S100A12 is associated with response to therapy in Juvenile Idiopathic Arthritis

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Abstract

Objectives

Around one third of patients with juvenile idiopathic arthritis (JIA) fail to respond to first line methotrexate or anti-TNF therapy, with even fewer achieving ≥ ACRpedi 70, though individual responses cannot yet be accurately predicted. As change in serum S100-protein MRP8/14 is associated with therapeutic response, we tested granulocyte-specific S100-protein S100A12 as a potential biomarker for treatment response.

Methods

S100A12 serum concentration was determined by ELISA in MTX (n=75) and anti-TNF (n=88) treated patients at baseline and follow-up. Treatment response (≥ ACRpedi50 score), achievement of inactive disease (ID) and improvement in JADAS-10 score were recorded.

Results

Baseline S100A12 concentration was measured in patients treated with anti-TNF (etanercept n=81, adalimumab n=7, median 200, IQR 133-440 ng/ml) and MTX (median 220, IQR 100-440 ng/ml). Of the patients in the anti-TNF therapy group, 74 (84%) were receiving MTX. Responders to methotrexate (n=57/75) and anti-TNF (n=66/88) therapy had higher baseline S100A12 concentration compared to non-responders: median 240 (IQR 125-615) ng/ml versus 150 (IQR 87-233) ng/ml p=0.021 for MTX, and median 308 (IQR 150-624) ng/ml versus 151 (IQR 83-201) ng/ml p=0.002 for anti-TNF therapy. Follow-up S100A12 could be measured in 44/75 methotrexate-treated (34/44 responders) and 39/88 anti-TNF-treated (26/39 responders) patients. Responders had significantly reduced S100A12 concentration (MTX: p=0.031, anti-TNF: p<0.001) at follow-up versus baseline. Baseline serum S100A12 in both univariate and multivariate regression models for anti-TNF therapy and univariate analysis alone for MTX therapy was significantly associated with change in JADAS-10.

Conclusion

Responders to MTX or anti-TNF treatment can be identified by higher pre-treatment S100A12 serum concentration levels.

Abbreviations

ACRpedi50 American College of Rheumatology (ACR) paediatric-50 criteria for response, **ELISA** enzyme linked immunoabsorbant assay, **ID** inactive disease, **IQR** inter-quartile range, **JADAS-10** Juvenile Arthritis Disease Activity Score, **MTX** methotrexate, **anti-TNF** anti-Tumour Necrosis Factor.

1. Introduction

Juvenile idiopathic arthritis (JIA) is a clinically heterogeneous condition, frequently requiring therapy with conventional disease-modifying anti-rheumatic drugs (cDMARDs) such as methotrexate (MTX). Combination therapy increasingly also includes biological DMARDs (bDMARDs) with TNF-inhibitors (e.g. etanercept and adalimumab).[1–3] However up to 40%, or even higher depending on the definition used, of patients will not respond to treatment with bDMARDs.[4–6] Using biomarkers, alongside known predictive demographic and clinical factors, could help improve the prediction of response.[1,7,8]

S100A12 and myeloid related protein complex 8/14 (MRP8/14 or S100A8/A9) are S100 protein family members. Both proteins are calcium binding proteins and phagocyte activation markers acting as pro-inflammatory ligands of Toll-like receptor-4 (TLR-4), which are constitutively expressed predominantly in phagocytic myeloid cells (i.e. granulocytes and monocytes). It is thought that both proteins are secreted in a similar mechanism, either by non-classical secretion from active cells or passively released from necrotic cells.[9] Both S100A12 and MRP8/14 are both validated predictors of relapse risk and disease activity in JIA.[10–12] S100A12 concentration measured at the time of treatment withdrawal in patients with JIA predicted the development of flare better than MRP, with the combination of S100A12 plus high-sensitivity c-reactive protein (CRP) performing best.[13] This suggests differences exist in the performance of S100A12 and MRP8/14 as biomarkers, despite their many apparent similarities. Baseline MRP8/14 has already been shown to predict response to MTX and anti-TNF treatment in JIA patients. However, the association of serum S100A12 with response to therapy in JIA has not yet been evaluated.[14,15]

2. Material and Methods

2.1 Study population

Data were analysed from three prospective cohort studies which were designed to study either the response to starting MTX or starting anti-TNF treatment (alone or in combination with other therapy including MTX, see Table 1) in patients with JIA diagnosed according to the International League of Associations for Rheumatology (ILAR) criteria.[3] The study was open for patients with undifferentiated JIA but no patient was enrolled. The prediction of response by MRP8/14 in these cohorts has already been published in detail [14,15] and here we focus on reporting the associations of S100A12. Response to MTX was analysed using data from the UK Childhood Arthritis Response to Medication Study (CHARMS, n=75 patients). Data on response to anti-TNF treatment were collected in the Dutch Arthritis and Biologicals in Children (ABC) Register (n=68), the German Registry for Biologics in Paediatric Rheumatology (BIKeR, n=12) and the CHARMS study (n=8). Each of these studies recruited patients with all subtypes of JIA who fulfilled ILAR criteria and started either new

DMARDS or biologic therapy for active arthritis (CHARMs). ABC and BIKER cohort data were combined to increase statistical strength. MTX and anti-TNF therapies were prescribed at the dose according to the previously published study protocols.[4,6,14]

The BIKeR register was approved by the ethics committee of the Aerztekammer Nordrhein Duesseldorf (ref 2/2015/7441), the CHARMS study was approved by the Institute of Child Health/Great Ormond Street NHS Trust (MREC-05/Q0508/95) and the ABC Register was approved by the Medical Ethics Committee at Erasmus MC Rotterdam (MEC-225.804/2003/51). The BIKER and ABC registries as well as the CHARMs study included provision in their ethical approvals for the collection, storage and analysis of biobanked samples. All three cohorts have been previously published in full elsewhere. Subjects were recruited with fully informed consent and child assent where appropriate.[4,6,14]

