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DR. CYRIELLE HOU (Orcid ID : 0000-0002-0258-4738)

DR. ISABELLE MELKI (Orcid ID : 0000-0002-8057-333X)

MR. MATHIEU PAUL RODERO (Orcid ID : 0000-0002-1300-0187)

DR. CYRIL GITIAUX (Orcid ID : 0000-0002-2190-6843)

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### **From diagnosis to prognosis: Revisiting the meaning of muscle ISG15 overexpression in juvenile inflammatory myopathies**

Cyrielle Hou, PharmD, PhD<sup>1</sup>, Chloé Durrleman, MD<sup>1,2</sup>, Baptiste Periou, MS<sup>1,3</sup>, Christine Barnerias, MD<sup>2</sup>, Christine Bodemer, MD, PhD<sup>4</sup>, Isabelle Desguerre, MD, PhD<sup>2</sup>, Pierre Quartier, MD, PhD<sup>5</sup>, Isabelle Melki, MD, PhD<sup>6</sup>, Gillian I Rice PhD<sup>7</sup>, Mathieu P Rodero, PhD<sup>8</sup>, Jean-Luc Charuel, MD<sup>9</sup>, Frédéric Relaix, PhD<sup>1</sup>, Brigitte Bader-Meunier, MD<sup>5</sup>, François Jérôme Authier, MD, PhD<sup>\*1,2,3</sup>, Cyril Gitiaux, MD, PhD<sup>\*1,2,10</sup>

#### **Authors' affiliations**

<sup>1</sup>UnivParis Est Créteil, INSERM, IMRB, F-94010 Créteil, France.

<sup>2</sup>Reference Centre for Neuromuscular Diseases "Nord-Est-Ile de France", FILNEMUS, France.

<sup>3</sup>AP-HP, Hôpital Mondor, Service d'histologie, F-94010 Créteil, France.

<sup>4</sup>Department of Dermatology, Necker Enfants Malades Hospital, AP-HP centre, Paris University, Paris, France.

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<sup>5</sup>Department of Pediatric Immunology-Hematology and Rheumatology, Necker Enfants malades Hospital, AP-HP centre, Paris University, Paris, France.

<sup>6</sup>Laboratory of Neurogenetics and Neuroinflammation, Imagine Institute, Paris, France.

<sup>5</sup>Division of Evolution and Genomic Sciences, School of Biological Sciences, University of Manchester, Manchester, UK.

<sup>8</sup>Chimie et Biologie, Modélisation et Immunologie pour la Thérapie (CBMIT), Paris University, CNRS, UMR8601, Paris, France.

<sup>9</sup>Department of Immunology, Pitié-Salpêtrière Hospital, AP-HP, Paris, France.

<sup>10</sup>Department of Pediatric clinical neurophysiology, Necker Enfants malades Hospital, AP-HP centre, Paris University, Paris, France.

\*: these two authors contributed equally to the work

**Corresponding Author:** Dr Cyril Gitiaux. Hôpital Necker-Enfants Malades, 149 rue de Sèvres, 75015 Paris, France. Email: [cyril.gitiaux@aphp.fr](mailto:cyril.gitiaux@aphp.fr).

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## ABSTRACT

**Objectives.** Juvenile idiopathic inflammatory/immune myopathies (JIIM) constitute a highly heterogeneous group with diagnostic difficulties and prognostic uncertainties. Circulating myositis-specific autoantibodies (MSAs) were first recognized as reliable tools for patient sub-stratification. Considering the key role of type 1 interferon (IFN-I) up-regulation in JIIM, we investigated whether the IFN-stimulated gene 15 (ISG15) could be reliable biomarker for stratification and diagnosis while shedding light on its role in JIIM pathophysiology. **Methods.** We included 56 patients, 24 Juvenile Dermatomyositis (JDM), 12 Juvenile Overlap Myositis (JOM), 10 patients with Duchenne Muscular Dystrophy (DMD) and 10 patients with Congenital Myopathies (CM). Muscle biopsies have been studied using immunohistochemistry, immunoblotting, and real-time quantitative PCR. The negative (*ISG15*, *USP18*) and positive regulators (*DDX58*, and *IFIH1*) of IFN-I were analyzed. **Results.** *ISG15* expression discriminated JIIM from non-immune myopathies, and among JIIM, JDM from JOM. Among JDM patients, IFN-I positive upregulation (*DDX58*, *IFIH1*) was similar whatever MSAs. In contrast, the highest level of *ISG15*, which is a negative regulator of IFN-I was observed in MDA5(+) patients compared to others. Finally, *ISG15* level was inversely correlated with the severity of muscle histological injuries, and was positively correlated with motor performance (CMAS, MMT). **Conclusions.** Muscle *ISG15* expression is strongly associated with diagnosis of JDM. Patients present a different muscle signature of *ISG15* according to their MSAs class. JDM MDA5(+) patients have a higher expression of *ISG15* (gene and protein) compared to the other subgroups. Moreover, our data showed that the IFN-I negative regulation correlates with milder muscle involvement.

