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Targeted proteomics reveals serum amyloid A variants and alarmins S100A8-S100A9 as key plasma biomarkers of rheumatoid arthritis

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ABSTRACT

Serum amyloid A (SAA) and S100 (S100A8, S100A9 and S100A12) proteins were previously identified as biomarkers of interest for rheumatoid arthritis (RA). Among SAA family, two closely related isoforms (SAA-1 and SAA-2) are linked to the acute-phase of inflammation. They respectively exist under the form of three (α, β, and γ) and two (α and β) allelic variants. We developed a single run quantitative method for these protein variants and investigated their clinical relevance in the context of RA. The method was developed and validated according to regulations before being applied on plasma coming from RA patients (n = 46), other related inflammatory pathologies (n = 116) and controls (n = 62). Depending on the activity score of RA, SAA1 isoforms (mainly of SAA1α and SAA1β subtypes) were found to be differentially present in plasma revealing their dual role during the development of RA. In addition, the weight of SAA1α in the total SAA response varied from 32 to 80% depending on the pathology studied. A negative correlation between SAA1α and SAA1β was also highlighted for RA early-onset (r = −0.41). SAA2 and S100A8/S100A9 proteins were significantly overexpressed compared to control samples regardless of RA stage. The pathophysiological relevance of these quantitative and qualitative characteristics of the SAA response remains unknown. However, the significant negative correlation observed between SAA1α and SAA1β levels in RA early-onset suggests the existence of still unknown regulatory mechanisms in these diseases.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that mostly affects women resulting in painful and swollen peripheral joints leading to irreversible cartilage and bone destruction. RA disease activity is monitored by the determination of composite indices such as the disease activity score calculated on 28 joints (DAS_{28}) which integrates the number of tender and of swollen joints, and the ESR or CRP serum levels. Disease activity is categorised as low ($2.6 < DAS_{28} < 3.2$), moderate ($3.2 \leq DAS_{28} \leq 5.1$) or severe ($DAS_{28} \geq 5.1$) [1].

Serum amyloid A (SAA) and S100 (S100A8, S100A9 and S100A12) proteins have previously been highlighted as potential biomarkers of interest of RA [2,3]. The SAA family is composed of four isoforms, among which two (SAA1 and SAA2) are associated with acute-phase of inflammation [4,5]. SAA3 is believed to be a pseudo gene and SAA4 is constitutively expressed in the liver. Structurally, SAA1 and SAA2 share up to 90% of sequence homology and therefore cannot be distinguished by immunoassays as the antibody reacts indistinctly with both SAA isoforms [6]. In addition, SAA1 and SAA2 proteins are polymorphic with three (α, β and γ) and two (α and β) major allelic variants, respectively [4,6] (Table 1). Plasma concentration of SAA is higher in RA patients than in healthy individuals [4,7]. Synovial fluid levels of SAA are significantly higher than in corresponding serum, suggesting local joint production [7,8]. Serum concentration of SAA increases with RA severity [9,10] and in various tumours [11,12], which has led researchers to conclude that SAA concentration was very sensitive but not specific for diagnosis, prognosis and monitoring of patients with inflammatory diseases [4,11–14]. Although SAA has been widely studied in many diseases, the quantification of its respective isoforms remains poorly known. Sung et al. used LC-MS to separate SAA1 and SAA2 in lung cancer patients [15]. Kim et al. discriminated SAA1α, SAA1β, SAA1γ, SAA2α, and SAA2β for the same disease and highlighted

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List of abbreviations

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AS	Ankylosing spondylitis
CRP	C-reactive protein
DAS	Disease activity score
DoE	Design of experiments
FA	Formic acid
HC	Healthy controls
IMID	Immune-mediated inflammatory diseases
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOQ	Limit of Quantitation
MeOH	Methanol
MRM	Multiple reaction monitoring
OA	Osteoarthritis
RA	Rheumatoid arthritis
SAA	Serum amyloid A
SLE	Systemic lupus erythematosus
TFA	Trifluoroacetic acid
UHPLC	Ultra-high-pressure liquid chromatography

differences in the expression of each subtype [6]. However, to the best of our knowledge, this is the first study investigating SAA variants in the context of RA.

S100A8 and S100A9 are produced by neutrophils and macrophages upon interaction with activated endothelial cells [3,16–19]. Physiologically, they are associated under the form of a dimer, which forms heterotetramers in the presence of calcium [18,20]. The dimeric form of S100A8/S100A9 (calprotectin) is a known biomarker for inflammatory bowel disease [21], although increases of S100A8/S100A9 concentrations have also been observed in RA, in psoriatic arthritis and in ankylosing spondylitis (AS) [3,22,23]. S100A12 is produced by granulocytes and acts independently of S100A8 and S100A9 to induce pro-inflammatory signals [24].

In this work, we present the development of a method analyzing SAA variants and S100A8, S100A9, and S100A12 proteins using a single targeted bottom-up proteomics LC-MS/MS. Compared to previously developed methods, we extended the analysis to other proteins (S100 variants). We also comprehensively present the development and optimization of the sample preparation and protein digestion as well as the challenges associated to multiplexed bottom-up proteomics. Furthermore, an innovative approach was employed to access the absolute concentration of proteins of interest using a digestion curve translating a concentration of peptide into a concentration of protein. This strategy was then fully validated according to regulation to ensure reliability of the results. Finally, this method was subsequently employed to study the differential expression of each of SAA variants and S100A8 and S100A9 in healthy controls (HC) and in patients suffering from various immune-mediated inflammatory diseases (IMID) including RA and osteoarthritis (OA).

2. Methods

2.1. Materials

Acetonitrile (ACN), methanol (MeOH), water (H_2O), formic acid (FA), trifluoroacetic acid (TFA) of ULC-MS grade were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate (ABC), trypsin from bovine pancreas, calcium chloride ($CaCl_2$) and ammonia were obtained from Sigma-Aldrich (Saint-Louis, MI, USA). Oasis MAX 10 mg 96 well plates were purchased from Waters (Dublin, Ireland). Standard and internal standards peptides (ISTD) were synthesised by Eurogentec (Seraing, Belgium) with a purity > 98%. SAA1α and Apo-SAA proteins (both with purity > 98%) were obtained from Peprotech (London, UK). S100A12, S100A9, and S100A8 proteins (purity > 90%) were obtained from Abcam (Cambridge, UK).

2.2. Preparation of standard solutions

Standard peptides, ISTD, S100A8, S100A9 and S100A12 proteins were individually dissolved in H_2O/ACN/FA (90:10:0.1, v/v) at a concentration of 100 µg mL^{-1}. Apo-SAA and SAA1α proteins were dissolved in the same solvent at a concentration of 500 µg mL^{-1} and

Table 1
Sequence of studied proteins. Differences among each family are highlighted in orange. Underlined sequences represent the peptides that are being generated by tryptic digestion and analysed by LC-MS/MS.

