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Muscle Microdialysis to Investigate Inflammatory Biomarkers in Facioscapulohumeral Muscular Dystrophy

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Abstract Recent progresses in the understanding of facioscapulohumeral muscular dystrophy (FSHD) genetics opened the way to the development of targeted therapies. However, knowledge about pathophysiology of muscle damage is still limited and there is increasing need to identify biomarkers of disease activity in the perspective of clinical trial readiness.

We analyzed inflammatory mediators in the interstitial fluid of muscles with different MRI signal in FSHD patients, comparing muscles displaying early lesions on short-tau inversion recovery (STIR) sequences with normal ones. Patients with one T1-weighted normal and STIR hyperintense (STIR+) and contralateral T1-weighted and STIR normal (STIR-) lower limb muscle were asked to enter the study. Twelve consecutive patients, five controls, and one non-penetrant gene carrier underwent prolonged muscle microdialysis with high cut-off membranes. Microdialysates were analyzed using xMAP technology with a wide panel for cytokines, chemokines, and growth factors. A small number of inflammatory mediators were dysregulated in STIR+ versus STIR- and control muscles: CXCL13, upregulated in STIR+ muscles compared with controls ($p < 0.01$); CXCL5, downregulated in STIR+ compared with STIR- muscles ($p < 0.05$); and G-CSF, downregulated in STIR+ muscles compared with controls ($p < 0.05$). CXCL13 was also upregulated in the STIR+ muscles compared with the contralateral STIR- muscles of the same patient ($p < 0.01$).

These results support the evidence of a selective inflammatory process taking place in STIR+ FSHD muscles. The application of microdialysis could provide insights on novel mechanisms involved in muscle damage in FSHD and in other myopathies. Further studies are needed to validate these investigated molecules as tissue and circulating biomarkers.

Keywords Facioscapulohumeral muscular dystrophy · FSHD · Microdialysis · CXCL13 · Cytokines · Biomarkers

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is an inherited myopathy whose genetic mechanisms have been largely elucidated but pathophysiological events downstream the genetic lesion are still far to be clarified. Recent evidences, based on muscle MRI [1–4], pathology [5, 6], and molecular biology data [7], point towards an active role of inflammation in the development of early muscle damage and therefore disease progression at single muscle level. To investigate these inflammatory changes in humans in vivo in a minimally invasive way and trying to acquire additional information than that obtained with muscle biopsy specimen analysis, we decided to develop a prolonged muscle microdialysis protocol with high cut-off membranes. Microdialysis is a molecular sampling procedure performed with the introduction of a cylindrical semipermeable membrane into a medium of interest [8, 9]. Probes can be introduced within the tissue and perfused with

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a buffer, which has a composition similar to the normal extracellular fluid. Molecules that have a higher concentration in the extracellular fluid cross the semipermeable membrane and are collected into a fraction collector [10].

The aim of the current study was to perform a comparison between the microenvironment of affected muscles in early disease stages identified on muscle MRI and the microenvironment of apparently unaffected muscles in the same FSHD patient. Concentrations of inflammatory mediators in the collected microdialysates were analyzed to characterize the pattern of inflammation. The cytokines locally produced in muscles displaying early features of involvement have the potential of constituting tissue biomarkers of disease activity, whose identification is a research priority for the FSHD community [11], and even, since these molecules would have the advantage to be released in the serum, possible circulating biomarkers.

Patients and Methods

Patients

Patients were enrolled during MRI follow-up studies in the period April 2014–February 2016. All the patients with (a) genetically confirmed FSHD; (b) age >18 and <65 years; (c) one externally accessible lower limb muscle showing normal signal on T1-weighted MRI sequences and hyperintense signal on short-tau inversion recovery (STIR) sequences (STIR+), i.e., one muscle showing edema changes but not yet replaced or in the earliest stages of replacement by fat tissue, together with the contralateral muscle showing normal signal on both T1-weighted and STIR sequences (STIR-); and (d) no systemic inflammatory diseases were asked to undergo the microdialysis procedure. One non-penetrant gene carrier, i.e., a clinically asymptomatic carrier of the 4q35 shortened fragment in a family in which the fragment clearly segregated with the disease, with no systemic inflammatory disease, was also asked to undergo the procedure. The patients who agreed started the procedure after maximum 2 weeks from MRI. Controls were healthy volunteers of both sexes, with age >18 and <65 years, and no systemic inflammatory disease.

Microdialysis

We used the commercially available 71 High Cut-Off Brain Microdialysis Catheter (M Dialysis AB, Stockholm, Sweden), which is a sterile disposable catheter approved for use in human brain. Its membrane is 30 mm long and allows an increase in the recovery of macromolecules compared with standard membranes. The catheter was introduced into the muscle after skin disinfection through an introducing needle that was immediately removed once the catheter was placed into the muscle belly. Needle insertion was performed under ultrasonographic guide, to minimize the risk of bleedings due to puncture of a blood vessel and to warrant the placement into the correct muscle (Video 1). Catheter placement was performed at the same time of the day for all the patients, between 7 p.m. and 8 p.m. Catheters were fixed to the skin with an adhesive transparent patch. A CT scan was performed in selected cases if there was the need to confirm the correspondence between the position of the tip of the catheter and the area of STIR hyperintensity.

