

Concise report

S100A12 and S100A8/9 proteins are biomarkers of articular disease activity in Blau syndrome

Lin Wang^{1,*}, Carlos D. Rosé^{2,*}, Kevin P. Foley¹, Jordi Anton³, Brigitte Bader-Meunier⁴, Philippe Brissaud⁵, Gaelle Chédeville⁶, Rolando Cimaz⁷, Jorge Fernández-Martín⁸, Catherine Guly⁹, Eric Hachulla¹⁰, Miroslav Harjacek¹¹, Friederike Mackensen^{12,†}, Rosa Merino¹³, Consuelo Modesto¹⁴, Antonio Naranjo Hernández¹⁵, Christine Pajot¹⁶, Athimalaipet V. Ramanan¹⁷, Akaluck Thatayatikom¹⁸, Caroline Thomée¹⁹, Sebastiaan Vastert²⁰, Bart J. Votta¹, John Bertin^{1,**} and Carine H. Wouters^{21,**}

Abstract

Objective. To identify biomarkers of articular and ocular disease activity in patients with Blau syndrome (BS)

Methods. Multiplex plasma protein arrays were performed in five BS patients and eight normal healthy volunteers (NHVs). Plasma S100A12 and S100A8/9 were subsequently measured by ELISA at baseline and 1-year follow-up in all patients from a prospective multicentre cohort study. CRP was measured using Meso Scale Discovery immunoassay. Active joint counts, standardization uveitis nomenclature for anterior uveitis cells and vitreous haze by Nussenblatt scale were the clinical parameters.

Results. Multiplex Luminex arrays identified S100A12 as the most significantly elevated protein in five selected BS vs eight NHVs and this was confirmed by ELISA on additional samples from the same five BS patients. In the patient cohort, S100A12 ($n=39$) and S100A8/9 ($n=33$) were significantly higher compared with NHVs ($n=44$ for S100A12, $n=40$ for S100A8/9) ($P=0.0000004$ and $P=0.0003$, respectively). Positive correlations between active joint counts and S100 levels were significant for S100A12 ($P=0.0008$) and S100A8/9 ($P=0.015$). CRP levels did not correlate with active joint count. Subgroup analysis showed significant association of S100 proteins with active arthritis (S100A12 $P=0.01$, S100A8/9 $P=0.008$). Active uveitis was not associated with increased S100 levels.

Conclusion. S100 proteins are biomarkers of articular disease activity in BS and potential outcome measures in future clinical trials. As secreted neutrophil and macrophage products, S100 proteins may reflect the burden of granulomatous tissue in BS.

Key words: S100 proteins, Blau syndrome

¹Pattern Recognition Receptor Discovery Performance Unit, Immuno-Inflammation Therapeutic Area, GlaxoSmithKline, Collegeville, PA, USA, ²Pediatrics, Nemours/Alfred I. duPont Hospital for Children, Wilmington, DE, USA, ³Pediatric Rheumatology, Hospital Sant Joan de Déu, Universitat de Barcelona, Barcelona, Spain, ⁴Service d'Immunologie-Hématologie et Rhumatologie Pédiatrique, Hôpital Necker-Enfants Malades, Institut Imagine, ⁵Médecine Interne Rhumatologie, Hôpital Bichat-Claude Bernard, Paris, France, ⁶Division of Rheumatology, The Montreal Children's Hospital, Montreal, Quebec, Canada, ⁷AOU Meyer, Meyer Children's Hospital, Florence, Italy, ⁸Internal Medicine, Hospital do Meixoeiro-Chuvi, Vigo, Pontevedra, Spain, ⁹Retina Unit, Bristol Eye Hospital, Bristol, UK, ¹⁰Service de Médecine Interne, Hôpital Claude Huriez, Lille, France, ¹¹Rheumatology, Children's Hospital Srebrnjak, Zagreb, Croatia, ¹²Interdisciplinary Uveitis Center, Universität Klinikum Heidelberg, Heidelberg, Germany, ¹³Unidad de Reumatología Pediátrica, Hospital La Paz, Madrid, ¹⁴Pediatric Rheumatology, Hospital Universitario Vall d'Hebron, Barcelona, ¹⁵Servicio de Reumatología, Hospital de Gran Canarias Dr Negrin, Las Palmas, Spain, ¹⁶Service Urgences & Service Médecine Interne-Rhumatologie, Hôpital des Enfants, Toulouse, France, ¹⁷Department of Paediatric Rheumatology, University Hospitals Bristol

