Validation of Relapse Risk Biomarkers for Routine Use in Patients With Juvenile Idiopathic Arthritis

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Objective. The myeloid-related proteins 8 and 14 (MRP-8/MRP-14) and neutrophil-derived S100A12 are biomarkers of inflammation. They can be used to determine the relapse risk in patients with juvenile idiopathic arthritis (JIA) after stopping antiinflammatory treatment. In this study, we tested the performance of different enzyme-linked immunosorbent assays (ELISAs) in order to validate systems available for routine use.

Methods. MRP-8/MRP-14 and S100A12 serum concentrations of 188 JIA patients in remission were analyzed. Commercially available test systems were compared to experimental ELISAs established in house. The ability of the assays to identify JIA patients at risk for relapse was analyzed.

Results. For MRP-8/MRP-14, the PhiCal Calprotectin and Bühlmann MRP8/14 Calprotectin ELISAs revealed hazard ratios of 2.3 and 2.1, respectively. For S100A12, the CircuLex S100A12/EN-RAGE ELISA revealed a hazard ratio of 3.1. The commercial assays allowed a JIA relapse prediction that was at least comparable to the experimental ELISAs.

Conclusion. For the prediction of JIA relapse after stopping medication, the biomarkers MRP-8/MRP-14 and S100A12 can be determined by using assays that are available for routine use. The tested commercial MRP-8/MRP-14 and S100A12 ELISAs showed a performance comparable to well-established experimental ELISA protocols when assay-specific cutoffs for the indication of relapse prediction are thoroughly applied.

Introduction
Juvenile idiopathic arthritis (JIA) is a relapsing inflammatory joint disease in children. Serum concentrations of phagocyte-derived S100 proteins correlate with inflammation in JIA (1,2). S100 proteins comprise a family of calci-
um-binding proteins. The subgroup of calgranulins is expressed in myeloid cells and consists of S100A8 (myeloid-related protein 8 [MRP-8]), S100A9 (MRP-14), and S100A12. Most S100 proteins can be found as homodimers, but S100A8 and S100A9 preferably form an S100A8/S100A9 heterodimer (MRP-8/MRP-14). It has been shown that these S100 proteins are released by activated phagocytes and have a function as proinflammatory “alarmins” (3–10).

In clinical remission, low-grade inflammatory activity may persist but remain undetectable by routine clinical or laboratory means. Former studies using assays established in our laboratory have demonstrated that MRP-8/MRP-14 and S100A12 can be used to stratify patients at times of low disease activity by relapse risk, i.e., risk of flare after withdrawal or tapering of antiinflammatory medication. Therefore, these biomarkers allow more accurate treatment decision making, personalized for the individual patient (8–10). Thus, our main goal was to translate these findings into clinical practice. Currently, experimental enzyme-linked immunosorbent assays (ELISAs) measuring MRP-8/MRP-14 and S100A12 are not globally accessible for routine use. However, commercial ELISAs are available but not validated for use in confirming stable remission in JIA or stratifying therapeutic decisions.

Therefore, we aimed to validate our findings on the predictive power of S100 protein analyses in inactive JIA using commercial ELISA kits. In this study, we showed that the biomarkers MRP-8/MRP-14 and S100A12 can be measured with commercial assays for use in stratifying JIA patients according to the risk of relapse.

### Patients and methods

**Patients.** In a previously published controlled trial, JIA patients with inactive disease for at least 3 months were randomized to withdraw all medication after a further 6 or
Figure 2. Relapse prediction using different assays for myeloid-related proteins 8 and 14 (MRP8/14) or S100A12. Receiver operating characteristic curve analyses (left panels) and Kaplan-Meier time to relapse curves (right panels) are shown for
A, in-house MRP8/14 enzyme-linked immunosorbent assay (ELISA; Munster), B, Bühlmann MRP8/14 Calprotectin ELISA, C, PhiCal Calprotectin ELISA, D, in-house S100A12 ELISA (Munster), and E, CircuLex S100A12/EN-RAGE ELISA. The Kaplan-Meier curves for the in-house ELISAs have been published previously but are shown here for comparison (8,9). Log rank tests revealed statistically significant differences between the relapse rates in patients with biomarker levels above versus below the assay-specific cutoffs as validated for each ELISA.
12 months of continuous inactive disease status. Study design, clinical definitions, and patient population data have been reported in detail previously (8,9).

**Laboratory measures.** As previously described, MRP-8/MRP-14 and S100A12 serum levels of 188 patients with JIA in stable remission had been determined by ELISA protocols established in our laboratory (8,10). Analysis was performed at the time when all antiinflammatory drugs were stopped.

For the present study, an aliquot of each sample was used to measure serum levels of both proteins by commercially available ELISA kits. For MRP-8/MRP-14, we applied the Bühlmann MRP8/14 Calprotectin ELISA (Bühlmann Laboratories) and the PhiCal Calprotectin ELISA (Immundiagnostik). For S100A12, we used the CircuLex S100A12/EN-RAGE ELISA (CycLex).