2.2 Definition of treatment response

Treatment responders achieved an ACRpedi-50 or better score at follow-up, equivalent to ≧ 50% improvement in a minimum of 3 out of 6 core variables, with no worsening in >1 remaining variables by >30%. Core variables are: 1) physician's global assessment score (PGA, using VAS: range 0-10 cm, 0=best score), 2) patient/parent global assessment of wellbeing (VAS: range 0-10 cm, 0=best score), 3) Childhood Health Assessment Questionnaire (CHAQ, range 0-3, 0=best score), number of joints with 4) active arthritis 5) limited motion and 6) erythrocyte sedimentation rate (ESR).[3,16] Disease activity and response were also quantified by parent/patient pain visual analogue scale (VAS), the achievement of inactive disease and change in JADAS-10, defined as the difference between baseline and follow-up JADAS-10.[17] The JADAS-10 score is quantified in four domains, three on a continuous scale (physician global, parent/patient global and number of active joints out of 10 specified) and the fourth being the presence of a normalized ESR.[18] The modified definition of inactive disease (ID, Wallace et al.[19]) requires the absence of active arthritis, systemic features, uveitis, normal ESR (≤20 mm/h) but accepts a higher acceptable PGA ≤1.0 cm (which in practice is rarely scored as 0) compared to the standard ID definition, As all patients achieving ID also fulfil ACR50, ACR50 was used as the measure of response because if any prediction of response was found with this lower threshold, it is likely the same or a higher response would be present with the use of ID. Baseline demographics and clinical scores including JADAS-10 are shown in **Table 1** and the follow up characteristics (responders and non-responders) are shown in **Supplement 1.**

2.3 S100A12 measurement

Serum concentrations were measured using a well described in-house ELISA assay as well as a commercial assay (*CircuLex*, *CycLex Co.Ltd*) on frozen samples.[11,13]. Both assays were utilised in order to investigate whether measured concentrations were reproducible in both assays and identify a suitable commercial assay, approved for research use, for use for further studies, which do not have access to this in-house ELISA. Reference internal control sera were used in each assay. S100A12 is a stable biomarker which is reliably measurable in samples sent at room temperature as well as in repeatedly thawed and frozen samples. All reported S100A12 values refer to in–house assay results unless specified. Results using the commercial assay are shown in **Supplement 2**. All assays were performed blind to the clinical diagnosis and results were not reported to treating clinical staff during the study. Results are presented as median (IQR).

2.4 Statistical analysis

Categorical characteristics were tested using Chi-squared, continuous variables using Mann-Whitney U and correlations with the Spearman (*rs*) or Pearson (*r*) test. Baseline and follow-up S100A12 was compared in paired analyses using the Wilcoxon signed rank test. Baseline S100A12 concentration was assessed for its prediction of ACRpedi outcome by binary logistic regression modelling and association with change in JADAS-10 by linear regression modelling. Multivariable linear models were also fitted for change in JADAS-10, allowing correction for other potential predictors and to assess the added value of S100A12 in predicting response. For this modelling, known predictive variables (gender, age at JIA onset, disease duration, baseline JADAS-10, baseline CHAQ, number of previously used DMARDs and ESR) were pre-specified.[7,8,20,21] Missing data were handled using the chained equations multiple imputation command *ice* in Stata/SE (v13.0). Anti-TNF (adalimumab or etanercept) treated patients were combined after being assessed as having identical characteristics. Cut-off values for baseline S100A12 as a predictive marker for treatment response were defined using receiver operator characteristic (ROC) analysis.[13] Other analyses were performed with SPSS (IBM for Windows V.21) and Prism (Graphpad v5).

3. Results

3.1 Baseline characteristics

Baseline median S100A12 concentration in patients before either therapy (MTX: n=75, anti-TNF: n=88) significantly correlated with baseline ESR (MTX rs 0.40, p<0.001; anti-TNF rs 0.38, p<0.001) and JADAS-10 (MTX rs 0.25, p=0.04; anti-TNF rs 0.22, p=0.04, **Table 1**). Subgroup analysis of S100A12 with number of active joints at start showed no correlation (Spearman's rho 0.19 (p=0.072). In MTX treated patients, there was no difference in baseline S100A12 among JIA subtypes (p=0.17, Kruskal Wallis test). However, in anti-TNF treated patients a difference among patients of different subtypes

was seen (p=0.024), with the highest concentrations in polyarticular RF positive JIA (median 411 ng/ml, n=13) and lowest in oligoarticular persistent JIA (median 56 ng/ml, n=5).

3.2 Clinical response to therapy

Follow-up was at a median of 6.6 (IQR: 5.8-7.6) months for MTX and 3.2 (2.6-5.0) months for anti-TNF treated patients. The clinical response of each treatment group was analysed separately, therefore this difference did not impact the results shown. Based on achievement of ACRpedi50 or better at follow-up, 57 of 75 MTX-treated patients and 66 of 88 anti-TNF treated patients were responders. Of the 66 anti-TNF treated responders, 46 had an ACRpedi70 or better response, while 31 patients were in clinical remission. The modified criteria for ID were fulfilled by 25/75 of MTX and 31/88 of anti-TNF treated patients. JADAS-10 at follow-up was median 3 (IQR 1-8) for MTX and 4 (1-9) for anti-TNF treated patients **(Supplement 1)**, improving from baseline **(Table 1)**. There were no significant differences between responders and non-responders for either treatment group in terms of baseline disease characteristics, excluding the variables included in the ID and JADAS-10 score **(Supplement 1)**.

