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Hypo-methylation of the TNF-α gene predicts methotrexate induced remission in RA.

DNA hypo-methylation of the *TNFA* gene predicts response to methotrexate in rheumatoid arthritis but not in psoriatic arthritis.

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Abstract

Objectives

The 'Treat-to-Target' approach significantly improves treatment outcomes in rheumatoid and psoriatic arthritis (RA/PsA). Methotrexate (MTX) is commonly used as a first-line treatment upon diagnosis. However, 50-70% of treated patients do not achieve clinical remission after 6 months. We previously developed a quantitative methylation-sensitive qPCR (qMSP) assay to quantify DNA-methylation levels of the *TNFA* gene. We demonstrated its predictive value for RA diagnosis. Here, we assessed the utility of this qMSP-assay for predicting MTX treatment outcome and investigated changes in DNA methylation levels after 6 months of MTX.

Methods

Early untreated RA (n=201) and PsA (n=77), were included with healthy controls (n=39). The *TNFA*-qMSP assay was performed by qPCR on bisulphite-converted DNA extracted from frozen peripheral blood mononuclear cells. The outcome predictive-value of DNA-methylation levels was analysed using binary logistic regression and area-under-the-curve (AUC).

Results

Pre-treatment DNA-methylation levels of the *TNFA* gene were reduced in RA ($p=1.8x10^{-13}$) and PsA (p=0.007) compared to controls. Higher DNA-methylation levels were associated with MTX-induced remission in RA (AUC=0.721) and substantially improved the performance of a predictive model when combined with clinical data (AUC=0.760) compared to clinical data only (AUC=0.699). Levels of methylation reduced over time, irrespective of response to MTX in RA. In PsA, DNA-methylation levels were not associated with treatment outcome.

Conclusion

The TNF qMSP assay has potential clinical utility as a biomarker for predicting MTX-induced remission specifically in early RA, but not in PsA. However, MTX does not appear to prevent the reduction of DNA-methylation levels at the *TNFA* loci over time.

Introduction

Chronic inflammatory arthritis (IA) such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA) affect approximately 1-2% of the population, and if untreated, results in reduced quality of life and increased mortality[1, 2]. The chronic inflammation occurring in both RA and PsA results in pain, swelling, stiffness affecting peripheral joints and/or spine with prevalent structural changes leading to loss of function and disability.

In early inflammatory arthritis, prompt and effective treatment significantly benefits both short- and long-term outcomes[3-5]. This principle is central to the "Treat-to-Target" (T2T) approach[6, 7], which aims to maximize the achievement of clinical remission (defined by the composite disease activity score, DAS28 in RA). This is achieved through drug escalation if DAS28 remission (<2.6) is not met at pre-specified time points. While this treatment approach has improved outcomes in early IA, more than half of patients do not achieve remission[8-10]. Mechanistically, methotrexate (MTX) is believed to exert some of its effects by interfering with DNA-methylation[11-14].

We previously reported that reductions in naive CD4+T-cell frequencies occur very early in RA disease progression[15] and in pre-clinical disease[16] and can predict both the likelihood of progression to RA and the occurrence of MTX-induced remission in early RA[8, 10, 16]. In addition, genetic associations with loci related to the epigenetic modification machinery have been observed in RA[17-19]. This led us to demonstrate that changes in DNA-methylation occur specifically in naive CD4+T-cells, across many genes and notably the tumour necrosis factor-alpha (*TNFA*), in early, untreated RA[20]. These findings provide a biological context for the role of naive CD4+T-cells remodelling in RA pathophysiology. Similarly, in synovial fibroblasts from RA patients, the *TNFA* gene was found to be demethylated[21].

Quantitative methylation specific qPCR (qMSP) is an innovative technology measuring levels of DNA-methylation at specific CpG-sites[22] which has potential as a biomarker test as it is easy to perform (based on PCR). This technology targets a specific CpG-dinucleotide and measures change in its methylation status, hence is designed for a specific single cytosine detecting whether it is methylated. In previous work, we developed an assay to quantify levels of DNA-methylation in the *TNFA* gene[23] and demonstrated the predictive value of this biomarker for RA classification[23] independently of anti-citrullinated protein antibodies (ACPA)-positivity, confirming our previous observation of change in DNA-methylation at the genome wide level[20]. Furthermore, we observed reduced *TNFA* DNA-methylation levels in 16 PsA patients compared

to healthy controls[23] and differential methylation of the *TNFA* gene has also been reported between PsA patients with and without skin involvement[24].

In this study, we aimed to assess the value of the *TNFA*-qMSP for predicting the achievement of MTX-induced remission in early drug naïve RA and investigate its potential value in MTX-treated PsA patients. In addition, we investigated the effect of MTX on DNA-methylation of the *TNFA* gene 6 months after MTX treatment.

Patients and Methods

Patients

All participants provided informed written consent prior to recruitment.

The Leeds Inflammatory Arthritis Disease Continuum (IACON, National Research Ethics Service, West Yorkshire Ethics Committee REC approval: 09/H1307/98) is a longitudinal cohort register of early IA symptoms with <24 months of duration, also, including healthy controls (HC). Data used for this project include age, sex, smoking, symptoms duration at inclusion, presence of rheumatoid factor and anti-citrullinated peptide autoantibodies (rheumatoid factor [RF]/ACPA), tender/swollen joints count (TJC/SJC), C-reactive protein levels (CRP), patient global health (VAS-GH) and a calculated disease activity score (DAS28). Patients were classified as RA using EULAR 2010 criteria or PsA with CASPAR criteria.

Newly classified RA patients included in this cohort, were prescribed MTX according to national guidelines, starting at 15mg/week and escalating to 25mg/week over 8 weeks if not achieving remission. We identified those who had recorded data over 6 months allowing us to assess disease activity and the achievement of remission (DAS28<2.6). Additional conventional synthetic disease-modifying anti-rheumatic drugs (cs-DMARDs: sulfasalazine [SLZ] or hydroxychloroquine [HCQ]) were allowed if low disease activity was not achieved at 3 months. In early RA, 65% of the patient not achieving remission did not escalate to multiple therapy before 6 months, 17% added a 2nd synthetic at 3 months due to lack of response and 3% started on MTX + HCQ (missing beyond baseline for 5%). Patients achieving remission at 3 month and 6 months escalated doses of MTX but did not add another DMARDs. The PsA cohort included DMARD naïve PsA patients who then received MTX, starting at 15mg/week and escalating to 25mg/week over 4 weeks following inclusion in the placebo arm of the GOLMePsA clinical trial (Ethical approval granted by the East Midlands, Northampton Committee; reference, 14/EM/0124; EudraCT 2013-004122-28[25]). Demographic data and clinical variables were retrieved from the trial database (age, sex, symptom duration, smoking, CRP, TJC/SJC, VAS-

GH). Psoriatic arthritis disease activity score (PASDAS) < 3.2 and ACR20 were the trial scores used to assess treatment response at 6 months.

