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Galectin-9 and CXCL10 as biomarkers for disease activity in juvenile dermatomyositis: a longitudinal cohort study and multi-cohort validation

Running title: Galectin-9 and CXCL10 as biomarkers for JDM

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Competing interests

All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author). Dr. Nistala is currently employed by GlaxoSmithKline as a physician. All other authors declare no other support from any organisation for the submitted work than the grants

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ABSTRACT

Objective: Objective evaluation of disease activity is challenging in patients with juvenile dermatomyositis (JDM) due to lack of biomarkers, but crucial to avoid both under- and overtreatment. Recently, we identified two proteins that highly correlate with JDM disease activity: galectin-9 and CXCL10. Here, we validate galectin-9 and CXCL10 as biomarkers for disease activity, assess disease-specificity and investigate their potency to predict flares.

Methods: Galectin-9 and CXCL10 were measured in serum samples of 125 unique JDM patients in three international cross-sectional cohorts and a local longitudinal cohort, by multiplex immunoassay. Disease-specificity was examined in 50 adults with (dermato)myositis and 61 patients with other systemic autoimmune diseases.

Results: Galectin-9 and CXCL10 outperformed the currently used marker creatine kinase (CK) to distinguish between JDM patients with active disease and remission, both cross-sectionally and longitudinally (area ROC curve: 0.86-0.90 for galectin-9 and CXCL10, 0.66-0.68 for CK). The sensitivity and specificity were 0.84 and 0.92 for galectin-9, and 0.87 and 1.00 for CXCL10. In 10 prospectively followed patients with a flare, continuously elevated or rising biomarker levels suggested an imminent flare up to several months before symptoms, even in absence of elevated CK. Galectin-9 and CXCL10 distinguished between active disease and remission in adults with (dermato)myositis and were suited for measurement in minimally-invasive dried blood spots.

Conclusions: Galectin-9 and CXCL10 were validated as sensitive and reliable biomarkers for disease activity in (J)DM. Implementation of these biomarkers into clinical practice, as tools to monitor disease activity and guide treatment, might facilitate personalized treatment strategies.

INTRODUCTION

Juvenile dermatomyositis (JDM) is a rare, chronic systemic immune-mediated disease with a high disease burden. JDM is characterized by inflammation of skeletal muscles and skin, leading to muscle weakness and a pathognomonic skin rash. Vital organs such as the lung and heart can also be involved. Although the pathogenesis is still largely unknown, environmental and genetic factors may predispose to the disease. (1–5) The autoimmune process is characterized by a type I interferon signature and by infiltration of immune cells such as plasmacytoid dendritic cells, B cells, CD4⁺ T cells and macrophages into skin and muscle tissue. (6–9)

Children with JDM are at risk of both under- and overtreatment due to lack of reliable biomarkers for disease activity. Current treatment guidelines recommend immunosuppression for at least two years, tapering steroids over the first year and withdrawing treatment if a patient has been off steroids and in remission on methotrexate (or alternative DMARD) for a minimum of 1 year. (10–12) However, for some patients this standardized regimen may not be optimal. Approximately 50% of patients do not respond to initial treatment or present with disease flares during follow-up, resulting in additional tissue damage and impaired physical recovery. (13–15) In the other half of patients some could likely benefit from shorter treatment duration, taking into account that overtreatment with steroids can result in serious side effects in children, such as Cushing's syndrome, osteoporosis and growth delay. (16–18)

To determine the rate of medication tapering and to avoid both under- and overtreatment, objective measurement of disease activity and subclinical inflammation is crucial. However, validated and reliable biomarkers for disease activity are lacking. (19) Disease activity is currently assessed by a combination of muscle enzyme testing and clinical evaluation; (10,20–22) the latter depends on the experience of the health care professionals and the patient's collaboration. Muscle enzymes, including serum creatine kinase (CK) activity, have been shown to correlate only moderately with disease activity in JDM and the erythrocyte sedimentation rate and C-reactive protein are rarely elevated in patients with JDM. (23–25) Lack of objective tools or biomarkers to monitor response to therapy also hampers clinical trial design. Thus, there is an unmet need for an objective and reliable measure for disease activity.

Recently, we have demonstrated that three proteins, galectin-9, CXCL10 and TNFR11, distinguish between active disease and remission in a cross-sectional cohort of patients with JDM, with galectin-9 and CXCL10 being the most discriminative markers. (26,27) CXCL10 and galectin-9 can be produced by a variety of cells, both immune and non-immune, upon stimulation with interferons. (28,29) CXCL10 has been reported as a biomarker in several human autoimmune diseases including myositis, (29–33) whereas galectin-9 has been mainly investigated as a biomarker in cancer and viral infections. (28,34) Reports on the role of galectin-9 in autoimmunity are conflicting, suggesting either an attenuating or aggravating effect on autoimmune manifestations in experimental models. (35,36) Its role in human autoimmune diseases has yet to be elucidated. Here, we aim to validate galectin-9 and CXCL10 as biomarkers for active disease in JDM, examine disease-specificity in adult patients with (dermato)myositis and patients with other systemic autoimmune diseases, assess

their potency to predict flares and test the applicability of the biomarkers in minimally-invasive dried blood spots, to aid broad implementation into clinical practice.

PATIENTS AND METHODS

Cohorts

125 unique patients with JDM from three independent cross-sectional international cohorts and one Dutch prospective cohort participated, which were included between May 2001 and May 2017. Two large cohorts from Utrecht and London were used for validation of the biomarkers; a third smaller cohort from Singapore was used to assess international generalizability. An overview of all cohorts is shown in table 1. The internal validation cohort (IVC) from Utrecht does not overlap with the previously reported discovery cohort.

(26) For specific questions including disease specificity, longitudinal follow-up and measurements in dried blood spots, a combination of samples from the IVC and new patients was used.

