Receptor activator of nuclear factor kappa-B ligand (RANKL) serum levels are associated with progression to seropositive/negative rheumatoid arthritis

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Abstract

Objective

The aim of this study was to establish whether serum RANKL levels in early inflammatory arthritis (IA) were associated with rheumatoid arthritis (RA) diagnosis at follow-up, and to evaluate the added value of RANKL for RA diagnosis.

Methods

Serum from 298 patients was collected. Demographic and clinical (swollen/tender joint counts, CRP, DAS28-CRP, RF, ACPA and shared-epitope data were recorded. Baseline ultrasound of 26 joints was performed, including total power Doppler (PD). An ELISA was used to measure RANKL. Predictors of progression were identified using multivariable logistic regression analysis. Area under the receiver operating characteristics (AUROC) was used to assess the performance of the prediction models and quantify the added value of RANKL in RA diagnosis.

Results

151 patients developed RA and 147 were non-RA (undifferentiated IA, other inflammatory diagnoses or non-persistent inflammation). RANKL levels were significantly higher in RA (median [IQR]: 474.1 [270.8–1430.6]) than in non-RA (median [IQR]: 301.0 [174.1–477.5]. Three clinical factors (age, SJC and PD) were identified by multivariable logistic regression with model performance AUROC of 77.9% (95% CI 72.1–83.8%). Adding RANKL resulted in a relative increase of 6.5% in the model classification performance of an AUROC of 83.0% (95% CI 77.9–88.1%).
In ACPA-negative patients, the model performance increased from 77.6% (95% CI 69.5–85.7%) with clinical data only to 81.9% (95% CI 73.7–89.8%) with added value of RANKL and imaging.

Conclusion

RANKL levels can predict RA diagnosis over clinical biomarkers alone, both seropositive and particularly in seronegative IA patients.

Key words
RANKL, rheumatoid arthritis, ultrasound, diagnosis

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Introduction

Making the earliest diagnosis of rheumatoid arthritis (RA) is crucial to initiate treatment and prevent further disease progression (1), including damage to the joints (2). The interactions between genes and environment are central in all stages of RA (3). Leukocytes infiltrating the synovial membrane (4), a network of soluble mediators (5, 6) and the development of novel synovial blood vessels (*i.e.* angiogenesis) (7) are key features responsible for the development of synovitis (8).

Despite recent advances with the discovery and integration of anti–citrullinated protein antibody (ACPA) in the RA classification criteria (9), there is still an unmet need for novel diagnostic biomarkers, notably for ACPA-negative (seronegative) disease. Seronegative inflammatory arthritis (IA) is a heterogeneous group, ranging from clinical presentations with a raised C-reactive protein (CRP) and erosions, to those with joint inflammation but not CRP or erosion.

Our previous work (10) and others (11) has shown the importance of power Doppler ultrasonography (PDUS) in predicting persistent disease, particularly in seronegative early IA. This is a sensitive tool which can detect synovitis at the sub-clinical stage (12) as well as identify erosions at the stage where they are not detectable by conventional radiographs (13). Synovitis can therefore be accurately detected using PDUS in the small and large joints of patients with RA (14), with good correlation with histology and magnetic resonance imaging (MRI) (15, 16). PDUS is well tolerated by patients and can image a large number of joints at multiple time points over a relatively short period of time, with good reproducibility of data at the hand and wrist (17). Despite its utility, PDUS cannot predict all progression to RA.