NHS Foundation Trust and University of Bristol, Bristol, UK, ¹⁸Division of Allergy/Immunology/Rheumatology, Department of Pediatrics, University of Florida, Gainesville, FL, USA, ¹⁹Service de Pédiatrie, Clinique Pédiatrique CHL, Luxembourg, Luxembourg, ²⁰Division of Pediatrics, Department of Pediatric Rheumatology and Immunology, UMC Utrecht, Utrecht, The Netherlands and ²¹KU Leuven, Department of Microbiology and Immunology, Pediatric Immunology, University Hospitals Leuven, Pediatric Rheumatology, Leuven, Belgium

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*Lin Wang and Carlos D. Rosé contributed equally to this study.

**Carine H. Wouters and John Bertin contributed equally to this study.

†Deceased.

Correspondence to: Carine Wouters, KU Leuven, Department of Microbiology and Immunology, Pediatric Immunology, University Hospitals Leuven, Pediatric Rheumatology, Leuven, Belgium.
E-mail: carine.wouters@uzleuven.be

Rheumatology key messages

- This study identified S100A12 and S100A8/9 as biomarkers for articular disease activity in Blau syndrome.
- In Blau syndrome, S100 proteins showed a positive correlation with active joint count, whereas CRP did not.

Introduction

Blau syndrome (BS) is a dominantly inherited autoinflammatory disease resulting from mutations in or near the nucleotide oligomerization domain of the gene encoding nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [1]. A clinical triad of arthritis, dermatitis and uveitis is characteristic, but involvement of various organ systems has become recognized [2–4].

As a symmetrical polyarthritis with uveitis, BS resembles polyarticular JIA, yet the synovitis in BS is more exuberant than destructive and the uveitis affects both compartments of the eye. Moreover, the presence of non-caseating giant cell granulomas in affected tissues reveals its distinct pathogenesis involving activated macrophages as precursors of the characteristic multinucleated cells [5]. To date, no biomarkers of disease activity have been identified in BS.

Current management of BS involving corticosteroids, DMARDs and biologic agents is less than satisfactory. Despite multiple therapies, both articular and ocular disease activity persists over time [6]. Specific drugs targeting the NOD2 pathway are being sought and biomarkers are likely to be required. S100A12, S100A8 and S100A9, three phagocyte-specific S100 proteins comprising the group of calgranulins, are secreted by activated neutrophils and macrophages and have been proposed as biomarkers in JIA [7, 8]. S100 proteins are candidates for BS, a disease of activating and proliferating neutrophils and macrophages.

One of the aims of our prospective BS cohort study is the search for disease activity biomarkers. We will show here that plasma levels of S100A12 and S100A8/9 proteins are associated with articular disease activity and correlate positively with active joint counts in BS.

Methods

Patients and control subjects

BS patients with sequence-verified NOD2 mutations were enrolled in the on-going BS multicentre prospective cohort study involving 18 centers from 11 countries [6]. The age range for 39 BS patients included in this study was 3–58 years (female/male: 18/21). Patients underwent annual visits for clinical evaluation and sampling. Measurement of active and limited joints was performed by a rheumatologist using the 75-joint count score. Patients underwent an ophthalmological evaluation within a month of study visits. Standardization of Uveitis Nomenclature classification was used to grade anterior uveitis cells (range 0–4+). Vitritis was assessed by the Nussenblatt scale (range 0–8). Blood samples were obtained at baseline and at 1-year follow-up. Participation in the biomarker studies was optional to centers and

participants. Normal healthy volunteers (NHVs) were recruited from a pool of adults (age range: 25–63 years; female/male: 17/19) at GlaxoSmithKline (Collegeville, PA, USA) and youth (age range: 5–22 years; female/male: 6/2) from Leuven University Hospital (Leuven, Belgium). This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the committee on Medical Ethics of the University Hospitals KU Leuven and Ethics committees and Institutional Review Boards at all clinical sites.