ELISAs were performed according to the manufacturer’s protocol, but for the PhiCal Calprotectin ELISA, some samples required an adjusted dilution because they were out of range. Validation tests performed for each assay included analyses of cross-reactivity, range of normal values, and linearity. Cross-reactivity was tested using 0.5–32 ng of the recombinant proteins (MRP-8/MRP-14 or S100A12) applied in serial dilutions. Sera from 10 adult healthy controls as well as 25 healthy children were used to confirm the range of normal values. To show linearity, we used 10 randomly chosen samples of JIA patients in serial dilution. Mean values were calculated to show the average deviation between expected and observed concentrations.

**Statistical analysis.** Descriptive statistics showed a different range of values in each ELISA kit compared to our experimental ELISA. Therefore, using receiver operating characteristic (ROC) curve analyses, we established assay-specific cutoff values that predicted a flare within 6 months after treatment withdrawal with a maximal Youden index (11). Moreover, assay-specific “high-risk” cutoff values were determined. High-risk cutoff was defined as a cut point for biomarker values above which the relapse rate within 6 months after treatment withdrawal was larger than twice the mean relapse rate across all patients. Survival analysis was used to evaluate a maximum followup period of 12 months from the day antiinflammatory medication was stopped to disease flare (8,9). Patients who had no flare during this period were considered to be in stable remission at the end of followup. For each ELISA kit, a proportional hazards model was established and corresponding hazard ratios (HRs) were estimated.

Statistical analyses were performed using SAS for Windows, version 9.2. Point estimates were generally supplemented by corresponding 95% confidence intervals (95% CIs). P values less than 0.05 were considered to be statistically significant. Standards for Reporting of Diagnostic Accuracy guidelines were used in this study.

**Results**

As with the experimental in-house assays (Munster), serum concentrations of MRP-8/MRP-14 and S100A12 analyzed with the Bühlmann MRP8/14 Calprotectin ELISA, PhiCal Calprotectin ELISA, and CircuLex S100A12/EN-RAGE ELISA measured at the time of stopping medication were elevated in patients who then relapsed after 3 months, 6 months, or 12 months compared to patients in stable remission after stopping therapy (Figure 1). High serum levels of MRP-8/MRP-14 and S100A12 were associated with higher relapse risk after medication withdrawal, especially within the first 3 months.

Cross-reactivity was not found in any ELISA tested. The mean value for the Bühlmann MRP8/14 Calprotectin ELISA was 2,540 ng/ml (95% CI 1,869, 3,207), for the PhiCal Calprotectin ELISA was 1,240 ng/ml (95% CI 837, 1,635), and for the CircuLex S100A12/EN-RAGE ELISA was 100 ng/ml (95% CI 60, 134). All values were within
the manufacturers’ specifications. Mean linearity for a serial dilution (1:100, 1:200, and 1:400) of the Bühlmann MRP8/14 Calprotectin ELISA was −38% (95% CI −52%, −24%). The PhiCal Calprotectin ELISA had a linearity of +47% (95% CI −27%, +121%) when diluted 1:50, 1:100, and 1:200. The CircuLex S100A12/EN-RAGE ELISA with dilutions of 1:200, 1:400, and 1:800 showed a mean linearity of −46% (95% CI −64%, −28%).

Figure 3. “High-risk” relapse prediction using different assays for myeloid-related proteins 8 and 14 (MRP8/14) or S100A12. A, in-house MRP8/14 enzyme-linked immunosorbent assay (ELISA; Munster), B, in-house S100A12 ELISA (Munster), C, Bühlmann MRP8/14 Calprotectin ELISA, D, PhiCal Calprotectin ELISA, and E, CircuLex S100A12/EN-RAGE ELISA. The black lines show for a certain cut point (x) the relapse rate among all patients whose biomarker values are greater than or equal to x. The gray lines show the relapse rate among all patients whose biomarker values are less than or equal to a certain cut point (x). The horizontal solid line shows the mean relapse rate (MRR), while the broken lines indicate half and double the MRR levels. None of the tested biomarkers provides reliable confidence of nonoccurrence of relapse because no valid lower cut point (x\textsubscript{low}) can be robustly determined, either because a low-risk subgroup does not exist at all [i.e., the gray line never falls below half the MRR across all patients] or because the gray line does not show a consistent positive slope in the whole x range. However, optimal cut points were determined that provide reliable probability of an occurrence of a relapse. The high-risk subgroup of patients has a relapse rate of 0.375, which is twice the mean relapse rate across all patients.
Assay-specific cutoff values that distinguish between patients at risk of flares within the following 6 months were established (indicated by the dotted lines in Figure 1). The specific cutoff values were 1,870 ng/ml for the Bühlmann MRP8/14 Calprotectin ELISA, 980 ng/ml for the PhiCal Calprotectin ELISA, and 200 ng/ml for the CircuLex S100A12/EN-RAGE ELISA, whereas the experimental ELISAs had cutoff values of 690 ng/ml for MRP-8/MRP-14 and 175 ng/ml for S100A12, as previously published (8,9). ROC curve analyses were performed to assess the diagnostic accuracy of the ELISAs when using the assay-specific cutoffs (Figure 2, left panels). The area under the curve was calculated (Table 1). ROC curve analyses for experimental MRP-8/MRP-14 and S100A12 assays have been published previously (8,9).