3.3 Baseline S100A12 and response to therapy

Baseline S100A12 concentration was higher in responders versus non-responders (**Figure 1A** MTX median 240 (IQR: 125-615) ng/ml versus 150 (87-233) ng/ml, p=0.02; **Figure 1B** anti-TNF median 308 (IQR: 150-624) ng/ml versus 151 (IQR: 83-201) ng/ml, p=0.002). Increased baseline S100A12 was associated with odds ratios >1 for the prediction of ACRpedi50 and improvement in JADAS-10 in univariate models at follow-up, for both treatments **(Table 2)**. For patients using anti-TNF and MTX therapy, logistic regression modelling was also performed with the additional variable “MTX at start” and the odds ratio for baseline S100A12 did not change, and concomitant MTX was not a significant factor in the combined model (OR 3,46 95% CI 0,93-12,85). Multivariate models constructed with known predictors of response, as detailed in the statistical methods above, tested their prediction of JADAS-10. Excluding S100A12, model variables explained 70% of the variance in change in JADAS-10 at follow-up for MTX-treated patients and 50% of the variance for the anti-TNF group. Including S100A12 as a variable improved the predictive models by 2% (not significant) for MTX and 5% (p=0.004) for anti-TNF therapy **(Table 2)**.

3.4 Follow-up S100A12

Follow-up S100A12 concentrations were determined for MTX (44/75) and anti-TNF (39/88) treated patients, limited only due to lack of serum for this analysis which was performed blinded (see section 2.3). Of these, 34/44 (77%) MTX and 26/39 (67%) anti-TNF treated patients were responders. At

follow-up, both responders and non-responders, irrespective of therapy, had comparable S100A12 concentrations: MTX responders median 165 (IQR: 113-273), non-responders 79 (46-213, p=0.08), and anti-TNF treatment responders 110 (53-254) and non-responders: 91 (42-235), p=0.55 (**Figure 1**). However, responders (those achieving ACRpedi50) had significant reduction from their baseline S100A12 concentration measured by the Wilcoxon signed rank test **(Supplement 1).** Sensitivity, specificity and likelihood ratios for prediction of response by S100A12 using ROC analysis are shown in **Table 3.**

3.5 Use of concomitant therapy

Concomitant therapy was given according to physician choice. The percentage of patients using concomitant MTX at start of anti-TNF therapy in the group of responders was 91% (60/66) and 63% (14/22) in the non-responders. Systemic corticosteroid use at the start of MTX treatment (n=25/61, 41%) was not associated with any significant differences in either baseline or follow-up S100A12. However, in the anti-TNF treatment group, those who were also receiving corticosteroids at the start of the treatment (n=25/88) had higher baseline S100A12 than those who did not (median 380 (IQR: 177-838) ng/ml versus 187 (128-331) ng/ml, p=0.006) and also greater change at follow-up (delta S100A12 -145 (-327 to -97) versus -84 (-149 to 13, p=0.034). However, there was no difference in corticosteroid use between patients characterised as responders or non-responders, therefore concomitant corticosteroid use was unlikely to be the major factor in patients reaching clinical response. Concomitant DMARD use (excluding MTX), were used by so few patients (MTX-treated= 3/66; anti-TNF-treated=3/88) that no conclusions could be drawn.

3.6 Measurement of S100A12 by commercial ELISA

S100A12 measured by commercial assay **(Supplement 2)** was comparable with in-house assay results and also showed significantly higher S100A12 in responders versus non-responders and higher baseline versus follow-up concentrations. However, while a good AUC was obtained for both therapy groups, this was lower with the commercial (MTX AUC 0.662, 95% CI 0.532-0.791; anti-TNF 0.675, 95% CI 0.550-0.800) versus in-house (MTX AUC 0.675, 95% CI 0.559-0.805; anti-TNF 0.734, 95% CI 0.662-0.846) assay. Sensitivity (commercial ELISA: MTX 45.6, anti-TNF 39.4; in-house ELISA: MTX 47.4, anti-TNF 58.6) and specificity (commercial ELISA: MTX 83.3, anti-TNF 86.4; in-house ELISA: MTX 88.9, anti-TNF 80.7) were also lower with the commercial ELISA. Absolute commercial assay concentrations were also higher than the in-house assay, approximately double, and the cut-off levels calculated for each therapy group were also much wider than with the in-house assay.

4. Discussion

Baseline serum S100A12 was associated with response to both MTX and anti-TNF therapy in patients with JIA who had a high baseline concentration that decreased significantly with either MTX or anti-TNF treatment. Patients with higher baseline S100A12 concentration had higher disease activity and ESR and were more likely to be treatment responders. Furthermore, the addition of S100A12 to multivariate models improved the prediction of response.

The aim of this study was not to directly compare level of response to MTX versus anti-TNF therapy, or consider their combined therapy versus individual use, but rather to determine whether S100A12 concentration can predict a response to therapy when a clinician initiates either of these medications. Further work and specific trials are needed to determine which therapy would be best initiated in which patients, and such studies would also require the availability of predictive markers of response, like S100A12 which is discussed here.