Many groups have shown that known regulators of bone homeostasis include bone and cartilage turnover biomarkers such as the Receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) (18, 19), pyridinoline (20), cartilage oligomeric matrix protein (COMP) (21), matrix metalloproteinase 3 (MMP-3) (22), carboxy-terminal telopeptide of type I collagen (CTX-I) (23) and carboxy-terminal telopeptide of type II collagen (CTX-II) (24). Some were related to radiographic progression and differ in levels according to ACPA status (25, 26). In the receptor-activator-nuclear-factor-κB axis, RANKL orchestrates bone destruction by inducing the differentiation of osteoclasts, whilst OPG is its decoy receptor and interferes with the binding of RANKL to its receptor RANK (27, 28). RANKL and OPG expression (at mRNA level) in the synovial tissue of patients with RA showed a significant decrease for OPG whilst RANKL was increased (29-31). The mRNA ratio of RANKL:OPG was therefore defined as an indicator of osteoclast activation, favouring osteoclastogenesis in RA (32, 33). Using serum protein level the RANKL:OPG ratio was shown to predict subsequent joint destruction (34) and annual radiological progression over 11 years in RA (35). Additionally, serum RANKL levels alone may predict anti-tumour necrosis factor therapy induced remission in RA (36), whilst synthetic disease-modifying anti-rheumatic drugs (DMARDs) treatment significantly reduces the RANKL:OPG ratio (37).

However, there are no studies which use ultrasound and bone turnover assessment for the prediction of progression to RA, especially in the ACPA negative group.

The aim of this study is to establish whether serum levels of bone turnover biomarkers in people with early inflammatory arthritis (IA) are associated with RA diagnosis at follow-up and to evaluate the added value of RANKL with PDUS for the diagnosis of early RA.

Methods

Patients

Consecutive patients attending the Leeds Early Arthritis clinic (EAC) were enrolled in the Inflammatory Arthritis disease CONtinuum register (IACON, approved by Local Ethics Committee REC: 09/H1307/98). This study recruited cases aged over 18, with an IA of less than 24 months symptom duration at first presentation,

enrolled between 2010 and 2015. Demographic data (age, gender, symptom duration), clinical data (swollen and tender joint counts (SJC, TJC), disease activity score (DAS28-CRP) and laboratory characteristics (CRP), rheumatoid factor (RF), ACPA and shared epitope (SE)) were collected. Patients were follow-up for 2 years and RA classification established over this period using the EULAR 2010 criteria. Alternative diagnosis included undifferentiated arthritis (UA), psoriatic arthritis (PsA), systemic lupus erythematosus (SLE), gout, non-persistent arthritis, osteoarthritis (OA) and palindromic rheumatisms (PAL) as well as non-persistent symptoms. No patients included in our study had received a DMARD previous to the baseline visit/samples. Clinical data were recorded in the register although not all patients had a full dataset (as detailed in Table I legend). All patients gave written, informed consent.

Imaging

Ultrasound was performed at inclusion by experienced sonographers (RJW, ALT) using a GE E9 machine and employing either a 6–15 or 8–18 MHz linear array transducer as appropriate. For power Doppler examinations, the pulse repetition frequency was adjusted to provide maximal sensitivity at the lowest possible value for each joint. Twenty-six joints (bilateral elbows, wrists, MCP 2–3, PIP 2–3, knees, ankles and MTP 1-5) were scanned using EULAR recommendations for image acquisition (38). Images were scored using the semi-quantitative scoring system previously published (17). Summative scores were then calculated for the following parameters: power Doppler (PD), grey-scale hypertrophy (GS) and erosions (ERO). Presence/absence of osteophytes at each site was also recorded.

Laboratory methods

The assessment of the laboratory biomarkers was divided into 2 phases. First, in an exploratory cohort, six biological markers were studied. These were CTX I, CTX II, COMP, MMP3, RANKL and OPG, using blood and urine samples from RA and non-RA patients from our in-house clinical cohorts (n=40).

Subsequently, RANKL, OPG and MMP3 were studied in a larger cohort (n=117), using samples from additional RA and non-RA patients to narrow down further the marker selection. In the last stage, a total of 298 serum samples were tested for RANKL only (including the 2 previous groups).