Participants

Patients with JDM were included if they met the Bohan and Peter criteria for definite or probable JDM. (37,38) The childhood myositis scale (CMAS; 0-52), manual muscle testing of 8 muscle groups (MMT-8; 0-80) and physician's global assessment (PGA; 0-10) were recorded as clinical measures of muscle and global disease activity. Cutaneous assessment tool scores measuring skin disease (CAT; 0-116) were additionally recorded in Dutch and Singaporean patients. Disease remission was defined according to the updated criteria for clinically inactive disease, and, in case of missing data by clinical description, as indicated in the table legends. (39) All other patients were considered active. Flares were defined as the combination of the following three items: a previous response to treatment with the decision

to start tapering steroids, worsening of at least one out of three clinical scores (CMAS, PGA, CAT) with two or more points and the decision to start new immunosuppressive treatment or increase the current dose.

Adult patients with dermatomyositis (DM) and non-specific myositis (NSM), were classified according to the ENMC criteria. (40) Myositis was confirmed by biopsy unless typical skin manifestations of dermatomyositis were present. Patients with cancer-associated myositis were excluded. Disease activity was determined by combined evaluation of muscle strength with the medical research council (MRC) scale, skin symptoms and muscle enzymes. To determine the disease specificity of the biomarkers, different disease controls were added in the study: Paediatric and adult patients with systemic lupus erythematosus (SLE), paediatric patients with localized scleroderma, adult patients with eosinophilic fasciitis (EF) and paediatric and adult patients with hereditary proximal spinal muscular atrophy (SMA). All controls had either systemic inflammation, inflammation of skin or muscles or had a non-inflammatory neuromuscular disorder. Patients with SLE fulfilled the ACR classification criteria for SLE. (41) Active disease was defined as a SLEDAI score ≥ 4 out of 105. (42) Patients with LoS were diagnosed based on the typical clinical picture. Active disease was defined as a modified LoS Skin Severity Index (mLoSSi) ≥ 5 out of 162. (43) Patients with EF were diagnosed based on the clinical picture and histopathological evaluation of skin biopsies containing the fascia. As the mLoSSi may stay high in these patients due to extensive irreversible sclerosis despite improved inflammatory symptoms, active disease was defined as a PGA for activity ≥ 5 out of 100. (43) Patients with hereditary proximal SMA, a progressive, non-inflammatory neuromuscular disorder, were diagnosed by genetic confirmation of a homozygous loss of function of the survival motor neuron 1 gene and served as disease controls. (44) Adult healthy volunteers were included as healthy controls.

Ethical approval

This study was approved by the institutional ethics committees of the involved centres (UMC Utrecht (METC 15-191 and 12-466), United Kingdom (MREC1/3/22), CHUV Lausanne, CHU Strasbourg, SingHealth centralized IRB, AMC Amsterdam) and conducted according to the Declaration of Helsinki. Written informed consent was obtained prior to inclusion in the study, from patients and from parents or legal representatives when the patient was less than 12 years old.

Patient material

Blood was collected in serum tubes, according to the local study protocol (all participating centers). At the UMC Utrecht, sodium-heparin tubes were collected in addition to serum. All samples were spun down and aliquoted within four hours after collection and subsequently stored at -80°C until analysis.

Dried blood spots

Dried blood spots (DBS) were made by application of 50 µL sodium-heparin full blood to each spot on Whatman® 903 filter paper within four hours after blood was drawn. Spotted filter papers were dried for two days at room temperature to mimic mail delivery times and subsequently stored with desiccant in individual air tight polyethylene bags at -80°C under constant monitoring of humidity levels until analysis. Two circles of 3.0 mm in diameter (containing approximately 3 µl of whole blood each) were punched from the central part of one spot and eluted in 100 µL buffer (PBS containing 5 mL/L Tween-20, 10 g/L bovine serum albumin and complete protease inhibitor cocktail with EDTA (Roche, one tablet per 25 mL buffer)) in 96-well plates. Plates were sealed and placed overnight at 4°C on a

microshaker (600 rpm) and were spun down at 2100g for 2 minutes. The analysis was performed on the obtained eluate.

Biomarker analysis

Galectin-9 and CXCL10 were measured in 50 μ L of serum, plasma or eluate by multiplex technology (xMAP; Luminex). CXCL10 was measured in undiluted material. Galectin-9 was measured in 10x diluted plasma or serum, except in the serum/plasma samples paired with DBS (here it was measured undiluted from eluate and serum/plasma). The multiplex immunoassay was performed as described previously. (45) Heterophilic immunoglobulins were pre-absorbed from all samples with HeteroBlock (Omega Biologicals). Acquisition was performed with a Bio-Rad FlexMAP3D in combination with xPONENT software version 4.2 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Bio-Rad). Between measurement of the internal and external validation cohorts in 2015, the recombinant protein for galectin-9 was replaced, which affected the standard curve. Therefore, absolute values between these cohorts may not be comparable. Since 2015, the inter-luminex variability has been negligible. (46) All biomarker analyses were performed at the UMC Utrecht, thereby minimizing inter-centre variation. Treating physicians were blinded for biomarker levels and technicians performing the multiplex assay were blinded for clinical data.

Statistical analysis

Basic descriptive statistics were used to describe the patient population. Statistical analyses were performed using either GraphPad Prism 7.0 or SPSS Statistics 21 (IBM). Correlations were assessed by Spearman rank correlation. For comparisons between two groups, the Mann-Whitney U test (unpaired analysis) or the Wilcoxon matched-pairs signed rank test (paired analysis) was used. For comparisons between multiple groups, non-parametric

variants of ANOVA with post hoc correction for multiple testing were used (Dunn's post hoc test for Kruskal-Wallis and Šídák's or Tukey's post hoc test for 2-Way ANOVA, as indicated in the figure legends). Multiplicity adjusted P values less than 0.05 were considered statistically significant. To assess diagnostic accuracy, the area under the curve (AUC) in receiver operating characteristics (ROC) curves were constructed. Cutoff values for galectin-9 and CXCL10 were determined based on the maximal Youden's index with a sensitivity of at least 80%.

