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ORIGINAL ARTICLE

Fetuin-A and thyroxin binding globulin predict rituximab response in rheumatoid arthritis patients with insufficient response to anti-TNFα

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Abstract

Objectives Rheumatoid arthritis (RA) is a debilitating disease, but patient management and treatment have been revolutionized since the advent of bDMARDs. However, about one third of RA patients do not respond to specific bDMARD treatment without clear identified reasons. Different bDMARDs must be tried until the right drug is found. Here, we sought to identify a predictive protein signature to stratify patient responsiveness to rituximab (RTX) among patients with an insufficient response to a first anti-TNFα treatment.

Methods Serum samples were collected at baseline before RTX initiation. A proteomics study comparing responders and nonresponders was conducted to identify and select potential predictive biomarkers whose concentration was measured by quantitative assays. Logistic regression was performed to determine the best biomarker combination to predict good or nonresponse to RTX (EULAR criteria after 6 months' treatment).

Results Eleven biomarkers potentially discriminating between responders and nonresponders were selected following discovery proteomics. Quantitative immunoassays and univariate statistical analysis showed that fetuin-A and thyroxine binding globulin (TBG) presented a good capacity to discriminate between patient groups. A logistic regression analysis revealed that the combination of fetuin-A plus TBG could accurately predict a patient's responsiveness to RTX with an AUC of 0.86, sensitivity of 80%, and a specificity of 79%.

Conclusion In RA patients for whom a first anti-TNFα treatment has failed, the serum abundance of fetuin-A and TBG before initiating RTX treatment is an indicator for their response status at 6 months. ClinicalTrials.gov identifier: NCT01000441.

Key Points

- *Proteomic analysis revealed 11 putative predictive biomarkers to discriminate rituximab responder* vs. *nonresponder RA patients.*
- *Fetuin-A and TBG are significantly differentially expressed at baseline in rituximab responder* vs. *nonresponder RA patients.*
- *Algorithm combining fetuin-A and TBG accurately predicts response to rituximab in RA patients with insufficient response to TNFi.*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10067-020-05030-6) contains supplementary material, which is available to authorized users.

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Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disease characterized by joint inflammation leading to joint destruction and functional disability [1]. Biologic disease-modifying anti-rheumatic drugs (bDMARD) such as tumor necrosis factor-alpha inhibitors (TNFi) have completely changed the outcome and prognosis for patients with RA. However, for unknown reasons, around 30–50% of patients fail to respond to bDMARDs [2]. For patients presenting an inadequate response to TNFi treatment, the recommended treatment strategy is to switch to another biologic. However, selection of a new therapeutic option is not well-defined when managing first-line bDMARD failure. Indeed, in addition to TNFi (infliximab, etanercept, adalimumab, golimumab, and certolizumab pegol), rheumatologists now have access to a broad range of bDMARDs targeting different immune cell types or molecular mechanisms involved in immunology—such as $CD20^+$ B cells (rituximab), activation of T cells (abatacept), IL6 receptors (tocilizumab and sarilumab)—and to targeted synthetic disease-modifying anti-rheumatic drugs (tsDMARD) directed against Janus kinases (tofacitinib and baricitinib). These new molecules are reported to be as effective as TNFi [3]. Whether the best approach is to select an alternative TNFi (rotating) or to opt for a biologic targeting a different molecular mechanism (changing) remains controversial [4], but recent evidence supports the efficacy of the latter approach, albeit without providing clear evidence on which individual mechanism of action to favor [5]. Identifying the right bDMARD for each patient is critical given that, for patients who have already been exposed to TNFi, the likelihood of a response to subsequent treatment with biologics declines as the number of previous TNFi treatments increases [6]. The reason why some patients respond to TNFi and others respond better to non-TNFi biotherapy remains elusive. In the absence of well-defined international recommendations, the available data favor a personalized approach, tailoring treatment to each individual patient. In a previous study, we identified predictive biomarkers of response to abatacept for RA patients not responding to a first TNFi treatment (TNF-IR) [7].

In this study, we aimed at extending our approach and focus our work on characterizing relevant blood biomarkers, which, when integrated into a multivariate model, could predict response to rituximab (RTX) as a second-line bDMARD treatment. To do so, patient samples, who have failed to respond to a first TNFi, were explored using discovery proteomics to reveal proteins that are differentially expressed between responders (R) and nonresponders (NR), as determined based on EULAR criteria at 6 months. This strategy identified a pool of candidate biomarkers that were then submitted to in vitro diagnostic (IVD) validated assay for validation. Finally, those results were combined in a multivariate analysis to generate a predictive model. This study paves the way for the generation of a predictive test to stratify patients for whom treatment with a first TNFi has failed.

Methods

Patient samples and study design

Patients from the "Rotation or change of biotherapy after first Anti-TNF treatment failure for rheumatoid arthritis" (ClinicalTrials.gov identifier: NCT01000441, [5]) cohort were selected according to these criteria: (i) RA patient with insufficient response to TNFi, (ii) DAS28 at baseline and 6 months, (iii) blood samples collected at baseline, and (iv) patients were treated with RTX. Two patient populations were studied. A first "discovery" population that included 10 patients was dedicated to the discovery of new protein biomarkers associated to drug responsiveness. A second "validation" population consisting of 25 patients was devoted for the validation of the identified biomarkers to discriminate responders from nonresponders to RTX.

Assessing clinical response

Demographic parameters and disease activity were recorded at baseline and 6 months after RTX initiation. Response to RTX treatment was assessed based on the EULAR criteria, with the 28-joint disease activity score (DAS28-ESR and DAS-CRP) [8, 9]. In this study, patients were considered as R if they showed a good EULAR response, and as NR if they showed a moderate response or lack of response. According to those criteria, there were 5 NR and 5 R in the discovery population and 20 NR and 5 R in the validation population. Intolerance status was not taken into account in our R/NR classification. Remission was defined as DAS28 < 2.6 and low disease activity (LDA) as DAS28 < 3.2 at 6 months.

Serum sample preparation for proteomic analyses

Crude and depleted serum samples from 5 R and 5 NR patients were used in the discovery proteomics analyses. Serum depletion was performed using the human Multiple Affinity Removal System (MARS) spin cartridge (Agilent Technologies). Crude and depleted sera were then prepared according to the Multiple Enzyme Digestion-Filter Aided

Sample Preparation protocol using a 3000-Da cutoff ultrafiltration device (Merck Millipore) as described [10].