Sample collection

Blood was collected the same day of the clinical evaluation. Corticosteroids were withheld for up to 24 h prior to sampling when medically feasible. Additionally, all samples were collected as close to the nadir of any biologic therapies as feasible. Peripheral blood was collected following each study site's approved venipuncture procedure using standard methods for plasma isolation. Plasma was stored in 1.5 ml protein LoBind tubes (Eppendorf) at -80°C until further analysis. SF samples were collected from both knees of a patient requiring therapeutic arthrocentesis.

Plasma protein profiling and cytokine measurement

Plasma protein profiling was performed at Myriad RBM (Austin, TX, USA) using the Human DiscoveryMAP 250+ multiplex Luminex array. The assay version used in these studies measured 261 different cytokines and circulating proteins. Plasma IL-8, IL-16 and CRP were measured using Singleplex kits from Meso Scale Discovery (Rockville, MD, USA). Plasma S100A12 was measured using the CircuLex S100A12/EN-RAGE ELISA kit (CycLex Co. Nagano, Japan). Plasma S100A8/9 heterodimer was measured using Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

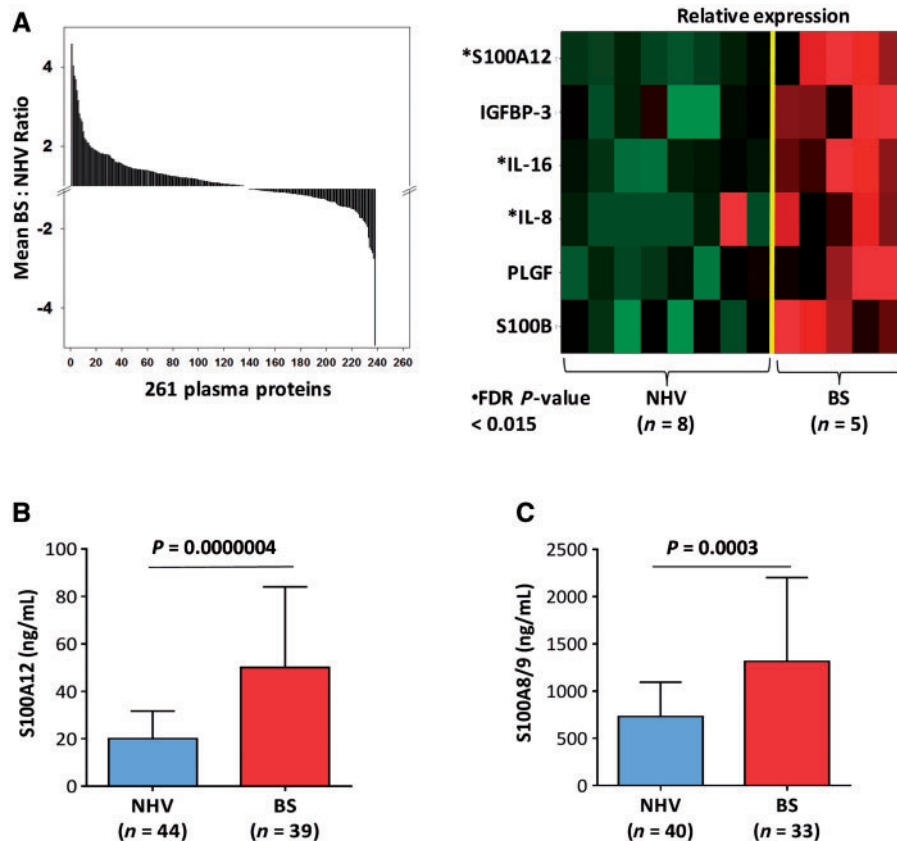
Bioinformatic analysis

Plasma protein profiles were analysed using a partial least squares discriminant analysis. Data was analysed using JMP 11.0.0 software (SAS Institute, Cary, NC, USA). Unpaired data endpoints that appeared to be approximately normal with similar variances were analysed using a two-sample, two-sided *t* test to compare group means, assuming unequal variances. Endpoints that violated the assumptions of normality and/or equal variance were analysed using the non-parametric Wilcoxon rank-sum test to compare groups.