S100A12 has already been shown to correlate with disease activity and concentrations >175 ng/ml potentially predict increased risk of flare in patients who have had treatment withdrawn.[10,13,22–24] The follow-up time of patients in this study was a median of five months. Most patients would be expected to show a treatment response within three months after initiation, with S100 concentrations shown to decrease in response to effective biological treatment within four weeks of beginning treatment.[25,26]

Moncrieffe et al. and Anink et al. identified MRP8/14 as being associated with MTX- and anti-TNF therapy response, and also suggested predictive modelling could be improved by including additional variables.[14,15] S100A12, like MRP8/14 has the advantage over other cytokines, e.g. IL-1beta, in having greater temperature stability, even withstanding storage and postage at room temperature. S100A12 measurement could therefore feasibly be incorporated into the routine laboratory work-up for JIA and therefore also be incorporated into in treatment prediction models.[7,21,27,28]

Whilst a well-established experimental ELISA S100A12 protocol exists, this is not yet in routine use. The commercial ELISA has already been demonstrated to perform well in analysing patient's serum.[11,26,29] Both assays require serial dilution of serum to obtain reliable results, due to the wide range of S100A12 concentrations in patients.[11] Therefore, while either assay can be used, results from each should not be directly compared and only used with assay-specific cut-offs. Although overall the same pattern of results were obtained with both assays, the in-house ELISA performed marginally better, as reflected by the slightly higher AUC and Youden Index values achieved for both MTX and anti-TNF treatment groups with the in-house assay compared to with the commercial assay.

Whereas S100A12 and MRP8/14 have some reported similarities in intra- and extracellular functions, the mechanism of release for each remains unknown. There are clear differences in the expression and functions between the two proteins.[9] A hallmark of MRP8/14 is its formation of a heterodimer,

whilst the hexamer is thought to be the active extracellular form of S100A12.[30] Adding S100A12 into the multi-variable models (investigated for MRP8/14 by Moncrieffe et al.) did result in a further increase in explained variance, though only a relatively small percentage (2%, non-significant) for MTX, but 5% ($p < 0.005$) for the anti-TNF group.[14]

In this cohort, baseline ESR and number of active joints already differentiated well between those patients who later became responders versus non-responders, which could be one factor why the addition of S100A12 to a multi-marker model added limited benefit. Other cohorts, particularly larger clinical cohorts, are required to ascertain whether S100A12 is a clinically useful predictive marker.

It is likely that no single biomarker can be sufficiently sensitive or specific for predicting response and multi-marker panels are increasingly being sought, such as the multi-biomarker disease activity test (MBDA) for rheumatoid arthritis.[1,31] It is also important to acknowledge that there is a lack of clinically viable alternative biomarkers that could replace S100A12 or MRP8/14, or add to their prediction in such multi-variable models at present. Additionally, heterogeneity within the same subgroup of JIA could be a further factor in variation in treatment response, and would further support the use of multi-marker panels to individualize management strategies. Small cohort size also increases the chance of clinical heterogeneity leading to statistically significant outcomes, and we combined two cohorts for the anti-TNF group to counter this. Larger studies would require greater multicentre collaboration and the use of inception cohorts. One factor that could be investigated is the presence and influence of TNF-alpha gene polymorphisms, which could be associated with the heterogeneity of response to anti-TNF treatment.[32]

Biological and MTX therapies are associated with potentially significant adverse effects and are expensive.[3,25,33] Most importantly, around a third of patients will show poor response to therapy.[4–7] In our study, the initiation of both MTX and anti-TNF treatment was effective and was associated with improvements in clinical disease activity measures, JADAS-10 score, attainment of ID and ACRpedi50 responses. Due to limitations in the size of the data set, we could not perform further subgroup analyses of response by each ACRpedi level, and instead used ACRpedi50 or better as the cut-off, using information from the JADAS score to supplement the measure of clinical improvement. Over 50 % of patients in each group reached an ACRpedi50 or better response, in line with published literature, including the study of etanercept efficacy by Quartier et al., where over half of treated JIA patients had over a minimum 50% improvement in their core set criteria at 3 months, which alongside the baseline characteristics suggested our patient population was an average group of patients.[25,34] However, the effect of concomitant therapy use by patients (MTX plus anti-TNF

therapy and/or other therapies such as corticosteroids) should be investigated specifically in more detail.

In conclusion, we have shown that high pre-treatment S100A12 serum concentrations of patients with JIA is associated with a good response to methotrexate or anti-TNF therapy. Further work to identify the ideal clinical scenarios where this biomarker could best be utilized (at onset of treatment in the absence of corticosteroid treatment for example, limited to anti-TNF treated patients or use in predicting patients who will respond to one drug rather than another or to combined therapies from the outset) should be performed. In addition, this work highlights that there is a significant clinical need for the clinical evaluation of predictive biomarkers. However, to achieve these objectives, validation cohorts with frequent longitudinal follow up is required.

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References

1. Hinze C, Gohar F, Foell D. Management of juvenile idiopathic arthritis: hitting the target. Nat Rev Rheumatol. 2015;11:290–300.

2. Beukelman T, Patkar NM, Saag KG, Tolleson-Rinehart S, Cron RQ, DeWitt EM, et al. 2011 American College of Rheumatology recommendations for the treatment of juvenile idiopathic arthritis: initiation and safety monitoring of therapeutic agents for the treatment of arthritis and systemic features. Arthritis Care Res (Hoboken). 2011;63:465–82.

3. Ringold S, Weiss PF, Beukelman T, DeWitt EM, Ilowite NT, Kimura Y, et al. 2013 update of the 2011 American College of Rheumatology recommendations for the treatment of juvenile idiopathic arthritis. Arthritis Rheum 2013;65:2499–512.

4. Horneff G, Schmeling H, Biedermann T, Foeldvari I, Ganser G, Girschick HJ, et al. The German etanercept registry for treatment of juvenile idiopathic arthritis. Ann Rheum Dis. 2004;63:1638–44.

5. Lovell DJ, Reiff A, Ilowite NT, Wallace CA, Chon Y, Lin S-L, et al. Safety and efficacy of up to eight years of continuous etanercept therapy in patients with juvenile rheumatoid arthritis. Arthritis Rheum. 2008;58:1496–504.

6. Prince FHM, Twilt M, ten Cate R, van Rossum MAJ, Armbrust W, Hoppenreijs EPAH, et al. Long-term follow-up on effectiveness and safety of etanercept in juvenile idiopathic arthritis: the Dutch national register. Ann Rheum Dis. 2009;68:635–41.