We introduced one catheter into the vastus lateralis muscle in control individuals and in the non-penetrant gene carrier, and two catheters simultaneously in two lower limb muscles chosen based on MRI features, one STIR+ and the contralateral STIR-, in FSHD patients. An adjustable flow rate microdialysis pump (107 MD pump, M Dialysis AB, Stockholm, Sweden) and a fixed flow rate pump (106 MD pump, M Dialysis AB, Stockholm, Sweden) were used to perfuse the membrane with Perfusion Fluid (M Dialysis AB, Stockholm, Sweden). The flow rate was set at 0.3 μl/min. The subjects were hospitalized throughout all the procedure and were asked to rest in bed as much as possible for 5 days but were allowed to walk for short distances inside the bedroom. Microvials were used as fraction collectors, exchanged every 4 h (8 h during the night) and stored at −80 °C until analysis.

Cytokine Analysis

The analysis of cytokine concentrations in the microdialysates was performed with a Luminex xMAP system (Bio-Rad 200 System, Bio-Rad Laboratories, Hercules, CA), using one commercially available multiplex kit (Bio-Plex Pro Human 40-plex Chemokine panel, Bio-Rad Laboratories) and one custom-premixed 14-plex kit. Fifty-four cytokines among the best-characterized in human immunology were quantified, namely: IL-1ra, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-16, IL-17a, MIF, G-CSF, GM-CSF, basic FGF, PDGF, VEGF, RANTES, CXCL5, CXCL1, CXCL2, CXCL6, CXCL8 (IL-8), CXCL9, CXCL10 (IP-10), CXCL11, CXCL12, CXCL13, CXCL16, CCL1, CCL2 (MCP-1), CCL3 (MIP-1alpha), CCL4 (MIP-1b), CCL7, CCL8, CCL11, CCL13, CCL15, CCL17, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CX3CL1, IFN-gamma, and TNF-alpha. Results were analyzed using a dedicated software from Luminex Technology and are expressed in picograms per milliliter [12, 13]. Serum samples from three patients (p5, p7, and p15) and one control (p13) collected in the early morning before breakfast at the 4th day after catheter insertion were also analyzed with the 40-plex assay.

Statistical Analysis

The assumption of normal distribution of data was checked using D'Agostino and Pearson omnibus normality test. Differences in cytokine concentrations between STIR+, STIR- and control muscles were tested using the Kruskall-Wallis test with Dunn's post hoc correction. Spearman's rho was used to explore the correlation between cytokine concentrations at the two different time points in the three different groups of samples. To compare the cytokine concentrations in STIR+ and STIR- muscles from the same patients, we used the Wilcoxon signed-rank test. Data are reported as mean ± standard deviation or median [interquartile range]. All p values were two-sided, and $p < 0.05$ was considered statistically significant. Data analysis was performed using the GraphPad Prism ver.5.01 for Windows.

Results

Patients

Twelve patients, five controls, and one non-penetrant gene carrier with normal MRI underwent the study (Table 1). Samples from 11 patients (six males and five females, mean age 43.2 ± 13.7 years, range 18–58, median EcoRI fragment length 23 kb [6], median clinical severity score 3 [0.5]), four controls (three males and one female, mean age 41.5 ± 16.4 years, range 24–60), and the non-penetrant gene carrier (female, age 47, EcoRI fragment length 33 kb) were available for analysis.

Microdialysis

No adverse events were reported during and after the procedure. The catheter insertion caused transitory pain comparable to an intramuscular injection and was well tolerated and completely painless after needle removal. Infusion and collection lasted the maximum expected time of 5 days in 13/18 subjects. The average duration was 113 ± 33 h and the average amount of collected fluid was 10.3 ± 3.3 μl/h. In one patient (p1) and one control (p3), there was premature interruption of the procedure due to membrane rupture at 27 and 26 h post insertion. For the same reason, in one patient (p16), only the STIR- muscle could be analyzed. A CT scan was performed in eight patients and confirmed the correct placement of the catheter in all of them (Fig. 1).

Cytokine Analysis

We performed a preliminary analysis on samples from one patient (p5) and one control (p2) in order to assess what was

Table 1 Summary of demographic, clinical, and genetic data of all subjects

Patient ID	Diagnosis	Age (years)	Sex	EcoRI fragment length (kb)	CSS	Muscle
p1	FSHD	45	F	23	4	Soleus[a]
p2	CTRL	24	M	N.A.	0	Vastus lateralis
p3	CTRL	55	F	N.A.	0	Vastus lateralis[a]
p4	FSHD	43	F	17	3	Gastrocnemius lateralis[b]
p5	FSHD	53	F	24	3.5	Peroneus
p6	FSHD	55	F	15	3.5	Vastus lateralis
p7	FSHD	29	M	20	2.5	Biceps femoris short head
p8	FSHD	18	M	23	1.5	Semimembranosus
p9	NPGC	47	F	33	0	Vastus lateralis
p10	FSHD	44	M	25	3.5	Gastrocnemius lateralis
p11	FSHD	58	F	23	3	Gracilis
p12	FSHD	53	M	24	3	Extensor digitorum longus[b]
p13	CTRL	60	M	N.A.	0	Vastus lateralis
p14	CTRL	50	M	N.A.	0	Vastus lateralis
p15	FSHD	34	M	19	3	Semitendinosus
p16	FSHD	58	M	37	3.5	Biceps femoris long head
p17	FSHD	30	F	18	3	Tibialis anterior
p18	CTRL	32	F	N.A.	0	Vastus lateralis