Clinical assessment and analysis

Spearman's rank test was used for correlations between S100 proteins and CRP levels with joint counts. Subgroups were stratified by articular and ocular disease. Briefly a joint count = 0 was required for inactive arthritis,

Fig. 1 S100A12 and S100A8/9 plasma levels in Blau syndrome



(A) Two hundred and sixty-one circulating proteins were examined in plasma from BS ($n = 5$) and NHV ($n = 8$) subjects using multiplex Luminex arrays (left). Six differentially expressed plasma proteins with FDR $P < 0.10$; of those, IL-8, IL-16 and S100A12 were significantly increased in BS relative to NHVs with FDR $P < 0.015$ (right). (B) Plasma levels of S100A12 in 39 BS [50.08 (34.01) ng/ml] were significantly increased vs 44 NHVs [20.08 (11.65) ng/ml] with $P = 0.0000004$. (C) Plasma levels of S100A8/9 heterodimer in 33 BS [1315 (886) ng/ml] were significantly increased vs 40 NHVs [730 (364) ng/ml] with $P = 0.0003$. The data are shown as means (s.d.). BS: Blau syndrome; FDR: false discovery rate; NHV: normal healthy volunteer.

a standardization uveitis nomenclature grade of 0 for inactive anterior uveitis and a Nussenblatt scale of 0 for inactive vitritis; the worse eye was considered when involvement was asymmetrical. Inactive eye disease required both inactive anterior uveitis and vitritis. Kruskal–Wallis and Mann–Whitney U tests were used for comparisons of S100 and CRP levels between activity groups.

