared to recent injury group, while HMGB1 was not associated to time after injury. The S100A8/A9 levels were also increased in OA. The initial inflammatory response was similar between the alarmins, and HMGB1 and S100A8/A9 shared 9 out of 20 enriched pathways. The alarmins displayed distinct response profiles, HMGB1 being associated to cartilage biomarkers while S100A8/A9 was associated to proinflammatory cytokines.
Conclusions: HMGB1 and S100A8/A9 are increased as an immediate response to knee trauma. While they share many features in inflammatory and immunoregulatory mechanisms, S100A8/A9 and HMGB1 are associated to different downstream responses, which may have impact on the OA progression after acute knee injuries.

Introduction

Osteoarthritis (OA) is a multifactorial disease, and several risk factors for developing OA have been identified including age, obesity, genetic factors and joint trauma[1–3]. Disease development involves both structural changes, inflammation and pain. It is well recognized that inflammation is an important element in OA development and proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1 act by stimulating synthesis of proteolytic enzymes, nitric oxide and prostaglandins[4]. However, the therapeutic benefits of TNF or IL-1 neutralization have only shown modest effects in OA[5]. Hence, other, alternative inflammatory mediators may be involved. One such class of mediators are alarmins, a group of endogenous molecules that are released into the extracellular space upon trauma, or by activation of cells. Extracellular alarmins initiate innate and adaptive immune responses that trigger inflammation[6].

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Two of the most well characterized alarmins are high mobility group box 1 protein (HMGB1) and S100A8/A9 (also known as MRP8/14 or Calprotectin), which both have been demonstrated to play a pivotal role for the inflammatory state of cells and tissues. When released from activated or dying cells, HMGB1 and S100A8/A9 bind cell surface receptors such as receptor for advanced glycation endproducts (RAGE) and toll like receptor 4 (TLR4). This initiates intracellular signaling pathways, via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which regulates chemokine and cytokine production, expression of adhesion molecules, cell migration and differentiation of stem cells[7–10].

A number of studies have shown that alarmins play an important role in different inflammatory diseases including sepsis, tissue-trauma and arthritis[11–14]. Elevated levels of HMGB1 and S100A8/A9 have been found in the synovial fluid of arthritis patients and preclinical studies have shown their association with inflammation and structural changes[15–19]. This indicates that alarmins may contribute to some of the pathophysiological aspects observed in OA, However, their role is not elucidated. Knee injury, with subsequent tissue damage and cell death is a major risk factor for developing OA[2,20]. We hypothesized that joint trauma may lead to the release of HMGB1 and S100A8/A9 initiating an inflammatory response, which may play a role in OA development.

The primary aim of this study was to investigate the levels of HMGB1 and S100A8/A9 in synovial fluid from patients with knee injuries compared to knee healthy reference subjects and OA patients. The secondary aim was to compare levels of HMGB1 and S100A8/A9 to clinical parameters including sex, age, time from knee trauma, type of knee injury and intraarticular hemolysis. The third aim was to investigate associations of alarmin levels with markers of inflammation as Wnt signaling, complement system, cartilage and bone degradation.

Materials and methods

Subjects and sampling

Synovial fluid was aspirated without lavage once per subject, centrifuged at 3000g for 10 min at 4 °C, aliquoted and stored at −80°C. Samples were from convenient cohorts of patients with knee injuries ($n = 210$, all injury group), reference subjects without a history of knee symptoms or knee injury and/or with normal clinical findings from radiographic and/or arthroscopic examinations ($n = 17$), and patients with knee OA ($n = 43$; Table I). In group-wise analyses knee injury subjects were stratified into two sub-groups based on time between injury and sampling: recent injury group (0–23 days after injury, $n = 145$), and old injury group (52–549 weeks after injury, $n = 45$) (Table I). For better separation between recent and old injury, the subjects with an intermediate sampling time point (29–334 days after injury, $n = 20$) were omitted in these sub-group analyses. Associations to other biomarkers was performed only in the recent injury group. The study is in accordance with the Helsinki Declaration and approved by the regional ethical review board (Lund, Sweden). Consent to participate were given by all subjects. The samples have been used in previous investigations[21–26].

Assays

HMGB1 ELISA

HMGB1 concentrations in synovial fluid samples were quantified with an ELISA based immunoassay (IBL International) according to the manufacturer's instructions. The immunoassay is developed for serum and plasma and the technical performance for synovial fluid samples is described in Table S1. Freeze-thaw effects, dilution linearity and spiking recovery was determined and the assay using synovial fluid samples behaved similar to serum and plasma samples reported by the manufacturer (data not shown).

S100A8/A9 ELISA

S100A8/A9 concentrations in synovial fluid samples were quantified using an in-house sandwich ELISA as described in Frosch *et al.*[27]. The ELISA detects both active heterodimers and inactive tetramers formed by S100A8 and S100A9. Increased local levels of S100A8/A9 complexes are thought to represent biological active S100A8/A9 heterodimers. Results are expressed as ng/ml S100A8/A9.

Heme Assay

The concentration of total heme in synovial fluid was determined by QuantiChrom™ Heme Assay kit (Nordic Diagnostica #DIHM-250) following the manufacturer's instructions. The technical performance of the assay for synovial fluid samples is described in Table S1.

Other Biomarkers

In the early injury group, we have previously analysed a number of biomarkers related to inflammation (both Olink Proximity extention assay (PEA) and Meso scale Discovery (MSD)) as well as biomarkers related to complement system, Wnt signaling, and cartilage and bone turnover (Table S2). In this study, the data was used to assess associations to HMGB1 and S100A8/A9 levels[25,26,28–30].

Statistical analyses

Shapiro–Wilk tests indicated skewed distributions of the alarmins HMGB1 and S100A8/A9. Comparison of alarmin levels between subject groups were done using Mann–Whitney tests and linear regression models with adjustments for age and sex. Differences in age and sex between groups were assessed by Pearson Chi-square and Students *t*-tests, respectively. For association analyses between alarmins and age or heme, Spearman's rank correlation (r_S) was used. For association analyses between alarmins and sex Mann–Whitney tests were used. For association analyses between alarmins and time after injury linear regression and Pearson correlation were used. Student's *t*-test or analysis of covariance (ANCOVA; adjusted for age, sex and days between injury and aspiration) were used for assessments of alarmins in relation to type of knee injury, and to ascertain normal distribution for these analysis, biomarker data were log10 transformed while confounders were expressed in linear data.

Spearman's rank correlation was used to assess associations between alarmins and other biomarkers. Missing values of the molecular biomarkers: i.e., a biological sample was obtained but the value was below lower limit of quantification (LLOQ) or there was no biological sample available, were imputed using a multiple imputation approach. For each outcome we created 10 imputed datasets using chained equations and simulation values below LLOQ[31]. In a sensitivity analysis, we repeated the main analysis on complete data, i.e., *excluding* persons with missing biomarker values.