For HC, only age and sex were reported.

Samples

All participants provided a 9 mL blood samples. Peripheral blood mononuclear cells (PBMC) were separated and cryo-preserved (10% DMSO at -150°C). Genomic DNA was extracted from frozen PBMC using a commercial QIAamp kit (DNA Blood Mini), according to manufacturer's protocol. DNA quality and concentration were asserted (spectrophotometer ND1000). Bisulphite conversion was performed using the DNA-Methylation Gold Kit (Zymo Research) following manufacturer's protocol.

qMSP assay

The development of qMSP assays was extensively described in our previous publication[23] including all validation steps relative to the conditions and optimization needed for a qMSP assay. This process is described in Supplementary Data S1. Primer and probes used were: for the *TNFA* gene assay: F-5-TTT**CG**GAAT**CG**GAGTAGGGAG, R-5'-ACCCTACACCTTCTATCT**CG**ATTTCTT, Probe-5'TTGGGTAGTTTTGGAGTTTTTAGTTG; for the GAPDH gene assay: F-5'-TTGGGTAG TTTTGGAGTTTTTAGTTG, R-5'-AATACAACATCTCCTTACCCCCAA, Probe-5'-AGTTAGGTTAGTTTGGTAGGGAA. The assay results are expressed as % of DNA-methylation at the specific CpG site in the *TNFA* gene.

Statistics

Data are described using median and interquartile range (IQR) as not normally distributed. Normality was assessed using Kolmogorov-Smirnov's test. To compare difference between groups Man-Whitney-U (MWU) were used for continuous variables, Chi^2 (X^2) were used for categorical variables. No adjustment for multiple testing was made at the univariate stage in descriptive tables. Covariance was analyzed using Spearman correlation. Missing data were limited and mainly related to missing GH (hence DAS28) for RA cases. Data were missing at random for all other variables in the 3 diseases (confirmed by Little's MCAR test). For multivariate analysis, binary logistic regressions using Enter or Forward methods were used to build models sequentially. Odd ratio (OR) was used to estimate the likelihood of an outcome occurring in one group compared to another, and area under the ROC curve (AUC) analysis was performed to establish predictive performances. A bootstrapping technique was used (500 permutations, automatic function) to calculate a discriminating index correcting for optimism

between the predicted and actual outcome for the best regression models (95% CI of the AUC). Regression models used both forced-entry (Enter) and forward-selection (Forward) procedures. The forced-entry approach included all variables simultaneously whereas the forward-selection procedure retained only variables providing a significant incremental contribution to the model. The comparison of these approaches enabled an assessment of the relative importance, stability, and predictive value of the candidate predictors. Additionally, descriptive performances of the models (sensitivity/specificity, positive/negative predictive values (PPV/NPV)) were calculated from the classification matrix of logistic regressions. Missing data imputation was performed before the regression (5 cycles of imputations). Then, a pooled dataset was created for analysis, and after verification that ORs were not affected by the imputation process, the pooled dataset was used in the regression. To account for possible overestimation of the model performance, a 95% CI of the AUC (using bootstrapping with 500 permutations) of each model was generated. A t-test to compare the distribution of 500 AUCs generated for the forward models, with (model 3) and without (model 2) the qMSP data was performed. A retrospective power calculation was also performed and suggested that an n=201 allowed for greater than 80% power. All the analyses were conducted using SPSS software version 29 (IBM, Armonk, NY, USA). The level of significance was set at $p<0.05$. Figures were generated using GraphPad Prism 8.2.0 software.

Results

DNA-methylation levels of the TNFA gene

We included 39 HC, 201 RA patients and 77 PsA patients. Despite intermediate median age and % of females in HC, the 3 groups were not significantly different for these 2 characteristics. We compared DNA-methylation levels of the *TNFA* gene (Figure 1A). As expected[23], DNA-methylation levels were highly significantly reduced in RA compared to HC ($p=1.8x10^{-13}$). In PsA, levels were also reduced but less significantly compared to health (Table 1, $p=0.007$) and with a wide distribution of values.

Similar to our previous report[23], DNA-methylation levels were not significantly correlated with age (trend rho=-0.200, not significant) or sex in RA. We observed no association between DNA-methylation level and any clinical variables (duration of symptoms, TJC, SJC, CRP, RF, or ACPA, DAS28, full details in Supplementary Data S1, n=201), despite possible trends for reduction with CRP, TJC or SJC (rho<-0.180). Of note, 15% of patients at referral to hospital for 1st visit, had received a local intramuscular steroid injection within the 3 months preceding referral, which had no detectable effects on DNA-methylation levels (as previously reported[23]). Similarly, there was also no association of DNA-methylation levels with any of the PsA related variables.

DNA-methylation levels after 6 months of treatment with MTX

From the IA register, we identified 32 patients with paired samples at baseline and 6 months post-MTX treatment for RA and 26 pairs for PsA (Figure 1B). Forty-three patients showed a reduction of DNA-methylation at 6 months and 15 had an increase. Significantly more reductions in DNA-methylation levels were observed in RA (4 rise, 28 loss) while not in PsA (8 rises/3 no change/15 losses, Chi^2 p=0.0106). At the group levels, (box plot on figure 1B), there was an overall reduction of DNA-methylation levels in RA samples over 6 months (MWU, p=0.025) while only a trend in PsA (p=0.093).

Individual paired-change in DNA-methylation between baseline and 6 months are reported in Figure 1B (dots-lines). Using paired-test, levels of DNA-methylation reduced over time in RA (Wilcoxon test, $p=8.4 \times 10^{-4}$) but not in PsA. Furthermore, the wide distributions of data observed at baseline in PsA were reflected in the breath of changes at 6 months (Figure 1C, increase of >+10% and reduction of -15%), while this was less marked in RA (+2.5%/-5%). The smaller baseline values in RA may, however prevent large reductions to be observed.