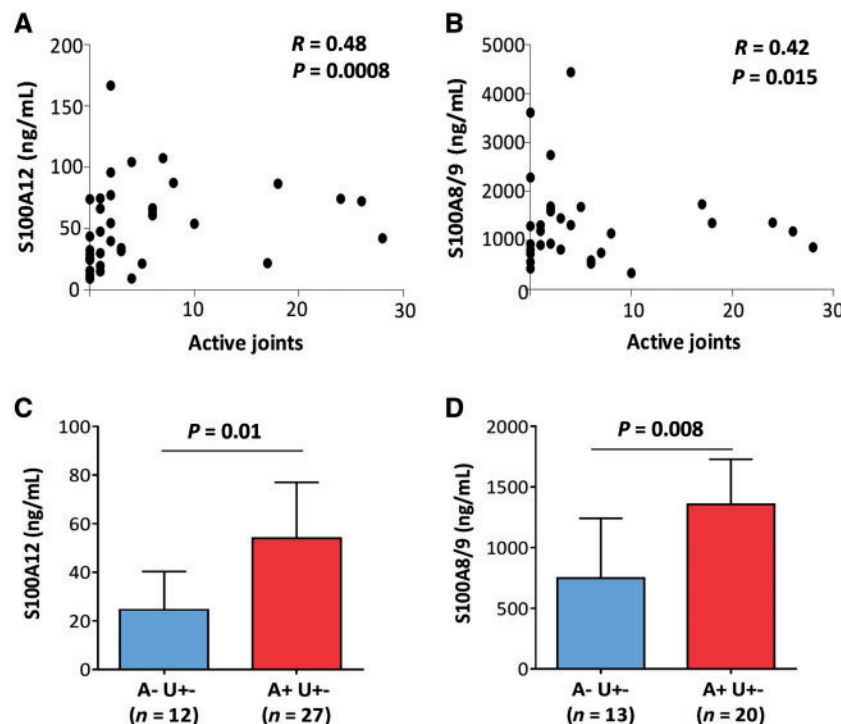
Results

Significantly increased plasma S100A12 and S100A8/9 in BS

To search for biomarkers of disease activity, we profiled an agnostic set of 261 cytokines and circulating plasma proteins from five BS patients and eight NHVs using multiplex Luminex technology. Six proteins, S100A12, IGFBP-3, IL-16, IL-8, PLGF and S100B, were more abundant in BS with false discovery rate $P < 0.1$. Of those, three proteins, IL-8, IL-16 and S100A12, represented

significant differences between BS and NHVs with false discovery rate $P < 0.015$ (Fig. 1A). To confirm these results, independent blood samples from these five BS patients were collected 6 weeks after the initial blood sampling and analysed using different immunoassays (Singleplex kits from Meso Scale Discovery for IL-8 and IL-16, ELISA for S100A12). The average levels of IL-8, IL-16 and S100A12 were significantly increased in BS by 2.5-, 1.8- and 4.4-fold, respectively, relative to NHVs, consistent with the initial Luminex results. Since S100A12 showed the highest increase in the five selected BS patients vs eight NHVs, it became the focus of further analysis.

Next, we measured plasma S100A12 in the whole cohort of BS patients at baseline. The mean values of S100A12 [50.08 (34.01) ng/ml vs 20.08 (11.65) ng/ml, $P = 0.0000004$] were significantly increased in BS ($n = 39$) compared with NHVs ($n = 44$) (Fig. 1B). Subsequently, we measured plasma S100A8/9 heterocomplexes in the cohort of patients at baseline because S100A8 and S100A9 have already been shown to be biomarkers in JIA. The results

Fig. 2 The correlation of plasma S100A12 and S100A8/9 levels with active joint counts in Blau syndrome

(A) Correlations between S100A12 ($n = 39$) and S100A8/9 ($n = 33$) levels with active joint counts at baseline in BS (Spearman's rank test). **(B)** S100A12 and S100A8/9 levels in BS with vs without active arthritis irrespective of uveitis activity (Mann-Whitney U -test). All data are shown as medians and interquartile ranges. BS: Blau syndrome; A: arthritis; U: uveitis; +: with active disease; -: without active disease.

showed significantly higher levels of S100A8/9 [1315 (886) ng/ml vs 730 (364) ng/ml, $P = 0.0003$] in BS ($n = 33$) compared with NHVs ($n = 40$) (Fig. 1C).

An additional observation was a robust elevation of S100A12 and S100A8/9 in SF (patient 4, supplementary Table S1, available at *Rheumatology* online). S100A12 and S100A8/9 concentrations in SF were 154- and 27-fold, respectively, higher than plasma concentrations obtained the same day. A collection of SF from the right knee of the same patient 2 year later confirmed the findings. Elevated S100A12 and S100A8/9 concentrations in SF may reflect local release from synovium into the joint space.

S100A12 and S100A8/9 correlate with articular disease activity in BS

Clinical activity scores and NOD2 mutation data on 39 BS patients are shown in supplementary Table S1, available at *Rheumatology* online. Thirty-nine BS samples from the baseline visit were available for S100A12 and CRP, and thirty-three for S100A8/9 measurements. S100A12 and S100A8/9 values exhibited a positive correlation with active joint count at baseline ($R = 0.48/P = 0.0008$, $R = 0.42/P = 0.015$, respectively) (Fig. 2A and B). At year 1, a positive correlation between S100A12 values and active joint count was confirmed ($R = 0.39/P = 0.03$). Plasma levels of CRP did not significantly correlate with active joint count, although the mean values of

CRP [8.92 (13.52) $\mu\text{g/ml}$ vs 2.73 (4.52) $\mu\text{g/ml}$] were higher in BS ($n = 39$) when compared with NHVs ($n = 40$).