Mass spectrometry-based quantitative proteomic analyses

Peptides were analyzed by online nano-liquid chromatography coupled to tandem mass spectrometry (UltiMate 3000 RSLCnano coupled to QExactive HF Quadripole-Orbitrap, Thermo Scientific) using a 120-min gradient. To do so, peptides were sampled on a 300-μm × 5-mm PepMap C18 precolumn and separated on a 75-μm × 250-mm C18 column (Reprosil-Pur 120 C18-AQ 1.9 μm, Dr. A. Maisch, HPLC-GmbH). Mass spectrometry (MS) and MS/MS data were acquired using Xcalibur.

Peptides and proteins were identified and quantified using MaxQuant (version 1.5.8.3) [11] through concomitant searches against the UniProt database (Ref Proteome human *Homo sapiens* taxonomy, January 2018 version) and the frequently observed contaminant database included in MaxQuant. Trypsin was chosen as the enzyme and two missed cleavages were allowed. Peptide modifications allowed during the search were as follows: carbamidomethylation (C, fixed), acetyl (Protein N-ter, variable), and oxidation (M, variable). Minimum peptide length was set to seven amino acids. Minimum number of peptides, razor + unique peptides, and unique peptides were all set to 1. Maximum false discovery rates—calculated by applying a reverse database strategy—were set to 0.01 at peptide and protein levels. Intensities of proteins were calculated from MS intensities of unique and razor peptides and used for statistical analyses using ProStaR [12].

Assessment of blood biomarkers

Baseline serum levels of thyroxin binding globulin (TBG) (Monobind Inc., 3525-300), fetuin-A (FetA) (Biovendor, RD191037100), and S100A8/A9 (ImmunoDiagnostik, K6939) were evaluated using commercial ELISA kits according to the manufacturers' instructions. Levels of C4-binding protein alpha chain (C4BPA) were quantified using an in-house sandwich ELISA targeting the complex C4BP as described in [7]. Baseline levels of lipoprotein(a) (Lp(a)), haptoglobin (Hp), serum amyloid A (SAA), and C1q were evaluated by nephelometry on a BNII system (Siemens Healthcare).

Statistics

Statistical analyses of quantitative LC-MS/MS proteomics data were performed using ProStaR [12]. Proteins identified in the reverse database, common contaminants, and proteins for which fewer than five intensity values were available in R or NR samples were discarded from the list. After log2 transformation, intensity values were normalized by median centering before imputing missing values (replacing missing values by the 2.5 percentile value for each column); statistical testing was conducted using a limma *t* test. Differentially expressed proteins were selected by applying a log2 (fold-change) cutoff of 0.5 and a *p* value cutoff of 0.05. For the validation study, baseline biomarker levels and demographic characteristics were compared using Fisher's exact tests and Wilcoxon–Mann–Whitney nonparametric tests. Associations between baseline biomarker levels and clinical response at 6 months were tested using univariate and multivariate logistic regression models after log transformation of biomarker values when necessary as described [7].

Results

Studied population

Among patients with RA with an insufficient response to a first TNF inhibitor included in the ROC study, 41 patients were treated with RTX. Thirty-eight baseline samples were available to carry out the study. Of these 38 samples, three patients had no DAS28 data at 6 months and are therefore excluded from our study. Demographic, clinical, and biological data are given at the time of treatment initiation (Table 1). The R and NR groups were not statistically different in terms of clinical and biological characteristics before the start of treatment apart from CRP, DAS28-ESR, and DAS28-CRP which are higher in the nonresponder group.

Discovery population: identification of differentially abundant serum proteins by mass spectrometry

To maximize our capacity to discover accessible proteins displaying differential abundance between R and NR patients, we compared serum samples from "extreme" R and NR patients who displayed high EULAR response and no response, respectively. Demographic, clinical, and biological data for cohort referred to as "Discovery" are given at the time of treatment initiation (Table 1). The R and NR groups were not statistically different in terms of clinical and biological characteristics before starting the treatment, including DAS28-CRP and DAS28-ESR. Only CRP was found significantly higher in NR patients compared to R patients, but it had no consequences on the disease score activity since DAS28-CRP was not statistically different between the R and NR groups.

Biomarker discovery was achieved through MS-based label-free quantitative analysis. To maximize analysis depth and serum proteome coverage, two types of sample preparation protocols were applied: (i) protein digestion from crude

Table 1 Baseline demographics and clinical assessments for the studied populations

	Discovery + validation population			Discovery population			Validation population		
	Responders $N = 10$ Median [Q1; Q3]	Nonresponders $N = 25$ Median [Q1; Q3]	p value (Wilcoxon or Fisher test)	Responders $N = 5$ Median [Q1; Q3]	Nonresponders $N = 5$ Median [Q1; Q3]	p value (Wilcoxon or Fisher test)	Responders $N = 5$ Median [Q1; Q3]	Nonresponders $N = 20$ Median [Q1; Q3]	p value (Wilcoxon or Fisher test)
Age, years	63 [49; 67]	57 [46; 71]	0.9	65 [49; 68]	72 [58; 75]	0.55	62 [50; 63]	56 [46; 68]	0.92
BMI, kg/m^2	27 [23; 32]	23 [20; 24]	0.054	29 [27; 32]	22 [22; 23]	0.095	26 [21; 26]	23 [20; 25]	0.45
ESR, mm/h	16 [7.2; 27]	28 [16; 63]	0.096	12 [8; 22]	29 [28; 66]	0.056	19 [6; 29]	21 [14; 63]	0.36
CRP, mg/L	2.7 [1.3; 4]	6.5 [3.8; 19]	0.018*	2.5 [2.2; 2.8]	15 [5; 20]	0.016*	4 [1; 5.9]	6.5 [2.8; 19]	0.16
DAS28-ESR, units	4.4 [4; 4.6]	5.2 [4.4; 6.6]	0.042*	4.6 [4; 5]	5.2 [5; 5.6]	0.22	4.3 [4.2; 4.5]	5.3 [4.3; 6.6]	0.11
DAS28-CRP, units	3.9 [3.6; 4.3]	4.6 [4.1; 5.3]	0.042*	3.9 [3.6; 4.3]	4.6 [4.4; 4.8]	0.22	3.8 [3.6; 4.3]	4.6 [4.1; 5.3]	0.11
Disease duration, years	9.5 [3; 18]	9.5 [4; 18]	1	11 [8; 16]	8 [7; 14]	0.60	2 [2; 18]	9.5 [4; 18]	0.47
Female, %	90	83.3	1	100	100	1	80	80	1
Rheumatoid factor positive, %	90	87	1	80	80	1	100	84	1
Anti-CCP positive, (%)	90	83	1	80	40	0.52	100	90	1
Res V0 IgG	11 [11; 13]	11 [9.2; 15]	0.89	12 [11; 13]	6.7 [5.2; 11]	0.15	11 [9.8; 12]	12 [10; 15]	0.58
Methotrexate, %	60	62.5	1	40	60	1	80	60	0.62
Leflunomide, %	0	12.5	0.54	0	40	0.44	0	10	1