Family	Name	Sequence
	Apo-SAA	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGVWAAEAISNARENIQRFFGRGAEDSLADQAANEWGRSGKDPN HFRPAGLPEKY
SAA1	SAA1α	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGVWAAEAISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPN HFRPAGLPEKY
	SAA1β	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGAWAAEVISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPN HFRPAGLPEKY
	SAA1γ	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGAWAAEAISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPN HFRPAGLPEKY
SAA2	SAA2α	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGRDP NHFRPAGLPEKY
	SAA2β	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGPGGAWAAEVISNARENIQRLTGRGAEDSLADQAANKWGRSGRDP NHFRPAGLPEKY
S100	S100A8	MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKGADVWFKELDINTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE
	S100A9	MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVIEHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEG DEGPGHHHKPGLGEGTP
	S100A12	MTKLEEHLEGIVNIFHQYSVRKGHFDTLSKGELKQLLTKELANTIKNIKDKAVIDEIFQGLDANQDEQVDFQEFISLVAIALKAAHYHTHKE

1 mg mL^{-1}, respectively. Solutions were aliquoted and kept at −80 °C until analysis. The preparation of calibration and validation solutions is presented in S2.

2.3. LC-MS conditions

Ultra-high-pressure liquid chromatography (UHPLC) separation was performed with a 1290 infinity system (Agilent Technologies, Waldbronn, Germany) on an Aeris C18 column (1.7 µm particle sise, 100 × 2.1 mm ID) (Phenomenex, Torrance, CA, USA) thermostated at 60 °C. The separation was achieved in gradient mode with mobile phase A (H_2O/FA, 100:0.1, v/v) and B (ACN/H_2O/FA, 90:10:0.1, v/v) at 0.5 mL/min. The gradient was as follows: 0–2.5 min, from 6% to 8.5% B; 2.5–3 min, from 8.5 to 18.25% B, 3–6 min, from 18.25 to 19% B, 6–8 min, from 19 to 60% B; 8–8.1 min, from 60 to 95% B; 8.1–9, 95% B; 9–9.1 min, from 95% to 6% B (2.2 min post-time). The injection volume was 5 µl. MS/MS detection was performed on a 6495 LC-MS Triple Quadrupole equipped with the iFunnel Technology (Agilent Technologies) and operated using positive electrospray ionization. The source conditions were optimized via the Agilent Source Optimizer software and were as followed. The capillary and the nozzle voltages were set at 3000 V and 1500 V, respectively. Nitrogen was used as drying (230 °C, 19 l/min) and sheath gas (250 °C, 12 l/min). The high and low-pressure funnels were operated at 70 V and 40 V, respectively. Multiple reaction monitoring (MRM) transitions were individually optimized for each standard and ISTD peptides using Skyline software.

2.4. Sample preparation

The optimized sample preparation protocol was the following. 25 µl of each calibration solution were mixed with 25 µl of bovine plasma and loaded in duplicate on a 96 well plate. 25 µl of sample were mixed with 25 µl of H_2O/ACN/FA (80:20:0.1, v/v). Then, 300 µl of 100 mM (pH 8.1) of ammonium bicarbonate (ABC) solution containing 33.3% of MeOH and 25 µl of 500 ng mL^{-1} ISTD solution were added in each well before incubation at 100 °C for 10 min. Then, 25 µl of 0.7 mg mL^{-1} trypsin solution (in 100 mM ABC) was added before incubation at 37 °C/600 RPM overnight. 25 µl of 25% ammonia was added to quench the reaction. Extraction was realized using an Oasis MAX SPE plate (10 mg sorbent, Waters Corporation, Dublin, Ireland). The protocol is present in S3A.

The second was of the SPE was optimized by varying the pH of the second wash (acid, neutral of basic); the proportion of MeOH in the second wash solution (from 20 to 80%) and the proportion of ACN in the elution solution (from 10 to 95% ACN) using 25 µl of 500 ng mL^{-1} peptide solution mixed with 25 µl of bovine plasma. Results were modelled using SAS JMP (Cary, NC, USA) to find the optimal conditions. To optimize the resuspension solution, 25 µl of 500 ng mL^{-1} peptide solution mixed with 25 µl of bovine plasma were extracted, evaporated to dryness and resuspended with 100 µl of solutions composed either of 0.1% FA or 0.1% TFA containing increasing amounts of ACN (from 0 to 40%).

2.5. Optimization of digestion conditions

Tryptic digestion was optimized using a design of experiment (DoE) achieved with 5 commercially available proteins (SAA1α, Apo-SAA, S100A8, S100A9, and S100A12) spiked at 1 µg mL^{-1} in bovine plasma processed as described in paragraph 2.1. The DoE was performed considering nature (MeOH or ACN) (X_1) and proportion of organic modifier in the digestion solution (5, 17.5 and 30%) (X_2), and the quantity of trypsin to use (17.5, 96.25 and 175 µg per sample, corresponding to 1:100 to 1:10 trypsin-to-proteins ratio) (X_3). Responses (peak areas of peptides) were modelled using SAS JMP (Cary, NC, USA). The equation is presented in supplementary data. The equation of the model (full factorial design) was as follows.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 + \beta_{11} X_2^2 + \beta_{22} X_3^2 + \varepsilon$$

where β_0 is the intercept, β_1, , and β_3 the main effect terms, β_{12}, β_{23}, and β_{13} the interaction terms, β_{11}, and β_{22} the quadratic terms, and ε the error term. Each experimental condition was run in triplicate and the central point was replicated 4 times.

2.6. Analytical validation

For the analytical validation, 8 independent calibration series (each composed of 8 levels) and 8 independent validation series (each composed of 6 levels) were realized. Within the calibration series, the peptides SAA1α, SAA1β, SAA1γ, SAA2, SAA2α and SAA2β were present as well as the Apo-SAA, S100A8 and S100A9 proteins. Within the validation series, the peptides SAA1β, SAA1γ, SAA2, SAA2α and SAA2β were present as well as the SAA1α, S100A8 and S100A9 proteins.

Each calibration series was composed of 8 levels (1, 5, 10, 50, 200, 500, 2000 and 5000 ng mL^{-1} for SAAs peptides; 2, 10, 20, 100, 400, 1000, 4000 and 10000 ng mL^{-1} for S100 proteins; 20, 100, 200, 1000, 4000, 10000, 40000 and 80000 ng mL^{-1} for Apo-SAA). Each validation series was composed of 6 levels (4, 20, 100, 500, 1250 and 5000 ng mL^{-1} for SAA peptides except SAA1α; 8, 40, 200, 1000, 2500 and 10000 ng mL^{-1} for S100 proteins; 50, 250, 1250, 8000, 2000 and 80000 ng mL^{-1} for SAA1α protein). For SAA1α only, the concentration of the generated peptide was first calculated using the quantitation curve built with SAA1α peptide in the calibration solution. Then, this concentration was transformed into the initial concentration of SAA1α protein using the digestion curve built with Apo-SAA. A statistical approach based on the total error concept for validation was employed. The E.Noval software (Arlenda, Liège, Belgium) was used to assess trueness, precision, accuracy and limit of quantification of the method. Acceptance limits were set at ± 20% except for LOQ where ± 30% was accepted. The selectivity of the method was evaluated by extracting and analyzing blank bovine plasma. The suitability of bovine plasma as surrogate of human plasma was proved by spiking internal standards at a concentration of 500 ng mL^{-1} in both fluids (n = 5). They were subsequently processed as described in paragraph 2.4. The peak areas obtained for both fluids were compared using Mann-Whitney t-tests (α = 0.05). The stability in the autosampler was evaluated by analyzing solutions of 500 ng mL^{-1} of peptides regularly during 45 h. Freeze-thaw stability was evaluated by freezing and defrosting bovine plasma solutions (n = 3) containing 5000 ng mL^{-1} SAA peptides, 80000 ng mL^{-1} SAA1α protein and 10000 ng mL^{-1} S100 proteins. Samples were left on ice for 2 h to defreeze. 3 cycles were realized. To evaluate the impact of the matrix, neat standards (A) were realized at three different concentrations (40, 400 and 4 µg mL^{-1}) for each analyte (peptides) in a mixture of H_2O/ACN/FA (80:20:0.1, v/v). Post-extraction spiked matrix (B) was realized by processing 25 µl of blank bovine plasma and reconstituting with the appropriate concentration of analyte. Pre-extraction spiked matrix (C) were realized by diluting stock solution to appropriate concentration in plasma and extracting 25 µl of this solution using the previously described protocol. The peaks were integrated and used to calculate the matrix effect (ME), extraction recovery (ER) and process efficiency (PE) according to the following equations:

$$ME(\%) = B/A \times 100$$

$$ER(\%) = C/B \times 100$$

$$PE(\%) = C/A \times 100$$

2.7. Choice of biological fluid

Paired plasma and serum coming from 9 RA patients and 10 controls were selected to choose the optimal fluid. Each of them was processed

in triplicate as detailed in paragraph 2.1 and injected twice in the LC-MS/MS system. Differences between groups were tested using parametric t-tests.

2.8. Clinical investigation

The developed method was used for clinical investigation of SAA and S100 variants in various patient groups suffering from immune-mediated inflammatory diseases (IMIDs) pathologies (age and sex-paired). The study included HC (n = 62), early-onset RA (disease duration less than 6 month) (n = 14), RA with weak/moderate ($2.6 \leq DAS_{28} \leq 5.1$) (n = 20) or severe disease ($DAS_{28} > 5.1$) (n = 12), ankylosing spondylitis (AS) (n = 30), systemic lupus erythematous (SLE) (n = 23), systemic sclerosis (n = 20) and osteoarthritis (OA) (n = 43). Samples were randomised and processed as described in paragraph 2.1. Statistics were realized using Graphpad Prism 7 (La Jolla, CA, USA). Results were $\log_{10}$ transformed and Kruskal-Wallis followed by Dunn's multiple comparisons ($\alpha = 0.05$) were used for statistical comparison. Pearson's correlation coefficients and ROC curves were computed using SAS JMP software (significance tested using two-tailed t-tests, $\alpha = 0.05$) using $\log_{10}$ transformed concentrations.

3. Results and discussion

The objective of this study was to evaluate the clinical interest of 8 potential biomarkers of rheumatoid arthritis, namely SAA1α, SAA1β, SAA1γ, SAA2α, SAA2β, S100A8, S100A9, and S100A12. A targeted bottom-up approach was optimized for the quantitation of those protein biomarkers, including the digestion of the proteins in proteotypic peptides and their analyses by LC-MS/MS. Special attention was paid to select peptides specific to each SAA variants, which only differ from one another by few amino acids (Table 1). An original analytical method was thus developed and validated, and then the clinical relevance of those biomarkers was investigated.

3.1. Optimization of biomarker separation

The beginning of the LC run permitted to separate SAA2β and SAA2α peptides, which was crucial to prevent cross talk since their product ion is common (Fig. 1). Then, the gradient slope increased to separate S100A8, SAA1γ, Apo-SAA, SAA1α, SAA2, and SAA1β peptides. Apo-SAA is a fusion protein whose sequence is hybrid between SAA1α and SAA2 (Table 1). It was used for quantitation purpose (see section 3.4). Despite being different SAA1γ and S100A8 peptides could not be resolved. However, their coelution was not considered as problematic since their MRM transitions were different (Supplementary data S1). The next gradient step was the most critical as SAA1α and SAA1β are isobaric and differ from SAA2 by 0.4 Da. Finally, S100A9 and SAA-tot (representing the SAA regardless of the variant) peptides were eluted. Noticeably, the presence of ammonia in the sample preparation phase did not cause deamidation to peptides containing sensitive amino acids such as asparagin (N) or glutamine except for the peptide coming from Apo-SAA, where a mass shift of +1 Da was observed on the doubly-charged precursor (+ 0.5 *m/z*). The y10 fragment ion used as quantifier was observed at 1089.4 instead of 1088.4 (+1 mass shift). Similarly, the y9 fragment ion used as qualified was observed at 903.4 *m/z* instead of 902.4 (+1 mass shift). However, this was not considered as problematic since it did not hamper the quantitation process.

3.2. Optimization of sample preparation

The acidic character of the studied peptides (median of isoelectric points: 4.54) indicated that the anion-exchange sorbents would yield better extraction yields than cation-exchange ones. Consequently, ammonia (at a final concentration of 5%) was first added to the solution before loading on the SPE plate to quench the trypsin reaction and ensure the negative ionization of the peptides. The first wash solution (composed of 5% ammonia in water) removed unspecific components and the nature of the second wash solution was optimized to remove as many unwanted interferants as possible. The use of slightly acidic conditions (0.01% FA) was investigated since it could have removed weak bases and improved the overall sample clean up. However, its presence, albeit at a low concentration, was found to be detrimental to the desirability (representing the goodness of compromise between the individual responses). (Supplementary data S3B). No significant differences were observed between neutral and basic conditions hence a mixture of MeOH/H_2O (80:20) was selected as second wash since it provided the highest desirability (0.79 ± 0.04). For the elution solution, a mixture of ACN/H_2O/FA (85:15:2, v/v) yielded the optimal desirability (0.88 ± 0.03) and was consequently selected for further experiments (Supplementary data S4B). Finally, the resuspension solution was optimized to maximise the retention of highly hydrophilic peptides (S100A12, SAA2α and SAA2β) and prevent the adsorption of hydrophobic peptides (S100A9 and SAA-tot). The presence of 0.1% TFA combined to 20% ACN was found to be the optimal conditions since TFA improved the retention of hydrophilic peptides and 20% ACN prevented the adsorption of SAA-tot peptide (Supplementary data S3C).

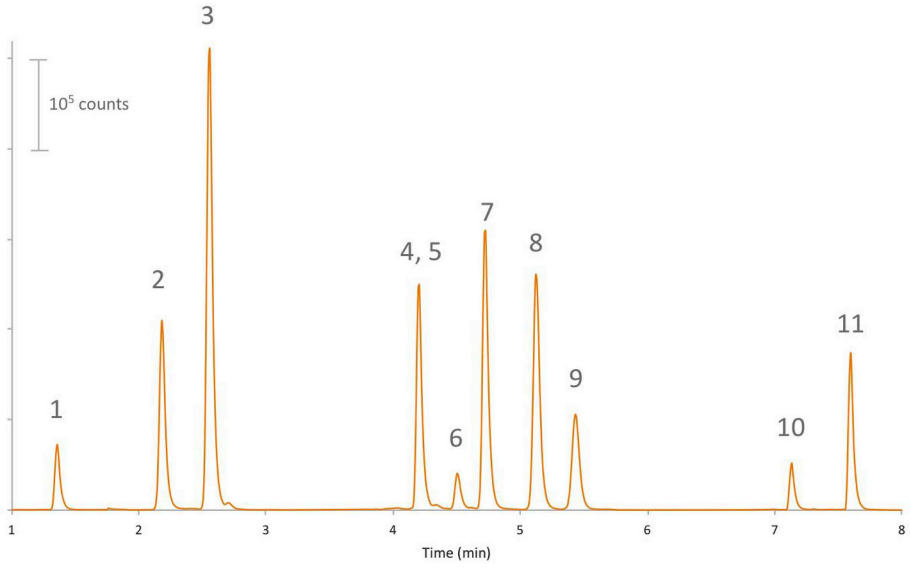

Fig. 1. Typical chromatogram (combined MRM traces) obtained after processing of calibration solution in bovine plasma and injection in LC-MS/MS. Peaks are unique peptides originating from S100A12 (1), SAA2β (2), SAA2α (3), S100A8 and SAA1γ (4, 5), Apo-SAA (6), SAA1α (7), SAA2 (8), SAA1β (9), S100A9 (10) and SAA-tot (11).