Higher S100A12 and S100A8/9 values in subgroups of BS patients with active arthritis

Patients were then stratified in subgroups according to presence or absence of active arthritis (A+, A-) and active uveitis (U+, U-) based on the definitions described in the methods both at baseline and at 1-year follow-up. Four subgroups were identified at baseline: A+U+ ($n = 17$), A+U- ($n = 10$), A-U+ ($n = 5$) and A-U- ($n = 7$). The median (range) values for S100A12 in each group were 66 (21-166), 38 (9-104), 25 (15-73) and 24 (9-50) ng/ml, respectively. The Kruskal-Wallis test showed a significant difference among the four groups ($P = 0.02$). Subgroup analysis showed higher S100A12 values in patients with active arthritis vs patients without, independent of uveitis activity ($P = 0.01$; Fig. 2C). S100A12 values in patients who had both active arthritis and uveitis were not significantly higher compared with values in patients with arthritis but no uveitis, suggesting a predominant effect of articular inflammation on S100A12 levels. For S100A8/9, median (range) values in nanogram per millilitre were 1361 (595-3611) for A+U+ patients ($n = 11$), 1355 (810-4434) for A+U- ($n = 9$), 970 (427-1686) for A-U+ ($n = 6$) and 731 (334-1292) for A-U- ($n = 7$). Subgroup analysis

revealed higher S100A8/9 values in patients with active arthritis vs patients without, independent of uveitis activity ($P=0.008$; Fig. 2D).

Discussion

We showed that S100A12 and S100A8/9 are associated with active arthritis in BS. Further, plasma levels of S100A12 and S100A8/9 correlate positively with the number of active joints. Conversely, S100 protein levels were not associated with active uveitis.

These findings suggest that the synovium is the major contributor to circulating S100 protein levels in BS, which is further supported by the prominently increased S100 levels in SF. The absence of association with uveitis activity is different from what has been reported in JIA and idiopathic uveitis [8]. This could be a consequence of the few patients without arthritis in our cohort, hampering our ability to isolate the effect of uveitis. CRP levels did not show association with articular disease activity, a fact that matches the absence of overt systemic inflammatory features in BS.

The S100A8/9 dimer (also known as calprotectin) and S100A12 proteins are multifunctional secreted proteins involved in cell–cell signalling that promote myeloid proliferation and modulation of the inflammatory response. S100A12 and S100A8/9 are produced by activated neutrophils and macrophages, and are chemotactic for both [9]. Although the evidence in support of gain function mutations of the NOD2 gene in BS is considerable [10], *ex vivo* stimulation of NOD2 mutated peripheral blood cells with muramyl dipeptide, its selective ligand, shows an attenuated nuclear factor κ B (NF κ B)-dependent cytokine response in supernatant [11, 12]. Correspondingly, the phenotype of the majority of BS patients is characterized by inflammatory granulomatous organ involvement, rather than overt systemic inflammation. Since S100A12 and S100A8/9 reflect the proliferation of activated neutrophils and macrophages in the inflamed hypertrophic synovium, it was not surprising to find a strong correlation with articular disease activity but not for CRP, a hepatic product secreted upon systemic stimulation by NF κ B-dependent circulating cytokines.

We recognize a number of weaknesses in our study. The fewer samples available at the year 1 visit may have impaired our ability to confirm the differences between clinical subgroups in S100 levels.

At this stage, S100 proteins may not be suitable for monitoring disease activity in individual patients. Our correlation analysis showed a lower performance of S100 proteins at the extreme values of joint counts. At present, no composite clinical activity scores have been validated for BS, and accordingly we used the active and limited joint counts as clinical measures.

This is the first prospective study focused on biomarker discovery for disease activity in BS. The design of our study allowed us to obtain reliable clinical evaluations at the time of blood sampling on a substantial number of patients for this rare disease.

To conclude, we have successfully identified S100A12 and S100A8/9 as biomarkers for articular disease activity in BS. Future efforts will include the search for an appropriate marker for uveitis activity as well as the collection of more longitudinal data to confirm the efficiency of the S100 biomarkers over time.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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