Data are expressed as median (interquartile range) unless otherwise stated. The threshold for significance * was set at $p < 0.05$

BMI, body mass index; *DAS28*, Disease Activity Score 28; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *Anti-CCP*, anti-cyclic citrullinated peptide

(nondepleted) serum and (ii) protein digestion after serum depletion of the most abundant proteins, to improve the detection of low-abundant proteins. Stringent computing and statistical analysis of the data generated allowed quantification of 206 different proteins from nondepleted and 295 proteins from depleted sera. Among these proteins, several were statistically found to be differentially abundant between R and NR patients (Fig. 1. Indeed, starting from crude serum, 7 proteins were found to be enriched in samples from R patients, whereas 6 proteins were more abundant in samples from NR patients. Using depleted samples, 43 proteins were identified as enriched in sera from R patients and 15 were more abundant in sera from NR patients. A list of 11 putative predictive biomarkers was selected for further analyses: fetuin-A, Lp(a), TBG, S100A8 and S100A9, C4BPA and C4BPB, haptoglobin (Hp), C1qA and C1qB, and serum amyloid A1 (SAA1) (Supplementary Table 1).

Following MS-based proteomics, we aimed at verifying the putative biomarkers in the same cohort using reliable quantitative immunoassays validated for IVD purpose. For C1qA and C1qB which share very similar expression pattern (Supplementary Table 1), we decided to measure the complex C1q (composed of A-, B-, and C-chains) which has an IVD assay kit. A similar strategy was employed for C4BPA and C4BPB for which we measured the C4BP complex and as well for S100A8 and S100A9 which were evaluated with an ELISA kit detecting the S100A8/A9 complex. Consistent with the proteomics data, quantitative immunoassays showed that FetA and TBG were more abundant in the R group, whereas S100A8/A9, Hp, and SAA were expressed at a higher level in the NR group. Only the expression pattern of C1q was not confirmed (Supplementary Fig. 1).

Validation population: confirmation of the relevance of biomarkers identified by proteomics to discriminate between R and NR populations at 6 months by quantitative assays

To validate the proteomic results, we used a second cohort of 25 RA patients in which 5 patients were classified as R and 20 as NR after 6 months of rituximab treatment. The demographic, clinical, and biological data did not differ globally between

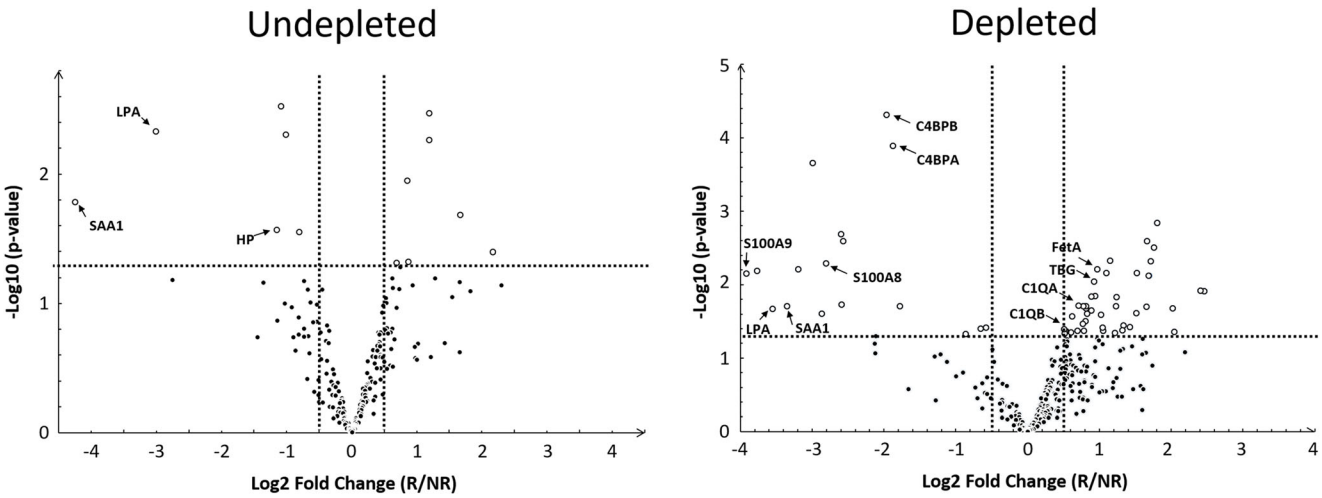

Fig. 1 Identification of differentially abundant proteins in sera from responder (R) and nonresponder (NR) patients. The volcano plots represent the −log10(*p* value) plotted against the log2(fold-change R/NR) for proteins identified and quantified by mass spectrometry from undepleted (left panel) or depleted (right panel) sera. Proteins identified as enriched (log2(fold-change) > 0.5 and *p* value < 0.05) in R and NR patients are shown in white circles. Arrowheads indicate the selected proteins for verification. FetA, fetuin-A; Lp(a), lipoprotein(a); TBG, thyroxin binding globulin; S100A8 and S100A9; C4BPA, C4-binding protein alpha; C4BPB, C4-binding protein beta; Hp, haptoglobin; C1qA, complement C1q subcomponent subunit A; C1qB, complement C1q subcomponent subunit B; SAA1, serum amyloid A1

validation and discovery cohorts except for the percentage of ACPA-positive patient (Table 2). However, this ACPA percentage was not significantly correlated with the EULAR R and NR status of the patients in the validation cohort as for the other studied parameters (Table 1). In this population, neither CRP nor DAS28 was significantly different between R and NR suggesting that the difference in CRP expression was mainly due to patients that were on the extreme opposite side of the EULAR response status but it could not differentiate moderate responders or nonresponders.