Finally, the resuspension solution used for further experiments was composed of ACN/H_2O/TFA (80:20:0.1, v/v).

3.3. Optimization of biomarker digestion

Preliminary experiments involving different trypsin-compatible denaturants (trifluoroethanol, solvents, heat, MS-compatible surfactants) did not significantly improve the digestion yield. However, they revealed that the presence of an organic solvent during tryptic digestion was required in order to prevent adsorption of peptides on surfaces and maximise their release from proteins. However, organic solvents lower the activity of trypsin [25]. DoE strategy was therefore employed to find the optimal conditions. DoE strategy was selected for optimization in this study since different proteins, having different characteristics, had to be digested. Furthermore, their signature peptides had different physico-chemical properties and some (SAA-tot and S100A9 peptides) were prone to adsorption. As a consequence, the DoE strategy permitted to optimize crucial parameters all-in-once.

As shown in Supplementary data S4, results indicate that MeOH is more desirable than ACN (desirability of 0.64 compared to 0.51, respectively) although ACN was more favourable for S100A9 and S100A8 proteins. Finally, the optimal conditions maximising sensitivity were 25% of MeOH combined to 17.5 µg of trypsin per sample.

3.4. Biomarker quantitation strategy

Two strategies coexist in the literature to quantitate proteins in sample in targeted bottom-up proteomics. Calibration curves can be built with the proteins (in our case, only Apo-SAA and SAA1α were commercially available) or with synthetic peptides from which the protein concentration can be subsequently deduced if the digestion step is completed. However, this was not the case since the tryptic digestion yield of 10 µg mL^{-1} SAA1α was calculated at 21.4 ± 0.2% (n = 3). To quantitate every variant at the proteins level, we developed a strategy employing the synthetic protein Apo-SAA to construct digestion curve transforming the concentration of SAA peptides in their initial protein concentration (Fig. 2A). Calibration solutions contained Apo-SAA, S100 proteins and the synthetic peptides specific for each SAA variant.

First, the concentration of the peptide generated by Apo-SAA after tryptic digestion was calculated using the calibration curve built with SAA2 peptide. The peak area of the Apo-SAA peptide was corrected by the peak area of the SAA2 internal standard prior to calculation using the calibration curve built with the peptide SAA2. Then, this concentration (in peptide) was plotted against the initial concentration of Apo-SAA (protein) to construct a curve which converts a concentration of peptide in a concentration of protein. This curve could subsequently be used to transform concentrations of SAA peptides into their initial protein concentrations. Apo-SAA was employed since it differs by SAA1α by only two amino-acids thus its solubility and resistance to tryptic digestion were expected to be close to those of other SAA proteins. In addition, the peptide generated after digestion is almost identical to those generated by the isoforms of SAAs. As a consequence, Apo-SAA was considered as a suitable surrogate to construct this digestion curve. This approach was employed to quantitate SAA1α, SAA1β, SAA1γ and SAA2 (Fig. 2B). However, this strategy could not be used for SAA2α and SAA2β since the followed peptides were coming from another part of the protein and were therefore not produced at the same yield as Apo-SAA peptide. Nonetheless, given that SAA2α and SAA2β both arise from SAA2 their individual concentrations were estimated by the following equation:

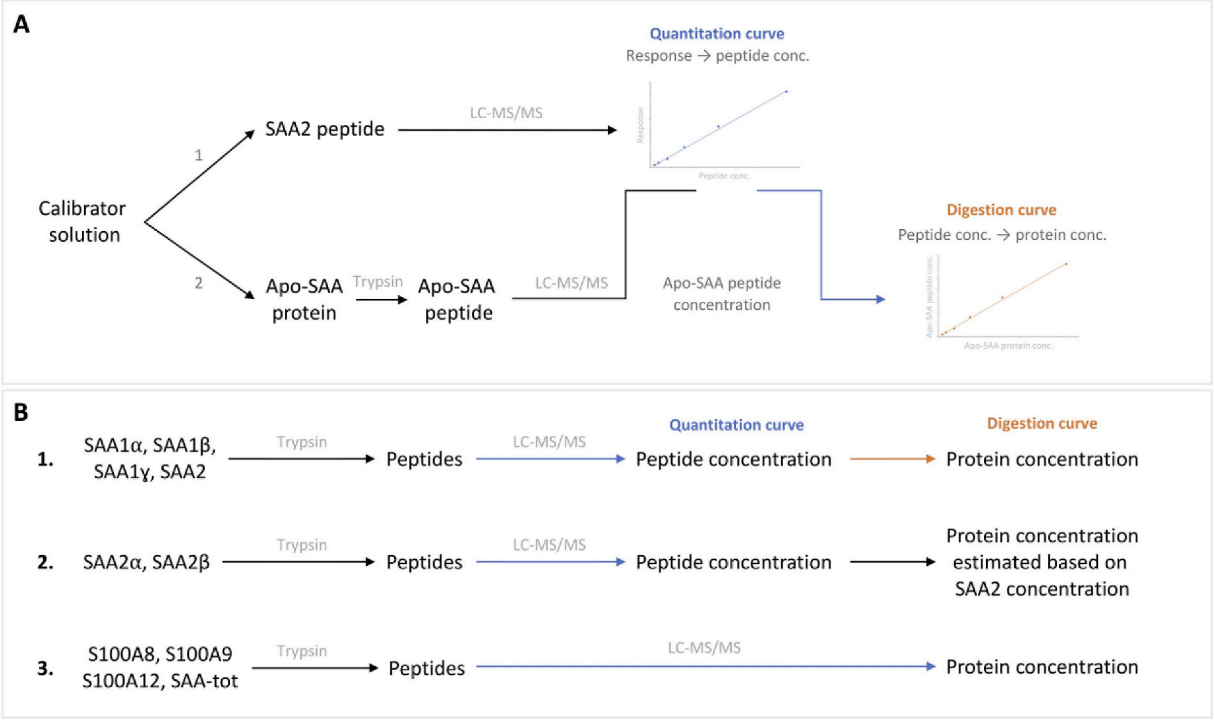

Fig. 2. Strategy employed for the quantitation of the studied proteins. (A) Construction of the digestion curve using Apo-SAA protein. The concentration of the peptide generated by the Apo-SAA protein was measured using a calibration curve built with SAA2 peptide. Then, this concentration was plotted against the initial concentration of Apo-SAA protein resulting in a digestion curve converting a concentration of peptide in a concentration of protein. (B1) strategy employed to quantitate SAA1α, SAA1β, SAA1γ and SAA2. Proteins were digested in peptide which were quantitated using their respective quantitation curves. Following this, the initial concentration of protein was calculated using the digestion curve. (B2) for SAA2α and SAA2β, the quantitation was only possible at the peptide level. The proportion of each peptide was used to estimate their absolute protein concentrations. (B3) S100A8, S100A9, S100A12 and SAA-tot were directly quantitated at the protein level owing the availability of the standard proteins.

