

An exploratory study of circulating cytokines and chemokines in patients with muscle disorders proposes CD40L and CCL5 represent general disease markers while CXCL10 differentiates between patients with an autoimmune myositis

Boel De Paep^{a,*}, Ken R. Bracke^b, Jan L. De Bleeker^a

^a Department of Neurology and Neuromuscular Reference Center, Ghent University Hospital, Ghent, Belgium

^b Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium

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ABSTRACT

Discriminating an autoimmune myositis from other disorders and subtyping of patient groups within this heterogeneous group of conditions remain diagnostic challenges. In our study we explored the potential of cytokine and chemokine typing in patient sera as an addition to the expanding set of blood-accessible diagnostic biomarkers available today. We selected sets of ten patients within well-characterized disease groups representing healthy controls, and patients with hereditary muscular dystrophies, immune-mediated necrotizing myopathy (IMNM) and sporadic inclusion body myositis (IBM). Prescreening using proteome arrays singled out three biomarker candidates, being the cytokine CD40L, and chemokines CXCL10 and CCL5. Enzyme-linked immunosorbent assays showed all three markers to be elevated in muscle disease irrespective of patient subgroup. CXCL10 levels on the other hand were higher in autoimmune myositis only, and levels were significantly higher in IBM compared to IMNM. The strong CXCL10 expression observed in the auto-aggressive inflammatory cells within IBM muscle tissues possibly represents a major source of circulating CXCL10. We conclude that CXCL10 levels could represent a convenient marker for autoimmune myositis indicative of patient subgroups.

Introduction

Myositis of autoimmune origin is a heterogeneous group of rare muscle conditions with varying clinical and myopathological characteristics. The major subgroups recognized today are immune-mediated necrotizing myopathy (IMNM), dermatomyositis, sporadic inclusion body myositis (IBM), polymyositis and myositis as part of the anti-synthetase syndrome [1]. Subtyping patients is highly relevant for disease management, as different subgroups require adapted treatment, and to counsel patient on disease prognosis. The subgroups of IMNM and IBM present in older patients and, due to the aging population and the widespread use of statins as cholesterol-lowering drugs, numbers of patients encountered in the clinic are steadily on the rise.

IMNM typically manifests with subacute predominantly proximal limb muscle weakness. Muscle biopsies often reveal no or only minimal inflammation and prominent muscle fiber necrosis and regeneration. Nonetheless, IMNM is irrefutably mediated by autoimmune responses

that are mostly driven by classically activated macrophage-mediated reactions. Disease phenotype, severity and treatment response can vary considerably, warranting further subtyping of IMNM via auto-antibody profiling [2]. Auto-antibodies directed against 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), an enzyme that catalyzes the conversion of HMG-CoA to the cholesterol precursor mevalonate, may be present. Part of these patients had been taking statins as a cholesterol-lowering drug [3]. The others are idiopathic or more rarely paraneoplastic. Presence of anti-HMGCR autoantibodies usually associates with moderate muscle weakness. Patients with auto-antibodies directed against signal recognition particle (SRP) usually display more severe and rapidly progressing muscle weakness. Autoantibody-negative IMNM forms a third subtype of patients, of whom a minority are paraneoplastic [4]. Standard treatment for IMNM are high doses of corticosteroids, yet therapeutic success varies between subtypes and individual patients. Some other immunomodulatory or immunosuppressive treatments are also used.

* Corresponding author at: Neuromuscular Reference Center, Ghent University Hospital, Route 830 - 3K5, Corneel Heymanslaan 10, 9000 Ghent, Belgium.

E-mail address: boel.depaepe@ugent.be (B. De Paep).

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IBM patients present with slowly progressive proximal and distal muscle weakness most often at an advanced age. Within the muscle tissue, nonnecrotic muscle fibers become actively invaded by auto-aggressive cytotoxic T-cells and macrophages. Muscle fibers additionally develop degenerative changes, with rimmed vacuoles and inclusions containing aggregates of ectopic proteins [5]. The disorder generally does not respond to conventional immunosuppressive treatment, and there is no standard cure for IBM at this time.