Peripheral blood and mid-stream urine samples were collected at the inclusion visit. After centrifugation, the serum and urine supernatant were stored in -80°C until analysis. Serum was used to measure markers using commercial kits (enzyme-linked immunosorbent assays (ELISA): RANKL (BioVENDOR, CZR), OPG (eBioscience, Ltd., UK), CTX-I (Immunodiagnostic Systems, UK), COMP and MMP3 (R&D systems, Biotechne, UK). Urine samples were used to measure urinary CTX-II (Immunodiagnostic Systems, UK) using creatinine (R&D systems, Biotechne, UK) for normalisation.

Statistical analysis

Continuous variables were not normally distributed and therefore data were described using median and interquartile range (IQR), and non-parametric tests were performed where appropriate. For exploring the data, Mann-Whitney U and Pearson's Chi square tests were used as well as Spearman correlations. The level of significance for *p*-values was set at 0.05. No adjustment was made for multiple testing in the exploratory analysis.

Logistic regression was used to derive the unadjusted odds ratios (OR) for each clinical variables. Model selection was performed to select clinical variables that predict the diagnosis of RA. Area Under the Receiver Operating Characteristics (AUROC) was used to assess the model performance for classification of RA/non-RA, and the added value of RANKL was evaluated based on the improvement of AUROC when RANKL was included in the prediction models. To minimise bias caused by a small proportion of missing data, multiple imputation by chained equation was used to produce 10 imputed datasets. Pooled modelling estimates and accompanying 95% confidence intervals were generated according to Rubin's rule. The statistical software SPSS Statistics v. 24 and R v. 3.5.1 (pROC package) was used for the analysis.

Results

Description of cohorts

A total of 298 consecutive patients attending the Leeds Early Arthritis clinic were recruited into this study. 151 were classified as RA using the 2010 ACR/EULAR criteria (delayed diagnosis was observed in 26 patients with a mean delay of 9 months (range 3–21) and 147 were non-RA, including UA (n=84), PsA (n=19), gout (n=2), SLE (n=2) inflammatory OA (n=18) and PAL (n=5) and non-persistent symptoms (n=17), with a mean delay for diagnosis of 18 months (range 0–24). A description of demographics and clinical parameters is detailed in Table I.

Exploratory biomarker analysis

Six markers (CTX I, CTX II, COMP, RANKL, OPG, MMP3) were initially tested on 40 samples including 26 RA and 14 non-RA patients. Analysis of serum CTX I, urine CTX II and COMP did not suggest any discrimination between RA/non-RA groups and these markers were not investigated further (Fig. 1A). RANKL, OPG and MMP3 showed non-significant trend for differences between groups and were therefore tested on 77 additional serum samples (Fig. 1B; altogether RA=78, non-RA=39). In this second analysis, higher MMP3 levels were associated with RA (p=0.045) as well as higher OPG (p=0.029), however, both with a large overlap of distribution and many outliers in the non-RA group. Higher RANKL concentrations were observed in RA with a clear shift in distribution and limited outliers, despite not reaching significance in this group (n=117, p=0.104). The median (IQR) values for the 1st and second analysis were similar for RANKL (RA: 335 [184-489] and 373 [185–979] in the 1st and 2nd cohort respectively; non-RA: 305 [170–418] and 301 [175–454]).

All 3 markers were significantly higher in ACPA-positive RA (n=53) (MMP3

Table I. Demographic, clinical and laboratory characteristics of patients: RA and non-RA.