Relationship between DNA-methylation levels and response to MTX in RA

Follow-up in RA patients receiving MTX, established that 111/201 (55%) achieved remission (DAS28<2.6) at 6 months, only escalating MTX doses. Patients not achieving remission also escalated MTX doses for 65% of case over 6 months, while adding SSZ or HCQ at 3 months in 17% of cases (3% started on MTX+HCQ and 5% had no follow-up information on added DMARDs). DNA-methylation levels were significantly higher in patients achieving remission compared to no remission (Figure 2A, MWU, $p=7.3 \times 10^{-8}$).

We investigated whether changes in DNA-methylation levels over 6 months of MTX treatment were more pronounced based on the response groups (Figure 2B). In RA, we observed a trend for larger reduction in the remission group (n=18, median -2.25%) compared to the non-response group (n=14, -0.87%, MWU p=0.101), and there were clearly more reductions (17/18) in the remission group than in the non-response group (11/14, Chi^2, p=0.075). Therefore, DNA-methylation levels were reduced at 6 months irrespective of response although more consistently in patients achieving remission, which most likely stem from the fact that levels at baseline were still high, while already reduced in the non-response group.

Relationship between DNA-methylation levels and response to MTX in PsA

Similarly, 29 PsA patients received MTX and 10/29 (35%) achieved PASDAS remission (<2.6) and 15/29 (52%) showed ACR20 response. *TNFA* DNA-methylation levels were not associated with response to MTX using either PASDAS-remission (Table 2 and Figure 2A), ACR20, or

even PASDAS response (<3.2, data not shown). Median change in methylation levels did not seem to differ with response groups (Figure 2B) and in fact, showed opposite direction for PASDAS and ACR20, confirming the lack of relationship. Furthermore, no single parameter was associated with response to MTX in PsA, preventing any further model development.

Impact of TNFA gene DNA-methylation levels on predicting treatment response in RA.

Individually, in addition to *TNFA* DNA-methylation levels, 4 clinical variables (TJC, SJC, VAS-GH, and DAS28), showed unadjusted associations with remission (Table 2, MWU), and predictive OR with significant AUC. DNA-methylation levels of the *TNFA* gene were the most strongly predictive of the remission outcome (Figure 3A, AUC=0.721, $p=7.3 \times 10^{-8}$).

We proceeded first, to a binary logistic regression analysis of all parameters to determine which may be of greater importance without (reference) and then including the *TNFA* qMSP data, for the prediction of remission induced by MTX. The reference model using all clinical variables (Table 3, Enter) has a relatively high accuracy (70%) and AUC=0.701, while only 2 variables significantly contributed (TJC and VAS-GH) to the prediction. A 2nd model (Forward, entering all variable but allowing the model to select the relevant ones) confirmed that TJC and VAS-GH were sufficient to build to the model, while with lower accuracy of 65.2% (suggesting that other variables have a non-significant contribution to the prediction) but a similar AUC=0.699 to Model-1 (Figure-3B). Adding the results of the *TNFA*-qMSP (model-3), modestly improved accuracy (+1.1%) over model-1 (Enter) but largely over model-2 (Forward +5.9%), with an increased AUC=0.760 (+0.059), clearly showing that these 2 clinical variables added to the predictive value of the *TNFA*-qMSP assay by itself. Despite TJC/VAS-GH being components of the DAS28 score, model-4 using DAS (instead of it 4 components) did not improve the overall accuracy (71.1%), although a further gain in AUC=0.774 (+0.014) was observed. A retrospective power calculation confirmed that an n=201 was providing >80% power for the regression. A T-test using the 500 permutations of the bootstrapping procedure used to generate the 95% CI of the AUC, was significant when comparing model 3 (Forward, clinical+qMSP) and model 2 (clinical only, p=0.038).

Discussion

Our study demonstrated the capacity of a recently developed qMSP assays to provide robust 1st time in human data for patient management. This is however disease specific, and the choice of the right gene to design the qMSP assay was critical. Our data suggests that the *TNFA*-qMSP could be a suitable biomarker in RA, predicting MTX-induced remission with better model performance and added-value over clinical parameters alone. This assay may not have value in PsA, despite being significantly reduced compared to health, as possibly not sufficiently powered to detect an association with MTX treatment outcome.

The mechanism of action of MTX is complex, and thought to be related to interferences with DNA-methylation[11-14], while also mediating multiple change in arthritis related events, including reduced neutrophil adhesion[26], inhibition of IL-1[27], IL-6[28], and IL-8 production[29], suppression of cell-mediated immunity[30] and inhibition of collagenase gene expression[31]. MTX inhibits the enzyme methionine-adenosine transferase, reducing levels of methionine and S-adenosyl-methionine, hence reducing the availability of the methyl donor for DNA-methylation by DNA Methyltransferases and is therefore expected to reduce DNA-methylation over time. MTX-treated RA patients showed better Treg suppressive function[32], which was indeed related to de-methylation of the *FOXP3* gene (bisulfite sequencing) in MTX-treated cells in tissue culture. In RA again, changes over of 4 weeks of MTX in 4 individual CpG sites (2 reduced/2 increased levels, *ADRA2C, GATA3, miR182, SNORD84* genes) were associated with good EULAR response[33], however no outcome predictive value was reported in this study. Therefore, while low-doses appear to reduce DNA-methylation in these RA studies, there is mixed evidence for both increase and reduction in DNA-methylation at the higher doses used in cancer[34-36]. Our results at 6 months are in line with those previous data in RA adding a T-cell specific CpG site in the *TNFA* gene, with continued reduction, whether or not patients respond to treatment. Furthermore, although the assay used measures methylation levels at a single CpG, this site is part of a larger region fully demethylated in RA as previously reported over at least 273 base pair with 8 consecutive CpG sites consistently showing ~90% demethylation (by sequencing)[20]. This region is specifically demethylated in early RA CD4+T-cells[20], while no evidence of demethylation over the whole *TNFA* gene was observed in monocytes in the same study. This was nonetheless associated with higher levels of circulating TNF being expressed in early RA[20] and it could be of interest to address whether continued demethylation of the *TNFA* gene has also an effect on TNF expression over time.