$$[\text{SAA2}\alpha\ \text{protein}] = \left(\frac{[\text{SAA2}\alpha\ \text{peptide}]}{[\text{SAA2}\alpha\ \text{peptide}] + [\text{SAA2}\beta\ \text{peptide}]}\right) * [\text{SAA2 protein}]$$

$$[\text{SAA2}\beta\ \text{protein}] = \left(\frac{[\text{SAA2}\beta\ \text{peptide}]}{[\text{SAA2}\alpha\ \text{peptide}] + [\text{SAA2}\beta\ \text{peptide}]}\right) * [\text{SAA2 protein}]$$

Finally, S100 and SAA-tot concentrations were directly calculated at the protein level using a quantitation curve constructed using the synthetic proteins as calibrators. Apo-SAA protein was employed as calibrator to quantitate SAA-tot since the peptide common to all SAA variants was also generated by Apo-SAA.

3.5. Importance of the biological fluid chosen for the assay

The choice of biological fluid was investigated using paired plasma and serum coming from control (n = 10) and RA patients (n = 9). On average, concentrations were slightly higher in serum compared to plasma for SAA1α (1.45 ± 0.38 times), SAA2 (1.87 ± 0.16 times), SAA1β (2.00 ± 0.51 times) and for SAA-tot (1.63 ± 0.44 times) although significant differences observed between RA and patient groups were similar between plasma and serum. On the contrary, for S100A8, differences observed were significant in plasma, but not in serum. S100A9 was uniquely detected in plasma. These differences might arise due to the formation of the heterodimer calprotectin in the presence of calcium in serum. Calprotectin then self-assembles into hetero-tetramers, which was shown to be extremely protease resistant [20,26,27]. However, the chelation of calcium by EDTA in plasma prevents the formation of calprotectin thereby consequently allowing the digestion of S100A8 and S100A9. Interestingly, a recent study revealed that calprotectin should also preferably be measured in EDTA-plasma when using ELISA because its concentration is overestimated in serum due to the release of calprotectin by neutrophils when coagulation occurs [28]. S100A8/S100A9 should therefore be measured in plasma regardless of the measurement technique employed. For LC-MS/MS, this is due to the fact that trypsin cannot generate the signature peptide in serum, although the concentration is in theory paradoxically higher than in plasma. For ELISA, this is due to the overestimation of calprotectin due to its massive release by neutrophils, which affects the quality of the final results. In the light of our results, plasma was selected for the assay of the biomarkers of interest.

3.6. Analytical validation

Validation was performed at protein level for S100A8, S100A9, SAA1α, SAA-tot, and at the peptide level for SAA1β, SAA1γ, SAA2, SAA2α, and SAA2β. Since SAA1α was the only commercially available protein, it was used to validate the quantitation strategy detailed in paragraph 3.3. Apo-SAA could not be employed in the validation since it is used to construct the digestion from which the concentrations of proteins are deduced. As shown in Supplementary data S4, no significant interferences were detected at the retention time of the peptides of interest when compared to blank bovine plasma therefore ensuring the selectivity of the method. Moreover, the stability inside the thermostated autosampler was found satisfactory since no significant degradation could be observed after up to 45 h stay (corresponding to the time needed to process a complete 96-well plate) in the tray. Additionally, the freezing/defrosting of the samples did not impact the results. Calibration solutions for SAA1α were built using signature peptide, and validation solutions were built with the protein. The resulting accuracy profile of SAA1α (S5) proves the reliability of the quantitation strategy based on a digestion curve to access the initial concentration of protein. Validation results concerning trueness, precision, accuracy, matrix effect, recovery, response function, linearity and stability are presented in supplementary data S5. The quantitation of S100A12 did not match acceptance criteria and S100A12 results were therefore not further considered for the study. For the other considered proteins, the maximum observed relative bias was 9.3% and the maximum precision variability was 19.4%. As beta-expectation tolerance limits never exceeded acceptance limits (± 20% except LOQ where ± 30% was accepted), the method was considered as accurate over the tested dosing range for the quantitation of S100A8, S100A9, and SAA variants.

As shown in Fig. 3, no significant interferences were detected at the

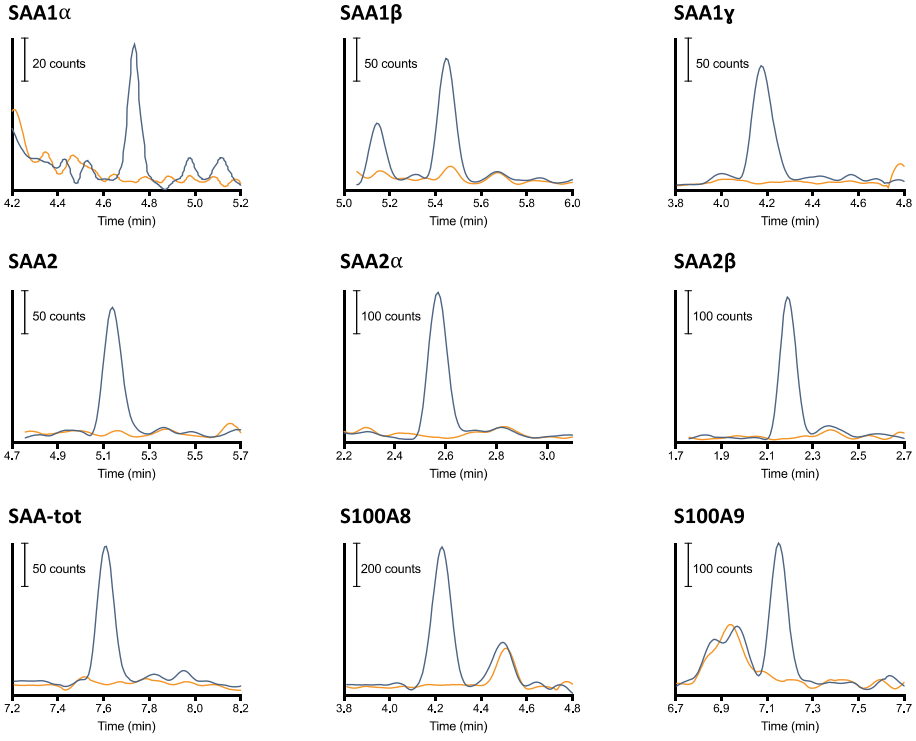

Fig. 3. Chromatograms showing the overlay of specific transition after injection of QC solution. Injected concentrations were 4 ng mL^{-1} for SAA1β, SAA1γ, SAA2, SAA2α and SAA2β (peptides); 50 ng mL^{-1} for SAA1α and SAA-tot (proteins) and 8 ng mL^{-1} for S100A8 and S100A9 (proteins) (blue trace) and blank bovine plasma (orange trace). Traces are extracted MRM transitions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