7. Otten MH, Prince FHM, Armbrust W, ten Cate R, Hoppenreijs EPAH, Twilt M, et al. Factors associated with treatment response to etanercept in juvenile idiopathic arthritis. JAMA. 2011;306:2340–7.

8. Geikowski T, Becker I, Horneff G. Predictors of response to etanercept in polyarticular-course juvenile idiopathic arthritis. Rheumatology (Oxford) 2014;53:1245–9.

9. Kessel C, Holzinger D, Foell D. Phagocyte-derived S100 proteins in autoinflammation: putative role in pathogenesis and usefulness as biomarkers. Clin Immunol. 2013;147:229–41.

10. Holzinger D, Frosch M, Kastrup A, Prince FHM, Otten MH, Van Suijlekom-Smit LWA, et al. The Toll-like receptor 4 agonist MRP8/14 protein complex is a sensitive indicator for disease activity and predicts relapses in systemic-onset juvenile idiopathic arthritis. Ann Rheum Dis. 2012;71:974–80.

11. Rothmund F, Gerss J, Ruperto N, Däbritz J, Wittkowski H, Frosch M, et al. Validation of relapse risk biomarkers for routine use in patients with juvenile idiopathic arthritis. Arthritis Care Res (Hoboken). 2013;66:949–55.

12. Foell D, Wulffraat N, Wedderburn LR, Wittkowski H, Frosch M, Gerss J, et al. Methotrexate

withdrawal at 6 vs 12 months in juvenile idiopathic arthritis in remission: a randomized clinical trial. JAMA. 2010;303:1266–73.

13. Gerss J, Roth J, Holzinger D, Ruperto N, Wittkowski H, Frosch M, et al. Phagocyte-specific S100 proteins and high-sensitivity C reactive protein as biomarkers for a risk-adapted treatment to maintain remission in juvenile idiopathic arthritis: a comparative study. Ann Rheum Dis. 2012;71:1991–7.

14. Moncrieffe H, Ursu S, Holzinger D, Patrick F, Kassoumeri L, Wade A, et al. A subgroup of juvenile idiopathic arthritis patients who respond well to methotrexate are identified by the serum biomarker MRP8/14 protein. Rheumatology (Oxford). 2013;52:1467–76.

15. Anink J, Van Suijlekom-Smit LWA, Otten MH, Prince FHM, van Rossum MAJ, Dolman KM, et al. MRP8/14 serum levels as a predictor of response to starting and stopping anti-TNF treatment in juvenile idiopathic arthritis. Arthritis Res Ther. 2015;17:200.

16. Giannini EH, Ruperto N, Ravelli A, Lovell DJ, Felson DT, Martini A. Preliminary definition of improvement in juvenile arthritis. Arthritis Rheum. 1997;40:1202–9.

17. Horneff G, Becker I. Definition of improvement in juvenile idiopathic arthritis using the juvenile arthritis disease activity score. Rheumatology (Oxford). Oxford University Press; 2014;53:1229–34.

18. Consolaro A, Ruperto N, Bazso A, Pistorio A, Magni-Manzoni S, Filocamo G, et al. Development and validation of a composite disease activity score for juvenile idiopathic arthritis. Arthritis Rheum. 2009;61:658–66.

19. Wallace CA, Giannini EH, Huang B, Itert L, Ruperto N. American College of Rheumatology Provisional Criteria For Defining Clinical Inactive Disease in Select Categories of Juvenile Idiopathic Arthritis. Arthritis Care Res. 2011;63:929–36.

20. Solari N, Palmisani E, Consolaro A, Pistorio A, Viola S, Buoncompagni A, et al. Factors associated with achievement of inactive disease in children with juvenile idiopathic arthritis treated with etanercept. J Rheumatol. 2013;40:192–200.

21. Bulatovic M, Heijstek MW, Van Dijkhuizen EHP, Wulffraat NM, Pluijm SMF, de Jonge R. Prediction of clinical non-response to methotrexate treatment in juvenile idiopathic arthritis. Ann Rheum Dis. 2012;71:1484–9.

22. Bae CB, Suh CH, An JM, Jung JY, Jeon JY, Nam JY, et al. Serum S100A12 may be a useful biomarker of disease activity in adult-onset still's disease. J Rheumatol. 2014;41:2403–8.

23. Yamasaki Y, Takei S, Imanaka H, Nerome Y, Kubota T, Nonaka Y, et al. Prediction of long-term remission of oligo/polyarticular juvenile idiopathic arthritis with S100A12 and vascular endothelial

growth factor. Mod Rheumatol. 2015;1–6.

24. Rahman MT, Myles A, Gaur P, Misra R, Aggarwal A. TLR4 endogenous ligand MRP8/14 level in enthesitis-related arthritis and its association with disease activity and TLR4 expression. Rheumatology (Oxford). 2014;53:270–4.

25. Quartier P, Taupin P, Bourdeaut F, Lemelle I, Pillet P, Bost M, et al. Efficacy of etanercept for the treatment of juvenile idiopathic arthritis according to the onset type. Arthritis Rheum. 2003;48:1093–101.

26. Choi IY, Gerlag DM, Herenius MJ, Thurlings RM, Wijbrandts CA, Foell D, et al. MRP8/14 serum levels as a strong predictor of response to biological treatments in patients with rheumatoid arthritis. Ann Rheum Dis. 2013;

27. Pomirleanu C, Ancuta C, Miu S, Chirieac R. A predictive model for remission and low disease activity in patients with established rheumatoid arthritis receiving TNF blockers. Clin Rheumatol. 2013;32:665–70.