Variable	Non-RA n=147	RA n=151	*p*-value
Demographics			
Age in years	49.0 (36.0–61.0)	59.0 (45.7–69.0)	<0.0001
Female	103 (70.1%)	101 (66.9%)	0.618
Duration (months)	4.0 (3.0–8.0)	4.0 (3.0–6.0)	0.663
Never smoker*	42 (42.9%)	45 (38.1%)	0.425
Serology/SE			
ACPA negative**	139 (95.2%)	54 (35.8%)	<0.0001
RF negative**	129 (89.6%)	65 (43.3%)	<0.0001
SE (negative/positive)**	79 (57.7%)	48 (32.9%)	<0.0001
Clinical			
SJC	1.0 (0.0–4.0)	4.0 (1.0–10.0)	<0.0001
TJC	2.0 (1.0–4.0)	5.0 (2.0–11.5)	<0.0001
CRP	5.0 (5.0–17.3)	9.3 (5.0–23.0)	0.008
DAS28-CRP[&]	3.1 (2.2–4.0)	4.2 (3.0–5.0)	<0.0001
Imaging**			
Power Doppler (PD)	1.0 (0.0–2.0)	4.0 (1.0–8.0)	<0.0001
Grey-scale (GS)	12.0 (6.5–18.0)	15.0 (9.5–24.0)	<0.0001
Osteophytes	2.0 (0.0–4.0)	3.0 (0.0–6.0)	0.042
Erosions (ERO)	0.0 (0.0–0.0)	0.0 (0.0–2.0)	0.002
Bone marker			
RANKL pmol/L	301.0 (174.1–477.5)	474.1 (270.8–1430.6)	<0.0001

Results are shown as median and (IQR) for quantitative data and as number of cases for qualitative data. * missing data in 82 participants; **missing data in 2 for ACPA, 4 for RF and 15 for SE; ***data available in 239 participants. [&] DAS28 used was 4 variables/CRP, missing the General Health visual analogue score in 73 cases due to non-relevance in early spondyloarthropathies.

Table II. Odds ratio (95% confidence intervals) for variables from unadjusted and adjusted logistic regression models applied to all patients (n=298). AUROC and 95% CI was reported for adjusted models with clinical factors and RANKL.

Variable in the model	Unadjusted	Adjusted (3 variables)	Adjusted (4 variables)
Age	1.03 (1.02–1.05)	1.03 (1.01–1.05)	1.03 (1.01–1.05)
Sex	1.16 (0.71–1.89)		
Duration	0.99 (0.94–1.04)		
Smoking (ever/never)	0.90 (0.44–1.84)		
ACPA	35.67 (16.6–89.09)		
RF	11.25 (6.18–21.68)		
SE	2.78 (1.72–4.54)		
SJC	1.22 (1.14–1.31)	1.13 (1.05–1.23)	1.15 (1.07–1.26)
TJC	1.10 (1.05–1.15)		
CRP	1.02 (1.01–1.03)		
DAS28-CRP	1.82 (1.45–2.32)		
Doppler (PD total)	1.25 (1.15–1.37)	1.14 (1.05–1.26)	1.10 (1.01–1.21)
Grey-scale (GS total)	1.05 (1.02–1.08)		
Osteophytes	1.09 (1.01–1.19)		
Erosions (ERO)	1.45 (1.17–1.89)		
RANKL (per 100 pmol/L)	1.13 (1.08–1.19)		1.17 (1.09–1.28)
AUROC		77.9% (72.1%–83.8%)	83.0% (77.9%–88.1%)

NS: non-significant *p*-value

p=0.048; OPG *p*=0.032; RANKL *p*<0.001). MMP3 levels correlated with age and many other clinical parameters with low rho values (<0.600) despite significant *p*-values: age (rho=0.413, *p*<0.0001), SJC (rho=0.271, *p*=0.003), DAS28-CRP (rho=0.246, *p*=0.012), CRP (rho=0.366, *p*=0.000), PDUS (rho=0.380, *p*=0.000), GS (rho=0.439, *p*=0.000), Osteophyte (rho=0.295, *p*=0.007). Similar correlations were observed for OPG: age (rho=0.389, *p*<0.0001), DAS28-CRP (rho=0.228, *p*=0.020), CRP (rho=0.232, *p*=0.012), PDUS (rho=0.217, *p*=0.019), Osteophyte (rho=0.509, *p*=0.000). In contrast, RANKL was not associated with age/gender or any clinical (TJC, SJC, CRP, DAS28) or imaging (GS, PD) parameters, suggesting independent value as a potential biomarker.