Diagnosing autoimmune myositis without the need for invasive procedures such as taking a muscle biopsy, remain a priority for these pathologies, aimed to subdivide patients into subtypes relevant to predict disease progression and patients' therapeutic response. In this respect, differences in the immunopathogenic characteristics of autoimmune myositis subgroups could be reflected by the differential inflammatory profile in the blood. To explore this possibility, we studied levels of cytokines and chemotactic cytokines termed chemokines in patient serum in a selection of well-characterized patients of two distinctive subgroups. IMNM on the one hand is characterized by low numbers of inflammatory cells and prominent muscle fiber necrosis, while on the other hand IBM muscle fibers are actively invaded by auto-aggressive immune cells and muscle fibers display degenerative characteristics. We also compared cytokine and chemokine levels to those present in sera from patients diagnosed with a hereditary muscular dystrophy. In the latter, the underlying genetic deficiency causes secondary inflammatory reactions in the skeletal muscle tissue that can be mistaken for autoimmune myositis.

Materials & methods

Patients and patient material

This retrospective study included muscle biopsies and sera from an established cohort of autoimmune myositis patients that had been clinically, serologically and myopathologically diagnosed, and patients with fully characterized hereditary muscle diseases, of which detailed information is given in Table 1. Controls were ten samples from healthy subjects obtained from Zenbio (Durham, NC). Sampling adhered to ethical and privacy regulations, all patients consented to participate to the study of which procedures had been approved by the Ghent University Hospital Ethics Committee (B670201836756, B670201938779).

Protein arrays

Cytokine expression was screened in six healthy control sera and three samples each from patients diagnosed with Becker muscular dystrophy (BMD) (HMD1,2,4), limb girdle muscular dystrophy (LGMD) (HMD5-7) and patients with an autoimmune myositis (IBM1, IBM5, and a patient diagnosed with polymyositis), using the Protein profiler Human XL cytokine arrays according to the manufacturer's specifications (R&D Systems - Bio-Techne, Minneapolis, MN). Protein spots were visualized with the Chemidoc and spot volumes were analyzed with Image Lab 6.0 software via the linear quantity regression method with local background subtraction (Bio-rad, Hercules, CA), and expressed relative to the mean level of six reference spots.

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) were performed with the Human CD40 ligand/TNFSF5, CCL5/RANTES and CXCL10/IP-10 Quantikine ELISA kits from R&D Systems (Bio-Techne, Abingdon, UK) according to the manufacturer's specifications. Values were calculated as the mean of duplicates and two dilutions tested, and reported as mean \pm sd. Shapiro-Wilk test showed measurement variables did not meet the normality assumption, hence significance of values obtained was tested with Kruskal-Wallis pairwise comparisons adjusted via Bonferroni correction for multiple tests. Wilcoxon Pearson's correlation

Table 1

List of patient clinical data.

#	DIAGNOSIS	GENDER	AGE	CK
C1	Healthy control	F	36	ND
C2	Healthy control	F	22	ND
C3	Healthy control	M	40	ND
C4	Healthy control	F	24	ND
C5	Healthy control	F	23	ND
C6	Healthy control	M	37	ND
C7	Healthy control	F	28	ND
C8	Healthy control	M	41	ND
C9	Healthy control	F	61	ND
C10	Healthy control	F	25	ND
IMNM1	IMNM; anti-HMGCR positive	F	67	1417
IMNM2	statin-induced IMNM; anti-SAE positive	M	67	4746
IMNM3	IMNM; auto-antibody negative	F	74	5500
IMNM4	IMNM; anti-HMGCR positive	F	60	5749
IMNM5	IMNM; anti-SRP positive	F	56	6144
IMNM6	IMNM; anti-Rho52 positive	F	68	150
IMNM7	IMNM; anti-PM-Scl75 positive	F	53	233
IMNM8	IMNM; auto-antibody negative	M	46	10,264
IMNM9	IMNM; autoantibody negative	M	57	400
IMNM10	IMNM; autoantibody negative	F	53	966
IBM1	IBM; anti-cN-1A positive	M	62	513
IBM2	IBM; auto-antibody negative	F	61	717
IBM3	IBM; anti-cN-1A positive	M	76	186
IBM4	IBM; auto-antibody negative	F	75	290
IBM5	IBM; anti-cN-1A positive	M	73	170
IBM6	IBM; auto-antibody negative	F	82	160
IBM7	IBM; auto-antibody negative	F	70	658
IBM8	IBM; anti-cN-1A positive	M	72	128
IBM9	IBM; auto-antibodies not determined	M	70	118
IBM10	IBM; auto-antibodies not determined	M	73	303
HMD1	BMD; DMD deletion exons 48–52	M	63	600
HMD2	BMD; DMD deletion exons 45–48	M	42	1108
HMD3	BMD; DMD deletion exons 45–48	M	29	1412
HMD4	BMD; DMD deletion exons 45–48	M	44	587
HMD5	LGMDR; FKRK homozygous c.826C > A	M	52	1968
HMD6	LGMDR; ANO5 homozygous c.191dupA	F	30	1557
HMD7	LGMDR; ANO5 homozygous c.191dupA	M	26	16,239
HMD8	LGMDR; BVES homozygous c.1A > G	M	39	4855
HMD9	X-linked EMD Emery-Dreifuss muscular dystrophy	M	32	2250
HMD10	FSHD type 1; deletion of chromosomal D4Z4 tandem repeats at the 4q35 location	F	31	366