All tests were 2-tailed and $P \leq 0.05$ was considered statistically significant. If not otherwise specified, expressions such as “higher” and “lower” in the text are based on statistically significant differences. All statistical analyses above were done using IBM SPSS Statistics (version 24) and Stata 15, StataCorp. 2017; Stata Statistical Software Release 15, College Station, TX; StataCorp LP and R version 3.5.1 (R core team, R Foundation for Statistical Computing, Vienna, Austria).

Main diagnostic groups	Sub-groups of injury	Time between injury and sampling	N (% women)	Age in years, median (range)	Differences in age, *P*-values		
					Reference *vs*	*Recent injury vs*	*Old injury vs*
Reference		–	17 (35)	34.8 (14.5–57.7)	–		
Knee injury[*]		0–549.4 weeks	210 (27)	28.6 (13.2–69.7)	0.130		
	Recent injury	0–23 days	145 (26)	24.5 (13.2–64.4)	**0.013**	–	–
	Intermediate injury	29–334 days	20 (15)	29.0 (16.9–48.7)	–	–	–
	Old injury	52.1–549.4 weeks	45 (36)	33.9 (14.0–69.7)	0.496	**<0.001**	–
Osteoarthritis[†]		–	43 (44)	59.2 (24.5–86.3)	**<0.001**	**<0.001**	**<0.001**

There was no difference in sex between knee healthy reference and the other subject groups (Chi square test, p between 0.425 and 0.985), nor between recent and old injury sub-groups ($p = 0.225$). The osteoarthritis (OA) group had a larger proportion women than the recent injury group ($p = 0.024$). There were differences in age (tested by Student's *T*-test) between subject groups: the recent injury group was younger than the old injury and reference group; the OA group were older than the other groups. Statistically significant group differences ($p < 0.05$) are shown in bold face type.

[*] Knee injuries were diagnosed by magnetic resonance imaging (MRI) and/or by clinical examination and were divided into following groups: isolated anterior cruciate ligament (ACL) or posterior cruciate ligament (PCL) rupture ($n = 19$), isolated meniscus injury ($n = 35$), ACL and meniscus injuries ($n = 47$), patella luxation ($n = 17$), isolated collateral ligament injury ($n = 7$), collateral ligament injury with ACL or PCL and/or meniscus co-injuries ($n = 77$), normal MRI ($n = 8$).

[†] OA patients ($n = 36$) were diagnosed radiographically and/or arthroscopically with a median disease duration of 2.2 years (range 0–22.6 years). According to a previous classification[50], $n = 22$ were early OA, $n = 14$ were late OA; $n = 7$ OA patient samples were collected from arthroplasty and considered end-stage OA.

Table I

Characteristics and demographics of the subject groups

Osteoarthritis and Cartilage

Complete linkage cluster analysis and enrichment analysis

Biomarkers included in the Olink PEA inflammation panel and other molecular markers measured in the synovial fluids from the early knee injury patients were analyzed by heatmap clustering (http://www.heatmapper.ca/expression/). For biomarkers that were analyzed both with PEA and another assay (i.e.., IL-6, TNF and IL-8) PEA data was used. Values were scaled by row and distance was measured by Spearman rank correlation. Rows and columns were clustered by complete linkage clustering. Proportions of gender and type of injury between the groups were compared by Chi-square test or Fisher exact test (Graphpad Prism 8.4.3 or RStudio® version 2021.09.0 + 351). Age, time from injury, heme levels, HMGB1 levels and S100A8/A9 levels between the groups were compared by Kruskal–Wallis multiple comparison test where *P*-values were corrected by Dunn's test (Graphpad Prism 8.4.3). Enrichment analysis of biomarkers significantly correlated with HMGB1 and S100A8/A9 levels, respectively, were performed by http://reactome.org/. Both interactors and project to human were included in the analysis. Pathways with *P*-values < 0.05 were considered significantly enriched.

Results

Synovial fluid alarmin concentrations are increased in patients with recent knee injuries

Based on median values, the synovial fluid HMGB1 and S100A8/A9 levels were approximately 2- and 300-times higher, respectively, in the *recent injury* group compared to the *reference* group; S100A8/A9 levels were approximately 7-times higher in the *OA* group compared to the *reference* group; S100A8/A9 levels were approximately 100-times higher in the *recent injury* group compared to the *old injury* group, and the S100A8/A9 levels were approximately 2-times higher in the *OA* group compared to the *old injury* group (Fig. 1(a) and (b), Table II). A second analysis with a linear regression model, showing the effect and 95% CI, adjusting for age and sex indicated similar results as the Mann Whitney tests, although the HMGB1 data was not statistically different (Table S3).

Synovial fluid concentrations of HMGB1 and S100A8/A9 are associated with age and heme

HMGB1 showed a negative correlation with age in the *old injury* group (Table S4). S100A8/A9 had a negative correlation with age in the *all injury* group, and a positive correlation with age in the OA group. The correlation between alarmin concentrations and age were not statistically significant for the *reference* group, nor between alarmin levels and sex in any of the subject groups (Table S4). In the *recent injury* and *OA* groups, there was a moderate to strong positive correlation between heme and the alarmins; in the *old injury* group, heme correlated positively to S100A8/A9 but not to HMGB1 (Table S4).

Synovial fluid S100A8/A9 concentrations are negatively associated with time after injury

For HMGB1, the correlation did not show significance to time after injury in any of the injury groups (Fig. 1(c) and (d), Table S5). In the *all injury* and *recent injury* groups, S100A8/A9 concentrations were associated with time after injury where levels were high early after injury and displayed a negative correlation with time from injury; this negative association remained after heme adjustments. Heme concentrations showed no association to time after injury (data not shown).

Soft tissue injuries have an impact on synovial fluid alarmin concentrations in the recent injury group

A sub-cohort of the recent injury group ($n = 114$) had magnetic resonance imaging (MRI) verified knee injury diagnoses, which were used for assessment of association between type of injury and synovial fluid concentrations of alarmins.