## INTRODUCTION

Juvenile idiopathic inflammatory/immune myopathies (JIIM) constitute a heterogeneous group of acquired muscle diseases of autoimmune origin. They include juvenile dermatomyositis (JDM), characterized by proximal muscle weakness and distinct skin rashes, juvenile overlap myositis (JOM) corresponds to patients that meet the criteria for JIIM as well as another autoimmune disease (systemic lupus erythematosus [SLE] or systemic sclerosis [SS]) and autoimmune necrotizing myopathy typically not associated with extra-muscular features (1–3). Although

modern treatment has improved JIIM outcome, numerous patients present with persistent chronic disease or damage, pointing out the need of reliable biomarkers to inform prognosis and orient treatment. Circulating myositis-specific autoantibodies (MSAs) were first recognized as reliable tools for patient sub-stratification (4). In JDM, which is basically characterized by muscle endomysial microangiopathy and ischaemic injuries, markers of vasculopathy severity were logically shown as predictive of poor prognosis. Indeed, ischaemic pattern at muscle biopsy correlated with more severe muscle weakness, more gastrointestinal involvement and the need for more aggressive treatment (5–7). Similarly, increased serum levels of galectin-9, CXCL10, TNFR2 and galectin-1, four markers related to endothelial dysfunction and inflammation, were found predictive of poor response to conventional treatment in two independent cohorts (8). Other important biomarkers investigated in JIIM relates to the selective activation of type 1 interferon (IFN-I, ie IFN $\alpha/\beta$ ) pathway, assessed by transcriptomic and proteomic analyses were performed on different tissues and cells: muscle, myogenic precursor cells, skin, blood, and circulating leucocytes. Such IFN-I signature has been identified in whole muscle extracts and blood samples of JDM and JOM patients and correlated with activity scores (9). Until now direct screening of IFN-I protein to stratify JIIM patients or to predict disease activity was not available. Very recently, we showed that serum IFN $\alpha$  concentrations correlated with disease activity in a cohort of JIIM patients by single molecule array (Simoa) digital ELISA technology assay (10). IFN-I is able to induce hundreds of interferon-stimulated genes (ISGs) (11). Among these ISGs, interferon-stimulated gene 15 (*ISG15*) is one of the most strongly and rapidly induced (12,13), one of the most overexpressed in JDM muscle tissue (14,15) and also selectively overexpressed in muscle from patients with adult dermatomyositis (DM) and perifascicular atrophy (16). The upregulation of IFN-I inducible transcripts is markedly and specifically increased in adult DM, compared to other myositis including anti-synthetase syndrome (ASS) and inclusion body myositis (IBM), and normal muscle (16–18). These results led us to investigate its specific contribution in pathophysiology of JIIM, mainly in JDM. In current work, first, we showed that muscle *ISG15* expression levels in children strikingly differentiated JIIM from other myopathies. In a second step, we showed that *ISG15* and other IFN-I-induced genes were differentially expressed according to JDM subtypes defined by MSAs. Protein *ISG15* expression is expressed differentially according to the MSAs, consistent with the gene results. Finally, we showed that, in the context of IFN-I pathway activation, the imbalance between *ISG15* (negative regulator) and positive regulators correlates with the severity of muscle injuries and disease.

## **MATERIAL AND METHODS**

### **Patients and samples**

Patients were recruited in the reference centers for Rare Pediatric Inflammatory Diseases (RAISE/FAI2R) and for Rare Neuromuscular Diseases (Nord-Est-Ile de France/FILNEMUS). Among the 122 patients with JIIM followed from June 2010 to June 2019, were enrolled in the study the patients newly diagnosed with JIIM for whom muscle biopsy, serum sampling and all clinical data were available (n=36). They were classified respectively as presenting JDM (n=24) according to conventional clinico-pathological criteria (6) and JOM (n=12), according to Troyanov criteria (1-3). Other muscle diseases have been included, patients with Duchenne Muscular Dystrophy (DMD, n=10) and Congenital myopathies (CM, n=10) for whom the diagnosis was based on genetic analysis and characteristic histopathological finding. Deltoid muscle biopsies were performed in the context of routine care in diagnostic work-up. For each patient presenting with JIIM, serum was screened in the same laboratory for myositis specific and myositis associated antibodies (MSAs/MAAs) at disease onset using immunodot assay (Euroline Autoimmune Inflammatory Myopathies 16 Ag, Euroimmun, Lübeck, Germany). Muscle strength testing included manual muscle testing (MMT) and childhood myositis assessment scales (CMAS) during 48h following admission. In accordance with current French legislation and hospital research ethics committee (Approval #12-009 at CPP Ile-de-France IX), parents or legal representatives gave their written informed consent for the participation of the children to the study.