Abbreviations: Becker muscular dystrophy (BMD), control (C), cytoplasmic 5'-nucleotidase 1A (cN1A), creatine kinase (CK), facioscapulohumeral muscular dystrophy (FSHD), female (F), hereditary muscle disease (HMD), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), sporadic inclusion body myositis (IBM), immune-mediated necrotizing myopathy (IMNM), recessive limb girdle muscular dystrophy (LGMDR), male (M), not determined (ND), small ubiquitin-like modifier-1 activating enzyme (SAE), signal recognition particle (SRP). CK is given in units per liter, age in years.

coefficients were calculated to analyze a possible association between cytokine levels and clinical characteristics. All analyses were done with SPSS software version 27 (IBM, New York, NY).

Immunohistochemical staining

8 μ m frozen sections were fixed in acetone and blocked in phosphate buffered saline with 5% bovine serum albumin and human and donkey serum added. Incubation with primary antibodies (4 μ g/ml mouse monoclonal anti-CD40L, sc-374635, Santa Cruz Biotechnology, Dallas, TX; 2 μ g/ml mouse monoclonal anti-CXCL10, MCA1693, Biorad, Hercules, CA; 10 μ g/ml mouse monoclonal anti-CCL5, MAB1036, Merck Millipore, Darmstadt, Germany) was carried out in the same solution, for 2 h at room temperature. Staining was achieved with the labelled streptavidin biotin horseradish peroxidase kit (Agilent, Santa Clara, CA) followed by chromogenic detection using 3,3'-diaminobenzidine (DAB) as the substrate, counterstained with hematoxylin.

Results

The preliminary proteome profiler arrays allowed simultaneous screening of 105 cytokines in sera of a smaller selection of patients and comparison with sera from healthy subjects. Out of the analyses came CD40L, CXCL10 and CCL5 as most promising candidate biomarkers. Low levels of CCL5 were present in controls, which were elevated in all patient subgroups. The levels of CD40L and CXCL10 appeared selectively elevated in patients with an autoimmune myositis (Table S1).

Subsequently, CD40L, CXCL10 and CCL5 protein levels were determined in sera from 30 selected patients (Table 1) using enzyme-linked

immunosorbent assays (Fig. 1A). CD40L levels were significantly elevated in IMNM, IBM ($p < 0.001$), and HMD ($p = 0.001$) compared to healthy controls. CXCL10 levels were significantly higher in IMNM ($p = 0.007$) and IBM ($p < 0.001$) than in healthy controls, with levels significantly higher in IBM compared to IMNM ($p = 0.04$). CCL5 levels were significantly elevated in IMNM, IBM and HMD ($p < 0.001$) compared to healthy controls. Pearson's correlation coefficients evaluated correlations between cytokines and patients' clinical characteristics (Supplementary Table S2, Fig. 1B). CD40L displayed a strong negative correlation with age in HMD (-0.8), with CD40L levels significantly higher in patients under 35 years of age ($n = 5$) compared to patients