RESULTS

Cross-sectional validation of galectin-9 and CXCL10

To validate the biomarker potential of galectin-9 and CXCL10, we measured the proteins in JDM patient samples from two independent validation cohorts: an external cohort (EVC) from London and an internal cohort (IVC) from Utrecht. The clinical characteristics of these cohorts are shown in supplementary table 1 and 2. As observed in the previously reported discovery cohort, (26) galectin-9 and CXCL10 levels were significantly higher in patients with active disease than in patients in remission ($p < 0.0001$; supplementary figure 1A and B). The levels were highest at diagnosis before treatment, decreased steadily under treatment and were comparably low in remission with or without medication (figure 1A and B). The wide range of biomarker levels in the active group under treatment ("AM") corresponds with a wide range of clinical disease activity within this group (CMAS range 3-44 in EVC, 10-52 in IVC; PGA range 2-8 in EVC, 1-9 in IVC). Both galectin-9 and CXCL10 were able to differentiate between patients with active disease on medication and patients in remission on medication, which is clinically important to assess response to treatment ("AM" versus "RM"; figure 1A and B). Paired analysis within individual patients comparing active disease and remission showed decreasing biomarker levels in response to therapy and confirmed the

high discriminative power of both proteins ($p=0.0078$ in EVC and $p=0.0002$ in IVC; figure 1C and D).

To further assess the discriminative power of galectin-9 and CXCL10 for active disease and remission, we examined the AUC in ROC curves in the two separate cohorts. Comparing active disease and remission regardless of treatment status, galectin-9 and CXCL10 had an AUC of 0.894/0.863 and 0.877/0.902 (EVC/IVC), respectively (figure 1E and F; supplementary table 3). To take into account the effect of treatment, we also assessed the AUC of active disease versus remission in patients on medication. During treatment, galectin-9 and CXCL10 had an AUC of 0.844/0.776 and 0.860/0.840 (EVC/IVC), respectively (supplementary figure 1C and D; supplementary table 3). Moreover, galectin-9 and CXCL10 performed better than the current standard laboratory marker CK in both cohorts (AUC of CK: 0.682/0.662 in EVC/IVC).

To calculate the optimal cut-off value for distinguishing active disease and remission, we analysed the ROC curves of the internal validation cohort, as this cohort was measured according to the most recently optimized and standardized protocol of the multiplex immunoassay. (46) Based on the coordinates of this ROC curve we determined cut-off values for galectin-9 (19396 pg/mL) and CXCL10 (805 pg/mL) with a high sensitivity (0.84 for galectin-9 and 0.87 for CXCL10) and high negative predictive value (0.83 for galectin-9 and 0.87 for CXCL10) for active disease, to ensure a low risk of ongoing inflammation in case of a test result below the cut-off (table 2). The specificity and positive predictive value were 0.92/1.00 and 0.93/1.00, respectively (galectin-9/CXCL10).

Consistent with the previously reported discovery cohort, (26) galectin-9 and CXCL10 correlated strongly with three clinical scores for global or muscle disease activity: PGA, CMAS and MMT-8. The correlation coefficients of both biomarkers, between 0.67 and 0.81 ($p < 0.0001$), were notably higher than those of CK ($r_s = 0.32-0.51$, $p < 0.01$; figure 1G and H; supplementary figure 1E, F and G). In conclusion, we have validated galectin-9 and CXCL10 in two independent validation cohorts as strong biomarkers for disease activity, which outperform the currently used laboratory marker CK.

To assess the international generalizability of galectin-9 and CXCL10, we tested the biomarkers in a small JDM cohort from a different geographic region (i.e. Singapore).

Observations in this cohort confirmed the discriminative potential of galectin-9 and CXCL10 between active disease and remission, and levels were comparable to the IVC ($p = 0.0006$ for galectin-9 and $p = 0.0025$ for CXCL10; “JDM Sing” and “JDM NL”; figure 2A and B).

Disease specificity of galectin-9 and CXCL10

Next, we investigated the disease specificity of galectin-9 and CXCL10 and explored their applicability as biomarkers in adult patients with (dermato)myositis and patients with other systemic autoimmune diseases. The biomarkers were first measured in a cohort of adult patients with dermatomyositis ($n = 36$), non-specific myositis ($n = 14$), eosinophilic fasciitis ($n = 18$) as well as 43 control patients with spinal muscular atrophy, a genetic neuromuscular disorder without systemic inflammation, and 22 healthy controls (supplementary table 4).

Both galectin-9 and CXCL10 were elevated in adult patients with active DM ($p < 0.0001$), NSM ($p < 0.0003$) and EF ($p < 0.05$) compared to healthy controls. Both biomarkers distinguished between active disease and remission in DM ($p = 0.0126$ and $p < 0.0001$); CXCL10 was also discriminative for disease activity in NSM ($p = 0.0139$) and EF ($p = 0.0497$;

figure 2A and B). As expected, the biomarkers were not elevated in SMA. A second cohort consisted of paediatric and adult patients with two other systemic immune-mediated diseases: localized scleroderma (n=15) and systemic lupus erythematosus (n=36) (supplementary table 5). In LoS and SLE the two biomarkers did not distinguish significantly between active disease and remission, but galectin-9 levels in SLE were elevated compared to healthy controls (p=0.0105; figure 2C and D). In conclusion, galectin-9 and CXCL10 are applicable as biomarkers for disease activity in both paediatric and adult patients with myositis.