### **Myopathological study**

For each JIIM patient, 7 µm cryostat cross-sections from deltoid muscle samples were processed for routine histological staining and immunohistochemistry, as previously described (Extended methods are available from the corresponding author upon request) (6). Age-matched controls were patients referred to our center for myological evaluation but without clinical signs of neuromuscular diseases and in whom diagnostic workup was normal. All biopsies were reviewed blindly for clinical and MSAs data by two of us (CG, FJA), using the validated score tool for muscle biopsy evaluation in patients with JIIM (19). This score includes a total score and some

sub-scores (muscle fiber domain, vascular domain, inflammatory score). It includes the overall muscle damage scoring through visual analogue scale (VAS), and specific scoring of inflammation, vascular damage, myofiber injuries and connective tissue changes (score=0: no abnormality; score=10: most abnormal). According to European research projects on neuromuscular diseases (ENMC) some markers in addition to the minimal battery used to establish the validated score of severity in JDM are recommended for the characterization of dermatomyositis (4), the following additional stains have been performed. The expression of major histocompatibility complex (MHC)-class I (HLA-ABC) and MHC-II (HLA-DR/DQ/DP) was checked, abnormal expression being defined by sarcolemmal staining associated or not with intracellular staining. Complement activation was assessed by evaluation the deposition of C5b-9 complex (MAC; membrane attack complex) on endomysial capillary walls and on the sarcolemma of non-necrotic myofibers (6). Immunohistochemical staining of myxovirus resistance protein A (MxA) protein which is one of type I IFN-induced proteins, was performed as previously described as (20).

### **Real-Time Quantitative PCR**

Total RNA was extracted from biopsy muscle sample using TRIzol and analyzed by Nanodrop. RNA was converted into cDNA by SuperScript III Reverse Transcriptase using the Invitrogen kit (18080-044, Thermo Fisher Scientific). Gene expression was quantified by quantitative real-time PCR using TaqMan Master Mix (Applied Biosystems) and the following TaqMan probes: *ISG15* (Hs01921425\_s1), *USP18* (Hs00276441\_m1), *IFIH1* (Hs00223420\_m1), and *DDX58* (Hs01061436\_m1) as key marker of negative and positive regulation of IFN-I overexpression. Expression of each gene was normalized to TATA Box Protein TBP (Hs00427620\_m1). The real-time qPCR was performed with the StepOnePlus real-time PCR system (Applied Biosystems). The relative quantification value was calculated using the 2- $\Delta\Delta$ CT method. Results are reported as relative gene expression (2- $\Delta\Delta$ CT) using healthy control muscle sample as reference. RT-qPCR in whole blood was performed as described in (21).

### **Western Blot analysis**

Western blot experiments were performed using control (n=7) and JDM muscles (n=12) to analyze ISG15 (Interferon-induced 15kDa protein or Ubiquitin-like protein ISG15) and USP18 (Ubiquitin-specific protease 18). Frozen muscles were cut and homogenized in lysis buffer (RIPA)

supplemented with protease inhibitor cocktail (1:100; Sigma P8340) and clarified by centrifugation. Proteins quantifications were performed by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) and an equal protein mass of 30ug in 10μL was subjected to NuPAGE™ 4-12 % Bis-Tris Midi Protein Gels (Invitrogen™, NP0335BOX) in Xcell4 Surelock tank (Life Technology SAS, WR0100) using NuPAGE™ MES SDS Running Buffer (20X) (Invitrogen™, NP000202). Protein transfer to polyvinylidene difluoride (PVDF) (Fisher Scientific, IB401032) membrane was performed using iBlot2 Dry Blotting System (Fisher Scientific, IB21001) and iBlot™ 2 Transfer Stacks (Invitrogen™, IB24001). Membranes were blocked in milk solution, then probed with mouse anti-ISG15 (F-9) (1:200; Santa Cruz, sc-166755), rabbit anti-USP18 (1:500; D4E7 Cell signaling) and rabbit anti-β-tubulin (9F3) (1:1000; #2128 Cell signaling) overnight at 4°C. Membranes were then washed and exposed 1 hour to HRP-conjugated goat anti-rabbit (1:5000; Santa Cruz, sc-2054) or anti-mouse (1:2500; PI-2000-1 ZC1212, Vector Laboratories) secondary antibodies. Proteins were visualized by a chemiluminescence assay kit (SuperSignal™ West Femto; Fisher scientific) using a c600 scanner (Azure Biosystems, Inc., Dublin, Ohio, USA) and signals were quantified using ImageJ software (V1.52s). To perform level expression comparison, the quantification of ISG15 and USP18 was divided by the β-tubulin quantification, the normalizing protein used in this study.