Higher global DNA-methylation (liquid chromatography and mass spectrometry) before treatment were observed in RA and associated with poor MTX response[37]. By contrast, our

results support that the loss of DNA-methylation at a specific CpG-site of the *TNFA* gene has biomarker value for response to MTX in early RA. Furthermore, this was not related to duration of symptoms at baseline, nor to the presence of systemic autoimmunity (ACPA/RF) and by extrapolation probably the genetic predisposition associated with ACPA-positivity; neither was it related to environmental factors (such as smoking) or disease burden (joint counts or CRP). Overall, these findings support the added-value of this biomarker as a predictor of MTX-induced remission, in addition to its validity as a stand-alone diagnostic biomarker, (i.e independent of other tools/tests used to classify RA as previously shown[23]). Nevertheless, our data also suggest that there is still room for improving the prediction of MTX-induced remission in early RA and that further research is needed to establish which particular combination of individual biomarkers can be used to personalize the use of MTX, for example combining qMSP for *TNFA* with data on naïve cell frequencies by flow cytometry[8, 10, 16].

Various mechanisms may drive this epigenetic remodeling. Our previous data suggested a role for IL-6[20], with a high specificity for remodeling for this particular CpG of the *TNFA* gene in early IA evolving to RA, but not towards other forms of IA[23]. Here, we did not identify a relationship with inflammation (not significant despite trends for reductions of DNA-methylation levels with CRP, JCs), however, remodeling may have happened before onset of inflammation (pre-RA phase) and as such correlations may no longer be observable. Therefore, the use of this test in pre-clinical RA samples would allow timing of such events (i.e. epigenetic remodeling) and may narrow the windows of time to focus the search for events triggering epigenetic remodeling and by extension, disease progression.

In PsA, the methylation levels observed in our study were very heterogeneous (at baseline and 6 months). These were also widely distributed in HC. These observations strengthen the conclusion that this particular CpG is probably not relevant as a biomarker in PsA, although they do not preclude the concept that a PsA-specific CpG site may be identifiable and could provide a biomarker for this disease alone. Our initial search was indeed focused on RA-specific CpGs, suitable to design qMSP assays, while the difficulties imposed by the local sequence resulted in many assays failing to achieve the requirements for qMSP[22, 23]. Of note, a commercial *IL-17A* gene qMSP-assay is available (surrogate of Th17 as % of CD4+T-cells) and we used it to show that it had value for RA classification[38]. This assay may be more relevant in PsA where Th17 cells have a more prominent role[39].

Our study has several strengths. During the optimization of qMSP-assays, the biological material source is extremely important as, in mixed cell type samples (like blood), the abundance of the

cells harboring the change in DNA-methylation levels is crucial. The *TNFA* assay was normalized to all cells in the sample (reference gene *GAPDH*), while in contrast the commercial *IL17A* assay used whole blood (WB) and normalization to the *CD4* gene[38], while there is no predictive value for the CD4 biomarker itself for classification or for MTX treatment outcome by flow cytometry (CD4+T-cells[8] and unpublished observations) or by qMSP (CD4/GAPDH)[38]. We already demonstrated the robustness of the *TNFA* assay using a CpG localized in a CD4+ T-cell tissue specific part of *TNFA* promotor (different from B-cells, monocytes and granulocytes[23]) that is furthermore specifically demethylated in early RA[20] and was detectable in purified total CD4+T-cells as well as in PBMC as the material source for DNA and also in WB (Supplementary Data S1). Therefore, even if CD4+T-cells represent only a small fraction of cells in WB, the differences between RA and controls previously observed in DNA from different sources (as detailed in our assay optimization paper)[23], suggest that a WB-DNA template, would still perform well using such CD4+T-cell specific CpG as in this *TNFA*-qMSP assay, WB being more suitable for a routine test in daily clinical practice. Nevertheless, it would be important to validate the robustness of the predictive value of our data using external cohorts, recruiting similar early (less 24 months symptom duration), DMARDs naïve RA patients, treated with MTX in a T2T approach and using DAS28<2.6 as outcome. Testing WB as a source of DNA, notably to facilitate a transfer of technology to hospital services where PBMC are not routinely separated will also be critical to limit variation introduced by other aspects of sample processing/handling.

On the other hand, it is important to note that the effect of drugs on lineage cell composition of PBMC could be interfering with qMSP measurements due to the tissue specificity used in the design of such assays (while not all designs may include this constrain), that could limit the interpretation of qMSP data. MTX is known to alter individual subsets of B-cells or monocytes cell surface marker shedding[40, 41] although it has no reported effect on the PBMC composition all lineages confounded[42, 43] and for CD4+T-cells data reported in previous publications on biomarkers from our group using flow cytometry[8, 10,] or using a *CD4* gene qMSP assay[38]. It is therefore unlikely, although not formally excluded, that the *TNFA*-qMSP assay may be influenced by such changes in our data (as we have not used the *CD4* gene qMSP assay here), while the assay was also designed in a CD4+T-cell specific region of the promoter[23] that would not be affected by methylation changes in other cell lineages.

Limitations of our work include the lower number of subjects available in the PsA group treated with MTX (compared to the RA group) which may limit the statistical power and generalisability of the results in this disease. Both cohorts were newly diagnosed, treatment naïve to disease

modifying therapies and of relatively short disease duration. MTX treatment escalation was faster in the PsA group than in the RA group, while this was not associated with a predictive value of the *TNFA*-qMSP in PsA. Whilst we could only include 201 RA patients, with 10 variables and 55% remission rate, the models developed had sufficient participants to achieve >80% power (calculated retrospectively), and model-3 and 4 clearly demonstrated added-predictive value over the use of clinical data only, showing high sensitivity (77/75%) and AUC (0.760/0.774). Despite the risk of overestimating the predictive performance of the models due to being developed and evaluated within the same dataset, bootstrapping was used to mitigate optimism bias and the increase in the AUC median (500 permutations (and 95% CI) was significant. External validations in independent cohorts nonetheless remain critical to confirm the generalisability and clinical applicability of this novel biomarker.

Furthermore, the results of the *TNFA*-qMSP could be dichotomized for easy decision-making (i.e. as proposed for RA classification of for ACPA-negative patients)[23]. Here, a cut-off set above 4% would have stratified 56/201 (28%) patients as high-chance of achieving MTX-induced remission, in whom 44/56 (80%) actually achieved remission. The test further predicted that 90/201 (45%) patients would not achieving remission and 78/90 (87%) did not indeed, whom could have been directed to a more aggressive treatment early, avoiding continued inflammation and the accumulation of damages. This suggests that a rule with 80% of chance for being right in choosing MTX and even better chance for not using it alone (adding further DMARDs early), could be designed for the management of early RA and tested prospectively to improve 1st line treatment outcome. Finally, the last hurdle in any biomarker research program, remains replication. A second (external) cohort is therefore needed to validate our findings before any clinical utility can be brough forward by our data (followed by a cost analysis) considering the benefit to patients/society of achieving remission as early as possible in the course of RA.