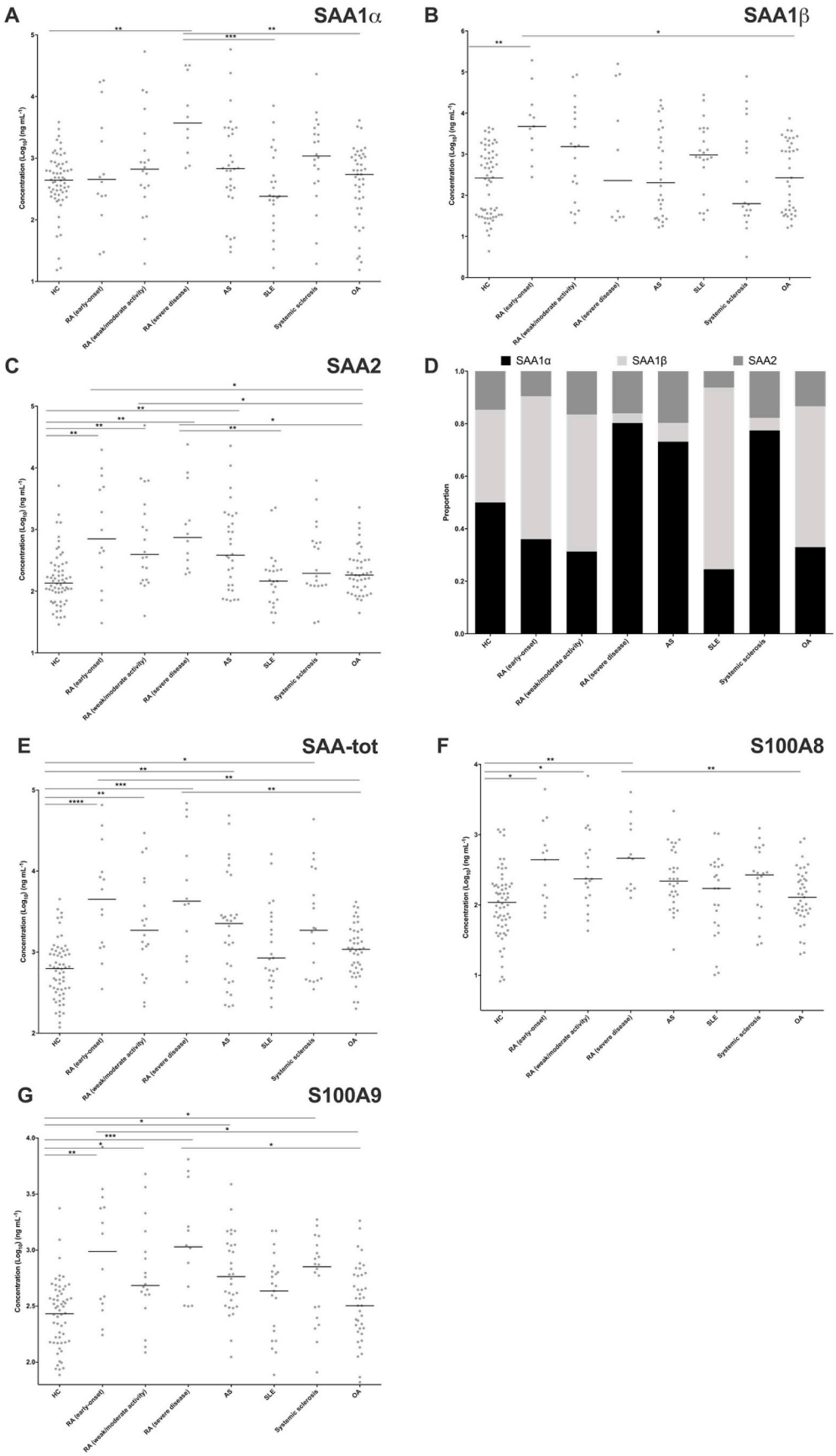

Fig. 4. Evolution of concentration of studied proteins sorted by pathology. (A) SAA1α; (B) SAA1β; (C) SAA2; (D) proportion of each isoform of SAA; (E) SAA-tot; (F) S100A8 and (G) S100A9. Bars represent median value. * represents p-value < 0.05; ** (p-value < 0.01); *** (p-values < 0.001) and **** (p-value < 0.0001) (Kruskal-Wallis followed by Dunn's test).

retention time of the peptides of interest when compared to blank bovine plasma therefore ensuring the selectivity of the method. Bovine plasma was considered as a suitable surrogate matrix for human plasma since no significant differences in terms of peak areas of the internal standards were observed between bovine and human plasma groups. Results are presented in S5H. Calibration and validation solutions were therefore mixed with bovine plasma prior tryptic digestion to ensure that calibrators were processed similarly to patient samples and hence ensure the reliability of the results generated for patients.

3.7. Clinical investigation

As shown in Fig. 4E, the clinical investigation showed that SAA-tot concentrations were significantly higher in the three subsets of RA