Serum concentrations of the selected biomarkers were then determined in the entire validation cohort ($n = 25$ patients). As observed for the discovery cohort, the pattern of protein expression was kept with a lower concentration of Hp, S100A8/A9, Lp(a), C4BP, and SAA and a higher expression of FetA and TBG in the R patient group (Fig. 2. As illustrated in Fig. 2,

Table 2 Comparison of the baseline demographics and clinical parameters between the discovery and validation populations

	Discovery cohort *N* = 10 Median [Q1; Q3]	Validation cohort *N* = 25 Median [Q1; Q3]	*p* value (Wilcoxon or Fisher test)
Age, years	66 [51; 73]	56 [46; 68]	0.32
BMI, kg/m^2	23 [22; 28]	23 [20; 26]	0.29
ESR, mm/h	28 [14; 37]	20 [13; 56]	0.8
CRP, mg/L	4 [2.6; 12]	5 [2.3; 12]	0.78
DAS28-ESR, units	5 [4.5; 5.3]	4.8 [4.2; 6]	0.87
DAS28-CRP, units	4.3 [3.8; 4.7]	4.5 [3.8; 5.2]	0.76
Disease duration, years	9.5 [7.2; 16]	9 [3; 18]	0.50
Female, %	100	80	0.29
Rheumatoid factor positive, %	80	87.5	0.62
Anti-CCP positive, (%)	60	92	0.043*
Res V0 IgG, g/L	11 [7.7; 13]	11 [9.8; 14]	0.56
Methotrexate, %	50	64	0.47
Leflunomide, %	20	8	0.56

Data are expressed as median (interquartile range) unless otherwise stated. The threshold for significance * was set at $p < 0.05$

BMI, body mass index; *DAS28*, Disease Activity Score 28; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *Anti-CCP*, anti-cyclic citrullinated peptide

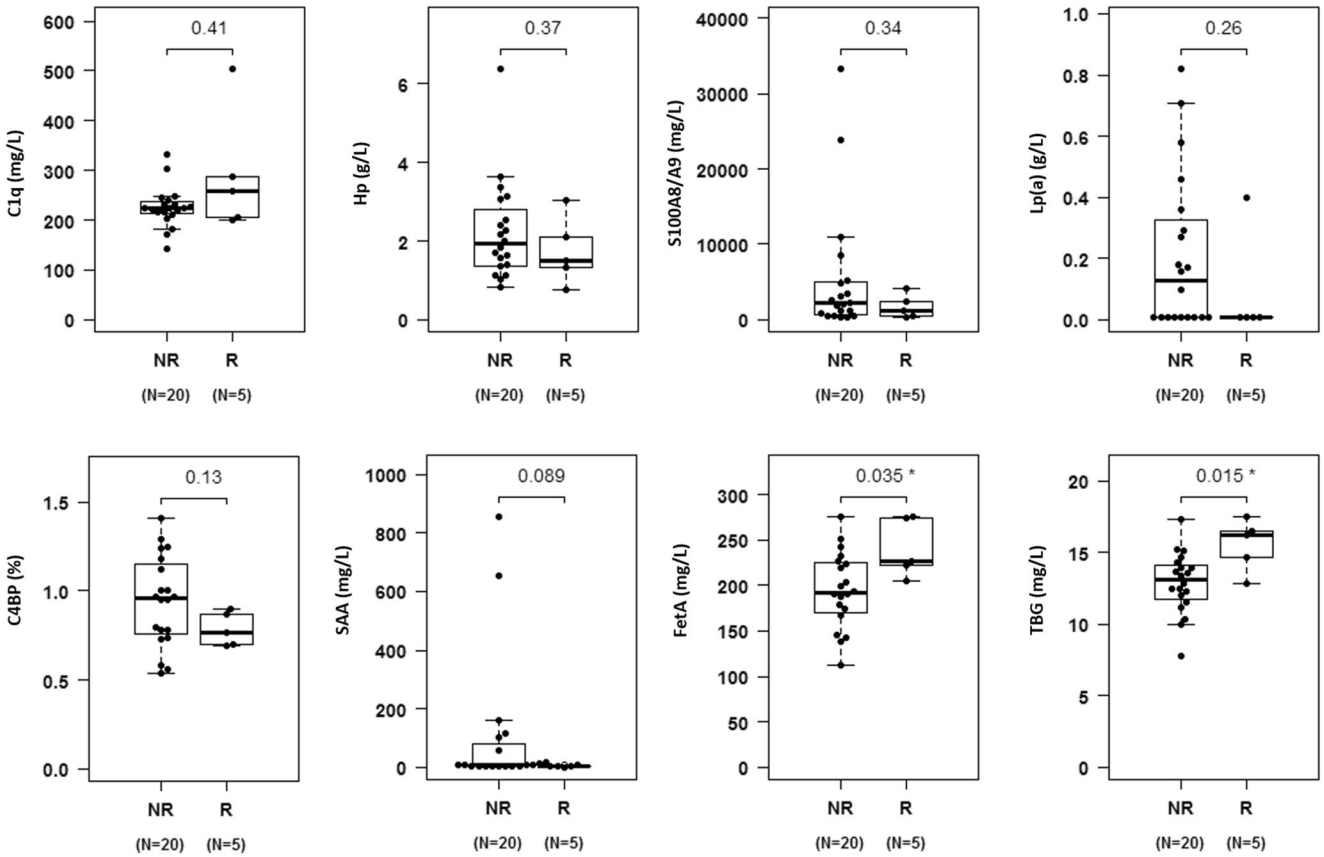

Fig. 2 Baseline biomarker concentrations for the combined cohorts classed as patients for whom a good (R) or poor (NR) EULAR response was measured after 6 months' RTX treatment. A Mann–Whitney non-parametric test was used to assess the significance of differences of biomarkers. The threshold for significance * was set at $p < 0.05$. FetA, fetuin-A; Lp(a), lipoprotein(a); TBG, thyroxin binding globulin; C4BP, C4-binding protein; Hp, haptoglobin; C1q, complement C1q; SAA1, serum amyloid A1

while the concentrations R vs. NR of Hp, S100A8/A9, Lp(a), C1q, C4BP, and SAA were not statistically different between the two groups, only FetA (226 and 192 mg/L, $p = 0.035$) and TBG (16 and 13 mg/L, $p = 0.015$) were significantly overexpressed in responders.

Investigating the predictive value of validated biomarkers

The predictive value of the most discriminant proteins identified within the discovery population and validated by absolute quantification in the validation population, i.e., FetA and TBG, was tested in univariate logistic regression analysis. Since discovery and validation cohort were similar in terms of demographic and clinical characteristics (Table 2), univariate logistic regression analysis was performed on the combined data set from both cohorts in order to gain power for predictive analyses. FetA and TBG displayed along with an AUC-ROC value of 0.84 and 0.80, respectively, indicating a high theragnostic potential. This predictive capacity was associated with high sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) (Table 3). The combination of those two biomarker into a multivariate regression model improved slightly the classification of patients in the R and NR groups with an AUC-ROC of 0.86 (Table 3, Fig. 3, sensitivity of 80% (CI 44–97%), specificity of 79% (CI 58–93%), PPV of 62% (CI 32–86%), NPV of 90% (CI 70–99%), positive likelihood ratio (LR+) of 3.84 (CI 1.66–8.89), and negative likelihood ratio (LR−) of 0.25 (CI 0.07–0.89). Although EULAR criteria is commonly used to evaluate clinical response, international recommendation mentioned that treatment should be aimed at reaching remission or LDA in every RA patient [13]. The combination of FetA and TBG showed also good predictive properties for both remission and LDA criteria (Table 3).