Prospective analysis and flare prediction

Next, to determine the prognostic value of galectin-9 and CXCL10 during clinical follow-up of JDM patients, we measured the biomarkers in a prospective cohort of 28 patients, with a median follow-up time of 2.8 years per patient (supplementary table 6). First, we established the biomarker dynamics after diagnosis in 15 patients who reached sustained remission within the first months of treatment and did not have a flare later. The biomarker levels quickly declined after start of treatment, reached levels below the previously determined cut-off within several months, and remained low in remission (“No flare”; figure 3A and B). The biomarker dynamics of patients with a flare after the first year (“Flare >12 months”; n=7) were similar to those of patients without flares (figure 3C and D). However, patients suffering from a disease flare in the first year after start of treatment (“Flare <12 months”; n=6) had significantly higher biomarker levels at diagnosis than patients with later flares (p=0.0254 for galectin-9 and p=0.0265 for CXCL10; middle panel figure 3C and D). In addition, these patients had elevated biomarker levels over the entire first year (figure 3C, D and E; right panel 3C&D shows total area under curve of first year). In contrast to the biomarkers, CK activity normalized in 5 out of 6 of these patients (figure 3E).

To assess the predictive value of the biomarkers for flares after the first year, we analysed 4 patients of whom longitudinal samples were available within 7 months before a flare (Figure 3F and supplementary figure 2). In patients #1 and #2, a raise of the levels of galectin-9 and CXCL10 (even while below cutoff) was observed from up to 7 months prior to flare, with levels above the cutoff up to 6 months prior for galectin 9 and up to 3 months prior for CXCL10. These biomarker fluctuations were observed even before clinical symptoms of a flare became apparent. In patients #3 and #4, persistently borderline cutoff values were observed for galectin-9 and CXCL10 in the 12 months prior to flare and biomarkers were elevated above the cutoff during flare. In contrast, CK did not increase prior to and during flare in patient #4, and did not demonstrate an increase until flare in patient #2 and #3. Only in patient #1 CK steadily increased by 3 months prior to flare. It was also observed that galectin-9 and CXCL10 stayed high during continued disease activity on medication after the start of flare, while in 3 out of 4 individuals CK decreased to within normal limits by the first timepoint following start of clinical flare despite continued disease activity.

In conclusion, persistently high or rising galectin-9 and CXCL10 levels above their cut-off values may be indicative of ongoing (sub)clinical inflammation or an imminent flare, even with a lack of clinical symptoms or elevated CK.

Measurement in dried blood spots

To facilitate minimally invasive (at-home) biomarker assessment and broad clinical applicability with centralization of diagnostic cores, we assessed galectin-9 and CXCL10 measurement in dried blood spots (DBS) and paired plasma and serum samples. Patient characteristics are shown in supplementary table 7. The correlation between the biomarker levels in the circulation and in DBS was higher for CXCL10 ($r_s=0.93/0.96$ (plasma/serum))

than for galectin-9 ($r_s=0.62/0.58$ (plasma/serum); figure 4A and B). Galectin-9 and CXCL10 levels were similar in plasma and serum (figure 4C). Both galectin-9 and CXCL10, as measured in DBS, were capable of discriminating between active JDM patients and healthy controls ($p=0.0040$ and $p<0.0001$, respectively; figure 4D), the latter having similar biomarker levels to JDM patients in remission (figure 2). In conclusion, both galectin-9 and CXCL10 are suited to be measured in dried blood spots as biomarkers for JDM.

DISCUSSION

In this study galectin-9 and CXCL10 were validated as strong, reliable and sensitive biomarkers for disease activity in JDM, and both identified as promising biomarkers for adult patients with (dermato)myositis. Galectin-9 and CXCL10 strongly distinguished between JDM patients with active disease and patients in remission, even under immunosuppressive treatment. Furthermore, we show that Galectin-9 and CXCL10 are relatively specific for auto-inflammatory myopathies in adult and paediatric patients, as they were not as highly increased or did not differentiate between active disease and remission in other autoimmune diseases, like LoS and SLE. Both cross-sectionally and longitudinally, galectin-9 and CXCL10 outperformed CK, which is commonly used as a laboratory marker for disease activity and is one of the current criteria for determining clinically inactive disease in JDM. (39,47) Continuously elevated or rising biomarker levels, as determined in a prospective patient cohort, may be indicative of an imminent disease flare up to several months before clinical symptoms, even in absence of elevated CK. The biomarkers may therefore be promising to use in longitudinal follow-up of patients for monitoring of disease activity. Lastly, galectin-9 and CXCL10 can be reliably measured in plasma, serum and minimally invasive DBS. It has recently been shown that capillary concentrations of CXCL10 correlate with venous concentrations; for galectin-9 this has not been established yet. (48) The

moderate correlation for circulating levels of galectin-9 and levels in DBS could either result from liberation of intracellularly stored galectin-9 and/or release from its carrier proteins upon elution and dilution.

Strengths and limitations of this study

This study has several strengths. Although many biomarkers are being identified for a variety of diseases, only few are implemented into clinical practice due to a lack of reproducibility and diagnostic accuracy. Galectin-9 and CXCL10 however have a high discriminative power and strong, reproducible correlation with disease activity. Thanks to a large international collaborative effort we have been able to extensively validate galectin-9 and CXCL10 in a large number of patients with JDM from three independent cross-sectional cohorts, despite the rarity of the disease. The additional analyses in a prospective JDM cohort with a long follow-up time add important information on the value of galectin-9 and CXCL10 in clinical follow-up. Next to the clinical validation in this study, the biomarkers have undergone a technical validation at the diagnostic department of the UMC Utrecht, which has demonstrated the stability of the biomarkers and reproducibility of the measurements. In addition, we have explored a minimally invasive diagnostic method of measuring the biomarkers in dried blood spots.

The findings of this study need to be interpreted keeping in mind the observational nature of the data and the use of a combination of clinical scores and CK (PRINTO criteria for clinically inactive disease) as gold standard for disease activity. (39,47) Importantly, galectin-9 and CXCL10 can complement, but not replace clinical assessment by experienced health care professionals. However, both biomarkers outperform the currently used marker CK, which underscores the gains that can be achieved by introducing the new biomarkers into clinical practice.