In conclusion, our data proposes a novel biomarker for predicting MTX induced remission, the *TNFA*-qMSP-assay, with potential clinical utility in early RA, but not in PsA where a qMSP-assay for other gene(s) may be more relevant. Further research is needed to test the value of this assay as a biomarker of response to TNF-inhibitors, which remains an area of unmet need in RA. Our past research has also identified other biomarkers of MTX-induced remission[8, 10, 38] and ultimately, multi-biomarker studies are needed to achieve sufficient discrimination towards defining stratification rules for personalizing the use of MTX in early RA. These findings should be interpreted as hypothesis-generating and require external validation in independent cohorts before clinical implementation.

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Conflicts of interest:

The authors have declared no conflicts of interest.

References

1. Pincus, T. and L.F. Callahan, The Side-Effects of Rheumatoid-Arthritis - Joint Destruction, Disability and Early Mortality. *Brit J Rheumatolo*, 1993. **32**: p. 28-37.
2. Wolfe, F., D.M. Mitchell, J.T. Sibley*, et al.*, The Mortality of Rheumatoid-Arthritis. *Arthritis Rheum.*, 1994. **37**(4): p. 481-494.
3. Emery, P., The Roche Rheumatology Prize Lecture. The optimal management of early rheumatoid disease: the key to preventing disability. *Br J Rheumatol*, 1994. **33**(8): p. 765-8.
4. Quinn, M., P. Conaghan, and P. Emery, The therapeutic approach of early intervention for rheumatoid arthritis: what is the evidence? *Rheumatology*, 2001. **40**(11): p. 1211-1220.
5. Quinn, M. and P. Emery, Window of opportunity in early rheumatoid arthritis: possibility of altering the disease process with early intervention. *Clin Exp Rheumatol.*, 2003. **21**: p. 154-157.
6. Smolen, J., FC Breedveld, GR Burmester*, et al.*, Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force. *Ann Rheum Dis*, 2015. doi.org/10.1136/annrheumdis-2015-207524.
7. Combe, B., R. Landewe, C.I. Daien*, et al.*, 2016 update of the EULAR recommendations for the management of early arthritis. *Ann Rheum Dis*, 2017. **76**(6): p. 948-959.
8. Ponchel, F., V. Goëb, R. Parmar*, et al.*, An immunological biomarker to predict MTX response in early RA. *Ann Rheum Dis*, 2014. **73**: p. 2047-53.
9. Hazlewood, G.S., C. Barnabe, G. Tomlinson*, et al.*, Methotrexate monotherapy and methotrexate combination therapy with traditional and biologic disease modifying antirheumatic drugs for rheumatoid arthritis: abridged Cochrane systematic review and network meta-analysis. *BMJ*, 2016. **353**: p. i1777.
10. Ponchel, F., A. Burska, N, , L. Hunt*, et al.*, T-cell subset abnormalities predict progression along the Inflammatory Arthritis disease continuum: implications for management. *Nature Scien Reports*, 2020. **10**: p. 3669-78.
11. Nesher, G. and T.L. Moore, The in vitro effects of methotrexate on peripheral blood mononuclear cells. Modulation by methyl donors and spermidine. *Arthritis Rheum*, 1990. **33**(7): p. 954-9.
12. Broxson, E.H., Jr., L.C. Stork, R.H. Allen*, et al.*, Changes in plasma methionine and total homocysteine levels in patients receiving methotrexate infusions. *Cancer Res*, 1989. **49**(21): p. 5879-83.
13. Nesher, G., T.L. Moore, and R.W. Dorner, In vitro effects of methotrexate on peripheral blood monocytes: modulation by folinic acid and S-adenosylmethionine. *Ann Rheum Dis*, 1991. **50**(9): p. 637-41.
14. Wang, Y.C. and E.P. Chiang, Low-dose methotrexate inhibits methionine S-adenosyltransferase in vitro and in vivo. *Mol Med*, 2012. **18**: p. 423-32.
15. Ponchel, F., A. Morgan, S. Bingham*, et al.*, Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. *Blood*, 2002. **100**. p.4550-56
16. Hunt, L., E. Hensor, J. Nam*, et al.*, T cell subsets: an immunological biomarker to predict progression to clinical arthritis in ACPA-positive individuals. *Annals of the rheumatic diseases*, 2016. **75**(10): p. 1884-1889.
17. Winchester, R., *The Molecular-Basis of Susceptibility to Rheumatoid-Arthritis*, in *Advances in Immunology, Volume 56*. 1994. p. 389-466.
18. Gregersen, P.K., J. Silver, and R.J. Winchester, The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum*, 1987. **30**: p. 1205-13.
19. Eyre, S., J. Bowes, D. Diogo*, et al.*, High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. *Nat Genet*, 2012. **44**: p. 1336-40.