### **Statistical analysis**

The comparisons between identified histopathological subgroups for other variables were performed using the Fisher's exact test for qualitative variables and the Mann-Whitney U test for quantitative variables. Pearson's Chi-squared was used to calculate clinical association between two categorical variables (JDM/JOM groups and histological markers). For qPCR and western-blot analysis between the different groups, all statistical analysis and graphs were performed using GraphPad Prism® Software (version 8.0). The results are presented as the median with range and examined by Mann-Whitney test, nonparametric equivalent to the independent t-test, or Wilcoxon test when there were a control group with the same values. Kruskal-Wallis test, Dunn's multiple comparisons test were used for the multiple-comparisons. Correlations have been calculated between two numerical values, *ISG15* level and performance score (MMT, CMAS) or histological score (VAS, JDM scoring tool). The Pearson correlation method was used to calculate the linear relationship. Differences were considered significant at  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*),  $p < 0.0001$  (\*\*\*\*).



## RESULTS

### Clinical and biological description of the JIIM cohort patients

Clinical and biological characteristics of the patients are listed in supplementary data (Table S1). Thirty-six patients with JIIM (sex ratio: 26F:10M; median age at disease onset 9.7 years [range 34m-179m]) were included. They were diagnosed JDM (n=24; 67%) and JOM (n=12; 33%). Most frequently found MSAs was anti-NXP2 and exclusively in JDM patients (n=8/24; 33%). Anti-MDA5 was found in both JDM and JOM patients (5/24 vs 6/12, respectively; p=0.07). VAS scores for histological muscle severity were significantly lower in JOM patients (p<0.01). Expression of MxA protein was identified in 71.4% on JDM samples and in 22.2% on JOM samples (p=0.01) (Figure S1 A and B). Patients with NXP2(+) autoantibodies had strong MxA expression (100% level 3) whereas patients MDA5(+) had no or weak MxA expression (Figure S1C). Myofiber MHC-I overexpression (associated with IFN-1 upregulation) was observed in almost all patients (33/36), myofiber MHC-II expression (associated with IFN $\gamma$  upregulation) was observed in only 9 patients and significantly associated with the diagnosis of JOM (7/12 in JOM vs 2/24 in JDM; p=0.001). (Figure 1). In control muscles, as expected, myofibers did not express MHC-I or MHC-II (Controls with histologically normal muscle are available from the corresponding author upon request).

### Muscle *ISG15* expression is differentially expressed according to JIIM subtypes

*ISG15* muscular expression was markedly increased in JIIM compared to DMD (p<0.0001) and CM (p<0.0001) (Figure S2). *ISG15* expression levels were specifically more increased in JDM (median 188.0 fold) as compared to JOM (median 50.9 fold), p=0.02 (Figure 2); or other myopathies: JDM vs DMD (median 7.9 fold): p<0.0001; JDM vs CM (median 3.0 fold): p<0.0001 (Figure S2). Furthermore, we confirmed the IFN-I regulation in JDM pathophysiology at protein level. *ISG15* protein was abnormally expressed in JDM muscle compared to control muscles where the protein was absent (Figure 3A, B). Ubiquitin-specific protease 18 (USP18) is essential for *ISG15*-mediated downregulation of IFN-I signaling, by directly regulating the stability of this ubiquitin for binding to the IFN receptor (IFNAR2) (22–24). USP18 was also undetectable in all histologically normal muscles but was expressed in all JDM muscles (p=0.002, Wilcoxon one sample test) (Figure 3A, C). There was a positive correlation (p=0.01) and association between *ISG15* and USP18 protein expression profiles in human muscle from JDM patients (Figure S3).

## High expression level of *ISG15* in muscle discriminate MDA5(+) JDM patients from JDM patients with other MSAs

Based on the highest expression level of *ISG15* in muscle of JDM patients compared to other studied myopathies, including JOM (Figure S2), we decided to focus on this patient's group for the rest of this study.