Comparison with other studies

A recent study using the SOMAScan assay also identified both galectin-9 and CXCL10 among the top upregulated proteins in JDM, correlating with disease activity assessed by PGA. (49) CXCL10 was previously shown to correlate with disease activity in JDM, (26,30–32,50) and is well known as an interferon-inducible chemokine that can be elevated in other types of myositis and autoimmune diseases. (29,33) In our study galectin-9 was a specific biomarker for inflammatory myopathies. In JDM patients, high circulating interferon-alpha levels have been found and in one group of JDM patients, more than 75% of patients had a positive interferon signature. (51,52) Circulating galectin-9 and CXCL10 could therefore be a direct reflection of active interferon-driven inflammation, which is supported by a recent study describing galectin-9 as a marker for the IFN signature in SLE and antiphospholipid syndrome. (53) Since the biomarkers correlate with disease activity in tissues, local tissue cells are the main candidate producers of the proteins. Indeed, galectin-9 cannot only be detected in the circulation, but also locally within inflamed muscle and skin, where it is mainly present in activated tissue macrophages and capillary endothelial cells (*data not shown*). A similar expression pattern, in tissue mononuclear cells and endothelial cells, was previously demonstrated for CXCL10. (54,55) Local biomarker production within inflamed tissues fits our previous observation that the biomarker levels only slowly decline after stem cell transplantation, as tissue infiltrating immune cells (and endothelial cells) are likely less affected by immune ablative pre-conditioning than circulating immune cells. (27)

Implications for clinical practice

Implementation of galectin-9 and CXCL10 into clinical practice, as tools to monitor disease activity and guide treatment, might enable personalized treatment strategies for patients with JDM. It is an advantage that both biomarkers perform equally well in our study, suggesting

that diagnostic centres can decide to use their biomarker of choice depending on availability and feasibility. Biomarker levels below the set cut-off reflect the absence of disease activity, which could allow tapering of immunosuppressive medication. Rising or persistently high levels might be indicative of an insufficient response to therapy and/or an imminent flare, even in the absence of clinical symptoms or elevated CK, possibly reflecting subclinical inflammation. Elevated biomarker levels might therefore indicate the need for intensification of treatment or slower tapering of steroids. With this envisioned personalized treatment strategy we respond to important patient-reported needs: a recently conducted patient survey by *CureJM*, the American patient organisation for juvenile myositis, has shown that “predictors for disease flares” and “new treatments, less side effects” are two of the top-three research priorities chosen by patients. (56) Galectin-9 and CXCL10 may also provide an objective outcome measure for response to therapy in future clinical trials assessing novel therapeutics. Our study has shown that galectin-9 and CXCL10 levels in DBS correlate with venous levels and can differentiate active JDM patients from healthy controls. Longitudinal assessment of these biomarkers via DBS, which requires further study, has potential for high utility in the future, as DBS can be sampled at home by simple capillary finger-prick. Since protein levels remain remarkably stable over time in DBS, even at room temperature, (57,58) they can be sent to a diagnostic centre through regular mail. This enables at-home diagnostics and centralization of diagnostic cores for both clinical care and multicentre studies. It also ensures maximum accessibility of the biomarker measurements for non-expert medical centres, which can also facilitate care in rural areas.

Unanswered questions and future research

Galectin-9 and CXCL10 could add important information in the complex differential diagnosis of muscle complaints during follow-up, and might aid to discriminate between steroid-induced myopathy, non-inflammatory muscle pain and muscle inflammation, all of which require different treatment strategies. Especially in these complicated cases, biomarkers may also abrogate the need for invasive diagnostic muscle biopsies or costly MRI scans, which can sometimes require sedation in young children. This specific potential will have to be further investigated in additional prospective studies. Additional prospective studies will also have to point out whether 1) one biomarker may be superior in answering specific clinical questions concerning JDM, 2) the biomarkers are able to detect mild disease activity, 3) the biomarkers also have prognostic value in adult patients with myositis and 4) biomarker-guided disease management will improve outcomes of patients with JDM.

Conclusion

Galectin-9 and CXCL10 were identified and extensively validated as strong, reliable and sensitive biomarkers for disease activity in JDM. The biomarkers might facilitate personalized treatment strategies for patients with JDM, by providing a diagnostic monitoring tool to guide treatment.

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Contributors

JW is the coordinator of the Dutch JDM cohort. JW, FBE, SK, EH, WA, MB, PHM collected and selected Dutch JDM samples and clinical data. JW and FBE analysed data and wrote the manuscript. CD, LW, KN and CP collected and selected JDM samples and clinical data in the UK. JL, AvdK, MV, JH and AM collected and selected adult DM/NSM samples and clinical data. JSM and TR collected and selected EF and LoS samples and clinical data. LLH, JT, RF, HO and JvR collected and selected SLE samples and clinical data. CAW and LP collected and selected SMA samples and clinical data. YJG and TA collected and selected JDM samples and clinical data. WJ supervised and performed the luminex analysis and helped with data analysis. SN supervised the dried blood spot analysis and advised on analyses. AvR and FW supervised JW and FBE and were closely involved in setting up the study, data analysis, writing and editing of the manuscript. All authors critically reviewed the manuscript.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author). Dr. Nistala is currently employed by GlaxoSmithKline as a physician. All other

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Transparency statement

The lead author (FW) has had the final responsibility for submission for publication and affirms that this manuscript is an honest, accurate, and transparent account of the study being reported, that no important aspects of the study have been omitted, and that any discrepancies from the study as planned have been explained.

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FIGURE AND TABLE LEGENDS

Table 1. Overview of JDM cohorts

Overview of the different JDM cohorts and analyses. *HC* = adult healthy control; *JDM* = juvenile dermatomyositis; *DM* = adult dermatomyositis; *NSM* = adult non-specific/overlap myositis; *SMA* = spinal muscular atrophy; *EF* = adult eosinophilic fasciitis; *LoS* = localized scleroderma; *SLE* = systemic lupus erythematosus; *NL* = Netherlands, *Sing* = Singapore; *EVC* = external validation cohort; *IVC* = internal validation cohort; *DBS* = Dried blood spots.