RANKL potential as a classification biomarker

In view of the previous results and the potential covariance between the level of OPG and MMP3 with demographic, clinical or imaging parameters, we choose to pursue RANKL. A final group including 298 individuals was selected from the early IA register, of whom 151 (51%) progressed to RA. The latter included alternative diagnoses (undifferentiated arthritis, other inflammatory arthritis) or non-persistent inflammation as established over 2 years of follow-up. All routinely used clinical parameters were individually associated with RA (Table I, ACPA, RF, SE, TJC, SJC, CRP, DAS 28, *p*<0.0001), as were imaging biomarkers (PDUS, GS, ERO, *p*<0.001; Osteophyte *p*=0.042). RANKL levels (pmol/L) were significantly higher in RA (median [IQR]: 474.1 [270.8–1430.6]) than in non-RA (median [IQR]: 301.0 [174.1–477.5] (Fig. 1C, *p*<0.0001). A ROC analysis for RANKL levels was performed and established that, with an AUROC of 0.680 (95% CI 0.619–0.740, *p*<0.0001), a cut-off at a value of 500 can classify RA/non-RA with a specificity of 79%, allowing for sensitivity of 47% PPV of 70% and NPV of 59% with an odds ratio of 2.23.

A multivariable logistic regression analysis was then performed to assess the value of RANKL for the classification of RA/non-RA. Autoantibodies (ACPA/RF) were not included in the multivariable regression as they are part of the criteria for RA classification. The components of the DAS28 rather than the score itself were prioritised in this modelling. After model selection, 3 clinical parameters were sufficient to account for all associations with RA: age, SJC, and PD with classification performance AUROC of 77.9% (95% CI 72.1–83.8%) (Table II). RANKL was a significant predictor for RA/non-RA (OR: 1.17, 95% CI 1.09–1.28). Adding RANKL to 3 previous parameters resulted a relative increase of 5.1% in the classification performance AUROC to 83.0% (95% CI 77.9–88.1%).