First, we analyzed by qPCR the expression levels of IFN-I-inducible genes among JDM subgroups defined by MSAs (no MSAs, TIF1 $\gamma$ , NXP2, and MDA5). We found a similar expression level of IFN-I positive regulators *IFIH1* (encoding MDA5) and *DDX58* (encoding RIG-I) among JDM patients ( $p=0.28$  and  $p=0.54$ , respectively; Kruskal-Wallis test) (Figure 4A, B). Conversely, a differential expression level of IFN-I negative regulator *ISG15* was observed ( $p=0.006$ , Kruskal-Wallis test). MDA5(+) JDM patients displayed the highest muscle *ISG15* expression levels (median 1074.5 fold) as compared to median values observed in others JDM subgroups (no MSAs: 86.5; TIF1 $\gamma$ : 248.1; NXP2: 118.1 fold) (Figure 4C). Moreover, protein ISG15 expression was differentially expressed according to MSAs, a finding consistent with the results of gene expression. Similarly, MDA5(+) JDM patients have higher ISG15 protein levels than other patient subgroups (no MSAs  $p=0.03$ ; NXP2  $p=0.03$ , Mann Whitney test) (Figure 4D). Then, because MDA5 specific autoantibody can be expressed by both JDM and JOM patients, we examined the level of *ISG15* gene in JOM MDA5(+) patients compared to the JDM MDA5(+) patients (Figure S2). Muscle *ISG15* expression levels were also higher in MDA5(+) JDM patients compared to MDA5(+) JOM patients ( $p=0.008$ ), in favor of different pattern of muscle IFN-I pathways activation among MDA5(+) JDM patients (Figure 4D). Consequently, highest ratios of negative vs positive regulators of IFN-I pathway were observed for MDA5(+) JDM suggesting a better muscle-specific negative regulation of IFN-I pathway in those patients and indicating that among JDM subsets, higher muscle *ISG15* expression is a specificity of the JDM MDA5(+) patients. In contrast, muscle USP18 expression was similar in JDM subsets (Figure S3B).

We recently showed that among JDM patients, those with anti-MDA5 autoantibodies presented more frequently with higher systemic IFN $\alpha$  levels (21). *ISG15* being checked as a component of IFN score, we went back to the raw data of this cohort (21) to specifically analyze the blood ISG15 concentrations in these patients. Expectedly, the highest ISG15 titers were observed in MDA5(+) patients compared to the other subgroups (MDA5(+) vs no MSAs,  $p=0.018$ ; MDA5(+) vs TIF1 $\gamma$ (+),  $p=0.036$ ; MDA5(+) vs NXP2+,  $p=0.021$ ; data not shown).

## Association between muscle *ISG15* expression and muscle performances in JDM

Lastly, we analyzed the potential correlations between in one hand different markers of disease severity and in the other hand the level of muscular *ISG15* gene expression. Disease severity was assessed by muscle performance scores and histological characteristics. The score MMT and CMAS at onset were positively correlated with *ISG15* muscle expression ( $p=0.03$ ;  $p=0.02$ ). Therefore, the higher *ISG15* gene level is, the better the muscle performance scores. (Figure 5A, B). Similarly, *ISG15* expression levels negatively correlated with the severity of histological changes, as assessed by inflammatory and vascular subscores ( $p=0.0015$  and  $p=0.04$ , respectively) (Figure 5C, D), overall muscle damage scoring through visual analogue scale (VAS) and total score (Figure 5E, F) ( $p=0.004$ , and  $p=0.0006$ , respectively).

## DISCUSSION

Juvenile idiopathic inflammatory myopathies (JIIM) are autoimmune diseases gathering dermatomyositis and overlap myositis. Each of them presents a great clinical heterogeneity, as much for their autoimmune profile as for their myopathological pattern. Understanding these conditions, specially what makes them different and what they have in common, provides a potential insight into differential pathophysiological mechanisms. This study focused on the regulation of type-I interferons (IFN-I) in the pathophysiology of JIIM. *ISG15* is a key negative regulator of IFN-I pathway and one of the most strongly upregulated in muscle tissue among all interferon-stimulated genes in dermatomyositis. We investigated whether quantifying its intramuscular expression could help to diagnose and classify JIIM, and how the level of *ISG15* expression may condition JDM clinical heterogeneity. From our results, it appears that muscle *ISG15* allowed sharply distinguishing JIIM from non-immune myopathies, and among JIIM, JDM from JOM. Importantly, we showed that *ISG15* muscle expression in JDM inversely correlated with the severity of the disease. This latter finding sheds new light on the significance of IFN-I pathway regulation in the course of dermatomyositis.