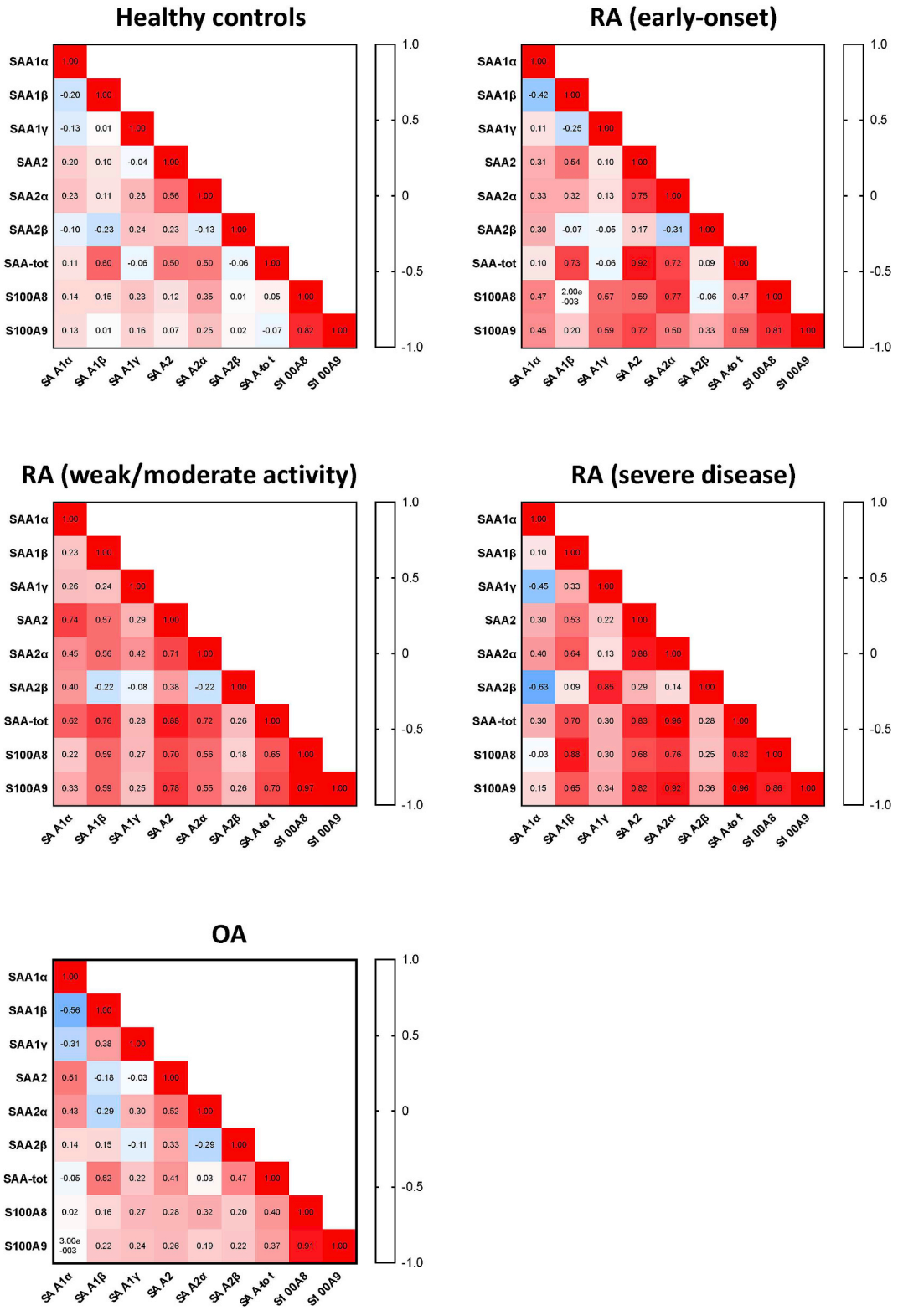

Fig. 5. Pearson's correlation coefficients obtained for each protein sorted by pathology.

patients, early-onset, weak/moderate and severe disease activity, in AS and in systemic sclerosis compared to HC. Similarly to CRP, SAA-tot levels were not increased in SLE [29]. SAA-tot levels also significantly increased in RA, early-onset and severe disease activity compared to OA patients.

Concerning SAA variants, significant differences were observed between groups for SAA1α, SAA1β, and SAA2. SAA1α levels were only significantly increased in RA patients with severe disease activity compared to HC, whereas not significantly different in RA early-onset and RA with weak/moderate disease activity (Fig. 4A), contrasting with what has been observed with SAA-tot levels in these three categories of RA compared to HC. They were also significantly higher in RA with severe disease activity than in SLE and OA (Fig. 4A). On the contrary, SAA1β levels were only increased in RA early-onset compared to HC and OA patients, but surprisingly not different than the levels observed in SLE (Fig. 4B). Opposite to SAA1α, SAA1β levels were not significantly different in RA patients with weak/moderate or severe disease activity compared to HC (Fig. 4B, Supplementary data S6). Interestingly, a statistically significant negative correlation was observed between SAA1α and SAA1β levels in RA early-onset ($r = -0.41$) and in OA ($r = -0.56$) patients (Fig. 5). The weight of SAA1α, representing the contribution of SAA1α in the total SAA response (sum of concentration of SAA1α, SAA1β and SAA2), varied among groups (min-max 32–80%) (Fig. 4D, Supplementary data S6). The weight of SAA1β also varied similarly (min-max 4–69%) (Fig. 4D, Supplementary data S6). SAA1α made up to 80% (median) of the total concentration of SAA variants in RA with severe disease activity, while SAA1β represented only 4% (median). Although the SAA response remains weak in SLE, SAA1β made up to 69% (median) of the total concentration of SAA variants while SAA1α represented only 25% (median). SAA1γ concentrations remained constant in all the groups (Supplementary data S6).

SAA2 levels were significantly higher in the 3 categories of RA as compared to HC (Fig. 4C, Supplementary data S6). SAA2 was also significantly increased in RA (early-onset) and RA (severe disease) compared to SLE and OA patients, implying that SAA2 might represent a marker of interest to differentiate RA from related pathologies. SAA2 levels were highly correlated to SAA1β, S100A8, and S100A9 at all stages of RA (Fig. 5). Within SAA2, SAA2α was the dominant subtype compared to SAA2β, the latter being detected at a constant level in each group (Supplementary data S6). SAA2α levels were also significantly higher in the three subsets of RA and in AS patients compared to HC (Supplementary data S6).

As a conclusion, the acute phase response was not only quantitatively different among the pathologies studied, but also qualitatively by different representation of isoforms. Indeed, SAA2 (mainly SAA2α subtype) concentrations were increased in the 3 subsets of RA patients, early-onset, weak/moderate and severe disease activity, while SAA1β only increased in RA early-onset and SAA1α only increased in RA patients with severe disease activity. Importantly, the weights of SAA1α and SAA1β levels in the SAA response were different according to the pathologies studied. Noticeably, no correlation with SAA1α was observed in any diseases. Compared to the literature, similar observations were realized by Kim et al. concerning SAA1γ and SAA2β for lung cancer patients, for which SAA1γ was mostly not detected and SAA2β significantly less present than SAA2α [6]. Even if the pathophysiological relevance of these quantitative and qualitative characteristics of the SAA response remains unknown, the significant negative correlation observed between SAA1α and SAA1β levels in RA early-onset (and OA patients) suggests the existence of still unknown regulatory mechanisms in these diseases.

Alarmins S100A8 and S100A9 concentrations were highly correlated in all categories studied, including HC (Fig. 5 and Supplementary data S6), due to their coexistence under the form of a dimer in physiological media. Their concentrations were significantly increased only in the 3 subsets of RA patients, compared to HC (Fig. 4F). S100A8 levels in RA with severe disease activity were also significantly higher than in OA patients (Fig. 4F). Lastly, S100A9 levels of RA patients with early-onset and with severe disease activity patients were also significantly higher than in SLE and in OA patients (Fig. 4G). Levels of alarmins were