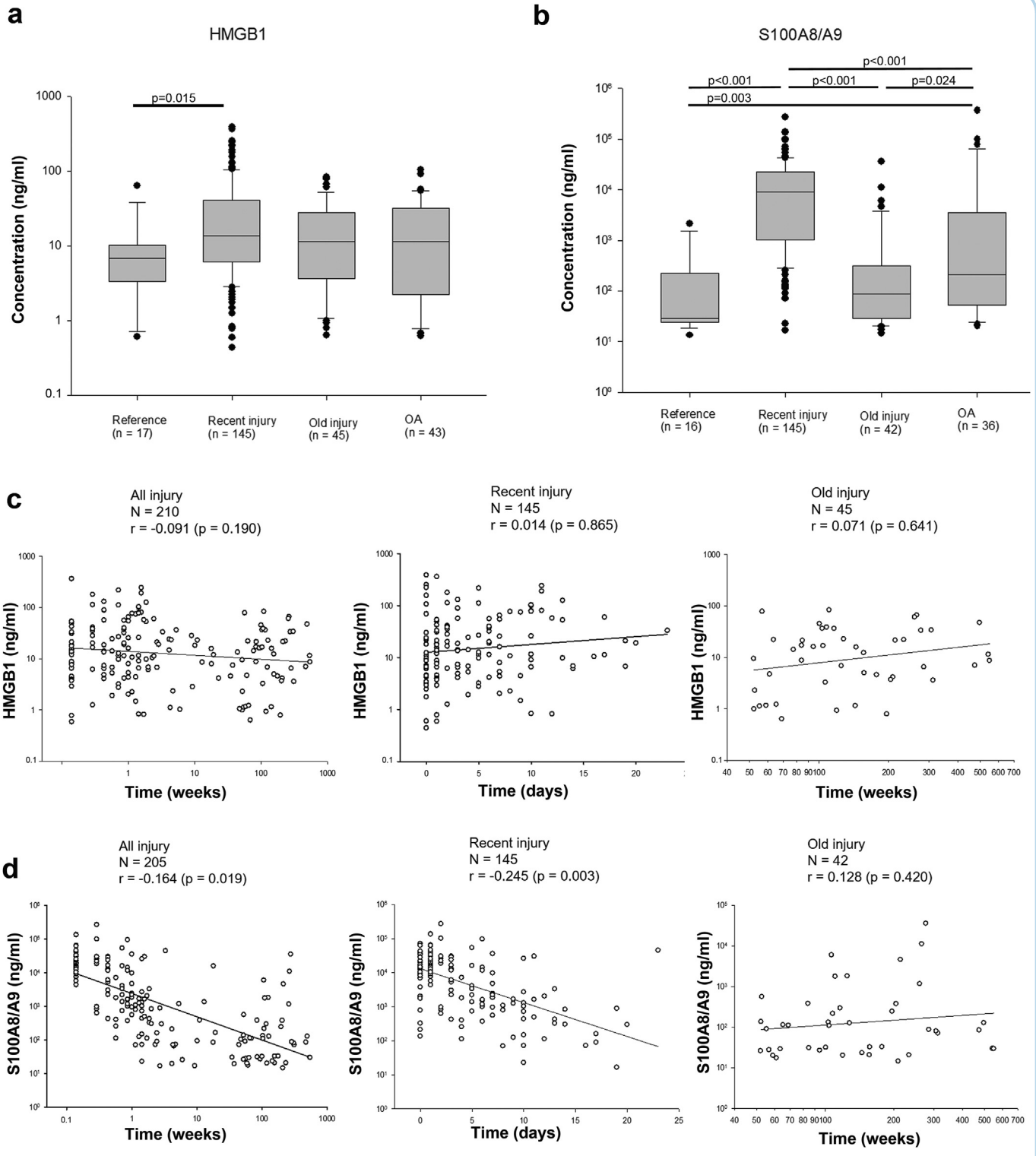


Fig. 1

Osteoarthritis and Cartilage

Synovial fluid concentrations of HMGB1 and S100A8/A9. a and b show box plots with subjects ordered by the diagnostic groups. Boxes show the quartiles (median, 25th and 75th percentiles) with error bars and whiskers for the 10th and 90th percentiles. c and d display bi-variate scatter plots with regression lines and association with time after injury (linear regression and Pearsons's r) for HMGB1(c) and S100A8/A9 (d). All injury = 0–549.4 weeks after knee injury, recent injury = 0–23 days after knee injury, old injury = 52.1–549.4 weeks after knee injury. *Y*-axes and the *X*-axes in the old injury panels are in log10 scales. All group level statistics and associations are presented in Tables S2 and S3.

	Groups	Median (2.5, 97.5 percentiles), ng/ml	N	n < LLOD (%)	Normalized vs reference		Normalized vs recent injury		Normalized vs old injury	
					Norm	P	Norm	P	Norm	P
HMGB1	Reference	6.9 (0.6, 63.6)	17	1 (6)	1	–				
	Recent injury	13.5 (0.8, 241.5)	145	2 (1)	1.96	0.015	1	–		
	Old injury	11.4 (0.8, 78.4)	45	4 (9)	1.67	0.221	0.85	0.125	1	–
	Osteoarthritis	11.4 (0.7, 90.6)	43	5 (12)	1.66	0.305	0.84	0.072	0.99	0.752
S100A8/A9	Reference	28.8 (13.4, 2135.0)	16	12 (75)	*1*	–				
	Recent injury	9142.0 (72.0, 100646.0)	145	2 (1)	317.54	**<0.001**	1	–		
	Old injury	88.5 (17.4, 11104.0)	42	17 (40)	3.07	0.090	0.01	**<0.001**	1	–
	Osteoarthritis	211.5 (20.8, 365345.0)	36	8 (22)	7.35	**0.003**	0.02	**<0.001**	2.39	**0.024**

Normalization are based on median values. Mann–Whitney tests (Exact sig. 2-tailed) were used for subject group comparisons. Statistically significant group differences ($p < 0.05$) are in bold face.

Table II

Osteoarthritis and Cartilage

Concentrations of HMGB1 and S100A8/A9 in synovial fluid

Osteochondral fracture

There were no differences in alarmin concentrations between patient-groups with or without osteochondral fractures, nor between groups in relation to osteochondral fracture with or without disrupted cortical bone (Table III; adjusted ANCOVA, adjusted for age at injury, sex and days between injury and aspiration); unadjusted ANOVA gave similar results (data not shown).

ACL injuries

The patient-group which had *no ACL-tear* had higher HMGB1 levels compared to the group which had ACL injuries (adjusted ANCOVA Table III, Table S6); unadjusted ANCOVA gave similar results (data not shown). Patients with patella luxation, which makes up 38% ($n = 16$) of the *no ACL-tear* group, had higher concentrations of HMGB1 compared to the levels in the ACL-tear group (Table S6). The effect size (η^2) of the ANCOVA models for HMGB1 and for these soft tissue injuries were moderate (ACL tear vs *no ACL-tear*, $\eta^2 = 0.077$) to strong (ACL tear vs patella luxation, $\eta^2 = 0.157$) (Table III, Table S6).