JIIM are highly heterogeneous disease in term of severity, tissue involvement and response to treatment. In autoimmune conditions, when patients demonstrate a sustained overexpression of type I interferon (IFN-I ie  $IFN\alpha/\beta$ ) signaling, the disease is considered as a multifactorial type I interferonopathy. IFN-I pathway dysregulation is acknowledged as a key factor for JDM/ adult DM pathogenesis, and has been shown to correlate with disease severity (25). Marked IFN-I

inducible gene overexpression have been identified *in vivo* in muscle, skin, circulating leukocytes and *in vitro* in myogenic precursor cells (MPCs) and endothelial cell isolated from patients (9,15,17,18,26,27). Among IFN-I inducible genes, muscle *ISG15* expression level was proposed to be used alone to reliably quantify the activation of the IFN-I pathway in adult inflammatory myositis (13). Our work supported similar view in JIIM patients and showed that highest muscle *ISG15* expression levels were selectively found in JDM patients. Muscle *ISG15* RT-qPCR may be considered as an additional biomarker for the discrimination of JIIM from non-immune myopathies, and among JIIM, JDM from JOM. Nowadays, MSAs have an important role in the classification scheme of IIM (4). Interestingly, in our cohort, MDA5 autoantibodies were detected in both JDM and JOM patients, but *ISG15* gene expression was higher in JDM MDA5(+) patients than JOM MDA5(+) patients. These results point out that a group of patients defined by the same type of autoantibody may present potential pathophysiological differences. So, it seems important to take into account all the clinical, biological and histopathological features for classify JIIM patients as accurately as possible. In this perspective, *ISG15* expression quantification could be helpful. However, prospective studies are still required to determine cut off values that will reliably guide the sub-stratification of JIIM subtypes.

The exact role of IFN-I in the JIIM pathophysiology is still not fully understood. Aouizerate *et al*, suggested that the more severe is muscle involvement, as typically observed in NXP2(+) DM/JDM patients, the higher IFN-I up-regulation (6). Recent data from muscle gene expression profiling studies demonstrated a prominent IFN-I signature in adult DM but failed to distinguish quantitative IFN-I dysregulations from patients with different DM-specific autoantibodies or associations with markers of clinical and histological disease activity/severity (13,17). We recently showed that among JIIM patients, those with anti-MDA5 autoantibodies presented more frequently with higher systemic IFN $\alpha$  levels (21). Expectedly, the highest blood *ISG15* titers were observed in MDA5(+) patients compared to the other subgroups, a finding reinforcing our present results.

IFN-I signaling is controlled at multiple levels by regulators. For example, *IFIH1* and *DDX58* code for nucleic acid receptor involved in the promotion of IFN-I signaling while *ISG15* and *USP18* inhibit IFNAR signaling. Our data showed that IFN-I positive upregulation (*IFIH1*, *DDX58*) was remarkably similar among JDM patients whatever the MSAs, a feature in accordance with previous results (28,29). In contrast, negative regulators strikingly differed according to the

subgroup, MDA5(+) patients having higher levels than others. As MDA5(+) JDM patients have more circulating IFN-I (21), IFN-I positive upregulation could only reflect more ISG in general. However, the specific IFN-I negative upregulation indicates that among JDM subsets, muscle *ISG15* overexpression is a specificity of the JDM MDA5(+) patients.

Our MDA5(+) JDM patients had milder muscle involvement compared to others, a well-known feature of this JDM subtype (21). In agreement with these observations, muscle *ISG15* expression levels in JDM negatively correlated with all scores used for quantifying the severity of muscle injuries. Most importantly, in JDM, muscle *ISG15* expression levels positively correlated with motor performance, as assessed by functional scales and muscle testing (CMAS, MMT). Muscle and blood *ISG15* transcription inversely correlated to muscle severity and could be complementary in order to assess JIIM.

In conclusion, our results confirm the significance of *ISG15* for stratifying JIIM. In the context of diagnostic workup, it could increase the diagnostic performance of muscle biopsy. Since histological findings sometimes may remain poorly conclusive, we believe that introducing the RT-qPCR quantification of muscle *ISG15* expression in the pathological panel could be of great help for routine stratification in combination with MSAs. Besides its contribution for JIIM diagnosis, our work provides new findings on JDM pathophysiology. Indeed, our results suggest that the meaning of IFN-I pathway activation depends on the positive/negative regulation balance. At last but not least, prominent IFN-I negative regulation correlates with milder tissue injuries, suggesting a potential therapeutic interest of regulating downstream IFN-I signaling pathways.

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## FIGURE LEGENDS

### **Figure 1: Representative immunohistochemical features of JIIM**

MHC-I (A, D, G), MHC-II (B, E, H), and NCAM (C, F, I). Immunoperoxidase technique performed on 7- $\mu$ m cryosections (see supplemental material for details).

A-C: NXP2(+) JDM with severe muscle involvement; A: Ubiquitous strong myofiber expression of MHC-I with perifascicular reinforcement in atrophic fibers; B: Lack of MHC-II myofiber expression, only capillaries and leukocytes being stained; immunostaining also disclosed marked capillary loss; C: Strong NCAM expression by numerous myofiber either perifascicular or grouped in large areas, witnessing the severity of muscle involvement.