Table 2. Sensitivity and negative predictive value of determined cut-off values for galectin-9 and CXCL10 in internal validation cohort.

Cut-off values for galectin-9 and CXCL10 were determined based on the maximal Youden's index with a sensitivity higher than 0.80, to ensure a low risk of ongoing active inflammation with a biomarker value below the set cut-off. Only 1 sample per patient per category ('active' or 'remission') was included in the analysis (i.e. cohort 'JDM NL' as shown in figure 2 and supplementary table 5). *NPV* = negative predictive value, *PPV* = positive predictive value.

Figure 1. Cross-sectional validation of galectin-9 and CXCL10 as biomarkers for disease activity in JDM in two independent validation cohorts.

(A&B) Galectin-9 and CXCL10 levels were measured in serum samples from JDM patients by multiplex immunoassay. Patients were stratified into four groups based on disease activity and use of medication: active disease before start of treatment (“A”), active disease on medication (“AM”), remission on medication (“RM”) and remission off medication (“R”). Medians and interquartile ranges are shown, on a log scale. Kruskal-Wallis with Dunnett’s post hoc test for multiple comparisons; multiplicity adjusted P values are reported. P values >0.999 are not shown. **(A)** External validation cohort (EVC) (A: n=12; AM: n=27, RM: n=28, R: n=12). **(B)** Internal validation cohort (IVC) (A: n=25; AM: n=30, RM: n=16, R: n=12). Of note: In the AM group, for 1 patient 3 samples (from different time points at least 3 months apart) were included and for 6 patients 2 samples (also from different time points, 2 to 11 months apart) were included. Exclusion of these extra samples did not change the statistical significance between the groups (*data not shown*). **(C&D)** Galectin-9 and CXCL10 levels in paired patient samples during active disease and remission (regardless of treatment) from EVC (**C**, n=8) and IVC (**D**, n=13). The median time between the two samples was 23 months in the EVC and 12 months in the IVC. Wilcoxon matched-pairs signed rank test. **(E&F)** ROC curves of galectin-9, CXCL10, and CK in the EVC (**E**) and IVC (**F**), regardless of medication. Only patients with a complete dataset for the specific ROC curve were included in the analysis. Statistic details of the ROC curve analysis are shown in supplementary table 3. **(G&H)** Correlation of galectin-9 and CXCL10 with CMAS in EVC (**G**, n=79) and IVC (**H**, n=61). Spearman rank correlation. Biomarkers are shown on a log scale. *A = active pre-treatment; AM = active on medication; RM = remission on medication; R = remission off medication; CMAS = childhood myositis assessment scale; r_s = Spearman r . Patient characteristics are shown in supplementary table 1 and 2.*

Figure 2. The biomarker potential of galectin-9 and CXCL10 in adult inflammatory myopathies and systemic autoimmune diseases with skin involvement

(A) Galectin-9 and (B) CXCL10 were measured in serum samples from patients with JDM (Dutch and Singaporean validation cohorts), adult patients with DM, NSM and EF, adult healthy controls, and a mixed cohort of adult and juvenile patients with SMA. Where applicable, patients were stratified into two groups based on disease activity, regardless of their treatment status. Only one sample per patient per activity group was included in the analysis; therefore the patient numbers of the internal validation cohort (here “JDM NL”) differ from figure 1. Medians and interquartile ranges are shown, on a log scale. (C) Galectin-9 and (D) CXCL10 were measured in serum samples from juvenile patients with JDM (Dutch cohort) and LoS, adult healthy controls, and a mixed cohort of juvenile and adult patients with SLE. Patients were stratified into two groups based on disease activity, regardless of treatment status. Only one sample per patient per activity group was included in the analysis. Medians and interquartile ranges are shown, on a log scale. (A-D) Statistics for comparison between active disease and remission within diseases are shown in regular text (upper p value): 2-Way ANOVA with Šídák’s post hoc test for multiple comparisons. Statistics for comparison of each disease group with HC are shown in italic text (bottom p value): Kruskal-Wallis with Dunnett’s post hoc test for multiple comparisons. Multiplicity adjusted P values are reported. P values >0.999 are not shown. *A = active disease regardless of treatment; R = remission regardless of treatment; HC = adult healthy control; JDM = juvenile dermatomyositis; DM = adult dermatomyositis; NSM = adult non-specific/overlap myositis; SMA = adult + juvenile spinal muscular atrophy; EF = adult eosinophilic fasciitis; LoS = juvenile localized scleroderma; SLE = adult + juvenile systemic lupus erythematosus; NL = Netherlands, Sing = Singapore. Patient characteristics are shown in supplementary table 4 and 5.*

Figure 3. Galectin-9 and CXCL10 in longitudinal follow-up of patients

Galectin-9 and CXCL10 were measured in longitudinal samples from JDM patients in a prospective cohort. (A&B) Dynamics of galectin-9 and CXCL10 up to 6 years after diagnosis of 15 patients without flares, with a first sample taken max. 6 months after start of treatment. Both patients with and without intensification of therapy within the first 3 months were included. Grey shading indicates the previously determined cut-off values for galectin-9 and CXCL10 (19396 pg/mL and 805 pg/mL, respectively). Each datapoint contains between 3 and 13 samples, which were pooled over the time span around the data point. The median interval between two samples from a patient was 3.6 months. Per patient, 4-14 samples were included in the analysis (median of 9 samples per patient). Means and standard deviations are shown, on a linear scale. (C&D) Galectin-9 and CXCL10 in longitudinal samples from JDM patients with a flare within the first year (n=6), after the first year (n=7) or without flares (n=15, same patients as figures A&B). Only patients with a first sample taken max. 6 months after start of treatment were included. Left panel: longitudinal data within the first year, means and standard deviation. Grey shading indicates the previously determined cut-off values for galectin-9 and CXCL10 (19396 pg/mL and 805 pg/mL). 2-Way ANOVA with Tukey's post hoc test for multiple comparisons. Multiplicity adjusted P values are reported: P values in regular text: flare <12 months versus no flare; P values in italic text: flare <12 months versus flare >12 months; P values >0.999 are not shown. Middle panel: Galectin-9 and CXCL10 levels at diagnosis before start of treatment, medians and interquartile ranges, 2-way ANOVA with Tukey's post hoc test corrected for all time points measured in left panel; Right panel: Total area under the curve (AUC) for each of the three groups in the graph shown in the left panel, calculated by the trapezoidal method. 1-way ANOVA with Tukey's post hoc test. Means and 95% CI are shown. (E) Galectin-9, CXCL10 and CK in the 6 individuals with a flare within the first year after start of treatment. Grey shading indicates