28. Marotte H, Miossec P. Biomarkers for prediction of TNFalpha blockers response in rheumatoid arthritis. Joint Bone Spine. 2010;77:297–305.

29. CircuLex S100A12/EN-RAGE ELISA Kit Ver.2 | Kits | MBL Life Sience -ASIA- [Internet]. [cited 2016 May 3]. Available from: http://ruo.mbl.co.jp/bio/g/dtl/P/?pcd=CY-8058V2#u-pub

30. Kessel C, Fühner S, Brockmeyer S, Wittkowski H, Föll D. OP0194 Hexameric S100A12 is Required for Pro-Inflammatory TLR4-Signalling. Ann Rheum Dis. 2015;74:144.4-145.

31. Centola M, Cavet G, Shen Y, Ramanujan S, Knowlton N, Swan KA, et al. Development of a multi-biomarker disease activity test for rheumatoid arthritis. PLoS One. 2013;8:e60635.

32. Scardapane A, Ferrante R, Nozzi M, Savino A, Antonucci I, Dadorante V, et al. TNF-alpha gene polymorphisms and juvenile idiopathic arthritis: Influence on disease outcome and therapeutic response. Semin Arthritis Rheum. 2015;45:35–41.

33. Tynjälä P, Vähäsalo P, Tarkiainen M, Kröger L, Aalto K, Malin M, et al. Aggressive combination drug therapy in very early polyarticular juvenile idiopathic arthritis (ACUTE-JIA): a multicentre randomised open-label clinical trial. Ann Rheum Dis. 2011;70:1605–12.

34. van Riel PLCM, Taggart AJ, Sany J, Gaubitz M, Nab HW, Pedersen R, et al. Efficacy and safety of combination etanercept and methotrexate versus etanercept alone in patients with rheumatoid arthritis with an inadequate response to methotrexate: the ADORE study. Ann Rheum Dis 2006;65:1478–83.

Figure 1: Baseline and follow-up S100A12 concentration by therapy used

Differences in baseline S100A12 concentrations in responders and non-responders to MTX (**A**) or anti-TNF therapy (**B**) measured by the in-house ELISA are shown. Change in S100A12 concentration after treatment with MTX and anti-TNF therapy is shown for responders **(C-D)** and non-responders **(E-F)**. Horizontal bars indicate the median concentration and vertical bars the IQR.

Figure 1

Methotrexate therapy (A,C,E) **Anti-TNF therapy (B,D,F)**

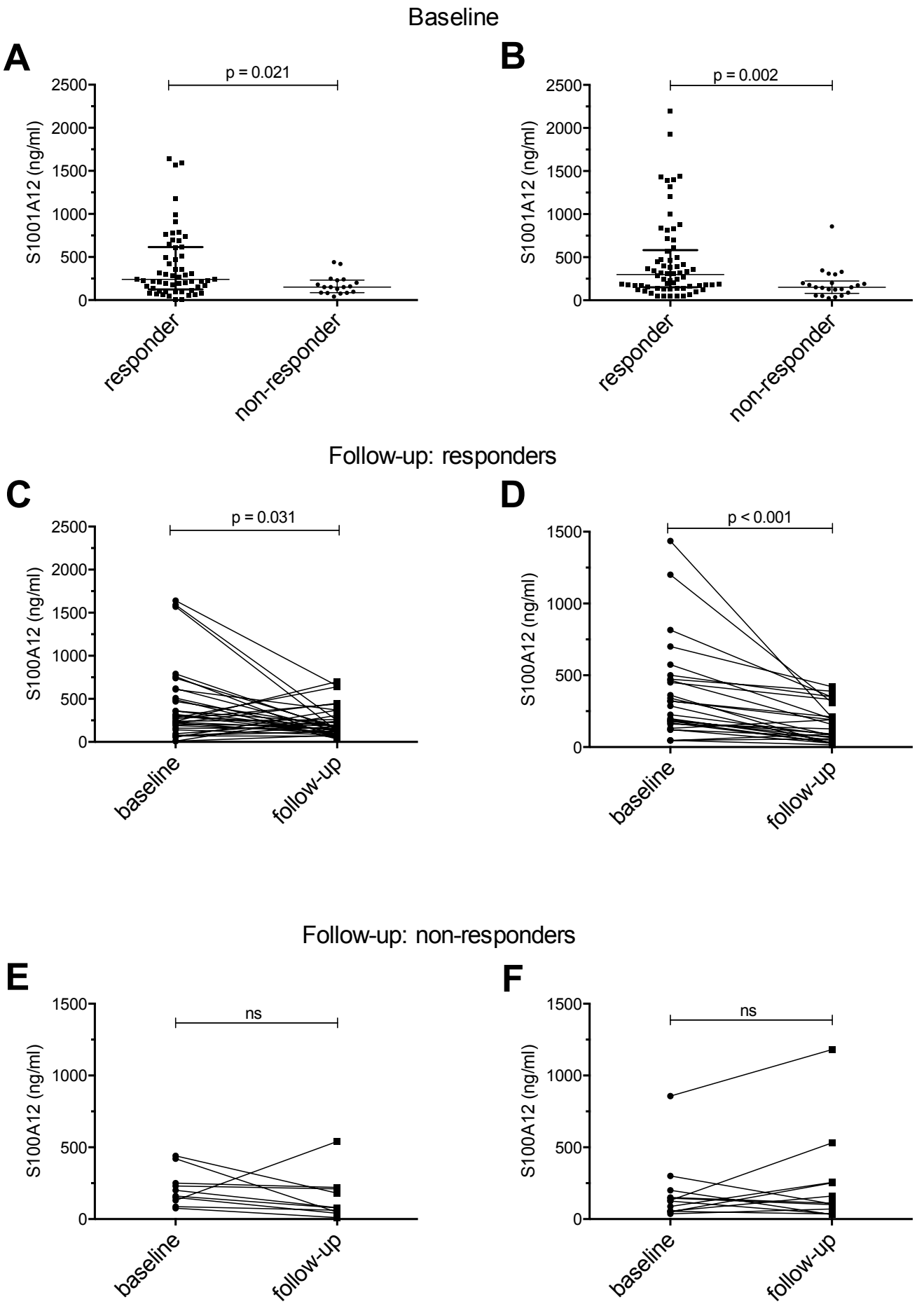

Table 1 Baseline demographics and characteristics of patients starting MTX and anti-TNF therapy