D-F: MDA5(+) JDM with mild muscle involvement; D: Ubiquitous myofiber expression of MHC-I with perifascicular reinforcement contrasting with the lack of conspicuous myofiber injury; E: Lack of MHC-II myofiber expression, only capillaries being stained; immunostaining also disclosed slight capillary loss in perifascicular areas; F: Presence of NCAM-expressing myofibers, mainly perifascicular or grouped, witnessing myofiber involvement.

G-I: MDA5(+) JOM; G: Ubiquitous MHC-I expression by myofibers with heterogenous intensity but without obvious perifascicular reinforcement; H: Perifascicular and focal MHC-II myofiber expression, characteristic of OM; I: NCAM-expressing regenerating fiber are either sparse and multifocal or grouped in small clusters.

### **Figure 2: Muscle *ISG15* expression is differentially expressed according to JIIM subtypes.**

The graph shows the fold change of *ISG15* in JIIM, by distinguishing JOM (n=12) and JDM patients (n=24). Statistics performed with Mann-Whitney test (\*, p<0.05).

### **Figure 3: *ISG15* and *USP18* protein were undetectable in all histologically normal muscles but was expressed in all JDM muscles.**

Representative Western blot results (A) and graphic presentation showed *ISG15* protein expression (B) and *USP18* protein expression (C) in different groups of children muscles as indicated, where one point corresponds to one patient. The relative protein levels of *ISG15* and *USP18* were determined after normalization with  $\beta$ -tubulin. Statistics performed with one sample

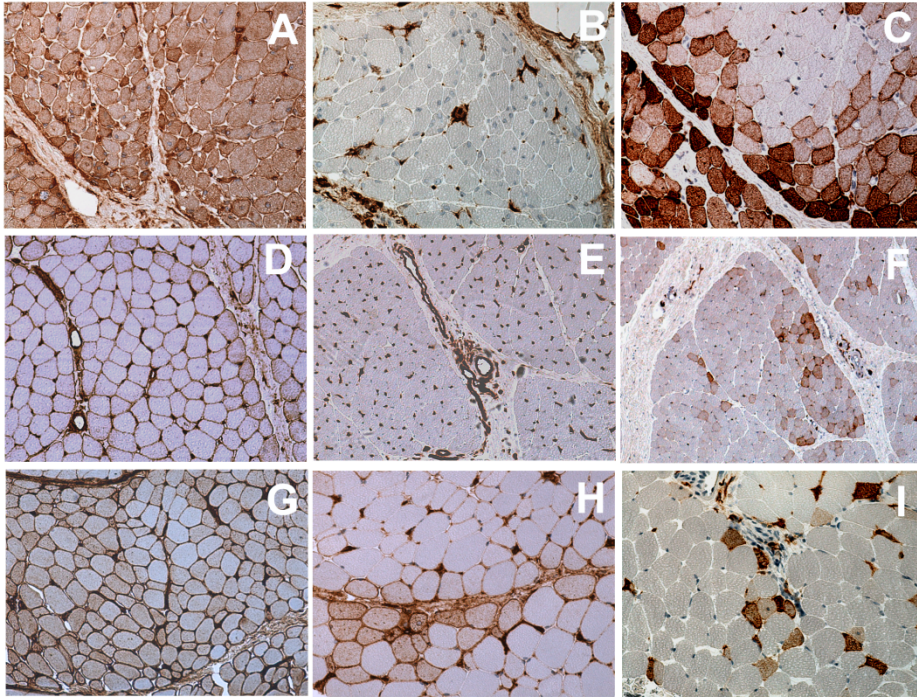
Wilcoxon test between control (n=7) and JDM group (n=12) (\*\*, p value=0.002).

**Figure 4: MDA5(+) JDM patients had a more efficient negative regulation of muscle IFN-I pathway signaling.**

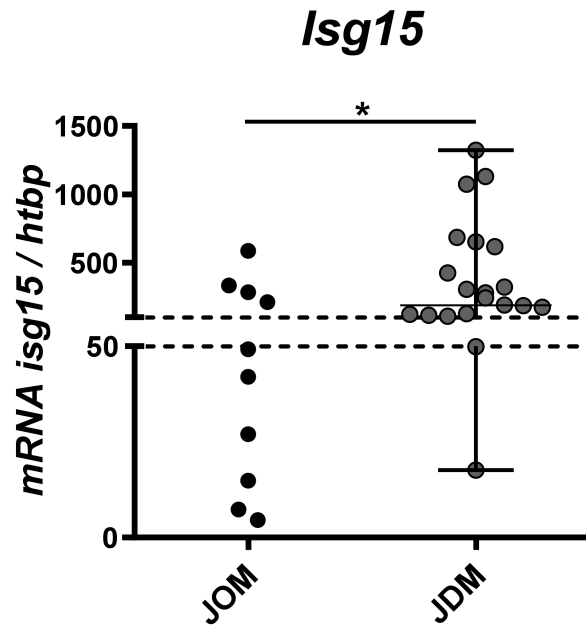
A-C: The positive regulators of IFN-I, *Ifih1* (A), *Ddx58* (B) and *Isg15* (C) for the negative regulation have been quantified by RT-qPCR. Graph shows the fold change in the subgroups of JDM patients distinguished by their autoantibodies: no MSAs (n=7), TIF1 $\gamma$  (n=4), NXP2 (n=8), MDA5 (n=5). D: Comparison of ISG15 protein expression between control muscles (n=7), JDM no MSAs (n=4), JDM NXP2(+) (n=4), and JDM MDA5(+) (n=4) patients. Statistics performed with Mann-Whitney test (ns; \*, p<0.05; \*\*, p<0.01).