the previously determined cut-off values for galectin-9 and CXCL10 and the standard cut-off of 150 IU/L for CK. (F) Longitudinal measurement of galectin-9, CXCL10 and CK in an individual with a disease flare after the first year. Dotted lines indicate the previously determined cut-off values for galectin-9 and CXCL10 (19396 pg/mL and 805 pg/mL, respectively). Grey shading indicates the cut-off for CK (150 IU/L). Biomarkers are shown on a log scale; time is in months. <12m = patients with a flare in the first year, >12m = patients with a flare after the first year; AUC = area under the curve, CK = creatine kinase, IU = international units, Dx = diagnosis, CMAS = childhood myositis assessment scale, PGA = physician's global assessment, CAT = cutaneous assessment tool, Pred = prednisone (mg/kg/day), MTX = methotrexate (mg/m²/week). Patient characteristics are shown in supplementary table 6.

Figure 4. Galectin-9 and CXCL10 measured in dried blood spots and paired plasma and serum samples.

Galectin-9 and CXCL10 levels in dried blood spots (DBS) were compared to levels in paired plasma and serum samples from both active JDM patients (n=10) and healthy controls (n=12). (A) Spearman correlation between biomarker levels in plasma and DBS, double log scale. (B) Spearman correlation between biomarker levels in serum and DBS, double log scale. (C) Paired representation of the biomarker levels in plasma, serum and DBS. Grey lines represent healthy controls, black lines represent active JDM patients, and dotted lines represent active JDM patients on medication. Biomarkers are shown on a log scale. (D) Biomarker levels in DBS compared between HC and active JDM. Mann-Whitney U test. Biomarkers are shown on a log scale. HC = healthy control, JDM A = Active JDM patients pre-treatment, JDM AM = Active JDM patients on medication. Patient characteristics are shown in supplementary table 7.

Table 1. Overview of JDM cohorts

	Cohort	Abbreviation	City, Country	Table no	Figure no	Patients	Samples	Paired samples Active-Remission	Material	Controls	Luminex analysis no.
(International) validation cohorts	External validation cohort	EVC	London, United Kingdom	Suppl 1	Fig 1 & S1	61	79	16	Serum	-	1
	Internal validation cohort	IVC	Utrecht, Netherlands	Suppl 2	Fig 1 & S1	47	83	26	Serum	Adult DM & NSM, adult EF, adult & paediatric SMA, adult HC	2
		JDM NL		Suppl 4	Fig 2	47	58	11			
	Asian cohort	JDM Sing	Singapore	Suppl 4	Fig 2	12	13		Serum		
Analysis-specific sub-cohorts from Utrecht	Systemic autoimmune disease cohort	-	Utrecht, Netherlands	Suppl 5	Fig 2	14	16	2	Serum	Paediatric LoS, paediatric & adult SLE, adult HC	3
	Longitudinal cohort	-	Utrecht, Netherlands	Suppl 6	Fig 3 & S2	28	286		Serum Plasma	Paediatric HC	5
	Dried blood spot cohort	-	Utrecht, Netherlands	Suppl 7	Fig 4	7	10		Serum Plasma DBS	Adult HC	4

EVC = external validation cohort; IVC = internal validation cohort; DBS = dried blood spots; HC = adult healthy control; JDM = juvenile dermatomyositis; DM = adult dermatomyositis; NSM = adult non-specific/overlap myositis; SMA = spinal muscular atrophy; EF = adult eosinophilic fasciitis; LoS = localized scleroderma; SLE = systemic lupus erythematosus; NL = Netherlands, Sing = Singapore.

Table 2. Sensitivity and negative predictive value of determined cut-off values for galectin-9 and CXCL10 in internal validation cohort.

Biomarker	Cutoff value	Sensitivity	Specificity	NPV	PPV
Galectin-9	19396 pg/mL	0.839	0.923	0.828	0.929
CXCL10	805 pg/mL	0.871	1.000	0.867	1.000

Cut-off values for galectin-9 and CXCL10 were determined based on the maximal Youden's index with a sensitivity higher than 0.80, to ensure a low risk of ongoing active inflammation with a biomarker value below the set cut-off. Only 1 sample per patient per category ('active' or 'remission') was included in the analysis (i.e. cohort 'JDM NL' as shown in figure 2 and supplementary table 5). *NPV = negative predictive value, PPV = positive predictive value.*