20. Pitaksalee, R., A. Burska, p. Chambers, *et al.*, Differential CpG methylation in peripheral naïve CD4+T-cells in early rheumatoid arthritis patients. *Clinical Epigenetics*, 2020. https://doi.org/10.1186/s13148-020-00837-1.
21. Karouzakis, E., K. Raza, C. Kolling, *et al.*, Analysis of early changes in DNA methylation in synovial fibroblasts of RA patients before diagnosis. *Sci Rep*, 2018. **8**(1): p. 7370.
22. Ponchel, F., R. Pitaksalee, and T. Sargent, Differential CpG DNA Methylation: qMSP a Promising New Approach toward Clinical Utility as Biomarkers *Annals of Clinical and Laboratory Research*, 2023. **11**: S4: 001
23. Pitaksalee, R., R. Parmar, R. Hodgett, *et al. DNA Hypomethylation in the TNF-Alpha Gene Predicts Rheumatoid Arthritis Classification in Patients with Early Inflammatory Symptoms*. Cells, 2023. **12**, DOI: 10.3390/cells12192376.
24. Natoli, V., A. Charras, S.R. Hofmann, *et al.*, DNA methylation patterns in CD4+ T-cells separate psoriasis patients from healthy controls, and skin psoriasis from psoriatic arthritis. 2023. 10.3389/fimmu.2023.1245876.
25. De Marco, G., P. Helliwell, D. McGonagle, *et al.*, The GOLMePsA study protocol: an investigator-initiated, double-blind, parallel-group, randomised, controlled trial of GOLimumab and methotrexate versus methotrexate in early diagnosed psoriatic arthritis using clinical and whole body MRI outcomes. *BMC Musculoskelet Disord*, 2017. **18**(1): p. 303.
26. Cronstein B.N., Eberle M.A., Gruber H.E., *et al.*, Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc Natl Acad Sci U S A*. 1991. **88**(6): p. 2441-5. doi: 10.1073/pnas.88.6.2441.
27. Connolly K.M., Stecher V.J., Danis E., *et al.*, Alteration of interleukin-1 production and the acute phase response following medication of adjuvant arthritic rats with cyclosporin-A or methotrexate. *Int J Immunopharmacol*. 1988. **10**(6): p. 717-28. doi: 10.1016/0192-0561(88)90025-2.
28. Sung J.Y., Hong J.H., Kang H.S., *et al.*, Methotrexate suppresses the interleukin-6 induced generation of reactive oxygen species in the synoviocytes of rheumatoid arthritis. *Immunopharmacology*. 2000. **47**(1): p. 35-44. doi: 10.1016/s0162-3109(99)00185-x.
29. Seitz M., Dewald B., Ceska M., *et al.*, Interleukin-8 in inflammatory rheumatic diseases: synovial fluid levels, relation to rheumatoid factors, production by mononuclear cells, and effects of gold sodium thiomalate and methotrexate. *Rheumatol Int*. 1992. **12**(4): p. 159-64. doi: 10.1007/BF00274936.
30. Cronstein B.N., Sitkovsky M., Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. *Nat Rev Rheumatol.* 2017. **13**(1): p. 41-51. doi: 10.1038/nrrheum.2016.178.
31. Firestein G.S., Paine M.M., Boyle D.L., Mechanisms of methotrexate action in rheumatoid arthritis. Selective decrease in synovial collagenase gene expression. *Arthritis Rheum*. 1994. **37**(2): p. 193-200. doi: 10.1002/art.1780370207.
32. Cribbs, A.P., A. Kennedy, H. Penn, *et al.*, Methotrexate Restores Regulatory T Cell Function Through Demethylation of the FoxP3 Upstream Enhancer in Patients With Rheumatoid Arthritis. *Arthritis Rheumatol*, 2015. **67**(5): p. 1182-92.
33. Nair, N., D. Plant, S.M. Verstappen, *et al.*, Differential DNA methylation correlates with response to methotrexate in rheumatoid arthritis. *Rheumatology (Oxford)*, 2019. 59(6):1364-1371.
34. Oosterom, N., P.H. Griffioen, M.A.H. den Hoed, *et al.*, Global methylation in relation to methotrexate-induced oral mucositis in children with acute lymphoblastic leukemia. *PLoS One*, 2018. **13**(7): p. e0199574.
35. Kim, Y.I., J.W. Logan, J.B. Mason, *et al.*, DNA hypomethylation in inflammatory arthritis: reversal with methotrexate. *J Lab Clin Med*, 1996. **128**(2): p. 165-72.
36. de Andres, M.C., E. Perez-Pampin, M. Calaza, *et al.*, Assessment of global DNA methylation in peripheral blood cell subpopulations of early rheumatoid arthritis before and after methotrexate. *Arthritis Res Ther*, 2015. **17**: p. 233.

37. Gosselt, H.R., B.D. van Zelst, M. de Rotte*, et al.*, Higher baseline global leukocyte DNA methylation is associated with MTX non-response in early RA patients. *Arthritis Res Ther*, 2019. **21**(1): p. 157.
38. Burska A., Thu A., Parmar R.*, et al.*, Quantifying circulating Th17 cells by qPCR: potential as diagnostic biomarker for Rheumatoid Arthritis. *Rheumatology*, 2019. 10.1093/rheumatology/kez162.
39. Marinoni, B., A. Ceribelli, M.S. Massarotti*, et al.*, The Th17 axis in psoriatic disease: pathogenetic and therapeutic implications. *Auto Immun Highlights*, 2014. **5**(1): p. 9-19.
40. Joly M.S., Martin R.P., Mitra-Kaushik S., *et al*., Transient low-dose methotrexate generates B regulatory cells that mediate antigen-specific tolerance to alglucosidase alfa. *J Immunol*, 2014. **193**(8): p.3947-58. doi: 10.4049/jimmunol.1303326.
41. Fuentelsaz-Romero S., Barrio-Alonso C., García Campos R., *et al*., The Macrophage Reprogramming Ability of Antifolates Reveals Soluble CD14 as a Potential Biomarker for Methotrexate Response in Rheumatoid Arthritis. *Front Immunol*, 2021. **12**: p.776-879. doi: 10.3389/fimmu.2021.776879.
42. Olsen N.J., Callahan L.F., Pincus T., Immunologic studies of rheumatoid arthritis patients treated with methotrexate. *Arthritis Rheum*. 1987. **30**(5): p.481-8. doi: 10.1002/art.1780300501.
43. Hedman A.K., Winter E., Yoosuf N., *et al.*, Peripheral blood cellular dynamics of rheumatoid arthritis treatment informs about efficacy of response to disease modifying drugs. *Sci Rep*, 2023. **13**(1): p.10058. doi: 10.1038/s41598-023-36999-0.

Figure legend

Figure 1: DNA-methylation levels of the *TNFA* gene

A) The *TNFA*-qMSP assay was performed on DNA from in 39 HC, 201 RA and 77 PsA patients. Levels were reduced in RA (MWU, $p=1.8x10^{-13}$), and in PsA (p=0.007) compared to HC.

B) The *TNFA*-qMSP-assay was performed in 58 paired baseline/6 months post MTX treatment samples, in 32 RA (left panel) and 26 PsA (righ). At the distribution levels (grey box-plots), there was an overall reduction in levels of DNA-methylation over 6 months in RA (MWU, p=0.025) while only a trend in PsA (p=0.093). Reductions in DNA-methylation levels at 6 months as paired-data were observed more often than increases in RA (Wilcoxon test, $p=8.4 x10^{-4}$). In PsA, no particular direction of change was observed.

C) Change in DNA-methylation levels (delta) between baseline and 6 months were calculated. There was no significant difference in the amplitude of changes between RA and PsA (MWU, NS). However, increases were observed only in 4/32 RA pairs, while 8/26 reductions were observed in PsA pairs in addition to 3 no-change, which represent a significant difference in the direction of changes between the 2 diseases (Chi^2, p=0.0106).