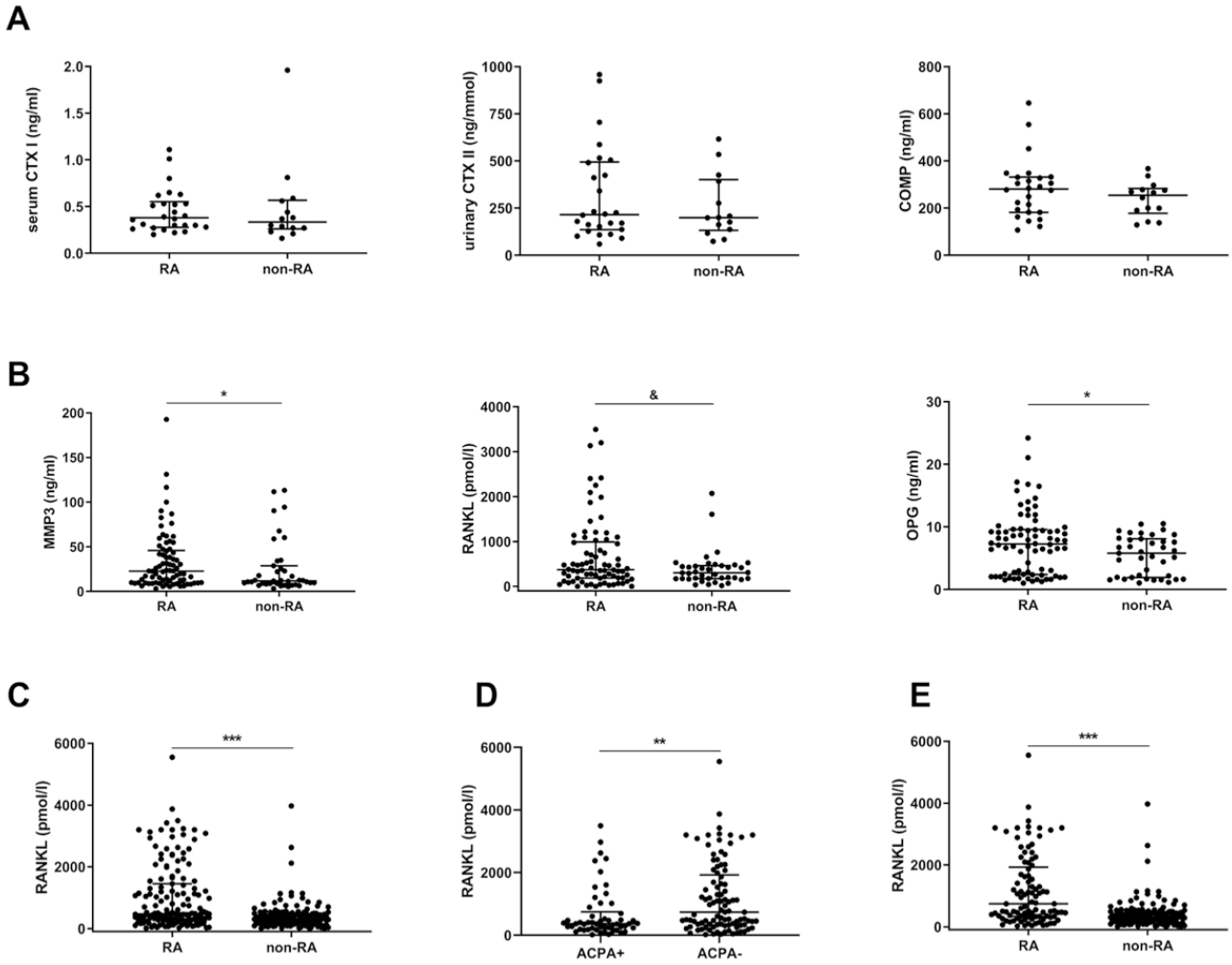

Fig. 1. Biomarker levels in RA compared to non-RA.
A: Tested 40 serum or urine samples as appropriate, RA=26, non-RA=14; **B**: Tested 117 serum samples, RA=78, non-RA=39.
C: RANKL levels in RA (n=151) and non-RA patients (n=147) including UA (n=84) PsA (n=19), gout (n=2) non-persistent (n=17) SLE (n=2) inflammatory OA (n=18) and PAL (n=5); **D**: RANKL levels in RA ACPA+ (n=54) and ACPA- patients (n=97); **E**) RANKL levels in ACPA– RA (n=97) and non-RA (n=147); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, $^{\&}$trend.

Table III. Odds ratio (95% confidence intervals) for variables from unadjusted and adjusted logistic regression models applied to sero-negative patients (RA n=140, non-RA n=54). AUROC and 95% CI was reported for adjusted models with clinical factors and RANKL.

Variable in the model	Unadjusted	Adjusted clinical	Adjusted clinical + imaging	Adjusted clinical + RANKL	Adjusted All
Age	1.04 (1.02–1.06)	1.03 (1.00–1.06)	1.03 (1.00–1.06)	1.03 (1.01–1.06)	1.03 (1.00–1.06)
Sex	0.90 (0.44–1.78)				
Duration	0.94 (0.87–1.02)				
Smoking (ever/never)	1.68 (0.5–5.34)				
SE	1.42 (0.74–2.72)				
SJC	1.27 (1.16–1.39)				
TJC	1.14 (1.08–1.20)				
CRP	1.01 (0.99–1.03)				
DAS28-CRP	2.36 (1.67–3.51)	2.08 (1.42–3.05)	1.94 (1.20–3.15)	2.44 (1.58–3.77)	2.06 (1.34–3.34)
Power Doppler (PD)	1.21 (1.11–1.35)		1.18 (1.01–1.38)		1.12 (1.03–1.24)
Grey-scale (GS)	1.05 (1.02–1.09)				
Osteophytes	1.12 (1.00–1.25)				
Erosions	1.31 (1.05–1.74)				
RANKL (per 100 pmol/L)	1.09 (1.03–1.16)			1.24 (1.13–1.38)	1.23 (1.12–1.39)
Overall % correct		77.6% (69.5%–85.7%)	78.4% (69.8%–86.5%)	81.3% (73.4%–89.1%)	81.9% (73.7%–89.8%)

NS: non-significant *p*-value.