Meniscal injuries

The concentration of S100A8/A9 was higher in the patient-group which had *no meniscal-tear* compared to the patient group which had meniscal tear (adjusted ANCOVA Table III, Table S6); the unadjusted ANCOVA gave similar results (data not shown). Patients with patella luxation, which makes up 23% ($n = 16$) of the group of *no meniscal-tear*, had higher concentration of S100A8/A9 compared to the levels in the meniscal-tear group (Table S6). The effect size

	Any OC fracture ($n = 65$)	Vs	No OC facture ($n = 29$)	vs	OC fracture with disrupted cortical bone ($n = 28$)	vs	OC fracture without disrupted cortical bone ($n = 37$)
	Concentration	P (η^2)	Concentration	P(η^2)	Concentration	P(η^2)	Concentration
HMGB1, ng/ml	13.8 (4.9–39.0)	0.294 (0.012)	18.5 (6.8–53.9)	0.309 (0.020)	14.3 (4.7–49.8)	0.469 (0.009)	12.3 (5.3–33.2)
S100A8/A9, µg/ml	14.2 (1.5–24.9)	0.723 (0.001)	11.7 (2.2–31.2)	0.651 (0.004)	9.5 (1.2–17.8)	0.505 (0.007)	16.6 (1.7–29.4)
	ACL tear ($n = 72$)	Vs	No ACL tear ($n = 42$)		Meniscal tear ($n = 43$)	vs	No meniscal tear ($n = 71$)
	Concentration	P (η^2)	Concentration		Concentration	P (η^2)	Concentration
HMGB1, ng/ml	10.7 (4.4–32.9)	0.003 (0.077)	22.4 (8.5–103.9)		12.3 (4.9–39.8)	0.939 (0.000)	17.6 (5.3–53.3)
S100A8/A9, µg/ml	9.8 (1.6–19.8)	0.382 (0.007)	18.8 (4.3–36.8)		9.2 (1.0–19.2)	0.039 (0.038)	15.0 (2.4–30.1)

Anterior crucial ligament (ACL) tear = ACL injury ± co-injuries. Meniscal tear = meniscus injury ± co-injuries. MRI = magnetic resonance imaging, η^2 = Eta Squared (estimated effect size), OC = osteochondral.

Table III

Osteoarthritis and Cartilage

Biomarker concentrations in the recent injury group in relation to osteochondral fracture, ACL tear and meniscal tear as visualized on MRI. Analysis between injury-type groups was performed using ANCOVA (adjusted for age, sex and days between injury and aspiration). To ascertain normal distribution for analysis, biomarker data were log10 transformed while confounders were expressed in linear data. Concentrations (linear data) are expressed as median (range: $25^{th} - 75^{th}$ quartiles). Statistically significant differences ($\boldsymbol{p} < 0.05$) are shown in bold face type

a

	HMGB1		S100A8/A9	
	N (% > LLOD)	r_S (P)	N (%)	r_S (P)
S100A8/A9	145 (90)	**0.245 (0.008)**		
Heme	145 (87)	**0.562 (<0.001)**	145 (96)	**0.471 (<0.001)**
IL-1β	105 (90)	-0.050 (0.595)	105 (100)	**0.610 (<0.001)**
IL-6	105 (90)	-0.109 (0.247)	105 (100)	**0.563 (<0.001)**
IL-8	105 (90)	**0.246 (0.008)**	105 (100)	**0.573 (<0.001)**
TNF-α	105 (90)	-0.121 (0.200)	105 (100)	**0.488 (<0.001)**
FRZB	100 (74)	-0.008 (0.935)	100 (81)	-0.116 (0.220)
DKK1	100 (89)	**0.257 (0.006)**	100 (97)	**0.200 (0.033)**
GREM1	100 (92)	**0.293 (0.001)**	100 (100)	0.180 (0.055)
C4d	105 (89)	0.076 (0.420)	105 (99)	**0.377 (<0.001)**
C3bBbP	105 (90)	0.062 (0.513)	105 (100)	0.108 (0.253)
sTCC	105 (90)	0.128 (0.174)	105 (100)	**0.361 (<0.001)**
Osteocalcin	105 (90)	-0.152 (0.108)	105 (100)	-0.113 (0.230)
Osteonectin	105 (90)	0.135 (0.152)	105 (100)	**0.466 (<0.001)**
Osteopontin	105 (80)	0.151 (0.110)	105 (89)	**-0.270 (0.004)**
sGAG	98 (89)	**0.310 (0.001)**	98 (100)	-0.153 (0.103)
ARGS	105 (90)	**0.223 (0.017)**	105 (100)	-0.108 (0.252)
COMP	105 (90)	0.173 (0.066)	105 (100)	-0.126 (0.181)
C2C	105 (90)	**0.492 (<0.001)**	105 (100)	**-0.238 (0.010)**