Discussion

In the current context of personalized medicine for RA management, the optimization of drug prescription is critical, particularly when failure to respond to several consecutive biologics considerably reduces the chances that the patient will find the right medication [6]. Consequently, a biologic should

Table 3 Receiver operating characteristic (ROC) curve analysis of potential theragnostic biomarkers

	Evaluation criteria	AUC	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
FetA	EULAR	0.84	1 (0.69–1.00)	0.71 (0.49–0.87)	0.59 (0.33–0.82)	1.00 (0.80–1.00)
TBG	EULAR	0.80	0.70 (0.35–0.93)	0.88 (0.68–0.97)	0.70 (0.35–0.93)	0.88 (0.68–0.97)
FetA plus TBG	EULAR	0.86	0.80 (0.44–0.97)	0.79 (0.58–0.93)	0.62 (0.32–0.86)	0.90 (0.70–0.99)
FetA plus TBG	Remission	0.71	0.67 (0.22, 0.96)	0.68 (0.48, 0.84)	0.31 (0.09, 0.61)	0.90 (0.70, 0.99)
FetA plus TBG	Low disease activity	0.78	0.73 (0.39, 0.94)	0.78 (0.56, 0.93)	0.62 (0.32, 0.86)	0.86 (0.64, 0.97)

The AUCs and associated standard errors and the 95% confidence interval as well as sensitivities, specificities, positive predictive value (PPV), and negative predictive value (NPV) are reported

FetA, fetuin-A; *TBG*, thyroxin binding globulin

be only prescribed to patients if it has a maximal chance of being effective. The challenge is then to provide evidence-based knowledge by identifying biomarkers which predict response to a drug before it is administered.

To date, few studies have investigated factors predicting response to RTX using different approaches. At the DNA level, patients carrying the FCGR2A rs1801274-TT genotype and FCGR3A rs396991-TT allele treated with rituximab showed higher EULAR response [14]. Few studies have highlighted the theragnostic potential of type I interferon pathway showing that the combination of IRF5 rs2004640, SPP1 rs9138, and TNFSF13B rs9514828 was strongly associated with good/moderate EULAR response to RTX at W24 [15]. A set of IFN type I response genes (LY6E, HERC5, IFI44L, ISG15, MxA, MxB, EPSTI1, and RSAD2) was associated with ΔDAS28 and EULAR response outcome [16]. At the cellular level, RF positivity, normal levels of $CD19^+$ B cells together with increased $CD19^+CD27^-IgD^-$ B cells have been suggested to predict response to RTX in RA [17]. Attempts in using clinical data to find predictive markers revealed that patients with RA presenting autoantibodies are likely to respond better to RTX [18] and particularly RF positivity [19]. A study also showed that RF positivity associated with elevated CRP may enhance the benefit of RTX treatment [20]. In our study, we did not observe a correlation between FR positivity and EULAR response status. On the other side, CRP was significantly different between the R and NR population, but this phenomenon seemed to be more pronounced for "extreme" patients (good responder vs. nonresponders). The difference in RF and CRP expression between our study and that of Lal et al. may be explained by the fact that the latter compares RTX responders vs. placebo patients in RA patients that were exposed or not to previous bDMARD while our study compares good EULAR responders vs. moderate and nonresponders in RA patients who failed to respond to a first TNFi only. S100A8/A9 is of particular interest since its expression has been described as predictive for RTX [21] as well as for TNFi [22, 23]. In our study, although S100A8/A9 proteins were identified by MS-based proteomics analysis, their relevance as prediction biomarkers was not confirmed in the validation cohort. The capability of S100A8/A9 to predict bDMARD response has been questioned since in the largest replication cohort, no theragnostic evidence was observed to support the use of S100A9 as a clinical biomarker predicting the response to etanercept, the TNF inhibitor biologic drug [24, 25]. Furthermore, plenty of assays were available with

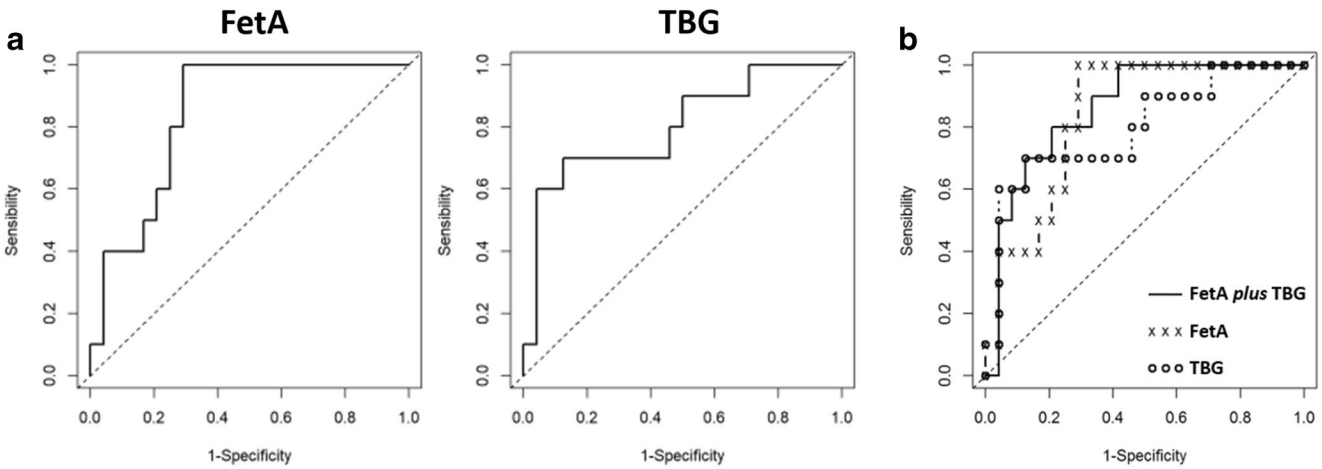

Fig. 3 Characteristic of the ROC curve analysis. **a** ROC curves analysis of each individual biomarker. **b** Overlay of the ROC curves for the predictive FetA plus TBG multivariate combined model and the univariate models (FetA and TBG). FetA, fetuin-A; TBG, thyroxin binding globulin

different performance and status (for research use only, for IVD or even homemade ELISA), and this heterogeneity and lack of standardization may have led to misinterpretation [26].