Survival analyses were performed to compare the time from treatment withdrawal to relapse within 12 months between patients with biomarker results above and below the assay-specific cutoffs. The HRs were 2.13 for the Bühlmann MRP8/14 Calprotectin ELISA, 2.26 for the PhiCal Calprotectin ELISA, and 3.07 for the CircuLex S100A12/EN-RAGE ELISA (Table 1). Log rank analyses confirmed the difference in relapse rates after stopping medication in patients with serum concentrations of MRP-8/MRP-14 and S100A12 above and below assay-specific cutoff values (Figure 2, right panels).

The diagnostic performance of the experimental ELISAs compared to the Bühlmann MRP8/14 Calprotectin ELISA, PhiCal Calprotectin ELISA, and CircuLex S100A12/EN-RAGE ELISA is shown in Table 1, demonstrating their accuracy in discriminating between high versus low relapse risk. The highest Youden index was achieved with the CircuLex S100A12/EN-RAGE ELISA and the lowest with the experimental in-house MRP-8/MRP-14 assay.

Cut points were explored that provide confidence that a relapse either will or will not occur. None of the tested biomarkers provided reliable confidence of nonoccurrence of relapse. However, optimal cut points were determined that provided reliable probability of an occurrence of a relapse. The high-risk subgroup of patients had a relapse rate of 0.375, which is twice the mean relapse rate across all patients. The resulting cut points (x_{high}) of all biomarkers are shown in the respective panels of Figure 3, as well as in Table 1. In case of the marker MRP-8/MRP-14, both the in-house ELISA and the commercial assays have higher high-risk cutoffs than the cutoffs providing the highest Youden index. In case of S100A12, both the in-house ELISA and the commercial assay have high-risk cutoffs that almost match the cutoffs with the highest Youden index.

Discussion

The S100 proteins MRP-8/MRP-14 and S100A12 have been demonstrated to be predictive biomarkers in determining relapse risk in JIA. These proteins are stable in serum, which facilitated the biomarker analyses to be performed centrally in our laboratory during previous multicenter studies (8,9). As a result, well-established experimental ELISA protocols exist for both MRP-8/MRP-14 and S100A12, but these are not available for use in routine laboratories. However, our translational research aimed for the broad application of these predictive biomarkers to allow decisions on the withdrawal of medication in JIA remission based upon the patient’s individual condition. Since commercial ELISA kits for MRP-8/MRP-14 and S100A12 are available but have never been validated for this purpose, it was important to provide reliable information on the feasibility of these analyses in clinical practice.

Our data demonstrated good performance by commercially available ELISA assay kits designed for analyzing patient serum samples. This is true for the relatively low S100 concentrations found in healthy controls and JIA patients in remission. However, all ELISA assays tested showed limited linearity in serum samples for higher concentrations. Therefore, serial dilution of individual sera is mandatory to obtain reliable results in the range of S100 concentrations found during active disease in different forms of JIA. In addition, the S100 protein concentrations analyzed with the individual assays varied substantially. Therefore, a direct comparison of the results obtained with one ELISA with a result from a different assay should not be made.

We can confirm assay-specific normal values that vary between assays. In the context of JIA patients in remission receiving medication, however, different cutoffs apply, since we aimed to establish risk profiles in 2 groups of patients: those with disease remission who will remain stable even after discontinuation of therapy and those with clinical but not immunologic disease remission. In the latter case, subclinical inflammation that is not detectable by other means but identified by MRP-8/MRP-14 or S100A12 will confer a risk for a disease flare after stopping medication. The biomarkers are especially useful in predicting early flares within 3 months after therapy withdrawal, which confirms previous findings. Applied as a snapshot analysis, the biomarkers inform about the stability of remission and the risk of flares, especially within a timeframe of a few weeks up to 3 months.

In the present study, we were able to determine assay-specific cutoff levels for determining the risk of disease flares in JIA. Compared to experimental assays, the tested commercial ELISAs produced a similar correlation between high serum levels of MRP-8/MRP-14 and S100A12 with the individual relapse risk. We therefore conclude that the risk-adapted stratification of decisions to stop or continue with therapy after achieving JIA remission using MRP-8/MRP-14 and S100A12 biomarker assays is feasible in clinical practice. However, there is no doubt that the results of our study, especially the cutoffs, need to be validated in an independent cohort. Therefore, we are currently running a prospective study to validate the potential of biomarkers for clinical practice.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Foell had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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