b

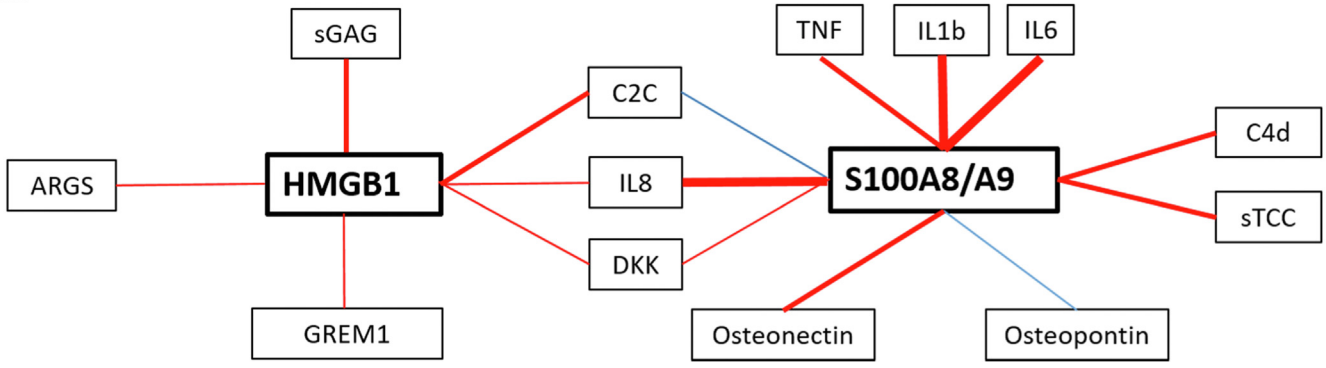
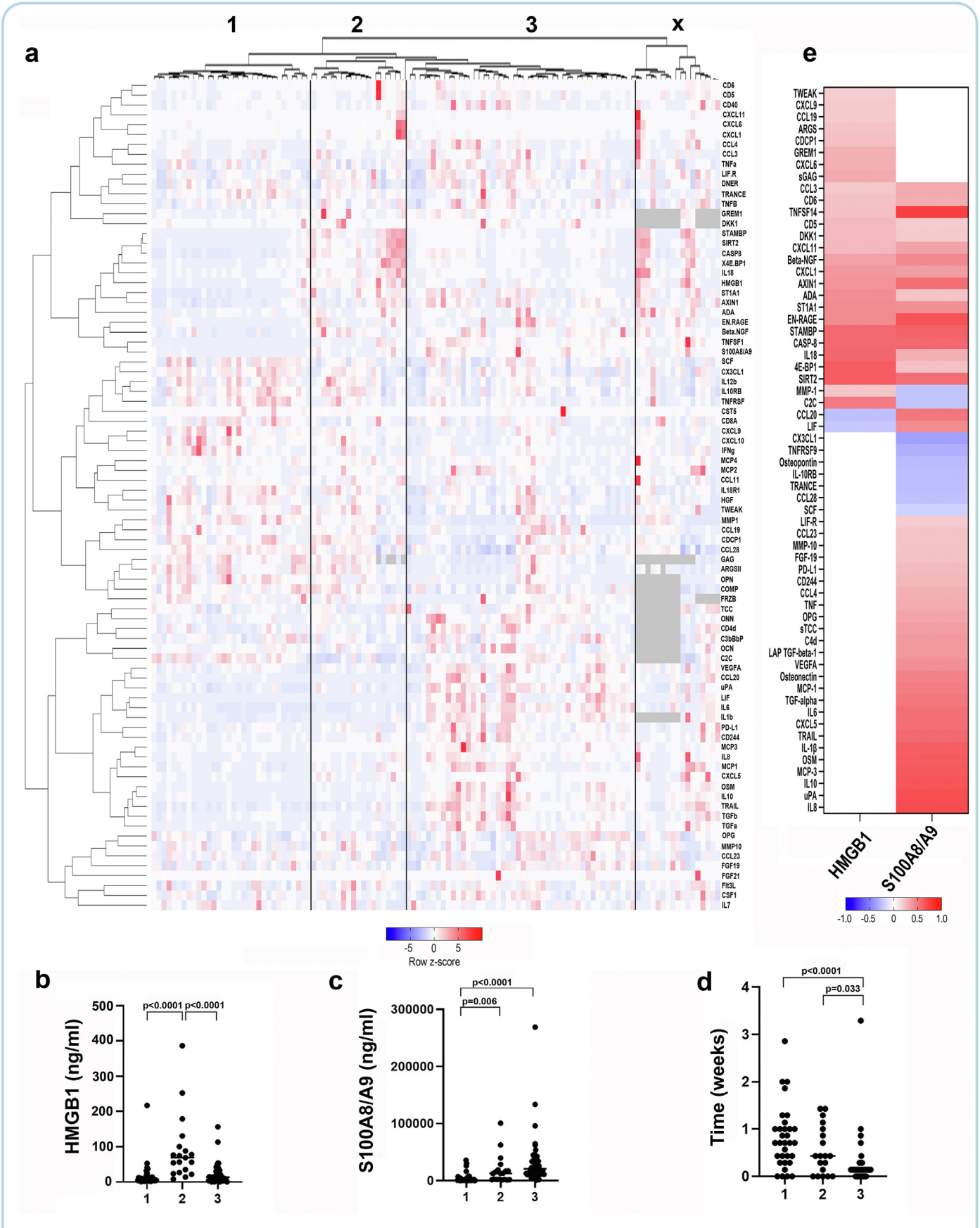


Fig. 2

Osteoarthritis and Cartilage

Correlation between different biomarkers and alarmins in the recent injury group. (a) Spearman rho (r_S) correlation between biomarkers and alarmins in synovial fluid samples were analyzed in a sub-group of the recent injury group. Amount of samples per analysis (N and %) of which were above lower limit of detection (LLOD) are shown. Significant correlations ($p < 0.05$) are shown in boldface type. IL = interleukin. TNF = tumour necrosis factor alpha, FRZB = frizzled-related protein. DKK-1 = dickkopf-related protein 1, GREM1 = gremlin 1, sTCC = soluble terminal complement complex, sGAG = sulfated glycosaminoglycan, ARGS = ARGS-aggrecan, COMP = cartilage oligomeric matrix protein, C2C = type II collagen epitope. (b) Red lines correspond to positive correlation and blue lines correspond to negative correlation. The thickness of the lines indicates the strength of correlation.


a
1
2
3
x
CD6
CD5
CD40
CXCL11
CXCL6
CXCL1
CCL4
CCL3
TNFa
LIF.R
DNER
TRANCE
TNFB
GREM1
DKK1
STAMBP
SIRT2
CASP8
X4E.BP1
IL18
HMGB1
ST1A1
AXIN1
ADA
EN.RAGE
Beta.NGF
TNFSF1
S100A8/A9
SCF
CX3CL1
IL12b
IL10RB
TNFRSF
CST5
CD8A
CXCL9
CXCL10
IFNg
MCP4
MCP2
CCL11
IL18R1
HGF
TWEAK
MMP1
CCL19
CDCP1
CCL28
GAG
ARGSII
OPN
COMP
FRZB
TCC
ONN
CD4d
C3bBbP
OCN
C2C
VEGFA
CCL20
uPA
LIF
IL6
IL1b
PD-L1
CD244
MCP3
IL8
MCP1
CXCL5
OSM
IL10
TRAIL
TGFb
TGFa
OPG
MMP10
CCL23
FGF19
FGF21
Flt3L
CSF1
IL7
-5 0 5
Row z-score
e
TWEAK
CXCL9
CCL19
ARGS
CDCP1
GREM1
CXCL6
sGAG
CCL3
CD6
TNFSF14
CD5
DKK1
CXCL11
Beta-NGF
CXCL1
AXIN1
ADA
ST1A1
EN-RAGE
STAMBP
CASP-8
IL18
4E-BP1
SIRT2
MMP-1
C2C
CCL20
LIF
CX3CL1
TNFRSF9
Osteopontin
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MMP-10
FGF-19
PD-L1
CD244
CCL4
TNF
OPG
sTCC
C4d
LAP TGF-beta-1
VEGFA
Osteonectin
MCP-1
TGF-alpha
IL6
CXCL5
TRAIL
IL-1β
OSM
MCP-3
IL10
uPA
IL8
HMGB1
S100A8/A9
-1.0 -0.5 0 0.5 1.0
b
HMGB1 (ng/ml)
500
400
300
200
100
0
p<0.0001 p<0.0001
1 2 3
c
S100A8/A9 (ng/ml)
300000
200000
100000
0
p<0.0001
p=0.006
1 2 3
d
Time (weeks)
4
3
2
1
0
p<0.0001
p=0.033
1 2 3

(η^2) of the ANCOVA models for S100A8/A9 and for these soft tissue injuries were weak (meniscal tear vs *no meniscal-tear*, $\eta^2 = 0.038$) to moderate (meniscal tear vs patella luxation, $\eta^2 = 0.077$) (Table III, Table S6).