Until now, most studies focused on available clinical data or already identified protein markers such as for S100A8/A9 [22]. Non-a priori studies based on large-scale approaches open promising perspectives to discover predictive protein biomarkers. A previous study from our group using a proteomic approach successfully identified COMP as a reliable predictor for the response to abatacept treatment [7]. So far, such strategy has never been undertaken for RTX. In this study, we used MS-based quantitative proteomics to identify several serum proteins (thyroxin binding globulin, fetuin-A, S100A8/A9, complement C4-binding protein, lipoprotein(a), haptoglobin, serum amyloid A, and complement C1q) that are differentially expressed between R and NR patients at baseline before initiating RTX treatment. Comparison of the concentrations determined for the R and NR groups by quantitative immunoassays showed a statistically significant difference in expression levels for FetA and TBG. These two biomarkers were all determined at a lower concentration in the NR group compared to the R group. Univariate and multivariate regression analyses revealed a high predictive potential for FetA and TBG individually. This potential was reinforced when they were combined with AUC-ROC values of 0.86. Altogether, our data suggest that patients with a first TNFi failure who have high serum concentrations of FetA and TBG will be the most likely to respond to RTX after 6 months.

TBG [27] binds circulating thyroid hormones; it transports T3 (triiodothyronine) and T4 (thyroxine). Variations in TBG concentration directly affect the amount of bioavailable thyroid hormone. So far, no study has specifically described the role of TBG in RA and its predictive potential in response to bDMARD treatments. However, thyroid dysfunction has been reported in RA. The frequency of thyroid dysfunction increases in RA patients [28]. A recent study reported that RA is more prevalent in patients with autoimmune thyroid diseases than the general population [29]. Interestingly, a study observed that RA patients with thyroid disorders had significantly poorer initial response to RA treatment compared with patients with isolated RA [30]. Thus, it may be of interest to investigate further the implication of TBG in the physiopathology of RA.

Fetuin-A, also known as alpha 2-Heremans Schmid glycoprotein (AHSG), is a circulating "carrier" protein secreted by the liver [31]. Fetuin-A acts as an acute phase protein. While fetuin-A is a positive acute phase protein during injury, it is conversely a negative acute phase protein in inflammation [32]. Fetuin-A expression decreases and its level is inversely correlated with CRP concentration in serum during inflammation. Some studies showed a different expression of FetA in RA patients compared to healthy subjects. While a study showed decreased expression of FetA [33] in RA, others reported an increase [34, 35]. More interestingly, the presence of FetA increases the rate of apoptotic cell uptake [36]. Considering that apoptosis and the subsequent clearance of apoptotic cells by phagocytes are pivotal processes in the resolution of inflammation [37], a high concentration of FetA in responders at baseline may increase the elimination of massive apoptotic B cell induced by RTX treatment and therefore favor toward a better resolution of inflammation in responder patients. The dysregulation of this process leading to persistent inflammation is thought to contribute significantly to tissue damage in chronic inflammatory diseases such as RA [38].

Despite a plethora of studies identifying predictive biomarkers for RA, none has delivered a biomarker tool for routine clinical practice. This lack of transfer may be due to the heterogeneity of the approaches used and the number of parameters to consider, *i.e.*, class of molecules *vs.* individual bDMARD, number of previous bDMARD treatments, nature of biomarker (cell type, DNA, RNA, proteins, etc.), and translational potential of the identified biomarkers into routine clinical practice. We therefore designed our study to facilitate a rapid implementation of the predictive model in daily practice. Thus, we narrowed our inclusion criteria to select (i) a homogeneous population by targeting only patients presenting failure to respond to a single TNFi. Indeed, it is likely that the serum proteome may be modified after one or more bDMARD treatments [39, 40] (ii) prioritize the selection of biomarkers for which validated and standardized diagnostic assays exist and are routinely used in clinical practice and commercially available. Accordingly, FetA and TBG expression levels were assessed using an IVD CE-accredited ELISA kits.

In conclusion, by applying a non-a priori stepwise approach (proteomics discovery, quantitative immunoassays, multivariate model generation), we identified novel robust baseline serum biomarkers such as TBG and FetA, which, when integrated into a predictive model, were able to stratify patients for whom a first TNFi treatment had failed. The model accurately predicted R and NR status after 6 months of RTX treatment. However, those results were generated in a limited number of patients and need therefore to be confirmed in an independent and a larger population. When validated, these tools should be rapidly translated into daily clinical practice to help clinicians to choose the most appropriate bDMARD for the patient.

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Authors' contributions PG and AB participated in the study design and coordination and critically revised the manuscript. AC participated in the study design and coordination, performed the statistical analysis, and analyzed the data. MVCN participated in the study design and

coordination of the sample dosages, performed ELISA assays, analyzed the data, and drafted the manuscript. AA and VB performed proteomic experiments and data analysis and revised the manuscript. YC performed proteomic statistical analysis and data analysis and revised the manuscript. FD performed C4BP dosages and nephelometry assays on a Siemens BNII system and data analysis and revised the manuscript. JEG supplied patient samples, collected clinical data, and revised the manuscript. LG and CDP revised the manuscript.

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Compliance with ethical standards

Ethics approval and consent to participate The "Rotation or change of biotherapy after first anti-TNF treatment failure for rheumatoid arthritis" trial (ClinicalTrials.gov identifier: NCT01000441) was approved by the Institutional Review Board of the Comité de Protection des Personnes-Est 1, Strasbourg, France. All patients gave written informed consent after receiving oral and written information about the trial.

Conflict of interest AB and PG are co-founders of Sinnovial company. MVCN, LG and AC are employees of Sinnovial company. JEG reported receiving grant support from Abbvie, Pfizer, and Roche and personal fees from Bristol-Myers Squibb, Merck Sharp & Dohme, UCB, GlaxoSmithKline, and Novartis. VB, YC, FD, LG, and CDP declare they do not have conflict of interest.