Figure 1

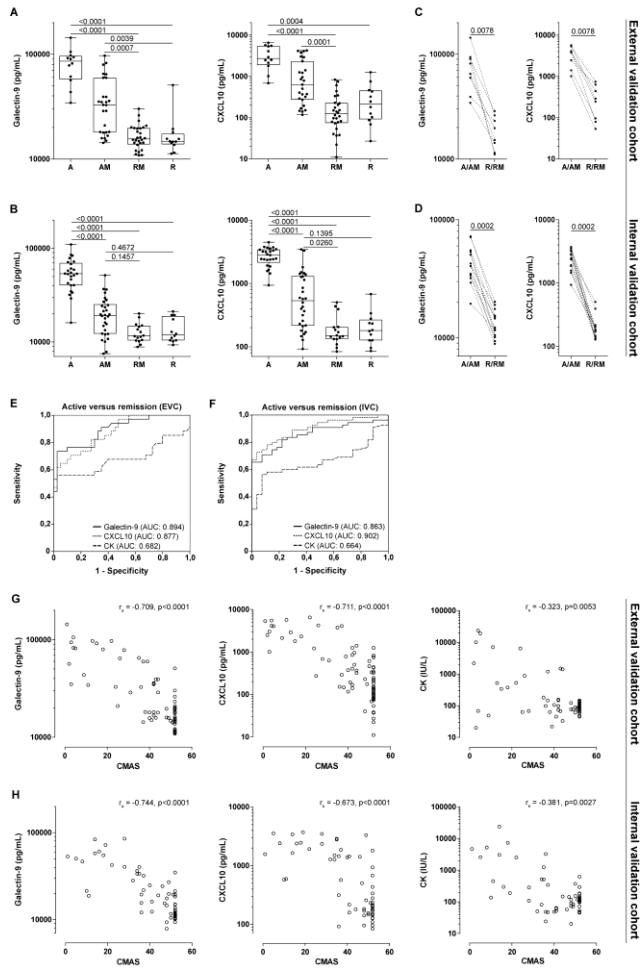


Figure 2

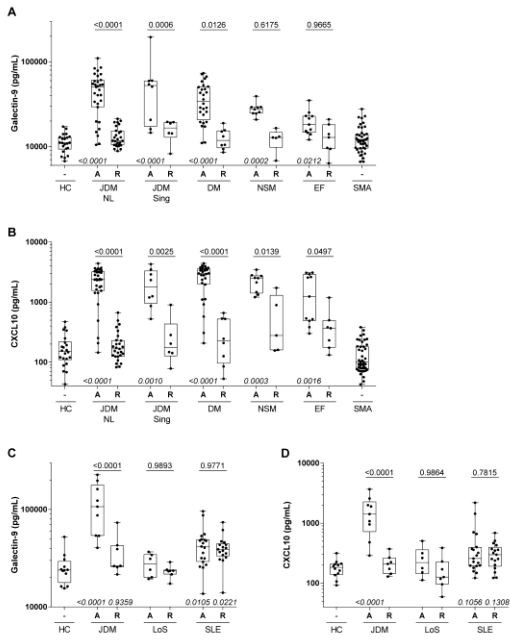


Figure 3

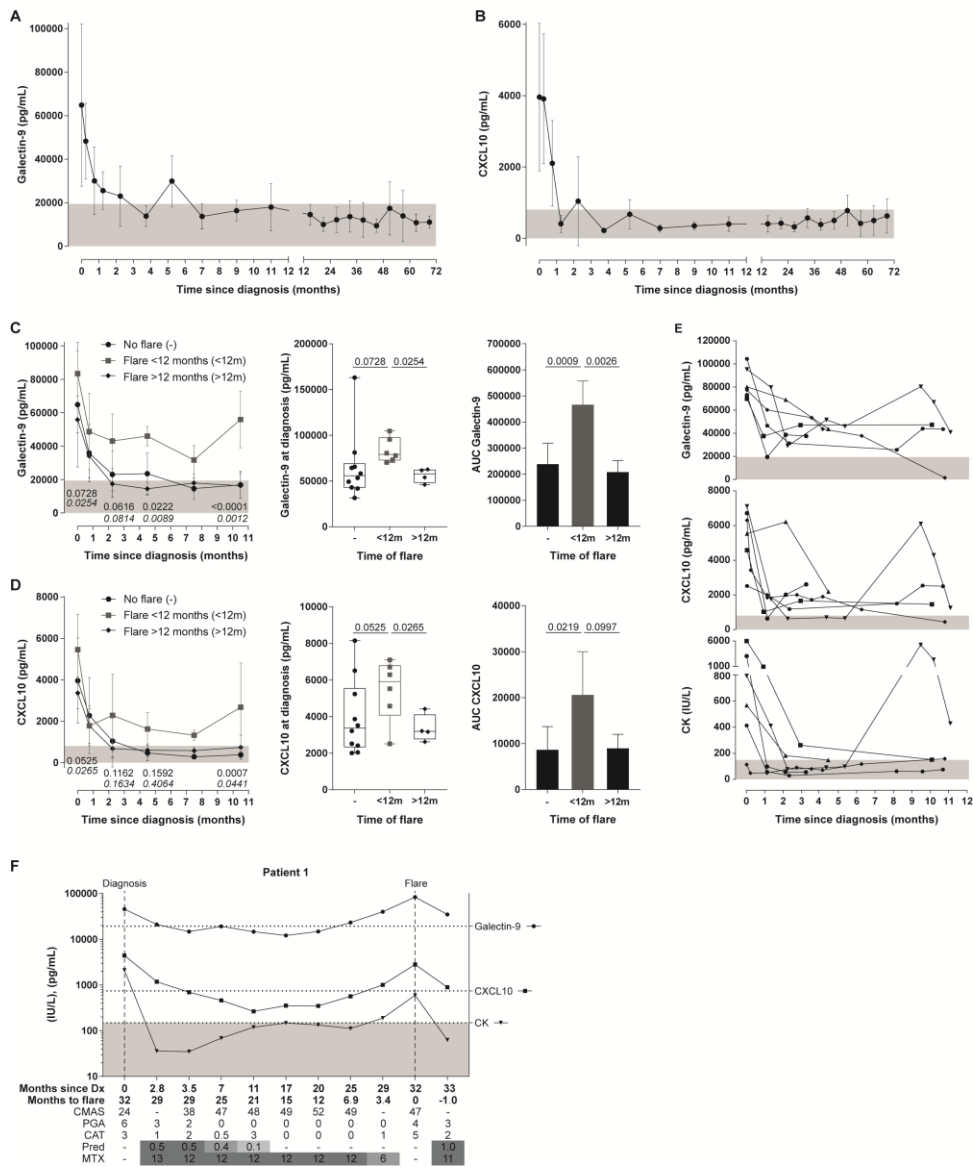


Figure 4