Baseline demographic	**MTX-treated patients (n=75)**	**Anti-TNF-treated patients (n=88)**
Age at JIA onset in years, median (IQR)	5.3 (2.5-10.5)	10.0 (3.9-12.3)
Disease duration at therapy start in years, median (IQR)	1.4 (0.5-3.8)	2.3 (0.9-6.0)
Female, n (%)	52 (69)	66 (75)
ANA positive, n/N (%)	48/72 (67)	25/76 (33)
RF positive, n/N (%)	10/71 (14)	13/80 (16)
JIA Category at MTX or anti-TNF start, n (%)		
Oligoarticular persistent	13 (17)	5 (6)
Oligoarticular extended	17 (23)	24 (27)
Polyarticular RF-	29 (39)	33 (38)
Polyarticular RF+	6 (8)	13 (15)
Enthesitis-related arthritis	6 (8)	4 (5)
Psoriatic	3 (4)	9 (10)
Not available	1 (1)	0
Clinical variables at therapy start		
Physician's VAS (0-100)	38 (22-56)	54 (30-68)
Active joints, n	5 (2-8)	10 (5-17)
Restricted joints, n	3 (2-6)	6 (2-14)
Parent/Patient VAS (0-100)	33 (14-56)	53 (5-70)
CHAQ score (0-3)	1.00 (0.25-1.75)	1.5 (0.8-2.1)
ESR (mm/h)	23 (10-63)	13 (8-27)
Concomittant therapy at therapy start		
Methotrexate	75 (100)	74 (84)
Anti-TNF therapy	0	88 (100)
Systemic prednisolone	25/61 (41)	25/88 (28)
JADAS-10 (0-40), median (IQR)	13 (8-20)	19 (14-23)
S100A12 (in-house) at start in ng/ml, median (IQR)	220 (100-440)	200 (133-440)
S100A12 (*CircuLex*) at start in ng/ml, median (IQR)	605 (318-1330)	348 (195-655)

Abbreviations: **MTX** methotrexate, **anti-TNF** anti-tumour-necrosis factor, **JIA** juvenile idiopathic arthritis, **IQR** inter-quartile range, **ANA** anti-nuclear antibodies, **RF** rheumatoid factor, **VAS** visual analogue scale, **CHAQ** Childhood Assessment Questionnaire, **ESR** erythrocyte sedimentation rate**,** **JADAS-10** Juvenile Arthritis Disease Activity

Table 2 Association of response to therapy to baseline S100A12 concentration

Logistic regression: *predicted minimum ACRpedi50 response*	**Unadjusted OR (95% CI)**	**P-value**
MTX therapy		
S100A12, per 50 unit ng/ml increase	1.213 (1.01-1.45)	0.034
Anti-TNF therapy		
S100A12, per 50 unit ng/ml increase	1.04 (1.01-1.08)	0.014
Univariate linear regression: *predicted change in JADAS-10*	**Beta (95% CI)**	**P-value**
MTX therapy		
S100A12, per 50 unit ng/ml increase	-0.453 (-0.726- -0.181)	0.002
Anti-TNF therapy		
S100A12, per 50 unit ng/ml increase	0.064 (0.025-0.102)	0.001
Multivariate linear regression: *predicted change in JADAS-10*	**Beta (95% CI)**	**P-value**
MTX therapy		
S100A12, per 50 unit ng/ml increase	0.197 (-0,397-0.003)	ns
Anti-TNF therapy		
S100A12, per 50 unit ng/ml increase	0.045 (0.015-0.076)	0.004

Abbreviations: **OR** odds ratio, **CI** confidence interval, **MTX** methotrexate, **anti-TNF** anti-tumour necrosis factor

Table 3 Sensitivity, specificity and likelihood ratios for the determined cut-off of S100A12 predicting response to MTX and anti-TNF therapy

Accuracy measure	MTX therapy	Anti-TNF therapy
Cut-off level S100A12 (ng/ml)	260	213
Sensitivity	47.4	58.6
Specificity	88.9	80.7
Positive likelihood ratio	4.3	3.0
Negative likelihood ratio	1.7	0.5
Youden index	0.363	0.392
AUC (95% CI)	0.675 (0.559-0.805)	0.734 (0.622-0.846)

Abbreviations: **AUC** area under the curve, **MTX** methotrexate, **anti-TNF** anti-tumour necrosis factor

Supplement File 1

Table S1 Baseline demographics and characteristics in all responders and non-responders