Alt text: This figure presents data of DNA-methylation (as individual data points representing % of DNA methylation) at the TNFA locus in healthy controls and patient with RA or PsA. Two further panels present data matched for each patient between results at baseline and after 6 months of treatment with MX in RA and in PsA. Another panel presents data of the difference in levels of methylation in RA and PsA calculated between levels after 6 months of treatment compared to baseline.

Figure 2. Association between DNA methylation levels on response to MTX

A) DNA-methylation levels in 201 RA patients received MTX were higher in 111/201 (55%) of patients achieving remission (Rem) compared to not (No rem) (MWU, $p=7.3 x 10^{-8}$). DNA-methylation levels were not associated with either response in 29 PsA patients who received MTX and achieved PASDAS-remission (10/29) or achieved ACR20 (15/29).

B) Changes in DNA-methylation levels at 6 months (delta) were not different between any of the response groups in PsA, while possibly showing a trend in larger reduction in RA in remission (MWU, p=0.101). Direction of changes (increase/reduction) was also not associated with response.

Alt text: This figure presents data of DNA-methylation (as individual data points representing % of DNA methylation) at the TNFA locus in patient with RA or PsA separated based on whether they achieved DAS-remission for RA and PASDAS remission or ACR20 for PsA after 6 months

of MTX treatment. A second panel present the same data but calculated as a difference between data, matched for each patient between results after 6 months of treatment with MTX compared to baseline.

Figure 3. Predictive value of the *TNFA*-qMSP assay

A) A ROC analysis was performed using DNA-methylation levels of the *TNFA* gene as predictor of remission at 6 months in RA. Area under the ROC (AUC) is used to display result (AUC=0.721, $p=7.3 \times 10^{-8}$).

B) A ROC analysis was performed using the individual patient's probability calculated in 4 regression models. Using the enter method the reference model (clinical data alone) had an AUC=0.701. A forward approach model showed a similar AUC=0.699. Combination of clinical data with the DNA-methylation levels of the *TNFA* gene increased the predictive value of the forward model (AUC=0.760). Using DAS28 (instead of TJC/SJC, CRP and VAS-GH), increased further the performance of the model (AUC=0.774).

Alt text: This figure presents ROC curve analysis of the value of DNA-methylation levels at the TNFA locus for predicting MTX-induced remission in RA patient at 6months. A second panel present the value of adding the data on DNA-methylation to clinical data in modelling the prediction of MTX-induced remission in RA.

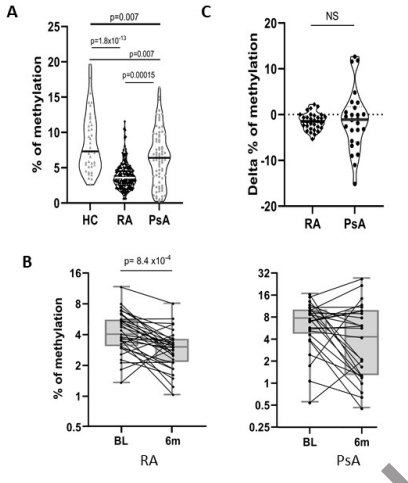

Figure 1

338x190mm (96 x 96 DPI)

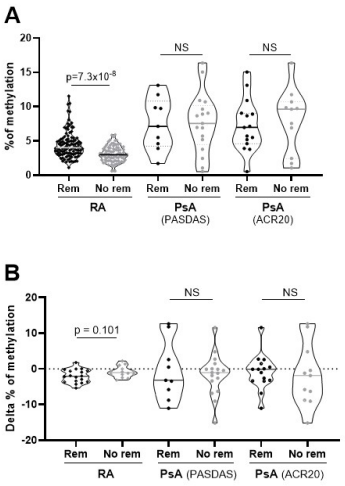

Figure 2

338x190mm (96 x 96 DPI)

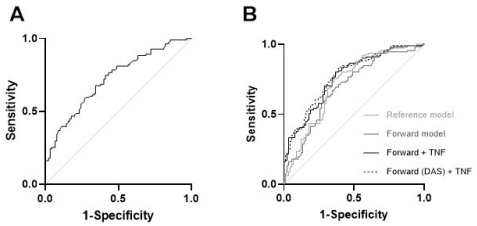

Figure 3

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Table 1: Patient characteristics

	Missing data (n)	RA (n=201)	PsA (n=77)	HC (n=41)
TNF methylation levels (%)*		3.6 [1.8]	6.4 [3.9]	7.3 [6.0]
Age * (years)		56 [22]	39 [12.7]	49 [17]
Female sex n (%)		150 (75%)	39 (51%)	25 (61%)
Symptoms duration (months)*	4	5.5 [8]	9.5 [30.9]	
Smokers (past & current) n (%)	6	117 (58%)	39 (51%)	
RF positive (%)	0	94 (47%)		
ACPA positive (%)	1	106 (53%)		
Tender joint count*		9 [12]	4 [5.1]	
Swollen joint count*		4 [7]	2 [2.7]	
CRP * (mg/L)	10	11 [45]	52 [26.6]	
VAS-GH *	32 in RA 3 in PsA	50 [45]	52 [26.6]	
DAS28*	37	4.8 [2.1]		
PASDAS	15		5.8 [1.2]	
BASDAI*	15		10 [8]	

Data are described using n (% of patients), or * median [Inter Quartile Range]. RA: rheumatoid arthritis, PsA: psoriatic arthritis, RF: rheumatoid factor, ACPA: anti-citrullinated peptide antibodies, TJC: tender joints count, SJC: swollen joints count, CRP: C-reactive protein, VAS: visual analogic scale, DAS28: disease activity score 28 joints, PASDAS: PsA Disease Activity Score. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index,

Table 2: Patient characteristics with respect to response to MTX (RA n=201, PsA n=29)