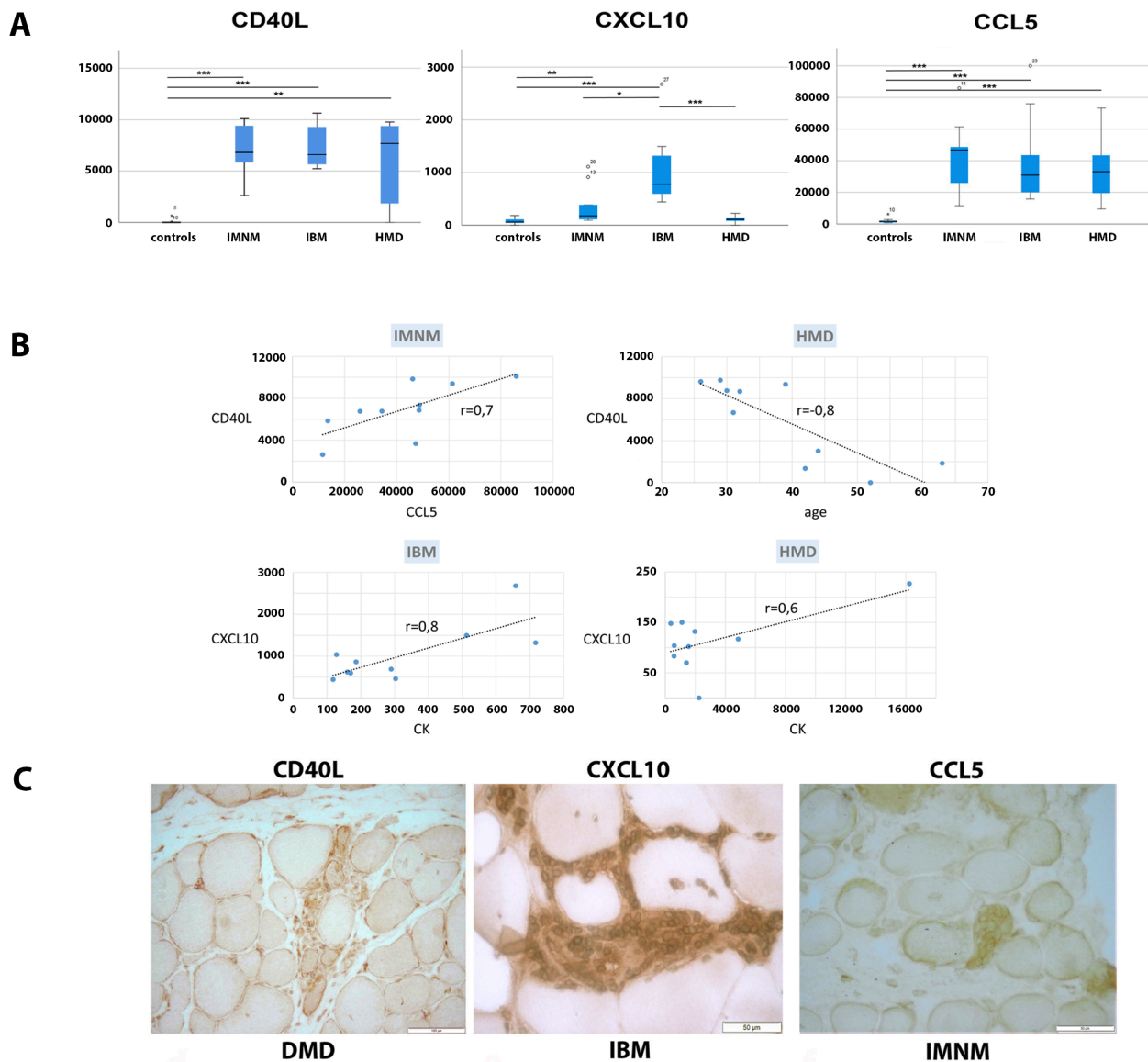


Fig. 1. CD40L, CXCL10 and CCL5 cytokine levels in patients with muscle disorders. A: Graphic representation of circulating cytokine levels. Levels of CD40L, CXCL10 and CCL5 determined using enzyme-linked immunosorbent assays and expressed in pg/ml serum are given in healthy controls, and in patients diagnosed with immune-mediated necrotizing myopathy (IMNM), sporadic inclusion body myositis (IBM), and hereditary muscular diseases (HMD). Kruskal-Wallis pairwise comparison between groups identified significant differences between diagnostic groups, with p-values as indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B: Correlations between cytokines and clinical features in muscle disorders. Strong positive correlations were found between CD40L and CCL5 levels in immune-mediated necrotizing myopathy (IMNM), between CD40L levels and age in hereditary muscle disorders (HMD), and between CXCL10 levels and creation kinase (CK) in sporadic inclusion body myositis (IBM); a strong negative correlation was observed between CXCL10 and CK in HMD, as indicated by respective Pearson's correlation coefficients (r). C: Cytokine immunohistochemical staining in skeletal muscle tissues. Cytokine staining was visualized with the 3,3'-diaminobenzidine chromogen (brown) and cell nuclei were counterstained with hematoxylin (blue). CD40L is observed in inflammatory cells and on muscle fibers in proximity of immune infiltrates in the muscle tissue from a Duchenne muscular dystrophy (DMD) patient. CXCL10 is expressed by the majority of immune cells surrounding and invading nonnecrotic muscle fibers in muscle tissue from a sporadic inclusion body myositis (IBM) patient. CCL5 is localized to the inflammatory cells inside a necrotic muscle fiber in muscle tissue from a patient diagnosed with immune-mediated necrotizing myopathy (IMNM). Scale bars: 100 μ m (CD40L) and 50 μ m (CXCL10, CCL5).