	MTX-treated patients (n=75)		**Anti-TNF-treated patients (n=88)**	
	Responders (n=57)	Non-responders (n=18)	Responders (n=66)	Non-responders (n= 22)
Baseline demographic				
Age at JIA onset in years, median (IQR)	6.1 (2.6-11.0)	4.5 (2.4-10.5)	10.0 (4.2-12.3)	9.4 (3.1-13.7)
Disease duration at therapy start in years, median (IQR)	1.3 (0.4-4.7)	2.2 (0.7-3.8)	2.4 (1.1-4.9)	2.3 (0.8-7.7)
Female, n (%)	39 (68)	13 (72)	48 (73)	18 (82)
Anti-TNF therapy,				
Etanercept, n (% of all Etanercept)	n/a	n/a	61 (75)	20 (25)
Adalimumab, n (% of all Adalimumab)	n/a	n/a	5 (71)	2 (29)
JIA Category at start, n (%)				
Oligoarticular persistent	7 (12)	6 (35)	2 (3)	3 (14)
Oligoarticular extended	15 (26)	2 (12)	16 (24)	8 (36)
Polyarticular RF-	23 (40)	6 (35)	26 (39)	7 (32)
Polyarticular RF+	5 (9)	1 (6)	11 (17)	2 (9)
Enthesitis-related arthritis	5 (9)	1 (6)	3 (5)	1 (5)
Psoriatic	2 (4)	1 (6)	8 (12)	1 (5)
Clinical variables at baseline				
Active joints, n	5 (2-10)	4 (2-5)*	11 (5-18)	8 (2-16)
CHAQ score (0-3)	1 (0.31-1.75)	0.81 (0.25-2.06)	1.49 (0.75-2.13)	1.35 (0.63-1.96)
ESR (mm/h)	25 (10-69)	19 (8-35)	16 (9-30)	12 (7-18)
JADAS-10 (0-40), median (IQR)	14 (8-23)	10 (7-14)	20 (14-23)	17 (11-22)
S100A12 at baseline, median (IQR), ng/ml	240 (125-615)	150 (87-233)*	308 (150-624)	151 (83-201)**

Abbreviations: **MTX** methotrexate, **anti-TNF** anti-tumour-necrosis factor therapy, **JIA** juvenile idiopathic arthritis, **CHAQ** Childhood Assessment Questionnaire, **ESR** erythrocyte sedimentation rate**, JADAS-10** Juvenile Arthritis Disease Activity

*/** indicates significance between responders and non responder within MTX treated patients, or within anti-TNF treated patients as follows: *p< 0.05, **p< 0.005 (Mann Whitney U)

Supplement 2 S100A12 concentrations measured by commercial *CircuLex* ELISA

Performance of in-house assay versus Circulex assay

MTX: S100A12 concentrations measured by the in house ELISA assay significantly correlate with *CircuLex* measured concentrations (Spearman's rho: 0.85, p< 0.001).

Anti-TNF: S100A12 concentrations measured by the in house ELISA assay significantly correlate with *CircuLex* measured concentrations (Spearman's rho: 0.687, p<0.001).

S100A12 levels at baseline and response to treatment

Baseline S100A12 serum levels were higher in responders (median 720 (IQR 320-1765) compared to non-responders (median 417, IQR 243-818, p=0.039) for MTX treated patients (Figure 1A).

For anti-TNF treated patients, baseline S100A12 serum levels were also higher in responders (median 407, IQR 212-710) compared to non-responders (median 239, IQR 150-436, p=0.020).

In a univariate logistic regression this resulted in an OR of 1.06 for MTX therapy (95%CI 1.004-1.115, and an OR of 1.14 (95% CI: 1.01-1.28) for achieving at least an ACRpedi 50 response per 50 units of S100A12 (*CircuLex*)(ng/ml) for anti-TNF therapy.

Prediction of response corrected for other variables

Baseline S100A12 serum levels were significantly associated with change in JADAS-10 in a univariate linear regression analysis (β = -0.149, 95% CI -0.298 to -0.0007, p=0.050 per 50 units change in S100A12 for anti-TNF treated patients. For MTX this was: β = -0.159 (95% CI -0.264 - -0.053) In the corrected multivariable analysis the corrected β was -0.089 per 50 units increase in ng/ml, 95% CI -0.212 to 0.034 for anti-TNF therapy. The change in explained variance was 1.4% (not significant). Multivariable analysis: corrected beta for MTX: -0.102 (95% CI: -0.139 - -0.039), the change in explained variance was 5.3% (p=0.002).

Multivariate models constructed with known predictors of response as shown in the method were performed to test their association of with JADAS-10 score for each treatment group. Without S100A12,

the variables in the model explained 70 % (equal to that as measured by in-house ELISA) of the variance in change in JADAS-10 at follow-up for MTX-treated patients, and 50 % (also the same as with the in-house ELISA) for the anti-TNF group. Including S100A12 as a variable increased the models prediction by 5.3% (more than the 2% with the in-house ELISA) for MTX and 1.4% (vs 5% with the in-house ELISA for anti-TNF treated groups.

Use of S100A12 as a prognostic marker for response to treatment

The *CircuLex* ELISA was less accurate compared to the in-house ELISA for predicting response to anti-TNF treatment and MTX, shown in Table S3.

Table S3: Sensitivity, specificity and likelihood ratios for the determined cut-off of S100A12 predicting response to MTX anti-TNF treatment, *CircuLex* ELISA

	CircuLex **ELISA: MTX**	*CircuLex* **ELISA: anti-TNF**
Cut-off level S100A12 (ng/ml)	846	508
Sensitivity	45.6	39.4
Specificity	83.3	86.4
Positive likelihood ratio	2.7	2.9
Negative likelihood ratio	0.7	0.7
Youden index	0.289	0.258
AUC	0.662 (0.532-0.791)	0.675 (0.550-0.800)

AUC= area under the curve

Supplement 2, Figure 1: Baseline and follow-up S100A12 concentration by therapy used, measured by *Circulex* ELISA

Differences in baseline S100A12 concentrations in responders and non-responders to MTX (**A**) or anti-TNF therapy (**B**) measured by *Circulex* ELISA are shown. Change in S100A12 concentration after treatment with MTX and anti-TNF therapy is shown for responders **(C-D)** and non-responders **(E-F)**. Horizontal bars indicate the median concentration, and vertical bars the IQR.