List of abbreviations ESR, Erythrocyte Sedimentation rate; TNFi, Tumor necrosis factor-alpha inhibitors; bDMARD, Biologic disease-modifying anti-rheumatic drugs; csDMARD, conventional synthetic DMARD; EULAR, European League Against Rheumatism; LDA, Low Disease Activity; PPV, positive predictive values; NPV, negative predictive values; LR+, positive likelihood ratio; LR-, negative likelihood ratio; RA, Rheumatoid Arthritis; CRP, C-reactive protein; ACR, American college of Rheumatology; RTX, Rituximab; AUC, Area Under the Curve; ROC, receiver operating characteristic; CI, 95% confidence interval; FetA, Fetuin-A; C4BPA, C4b-binding protein alpha chain; C4BPB, C4b-binding protein beta chain; Lp(a), lipoprotein (a); TBG, thyroxin binding globulin; C1qA, Complement C1q subcomponent subunit A; C1qB, Complement C1q subcomponent subunit B; SAA1, serum amyloid A1

References

1. Maillefert JF, Combe B, Goupille P, Cantagrel A, Dougados M (2003) Long term structural effects of combination therapy in patients with early rheumatoid arthritis: five year follow up of a prospective double blind controlled study. Ann Rheum Dis 62:764–766
2. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, Smolen JS, Weisman M, Emery P, Feldmann M, Harriman GR, Maini RN, Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group (2000) Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 343:1594–1602. https://doi.org/10.1056/NEJM200011303432202
3. Navarro-Millán I, Curtis JR (2013) Newest clinical trial results with antitumor necrosis factor and nonantitumor necrosis factor biologics for rheumatoid arthritis. Curr Opin Rheumatol 25:384–390. https://doi.org/10.1097/BOR.0b013e32835fc62e
4. Favalli EG, Raimondo MG, Becciolini A, Crotti C, Biggioggero M, Caporali R (2017) The management of first-line biologic therapy failures in rheumatoid arthritis: current practice and future perspectives. Autoimmun Rev 16:1185–1195. https://doi.org/10.1016/j.autrev.2017.10.002
5. Gottenberg J-E, Brocq O, Perdriger A, Lassoued S, Berthelot JM, Wendling D, Euller-Ziegler L, Soubrier M, Richez C, Fautrel B, Constantin AL, Mariette X, Morel J, Gilson M, Cormier G, Salmon JH, Rist S, Lioté F, Marotte H, Bonnet C, Marcelli C, Sellam J, Meyer O, Solau-Gervais E, Guis S, Ziza JM, Zarnitsky C, Chary-Valckenaere I, Vittecoq O, Saraux A, Pers YM, Gayraud M, Bolla G, Claudepierre P, Ardizzone M, Dernis E, Breban MA, Fain O, Balblanc JC, Aberkane O, Vazel M, Back C, Candon S, Chatenoud L, Perrodeau E, Sibilia J, Ravaud P (2016) Non-TNF-targeted biologic vs a second anti-TNF drug to treat rheumatoid arthritis in patients with insufficient response to a first anti-TNF drug: a randomized clinical trial. JAMA 316:1172–1180. https://doi.org/10.1001/jama.2016.13512
6. Rendas-Baum R, Wallenstein GV, Koncz T, Kosinski M, Yang M, Bradley J, Zwillich SH (2011) Evaluating the efficacy of sequential biologic therapies for rheumatoid arthritis patients with an inadequate response to tumor necrosis factor-α inhibitors. Arthritis Res Ther 13:**R25**. https://doi.org/10.1186/ar3249
7. Nguyen MVC, Adrait A, Baillet A et al (2019) Identification of cartilage oligomeric matrix protein as biomarker predicting abatacept response in rheumatoid arthritis patients with insufficient response to a first anti-TNFα treatment. Jt Bone Spine Rev Rhum 86:401–403. https://doi.org/10.1016/j.jbspin.2018.09.005
8. van der Heijde DM, van't Hof MA, van Riel PL et al (1992) Validity of single variables and composite indices for measuring disease activity in rheumatoid arthritis. Ann Rheum Dis 51:177–181
9. Fransen J, van Riel PLCM (2005) The disease activity score and the EULAR response criteria. Clin Exp Rheumatol 23:S93–S99
10. Wiśniewski JR, Mann M (2012) Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. Anal Chem 84:2631–2637. https://doi.org/10.1021/ac300006b
11. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372. https://doi.org/10.1038/nbt.1511
12. Wieczorek S, Combes F, Lazar C, Giai Gianetto Q, Gatto L, Dorffer A, Hesse AM, Couté Y, Ferro M, Bruley C, Burger T (2017) DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics. Bioinforma Oxf Engl 33:135–136. https://doi.org/10.1093/bioinformatics/btw580
13. Combe B, Landewe R, Daien CI, Hua C, Aletaha D, Álvaro-Gracia JM, Bakkers M, Brodin N, Burmester GR, Codreanu C, Conway R, Dougados M, Emery P, Ferraccioli G, Fonseca J, Raza K, Silva-Fernández L, Smolen JS, Skingle D, Szekanecz Z, Kvien TK, van der Helm-van Mil A, van Vollenhoven R (2017) 2016 update of the EULAR recommendations for the management of early arthritis. Ann Rheum Dis 76:948–959. https://doi.org/10.1136/annrheumdis-2016-210602
14. Jiménez Morales A, Maldonado-Montoro M, Martínez de la Plata JE, Pérez Ramírez C, Daddaoua A, Alarcón Payer C, Expósito Ruiz M, García Collado C (2019) FCGR2A/FCGR3A gene polymorphisms and clinical variables as predictors of response to tocilizumab and rituximab in patients with rheumatoid arthritis. J Clin Pharmacol 59:517–531. https://doi.org/10.1002/jcph.1341
15. Juge P-A, Gazal S, Constantin A, Mariette X, Combe B, Tebib J, Dougados M, Sibilia J, le Loet X, Dieudé P (2017) Variants of genes implicated in type 1 interferon pathway and B-cell activation