over 35 years of age ($n = 5$) ($p = 0.02$). Correlation of CD40L levels with age was only weak in healthy controls ($r = -0.3$), and did not present itself in autoimmune myositis, in which all patients were older than 45 (IMNM) and 60 (IBM) years of age. CXCL10 and blood CK levels correlated strongly in IBM ($r = 0.8$) and HMD ($r = 0.6$), but not in IMNM. Correlations between individual cytokines were inconspicuous, except for a strong positive correlation between CD40L and CCL5 levels in IMNM sera ($r = 0.7$).

Immunohistochemical staining showed muscle expression of CD40L, CXCL10 and CCL5 (Fig. 1C). Moderate levels of CD40L were found present in muscle-infiltrating immune cells in all diagnostic subgroups and muscle fibers near the inflammatory infiltrates. CXCL10 was absent from the muscle fibers in IBM and IMNM, and occasionally encountered in necrotic fibers of muscular dystrophy sections. Part of muscle-infiltrating immune cells were CXCL10 positive, with strongest expression observed in the inflammatory cells surrounding and invading nonnecrotic muscle fibers present in IBM muscle tissues. CCL5 was mostly absent from IBM muscle fibers, but more frequently observed in the necrotic muscle fibers in muscular dystrophy and IMNM tissues. Moderate CCL5 staining of inflammatory cells could be observed in all diagnostic groups.

Discussion

Accurate diagnosis of muscle disorders is not always straightforward, and requires a combination of clinical evaluation and pathologic and genetic analyses. The clinical picture of adult-onset hereditary muscular dystrophy may resemble IMNM or IBM, and uncertain genetic diagnosis often awaits confirmation by protein-based diagnostics. The need persists for improvement, especially to reduce the diagnostic delay and prevent exposing patients unnecessarily to inappropriate hence ineffective and potentially harmful therapies. In the presented study we developed the strategy of mining for circulating diagnostic biomarkers by first exploring a vast number of cytokines in a limited number of patients, and subsequently quantifying those cytokines and chemokines that were singled out in a set of well-characterized patients.