HMGB1 is associated with cartilage biomarkers while S100A8/A9 is associated with markers of inflammation

In the sub-cohort of the recent injury group ($n = 98–105$), we have previously analyzed a number of biomarkers related to inflammation, complement system, Wnt signaling, and cartilage and bone turnover[21,25,26,28,30], and herein we assessed their association with HMGB1 and S100A8/A9. HMGB1 was positively correlated with IL-8, Dickkopf-related protein 1 (DKK1), gremlin 1 (GREM1) and with the cartilage markers sulfated glycosaminoglycans (sGAG), ARGS-neoepitope of aggrecan (ARGS-aggrecan) and type II collagen epitope C2C [Fig. 2(a)]. S100A8/A9 was positively correlated with the proinflammatory cytokines TNF, IL-1β, IL-6 and IL-8; as well as with DKK1, osteonectin and the complement factors C4d and soluble terminal complement complex (sTCC). Negative correlations were found between S100A8/A9 and C2C and osteopontin. Both alarmins correlated positively with heme (Fig. 2). An illustration of the statistically significant correlations between the biomarkers and the alarmins is shown in Fig. 2(b).

Different inflammatory profiles were associated with HMGB1 and S100A8/A9

To further analyze the relationship between alarmins and the inflammatory milieu in the *recent injury group*, alarmins, inflammatory markers (PEA inflammation panel) and the previously assessed biomarkers related to inflammation, complement system, Wnt cell signaling, and cartilage and bone turnover (see above) were assessed. Complete linkage cluster analysis displayed four distinct clusters, although group X clustered due to missing data and were omitted in further analyses [Fig. 3(a)]. Compared to the other cluster groups, group 2 displayed the highest HMGB1 levels, group 3 the highest S100A8/A9 levels, and group 1 had low HMGB1 and S100A8/A9 levels (Fig. 3(b) and (c), Table S7). Group 3 constituted of early samples, with a mean sampling time point of 2 days (95% CI 0.64–2.74) compared to 6 (95% CI 4.0–7.4) and 4 (95% CI 2.1–5.5) days for group 1 and 2 [Fig. 3(d)]. While the inflammatory profiles were different between the three cluster groups, no differences in sex, age or type of injury were observed (data not shown). Association between PEA marker levels and alarmin concentrations were assessed by Spearman rho correlations (r_S). Sixty-eight inflammatory markers (out of total 92) were selected based on PEA-vales above LLOD in more than 50% of the samples (Table S8). When including PEA inflammation markers plus the biomarkers presented in Fig. 3 our analysis showed that twenty-two biomarkers were associated with both HMGB1 and S100A8/A9; eight biomarkers were uniquely associated with HMGB1 and thirty-one to S100A8/A9 [Fig. 3(e)]. Interestingly, MMP-1, C2C, CCL20 and LIF were correlated to both alarmins but in an opposite positive - negative direction.

Reactome enrichment analysis of the significantly associated biomarkers, displayed the same top six pathways for the two alarmins: Chemokine receptors bind chemokines, Interleukin-10 signaling, Immune System, Cytokine Signaling in Immune system, Peptide ligand-binding receptors and Interleukin-4 and Interleukin-13 signaling (Fig. 4). Additionally, five of the top 20 pathways were common: Signaling by interleukins, TRIF-mediated programmed cell death, TNFR1-induced proapoptotic signaling, TLR3-mediated TICAM1-dependent programmed cell death and Class A/1 (Rhodopsin-like receptors); the remaining nine pathways were distinct between the two alarmins (Fig. 4).