modulate the EULAR response to rituximab at 24 weeks in rheumatoid arthritis. RMD Open 3:e000448. https://doi.org/10.1136/rmdopen-2017-000448
16. Raterman HG, Vosslamber S, de Ridder S, Nurmohamed MT, Lems WF, Boers M, van de Wiel M, Dijkmans BA, Verweij CL, Voskuyl AE (2012) The interferon type I signature towards prediction of non-response to rituximab in rheumatoid arthritis patients. Arthritis Res Ther 14:R95. https://doi.org/10.1186/ar3819
17. Tony H-P, Roll P, Mei HE, Blümner E, Straka A, Gnuegge L, Dörner T, FIRST/ ReFIRST study teams (2015) Combination of B cell biomarkers as independent predictors of response in patients with rheumatoid arthritis treated with rituximab. Clin Exp Rheumatol 33:887–894
18. Ferraccioli G, Tolusso B, Bobbio-Pallavicini F, Gremese E, Ravagnani V, Benucci M, Podestà E, Atzeni F, Mannocci A, Biasi D, Manfredi M, Sarzi-Puttini P, Laganà B, Montecucco C (2012) Biomarkers of good EULAR response to the B cell depletion therapy in all seropositive rheumatoid arthritis patients: clues for the pathogenesis. PLoS One 7:e40362. https://doi.org/10.1371/journal.pone.0040362
19. Quartuccio L, Fabris M, Salvin S et al (2009) Rheumatoid factor positivity rather than anti-CCP positivity, a lower disability and a lower number of anti-TNF agents failed are associated with response to rituximab in rheumatoid arthritis. Rheumatol Oxf Engl 48:1557–1559. https://doi.org/10.1093/rheumatology/kep314
20. Lal P, Su Z, Holweg CTJ, Silverman GJ, Schwartzman S, Kelman A, Read S, Spaniolo G, Monroe JG, Behrens TW, Townsend MJ (2011) Inflammation and autoantibody markers identify rheumatoid arthritis patients with enhanced clinical benefit following rituximab treatment. Arthritis Rheum 63:3681–3691. https://doi.org/10.1002/art.30596
21. Choi IY, Gerlag DM, Herenius MJ, Thurlings RM, Wijbrandts CA, Foell D, Vogl T, Roth J, Tak PP, Holzinger D (2015) MRP8/14 serum levels as a strong predictor of response to biological treatments in patients with rheumatoid arthritis. Ann Rheum Dis 74:499–505. https://doi.org/10.1136/annrheumdis-2013-203923
22. Obry A, Lequerré T, Hardouin J et al (2014) Identification of S100A9 as biomarker of responsiveness to the methotrexate/etanercept combination in rheumatoid arthritis using a proteomic approach. PLoS One 9:e115800. https://doi.org/10.1371/journal.pone.0115800
23. Obry A, Hardouin J, Lequerré T, Jarnier F, Boyer O, Fardellone P, Philippe P, Marcelli C, Loët XL, Vittecoq O, Cosette P (2015) Identification of 7 proteins in sera of RA patients with potential to predict ETA/MTX treatment response. Theranostics 5:1214–1224. https://doi.org/10.7150/thno.12403
24. Collison J (2017) Rheumatoid arthritis: S100A9 does not predict response to etanercept. Nat Rev Rheumatol 13:130. https://doi.org/10.1038/nrrheum.2017.13
25. Smith SL, Plant D, Eyre S, Hyrich K, Morgan AW, Wilson AG, Isaacs JD, Barton A (2017) The predictive value of serum S100A9 and response to etanercept is not confirmed in a large UK rheumatoid arthritis cohort. Rheumatol Oxf Engl 56:1019–1024. https://doi.org/10.1093/rheumatology/kew387
26. Romand X, Bernardy C, Nguyen MVC et al (2019) Systemic calprotectin and chronic inflammatory rheumatic diseases. Jt Bone Spine Rev Rhum. https://doi.org/10.1016/j.jbspin.2019.01.003
27. Refetoff S, Murata Y, Mori Y, Janssen OE, Takeda K, Hayashi Y (1996) Thyroxine-binding globulin: organization of the gene and variants. Horm Res 45:128–138. https://doi.org/10.1159/000184775
28. Li Q, Wang B, Mu K et al (2019) Increased risk of thyroid dysfunction among patients with rheumatoid arthritis. Front Endocrinol 9. https://doi.org/10.3389/fendo.2018.00799
29. Abd-Elhafeez HA, El-Meghawry E-S, Al-Azhary S et al (2018) Frequency of rheumatoid arthritis in patients with autoimmune thyroid disease: a case–control study. Egypt J Obes Diabetes Endocrinol 4:5–10. https://doi.org/10.4103/ejode.ejode_1_18
30. Emamifar A, Hangaard J, Jensen Hansen IM (2017) Thyroid disorders in patients with newly diagnosed rheumatoid arthritis is associated with poor initial treatment response evaluated by disease activity score in 28 joints-C-reactive protein (DAS28-CRP): an observational cohort study. Medicine (Baltimore) 96:e8357. https://doi.org/10.1097/MD.0000000000008357
31. Dabrowska AM, Tarach JS, Wojtysiak-Duma B, Duma D (2015) Fetuin-A (AHSG) and its usefulness in clinical practice. Review of the literature. Biomed Pap Med Fac Univ Palacky Olomouc Czechoslov 159:352–359. https://doi.org/10.5507/bp.2015.018
32. Wang H, Sama AE (2012) Anti-inflammatory role of fetuin-A in injury and infection. Curr Mol Med 12:625–633
33. Papichev EV, Zavodovsky BV, Polyakova YV, Seewordova LE, Akhverdyan YR (2018) Novel hepatokine in rheumatoid arthritis laboratory diagnostics. Klin Lab Diagn 63:756–760. https://doi.org/10.18821/0869-2084-2018-63-12-756-760
34. Harman H, Tekeoğlu İ, Gürol G, Sağ MS, Karakeçe E, Çİftçİ I, Kamanlı A, Nas K (2017) Comparison of fetuin-A and transforming growth factor beta 1 levels in patients with spondyloarthropathies and rheumatoid arthritis. Int J Rheum Dis 20:2020–2027. https://doi.org/10.1111/1756-185X.12791
35. Tekeoğlu İ, Harman H, Sağ S, Altındiş M, Kamanlı A, Nas K (2016) Levels of serum pentraxin 3, IL-6, fetuin A and insulin in patients with rheumatoid arthritis. Cytokine 83:171–175. https://doi.org/10.1016/j.cyto.2016.04.009
36. Jersmann HPA, Dransfield I, Hart SP (2003) Fetuin/alpha2-HS glycoprotein enhances phagocytosis of apoptotic cells and macropinocytosis by human macrophages. Clin Sci Lond Engl 1979 105:273–278. https://doi.org/10.1042/CS20030126
37. Savill J, Fadok V, Henson P, Haslett C (1993) Phagocyte recognition of cells undergoing apoptosis. Immunol Today 14:131–136. https://doi.org/10.1016/0167-5699(93)90215-7
38. Edwards SW, Hallett MB (1997) Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. Immunol Today 18:320–324
39. Allanore Y, Kahan A, Sellam J et al (2006) Effects of repeated infliximab therapy on serum lipid profile in patients with refractory rheumatoid arthritis. Clin Chim Acta Int J Clin Chem 365:143–148. https://doi.org/10.1016/j.cca.2005.08.010
40. Takeuchi T, Kotani T, Nakanishi T, Tabushi-Matsumura Y, Takubo T, Makino S (2010) Proteomic analysis of changes in the serum protein profile by anti-TNF-alpha therapy. Rinsho Byori 58:332–336