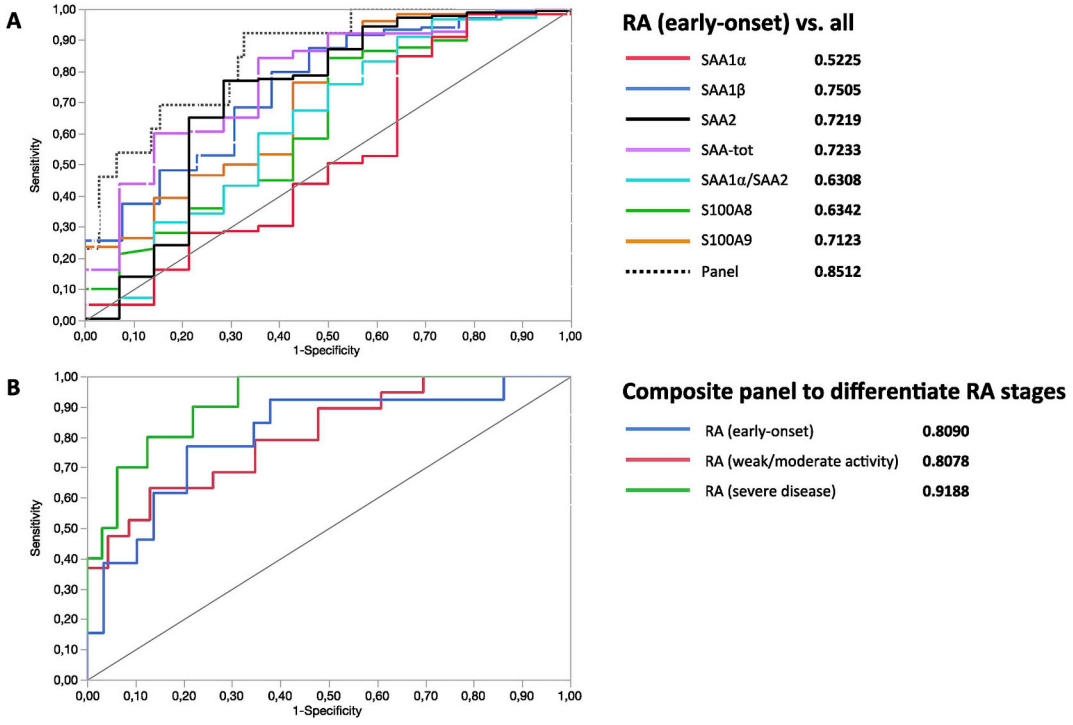

Fig. 6. Representation of ROC curves. (A) Discrimination of RA (early-onset) from other IMID pathologies using biomarkers taken individually and the composite panel. (B) Ability of the same composite panel to correctly assign the severity grade of RA. AUCs are indicated in bold and represent how well the model is performing at distinguishing between groups.

unrelated to any of the SAA variants levels in controls (Fig. 5). On the contrary, complex correlations were observed in diseases. First, their concentrations were strongly correlated with SAA2 concentrations in the 3 stages of RA, moderately correlated in SLE, systemic sclerosis, and in AS patients and not in OA patients. Second, their concentrations were strongly correlated to SAA1β in established RA, whether low/moderate or severe disease activity. Third, curiously, SAA1α, a major component of the acute phase response in RA with severe disease activity, is never correlated with S100A8/9 levels neither in RA, nor in any others diseases. Fourth, S100A8 and S100A9 levels were significantly correlated with SAA-tot in RA patients with weak/moderate or with severe disease activity as well as in OA patients (Fig. 5) whereas S100A9 alone did in early-onset RA. These observations suggest reciprocal influence of regulatory mechanisms between SAA variants and S100A8/A9 proteins. This elevation of S100A8 and S100A9 levels in plasma RA in the present work contrasts with other work that identified S100A8 and S100A9 in the serum of RA patients at equal levels as compared to OA, AS and SLE patients [30]. Differences may be explained either by differences in the proteomic technique and the biological fluids used. This study confirms previous results obtained by our group where S100A8, S100A9, and SAA protein levels were significantly higher in RA sera (and in AS and psoriatic arthritis) as compared to HC using semi-quantitative SELDI-TOF-MS [3]. In addition, this work extends to the isoforms the correlation previously highlighted between S100A8 and S100A9 levels and SAA variants found in the serum of RA, AS and psoriatic arthritis patients.

Finally, the ability of each biomarker to discriminate between RA (early-onset) and other studied pathologies (considered as non-RA) was tested with ROC curves. Curves and individual area under curves (AUC) for each biomarker are given in Fig. 6A. The AUC of the panel was 0.8512. The probability of correctly classify patients was higher with the ratio of SAA1α/SAA2 (AUC 0.8512) than without (AUC 0.7848). Interestingly, the composite panel of biomarkers increased the probability of correctly classify patients compared to the best biomarker (SAA-tot) considered individually, thereby underlying the interest of selectively quantitating SAA variants to differentiate between RA at early-onset and other related inflammatory pathologies.

The panel including the ratio of SAA1α/SAA1β was introduced to discriminate the three grades of RA. AUC were respectively 0.8090, 0.8078, and 0.9188 for RA (early-onset), RA (weak/moderate activity), and RA (severe disease) (Fig. 6B). Without introducing the ratio of SAA1α/SAA1β in the panel, the AUC were respectively 0.7454, 0.7712, and 0.8438 for RA (early-onset), RA (weak/moderate activity), and RA (severe disease). The ratio of SAA1α/SAA1β added to the previous panel therefore significantly enhanced the probability of classify patients in the correct severity stage. Although in this case clinical signs might be sufficient to differentiate the three stages of RA, it is interesting to notice that this classification can be achieved based solely on the quantitation of the biomarkers. However, this needs to be confirmed on larger cohorts to train and confirm the classification algorithm.

These results suggest that SAA isoforms, especially SAA2 and SAA1β, as well as S100 proteins could serve as biomarkers prior the onset of symptomatic RA thereby joining other pre-clinical biomarkers such as the rheumatoid factor (RF), auto-antibodies (*anti*-citrullinated proteins – *anti*-CCP), cytokines and CRP, for instance. However, a negative RF or *anti*-CCP does not necessarily rule out RA as it is the case for approximately 15–25% of patient [31,32]. As a consequence, SAA isoforms might be used in combination with existing blood tests to provide additional specificity. Furthermore, the ability of SAA isoforms to fluctuate (in terms of nature and concentration) in accordance with the severity could be advantageously used for treatment follow-up studies.

4. Conclusion and perspectives

In conclusion, we have developed an assay to selectively quantitate SAA variants and alarmins S100A8 and S100A9 using tryptic digestion, SPE and LC-MS/MS. A novel approach was employed to access the absolute concentrations of non-commercially available proteins. The method was developed using DoE and subsequently validated according to regulations. Successively, SAA variants and S100A8/S100A9 were studied in plasma of HC and patients suffering from several inflammatory diseases. Results indicated that SAA-tot levels were significantly higher in systemic inflammatory pathologies such in the 3 subsets of RA patients, in AS, and in systemic sclerosis than in OA patients or in HC. Interesting profiles were discovered in RA patients where quantitative and qualitative differences among SAA variants were highlighted. SAA2 (mainly SAA2α subtype) concentrations were increased in the 3 subsets of RA patients, early-onset, weak/moderate and severe disease activity, while SAA1β only increased in RA early-onset and SAA1α only increased in RA patients with severe disease activity. The weights of SAA1α and SAA1β levels in the SAA response were different according to the pathologies studied. Compared to controls, S100A8 and S100A9 levels were exclusively increased in the 3 subsets of RA patients. Further, while S100A8 and S100A9 levels were unrelated to any of the SAA variants levels in controls, complex correlations were observed with SAA1β, SAA2 and SAA-tot in diseased patients. Noticeably, no correlation with SAA1α was observed in any diseases. These proteins performed better at discriminating RA (early-onset) than other IMID when in composite panel as compared to proteins put individually. These results underline the benefits of using LC-MS/MS to discriminate closely-related isoforms, which would not have been differentiated otherwise. The careful optimization of the protocol allowed to analyze these proteins in a single method thus taking advantage of the multiplexed capacities of LC-MS/MS.

Altogether, these results highlight the added-value of the selective quantitation of SAA variants in the context of RA and pave the way for treatment follow-up studies and theranostic applications since the expression of the studied biomarkers seem to be related to the severity of the pathology. For instance, regular monitoring of SAA and S100 proteins could provide insights on the effectiveness of the given treatment which could be modified and adapted accordingly if needed.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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