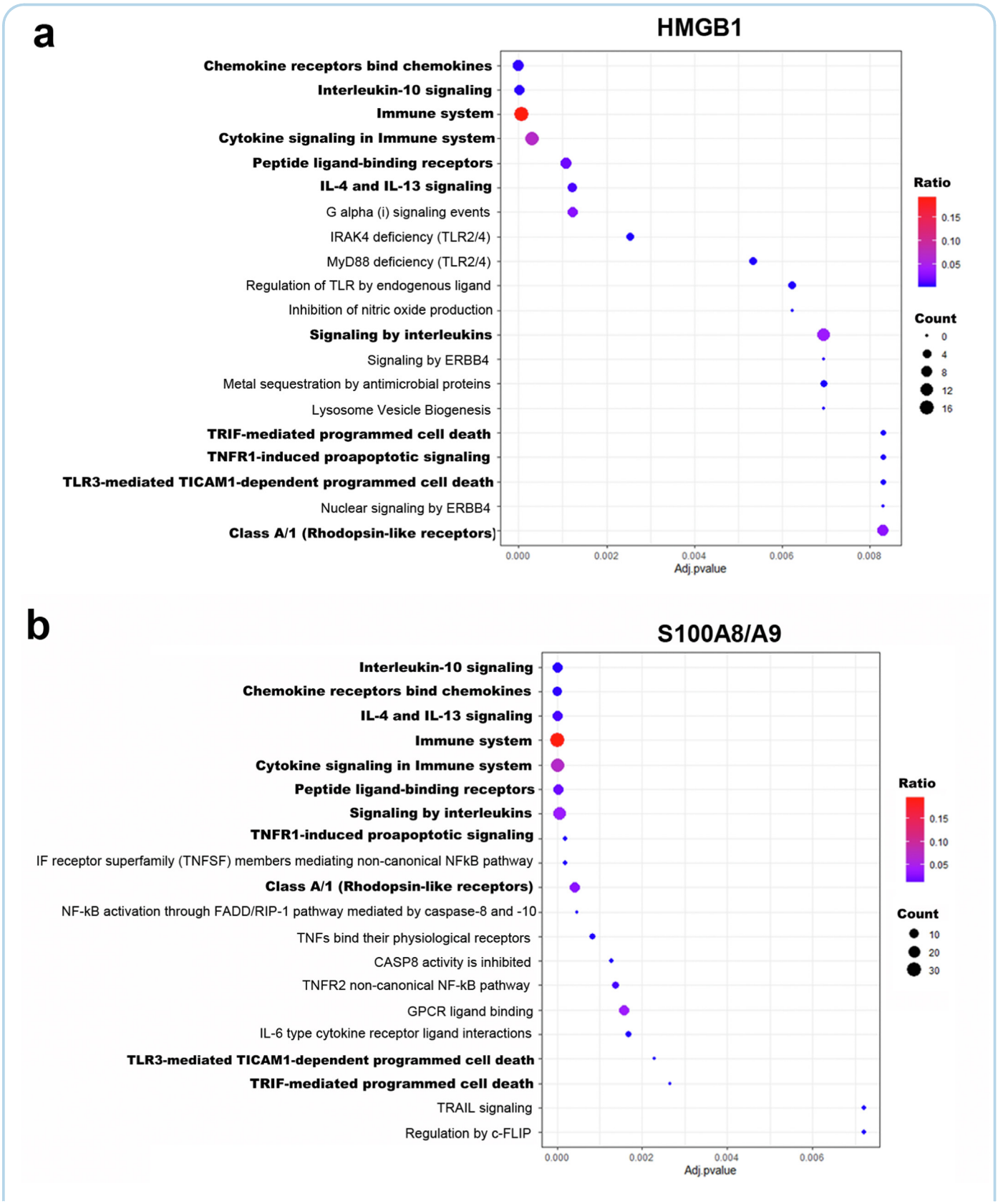
Discussion

In the present study, we investigated the levels of the alarmins HMGB1 and S100A8/A9 in synovial fluid from knee injured and OA patients and from knee healthy reference subjects. We found that HMGB1 and S100A8/A9 levels were increased early after knee injury (0–3 weeks) and that S100A8/A9 levels were negatively associated with time from injury and lower in the old vs recent injury groups, while HMGB1 was not associated to time from injury. The inflammatory profile of a subgroup of patients with synovial fluid aspiration early after knee injury was further analyzed by PEA inflammation panel and immunoassays of inflammation, Wnt signaling, complement system, cartilage and bone degradation. Multiple correlations and enrichment analysis revealed similar inflammation pathways for both alarmins, but also distinct features. In line with this observation, HMGB1 was associated to cartilage related biomarkers and TLR signaling, while S100A8/A9 was associated to inflammation and chemokine activity.

The median synovial fluid concentration of S100A8/A9 in the recent injury group (0–23 days after knee injury) was approximately 300-times higher than in the knee healthy reference subjects. Patients with OA had 7-times higher S100A8/A9 concentrations compared to the knee healthy reference subjects. Since alarmins are known to be initiators of the inflammatory cascade, high levels early after injury that decrease when inflammation is resolving would be expected and was observed for S100A8/A9 but not for HMGB1 in this study. We have previously observed a similar time-association of synovial fluid concentrations of alarmins in children diagnosed with juvenile idiopathic

Fig. 3 Osteoarthritis and Cartilage

Linkage Cluster analysis and correlations of inflammatory markers, alarmins and other biomarkers in early knee injuries. (a) Heat map diagram of complete linkage hierarchical clustering from the early knee injury subgroup presented four major clusters. (b–d) Scatter plots displaying differences in HMGB1 levels (b), S100A8/A9 levels (c) and time from injury (d) between clusters. Group x clustered due to missing data and were omitted in further analyses. Each column represents one sample, each row represents one protein. Red indicates high levels of the protein in the sample, blue indicates a lower level and gray represents missing data points. The analysis was based on the 68 PEA inflammation panel biomarkers with levels > LLOD (Table S7), HMGB1, S100A8/A9, heme and the previously assessed biomarkers IL-1β, TNF, osteocalcin, osteonectin, osteopontin, sGAG, C2C, ARGS-aggrecan COMP, C4d, C3bBbP, sTCC, DKK1, FRZB and GREM1. (e) Spearman rho correlation between HMGB1 or S100A8/A9 and all the biomarkers assessed in this study. Twenty-two biomarkers were associated to both HMGB1 and S100A8/A9; eight biomarkers were uniquely associated to HMGB1 and thirty-one to S100A8/A9. Four biomarkers (MMP-1, C2C, LIF and CCL20) were correlated to both alarmins but in opposite directions. Red = positive association, blue = negative association, white = not correlated. Correlation coefficients and description of the abbreviations are presented in Fig. 3 and in Table S7.


a
HMGB1
Chemokine receptors bind chemokines
Interleukin-10 signaling
Immune system
Cytokine signaling in Immune system
Peptide ligand-binding receptors
IL-4 and IL-13 signaling
G alpha (i) signaling events
IRAK4 deficiency (TLR2/4)
MyD88 deficiency (TLR2/4)
Regulation of TLR by endogenous ligand
Inhibition of nitric oxide production
Signaling by interleukins
Signaling by ERBB4
Metal sequestration by antimicrobial proteins
Lysosome Vesicle Biogenesis
TRIF-mediated programmed cell death
TNFR1-induced proapoptotic signaling
TLR3-mediated TICAM1-dependent programmed cell death
Nuclear signaling by ERBB4
Class A/1 (Rhodopsin-like receptors)
0.000
0.002
0.004
0.006
0.008
Adj.pvalue
Ratio
0.15
0.10
0.05
Count
0
4
8
12
16
b
S100A8/A9
Interleukin-10 signaling
Chemokine receptors bind chemokines
IL-4 and IL-13 signaling
Immune system
Cytokine signaling in Immune system
Peptide ligand-binding receptors
Signaling by interleukins
TNFR1-induced proapoptotic signaling
IF receptor superfamily (TNFSF) members mediating non-canonical NFkB pathway
Class A/1 (Rhodopsin-like receptors)
NF-kB activation through FADD/RIP-1 pathway mediated by caspase-8 and -10
TNFs bind their physiological receptors
CASP8 activity is inhibited
TNFR2 non-canonical NF-kB pathway
GPCR ligand binding
IL-6 type cytokine receptor ligand interactions
TLR3-mediated TICAM1-dependent programmed cell death
TRIF-mediated programmed cell death
TRAIL signaling
Regulation by c-FLIP
0.000
0.002
0.004
0.006
Adj.pvalue
Ratio
0.15
0.10
0.05
Count
10
20
30

arthritis[32]. The high S100A8/A9 levels were observed in patients with short disease duration while HMGB1 levels were increased irrespective of disease duration. In the present study, our data may reflect an acute inflammatory state in the joint where both alarmins are expressed as a response to the trauma, potentially influenced by infiltrating neutrophils, tissue damage and bleeding. S100A8/A9 is mainly expressed in neutrophils and monocytes, and S100 proteins constitutes up to 40% of all cytosolic proteins in neutrophils and 4–5% in monocytes[33]. Hence, the decreasing levels of S100A8/A9 in the old injury group could potentially reflect a termination of the acute inflammatory phase, where the infiltration and presence of neutrophils are lower compared to early after injury. The increased levels of S100A8/A9 in OA group could reflect a state of low grade inflammation with S100A8/A9 being produced from activated resident neutrophils or macrophages. HMGB1 on the other hand is known to be expressed by joint tissue cells such as activated fibroblasts, tissue resident macrophages and chondrocytes[34–36]. The diverse cellular source of HMGB1 and S100A8/A9 may explain the observed differences in the recent and old knee injury samples.