**Figure 5: Association between muscle *ISG15* expression and muscle performances in JDM.**

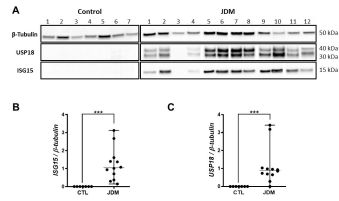
Clinical severity at disease onset (A, B, C) and histological muscle (D, E, F) parameters have been evaluated in JDM patients. Positive correlations between *ISG15* mRNA level and performance score (MMT, CMAS). Negative correlations between *ISG15* mRNA level and muscle damage (inflammatory score, vascular scores, VAS, total score). Total score is obtained by adding inflammatory and vascular score. The Pearson correlation method was used to calculate the linear relationship (\*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001).



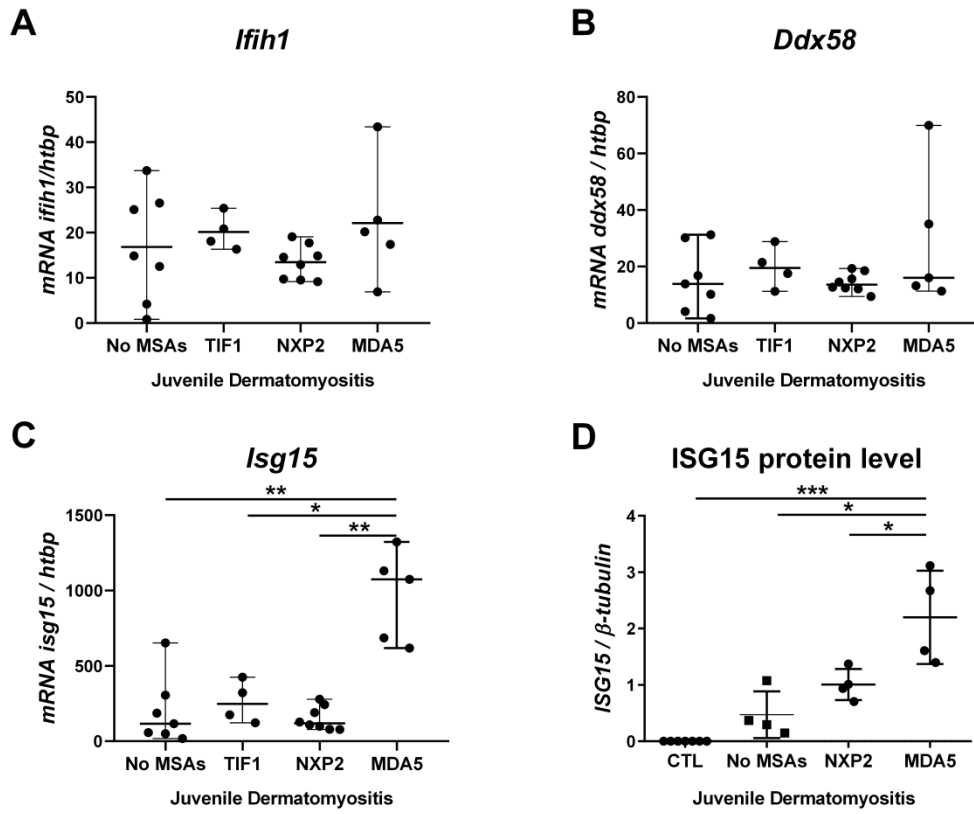
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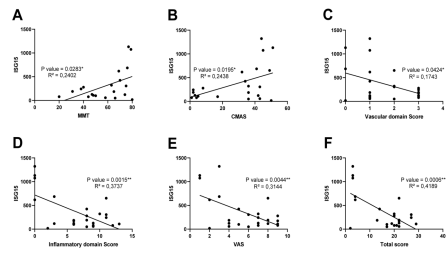
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