Determining cytokine and chemokine levels in a blood sample represents a convenient approach for diagnosing muscle disorders with important advantages. Compared to a muscle biopsy, blood sampling is a more convenient diagnostic procedure, as it is minimally invasive, simple and rapid. Blood samples are routinely available, and serum CK levels especially represent an excellent diagnostic marker indicative of disease activity that has been in use in the clinic for decades. CK leaks into the circulation from damaged muscle, however, levels are dependent on gender, muscle mass, and physical activity. Patients with muscular dystrophy and IMNM often display highly elevated CK levels, yet in IBM patients levels can be only slightly elevated or normal and are independent of muscle weakness or disease severity. Diagnostic testing for autoantibodies is also routinely performed on a blood sample, as auto-antibody profiles are of high diagnostic performance in autoimmune myositis to identify distinct clinical patient subsets.

Muscle disorders of autoimmune and of genetic origin share the pathogenic mechanism of infiltration by activated immune cells in the skeletal muscle tissue. However, as a particular variety of immune cells accumulate in muscle disease subgroups, it is warranted to decipher their involvements and identify those individual cytokines with key pathogenic functions. Advanced knowledge of cytokine-mediated mechanisms and interactions will help to further elucidate pathophysiological pathways, mapping out the differences in innate and adaptive, and primary and secondary immune responses underlying these complex human disorders. These shared and selective involvements of cytokines and chemokines are illustrated by the significant increase of CD40L and CCL5 we report in autoimmune myositis and muscular dystrophy patients alike, while CXCL10 is increased only in the former. CXCL10 is a chemokine known to be involved in autoimmune disease [6,7] and our results are in line with previous studies. High CXCL10

serum levels have been reported in IBM before, with CXCL10 among the ten cytokines identified as diagnostic markers able to discern patients from healthy controls and from other neuromuscular disorders [8]. A large study describing plasma levels in a hundred patients belonging to different subgroups of autoimmune myositis, which included IMNM and IBM, also reported CXCL10 levels could distinguish patients from those with a hereditary muscle disorder with high sensitivity and specificity [9]. In addition, sera of dermatomyositis and polymyositis patients display highly elevated levels of circulating CXCL10 [10–12]. However, comparisons between the different subgroups of autoimmune myositis have not been made. In this exploratory study, we report CXCL10 serum levels to be significantly higher in IBM patients as compared to patients diagnosed with IMNM. The muscle fibers themselves are a potent source of circulating inflammatory factors in response to tissue damage and inflammation termed myokines, which allow active modulation of the pathogenesis of myositis. Muscle fibers can be induced to produce a broad spectrum of factors including cytokines (transforming growth factor- β (TGF- β), interleukin (IL)-6, IL-15, IL-18) and chemokines (CXCL10, CCL2, CCL4, CCL5, CCL20) [13,14]. The higher levels in IBM compared to the other patient groups could be explained by more prominent accumulation of muscle-infiltrating inflammatory cells in IBM, which display pronounced CXCL10 staining [15] less frequently observed in inflammatory cells in muscular dystrophy tissues [16]. Strongest CXCL10 expression could be shown in the CD68+ and CD3+ cells actively invading nonnecrotic muscle fibers [17], a diagnostic feature observed in IBM and polymyositis. Our observation of significantly higher CXCL10 levels in IBM compared to IMNM thus points to CXCL10 as a potential circulating marker for overt inflammation and active invasion of muscle fibers, which can only be determined by taking an invasive muscle biopsy. This observation needs to be confirmed in larger cohorts of patients that include other patient subgroups.

To conclude, we propose circulating cytokines and chemokines may be developed further as multi-biomarkers for muscle disorders, complementing the diagnostic arsenal of patient-friendly blood sampling already in place today for determining muscle enzymes and autoantibodies. Cytokine profiling may orient diagnosis toward or away from a genetic muscle disorder or autoimmune myositis. CXCL10 surfaces as a biomarker which could be developed further to diagnose autoimmune myositis, and the detailed description of cytokine and chemokine profiles may offer new insight into the complex immunopathogenesis of this heterogeneous group of inflammatory muscle disorders.

CRediT authorship contribution statement

Boel De Paepe: Conceptualization, Project administration, Methodology, Formal analysis, Visualization, Writing – original draft. **Ken R. Bracke:** Resources, Supervision, Writing – review & editing. **Jan L. De Bleecker:** Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cytox.2022.100063>.

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