To assess whether high alarmin levels were due to hemolysis, total heme was measured in the samples. In the early injury group, alarmins were positively associated to heme levels suggesting that hemolysis and bleeding contribute to release of both HMGB1 and S100A8/A9 into the synovial fluid early after injury. It is well known that hemolysis is associated to both HMGB1 and S100A8/A9 expression, although the effects of their upregulation in the context of hemolysis is not fully understood and *ex vivo* hemolysis may also affect the levels of HMGB1 detected in a sample[37–40]. The lack of association between HMGB1 and heme in old injury indicate that cell death, bleeding or hemolysis is not the primary source of HMGB1 in later stages of knee injury. HMGB1 has been described as a late mediator in diseases such as sepsis and trauma, where the first inflammatory insult results in an immediate release of alarmins, including S100A8/A9 and HMGB1, but in later stages a second wave of HMGB1 can be observed as a result of increased release of HMGB1 from activated immune and tissue cells[12,41]. Although the pathophysiology differs from OA, the mechanism of a second wave of HMGB1 release could be similar. Interestingly, we found an association between heme and alarmins in the OA group. Many joint trauma patients suffer from hemarthrosis. In the early knee injury group bleeding could be considered part of the pathology, while in OA joint bleeding is less common. On the other hand, it is known that hemarthrosis in the knee is a risk factor for OA, and heme may be considered a contributor to disease development in itself[42]. The connection between heme, alarmins and OA after knee injury was not possible to study within the current context, but this finding warrants further investigations.

Since we detected similar trend of HMGB1 and S100A8/A9 levels early after injury, but not in late stages, we extended to investigate the relationships between alarmins and several previously measured biomarkers related to inflammation, Wnt signaling, complement system and bone and cartilage degradation. Linear regression analyses of these biomarkers showed, that HMGB1 and S100A8/A9 were associated to different types of molecules. HMGB1 levels correlated with sGAG, ARGS-aggrecan and C2C, which are related to extracellular matrix turnover in line with previous observations by us and others[15,43,44]. Furthermore, HMGB1 was associated to GREM1 and DKK1, which have been reported as natural brakes on hypertrophic differentiation in articular cartilage, indicating that HMGB1 is associated to cartilage remodeling and chondrocyte regulation[45]. S100A8/A9 on the other hand, was associated with proinflammatory proteins and bone remodeling/calcification processes. When adding data on inflammation associated proteins from PEA and analyzing the association between these biomarkers and HMGB1 and S100A8/A9, complete hierarchical cluster analysis displayed three distinct clusters. Interestingly, the clusters were separated by time from injury and by high or low HMGB1 levels. S100A8/A9 clustered mainly with samples early after injury and high HMGB1, while age, sex or type of injury did not differ between the main clusters. Patients with patella luxation had the highest synovial fluid alarmin levels, although the number of patients were too low to analyze the subgroup in more detail.

Reactome enrichment analysis of the HMGB1 or S100A8/A9 associated markers, indicated that both alarmins share many features. Many of the enriched pathways common for both alarmins included broad inflammatory and immune regulatory pathways. IL-4 and IL-13 signaling has been described as anti-arthritic cytokines in RA, involved in macrophage polarization, inhibition of cartilage damage and osteoclastogenesis[46]. IL-10 signaling is described as anti-inflammatory and immune regulatory[47]. Hence, both proinflammatory and resolving pathways are upregulated after knee injury. The enriched pathways displayed for HMGB1 (but not for S100A8/A9) included TLR pathways, metal sequestration and reduced nitric oxide production. They are all pathways associated to innate immune response and host defense against microbes and sterile inflammation. TLRs activate downstream signaling pathways that trigger inflammation by inducing signal transduction pathways that lead to the activation of NF-κB, interferon regulatory factors and mitogen-activated protein (MAP) kinases, which regulates the expression of cytokines, chemokines, and type I interferons[48]. But also to prime antigen-specific adaptive immune responses[49]. On the other hand, the enriched pathways displayed for S100A8/A9 (but not HMGB1) included classical pro-inflammatory signaling pathways such as TNF and IL-6 signaling. TNF is important in the typical immune response through the regulation of a number of pathways including an immediate inflammatory response with both innate immune involvement and cellular activation with proliferation and programmed cell death or necrosis. Hence, HMGB1 and S100A8/A9 are both involved in the initiation and perpetuation of inflammation but potentially via distinct pathways.

This study has some limitations. The samples were stored in −80°C for different lengths of time and the stability of HMGB1

Fig. 4 Osteoarthritis and Cartilage

Top 20 enriched pathways of HMGB1 and S100A8/A9. Dot plot showing the top 20 pathways from the reactome enrichment analysis performed for the biomarkers that were significantly correlated to HMGB1 (a) and S100A8/A9 (b) levels, respectively. The higher up and further left a dot is, the more enriched the pathway is. Dot color indicates k/n ratio, where k is the number of input proteins (correlated to HMGB1 and S100A8/A9 respectively) participating in the current reactome pathway and n is the total number of proteins annotated as participants of that same reactome pathway. I.e., the larger the dot, the greater is the number of input proteins that participates in the pathway irrespective of how many proteins in total are part of that pathway. Furthermore, the warmer (red) the dot is, the higher is the proportion of input proteins that participates in the pathway in relation to total number of proteins in the pathway. The top six pathways and additionally five (marked in boldface) among the top 20 were the same for both alarmins while 9 enriched pathways differed between HMGB1 and S100A8/A9.

and S100A8/A9 during long-term storage is unknown and may impact the data. The data indicated that the alarmins differ in kinetics and activation responses. Our samples are cross-sectional and longitudinal data following individual patients are needed to confirm these findings. It is well known that the redox state of HMGB1 cysteines are of importance for its function. However, the ELISA does not distinguish between isoforms and this could not be addressed in the current setting. The reference group is small which decreases the power of statistical analyses. The cluster analyses and enrichment analyses are performed in one subgroup (early injury), without reference to healthy samples.

In conclusion, early after knee injury, synovial fluid concentrations of HMGB1 and S100A8/A9 are increased as a response to the insult on the joint. One year after injury S100A8/A9 levels have decreased and are similar to reference levels. Furthermore, S100A8/A9 levels were increased in OA while HMGB1 was not. Data from inflammatory and structural biomarkers associated to the alarmins in the early injury subgroup indicated that although HMGB1 and S100A8/A9 share many features in inflammatory and immunoregulatory mechanisms, some of their associated effects are distinct. For example, HMGB1 was associated to biomarkers of cartilage while S100A8/A9 was associated to pro-inflammatory cytokines and bone related markers. Taken together, our study showed that alarmins are increased as an immediate response to knee trauma. While they share many features in inflammatory and immunoregulatory mechanisms, S100A8/A9 and HMGB1 are associated to different molecular processes, and their impact on OA progression after acute knee injuries may have an impact on OA progression and warrants further investigations.

Author contributions

CA, HEH, AS designed the study. CA, SL, TV, JR, PS, RH, AÅ and AS collected and assembled the data. CA, SL, PS, RH, HEH and AS analyzed and interpreted the data. AÅ provided statistical expertise. CA and AS drafted the article. All authors contributed and critically revised the final version of the article. All authors approved the final version.

Conflict of interest

The authors declare that they have no competing interests.

Role of the funding source

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Supplementary data

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