RA	Remission N=111	Not in remission N=90	MWU/Chi2 p-value	AUC (95% CI) p-value
TNF methylation levels*	4.0 [1.9] (1.10-11.56)	3.0 [1.7] (0.60-5.92)	7.3 x 10-8	0.721 (0.652-0.790) 7.3 x 10-8
Age (years)*	58 [23] (20-86)	56 [18] (19-86)	0.548	0.525 (0.444-0.615) 0.549
Female sex n (%)	79 (71.2)	71 (78.9)	0.211	0.461 (0.382-0.541) 0.347
Symptoms duration (months)*	5.5 [7.5] (0.5-24)	6.0 [8.3] (0.69-24)	0.912	0.495 (0.414-0.577) 0.912
Smokers n (%)	59 (54.1)	58 (67.4)	0.060	0.525 (0.444-0.615) 0.549
RF positive (%)	45 (40.5)	49 (54.4)	0.049	0.433 (0.353-0.514) 0.111
ACPA positive (%)	58 (52.7)	48 (53.3)	0.932	0.497 (0.416-0.578) 0.941
Tender joint count*	6 [11] (0-25)	12 [10.25] (0-27)	1,5x10-4	0.344 (0.269-0.420) 1,5x10-4
Swollen joint count*	3 [7] (0-21)	6 [7.5] (0-24)	0.022	0.407 (0.328-0.485) 0.023
CRP (mg/L)*	9.8 [22] (0-92)	13.7 [26.2] (0-201)	0.119	0.428 (0.346-0.510) 0.087
VAS-GH	47 [35] (0-100)	52 [28] (2-100)	6.4x10-5	0.333 (0.250-0.417) 6.6x10-5
DAS28*	4.21 [2.00] (1.10-6.81)	5.32 [1.64] (1.38-7.85)	2x10-6	0.310 (0.228-0.393) 2x10-6
PsA	Response N=10	No response N=19	p-value	AUC (95% CI)
TNF methylation levels*	8.4 [6.6] (1.73-13.12)	7.6 [5.9] (0.54-16.38)	0.839	0.526 (0.301-0.751) 0.819
Age (years)*	40 [26.5] (28-65)	41 [29] (23-60)	0.456	0.587 (0.360-0.813) 0.449
Female sex n (%)	3 (30%)	13 (68%)	0.064	0.308 (0.101-0.515)

				0.094
Symptoms duration (months)*	8 [15.6] (2-89)	10 [28] (3-198)	0.403	0.400 (0.175-0.625) 0.383
Smokers n (%)	4 (40%)	10 (53%)	0.700	0.437 (0.214-0.659) 0.083
Tender joint count*	4 [7.3] (0-11)	6 [7] (0-22)	0.769	0.466 (0.242-0.690) 0.766
Swollen joint count*	3.5 [4.3] (0-11)	3 [2.0] (0-7)	0.353	0.608 (0.366-0.850) 0.347
CRP (mg/L)*	5.5 [2.3] (5-25)	6 [11] (5-45)	0.456	0.411 (0.200-0.621) 0.435
VAS-GH*	26 [39] (5-78)	51 [27] (24-97)	0.016	0.229 (0.031-0.427) 0.018
PASDAS*	5.5 [1.8] (3.3-6.1)	5.7 [1.3] (4.6-7.4)	0.069	0.289 (0.092-0.486) 0.066
BASDAI *	3 [9.3] (1-14)	9 [8] (4-19)	0.011	0.213 (0.013-0.414) 0.012

Data are described using n (% of patients), or * median [Inter Quartile Range] and (min-max). RA rheumatoid arthritis, PsA psoriatic arthritis, RF rheumatoid factor, ACPA anti-citrullinated peptide antibodies, CRP C reactive protein, VAS-GH visual analogue scale general health, DAS28 disease activity score 28 joints. PASDAS: PsA Disease Activity Score psoriatic arthritis. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index. MWU Man Whitney-U, AUC area under the curve,

Table 3: Predictive model of response to MTX in RA.

RA	Unadjusted	Model 1 (Reference)	Model 2 Forward	Model 3 Forward+TNF	Model 4-DAS Forward+TNF
TNF methylation levels*	1.886 (1.466-2.425) 7.3×10^{-7}			1.825 (1.400-2.378) 8.0×10^{-6}	1.933 (1.453-2.571) 6.0×10^{-6}
Age (years)*	1.004 (0.986-1.022) 0.689	1.007 (0.987-1.028) 0.489	Not selected	Not selected	1.021 (1.000-1.044) 0.051
Female sex n (%)	1.514 (0.789-2,905) 0.213	0.639 (0.304-1.346) 0.239	Not selected	Not selected	Not selected
Symptoms duration (months)*	1.004 (0.956-1.055) 0.867	1.001 (0.947-1.058) 0.960	Not selected	Not selected	Not selected
Smokers n (%)	0.570 (0.317-1.025) 0.061	0.563 (0.296-1.072) 0.081	Not selected	Not selected	Not selected
RF positive (%)	0.571 (0.325 -1.001) 0.050	0.527 (0.271-1.024) 0.059	Not selected	Not selected	Not selected
ACPA positive (%)	0.976 (0.558-1.706) 0.932	1.238 (0.624-2.454) 0.542	Not selected	Not selected	Not selected
Tender joint count*	0.937 (0.902-0.974) 0.001	0.939 (0.895-0.986) 0.011	0.946 (0.910-0.984) 0.006	0.954 (0.915-0.996) 0.030	
Swollen joint count*	0.953 (0.908-1.001) 0.053	1.016 (0.952-1.084) 0.635	Not selected	Not selected	
CRP (mg/L)*	0.988 (0.977-0.999) 0.031	0.989 (0.977-1.001) 0.074	Not selected	Not selected	
VAS-GH	0.979 (0.967-0.991) 0.001	0.985 (0.972-0.998) 0.027	0.982 (0.969-0.994) 0.004	0.984 (0.971-0.997) 0.016	
DAS28*	0.618 (0.482-0.794) 0.000013				0.639 (0.499-0.820) 0.000413
Accuracy (95% CI)		70.1 (63.3-75.7)	65.2 (54.4-73.9)	71.1 (64.4-76.6)	71.1 (66.7-74.8)
SEN % (95% CI) SPE % (95% CI)		76 (71-81) 63 (58-69)	74 (69-79) 54 (49-60)	77 (72-82) 64 (59-70)	75 (70-80) 67 (61-72)
PPV % (95% CI) NPV % (95% CI)		72 (67-77) 68 (62-73)	67 (61-72) 63 (57-68)	73 (67-78) 69 (64-74)	73 (68-79) 68 (63-74)
AUC (95% CI) p		0.701 (0.650-0.795) 6.2×10^{-8}	0.699 (0.621-0.768) 2×10^{-6}	0.760 (0.700-0.830) 1.0×10^{-10}	0.774 (0.710-0.838) 2.4×10^{-11}

Data are described using OR (95% CI) p-value. RF rheumatoid factor, ACPA anti-citrullinated peptide antibodies, TJC tender joints count, SJC swollen joints count, CRP C-reactive protein, DAS28 disease activity score 28 joints. Accuracy % of correctly predicted cases, SEN/SPE sensitivity and specificity; PPV/NPV positive and negative predictive values AUC.