Sero-negative group analysis

The same strategy was then used to investigate the value of RANKL in classification of RA (n=140) /non-RA (n=54) in ACPA-negative patients, as our ability to predict those who will develop persistent, progressive disease is currently limited in this group. A ROC analysis showed an AUROC of 0.592 (95% CI 0.496–0.681, p=0.05) a cut-off at a value of 450 can classify RA/non-RA with a specificity of 71.5%, allowing for sensitivity of 37% PPV of 33% and NPV of 75% with an odds ratio of 1.30.

Logistic models were performed to sequentially demonstrate the independent value of adding imaging or RANKL to clinical data and then of combining clinical/imaging/RANKL. DAS28 was chosen to limit the number of parameters used (Table III). A model using only clinical data (age and DAS28) with the classification performance AUROC of 77.6% (95% CI 69.5–85.7%) was 1st defined. Adding imaging improved the model performance to 78.4% (95% CI 69.8–86.5%). RANKL added to clinical data improved the model up to 81.3% (95% CI 73.4–89.1%). RANKL added to clinical and imaging data improved the model performance further to 81.9% (95% CI 73.7–89.8%).

Discussion

Following an exploratory phase aiming at selecting the bone turnover biomarker with the best discriminative capabilities, our data showed that in early IA, progression to RA could be predicted with good accuracy using 4 variables combining demographic (age), clinical (SJC), imaging (PDUS) and serological biomarkers (RANKL). Furthermore, in ACPA-IA patients, who currently represent the group with the unmet need for a novel classification test, data suggest that progression can be best predicted with 3 variables: clinical (DAS), imaging (PDUS) and serology (RANKL).

Previous work has focussed on predicting the outcome of early IA/UA using conventional clinical markers (39). More recently ACPA and ultrasound/MRI have also been added in order to improve the sensitivity and specificity of such predictive models (10, 11, 40-42). However, ACPA as a biomarker strongly predicts RA and, as such, other biomarkers add very little improvement in a combined model. Furthermore, ACPA status being included in the classification criteria (43) probably explain why models containing ACPA do not detect the potential value of other markers. As such, our study showed a detectable +5.1% improvement in classification adding RANKL over current classification using clinical/demographic/imaging data. Despite overlapping CI, these data suggest that an ELISA for RANKL may offer earlier classification for some patients. The value of US may results from detecting more patients with an ACPA- status. Indeed, it remains difficult to predict a patient's clinical course, especially early on in the seronegative disease, but the presence of positive PDUS was shown to significantly increase confidence in a diagnosis of persistent disease (10), while MRI has limited practicability as a predictive tool (44). In our study, we confirmed that PD had a small +0.8% improvement on classification of seronegative patients while RANKL showed a +3.7% increase. The combination of both only added a further +0.6% to the improvement brought in by RANKL alone. In this group, performing an ELISA for RANKL offer a small but none the less detectable improvement on classification.

The implications of using such a model, particularly in the ACPA negative group, lies in the ability to identify in the Early Arthritis Clinic those with a good prognosis, thus avoiding overtreatment and potential drug related side effects. The limitation of our strategy was that we needed a large number of patients and achieved the selection of RANKL using the same patients for the discovery (cohort 1 and 2) and final analysis (cohort 3). In terms of feasibility, tender and swollen joint counts are standard clinical practice and US is increasingly used alongside the clinical assessment of patients. RANKL testing uses a simple serum assay and can easily be performed by routine laboratory services.

In conclusion, the value of RANKL over current biomarkers alone has been demonstrated, in both the seropositive (+5.1%) and sero-negative (+4.3%) IA groups. It is in this diagnostically challenging sero-negative group that new advances are especially welcome and the utility of RANKL shows encouraging preliminary results on which to build further work.

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