FSHD facioscapulohumeral muscular dystrophy, *CTRL* control, *M* male, *F* female, *CSS* clinical severity scale, *N.A.* not assessed, *NPGC* non-penetrant gene carrier

[a] Not analyzed, early membrane rupture

[b] Analyzed only at 56 h post catheter insertion

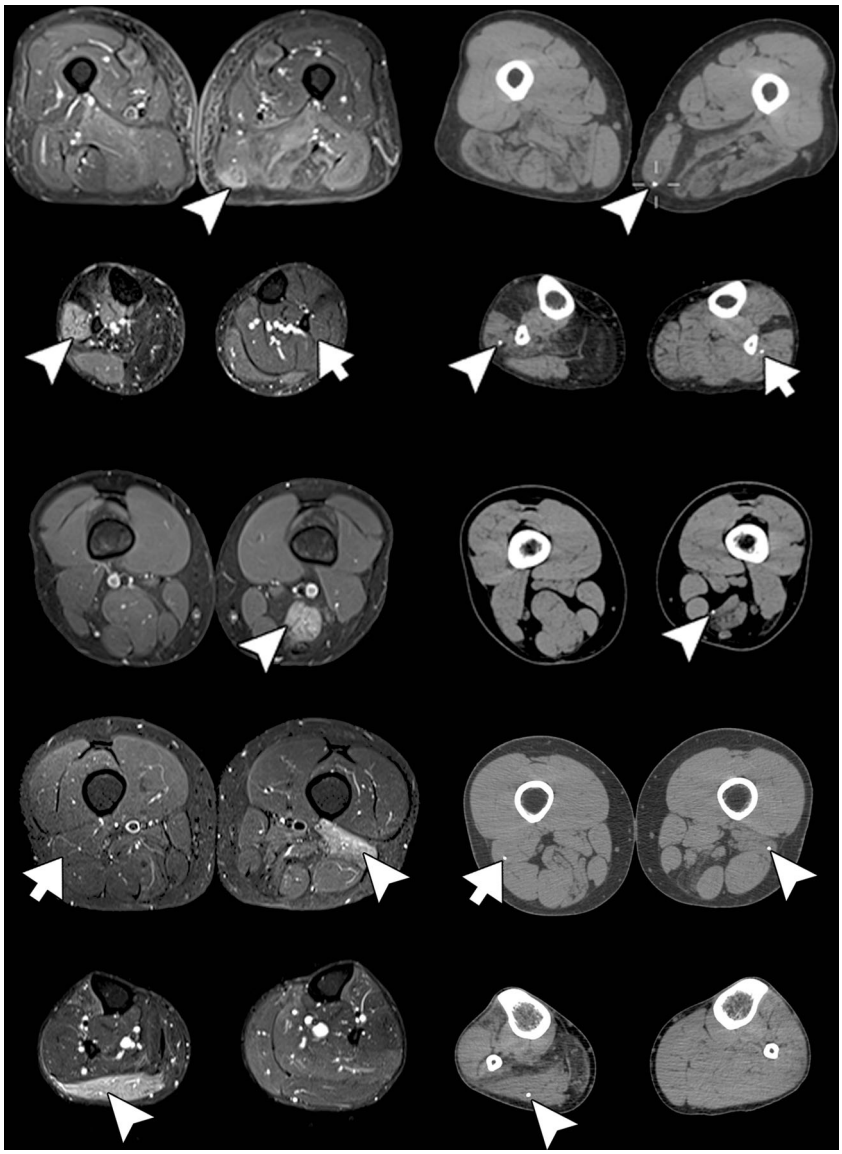

Fig. 1 Examples of ct scans (*right*) and corresponding STIR MRI images (*left*) of targeted muscles. The edge of the catheter is visible on the CT as a *white dot* corresponding to its gold tip. STIR- muscles are marked with an *arrow* and STIR+ muscles with *arrowheads*. *From top to bottom*: patients 11, 5, 8, 7, and 10

the time course to allow the return to the basal level of inflammatory mediators after the microinjury caused by needle insertion. To address this issue, we performed the Luminex assay on sequential samples (i.e., liquid collected at different time points during 5 days).

As expected, we observed a quick rise with a peak of cytokine concentrations in the first hours post insertion and a return to basal level after 48 h for most of the tested cytokines (Fig. 2).

Therefore, for subsequent analyses, we chose a time point corresponding to an interval of approximately 8 h around the 56th hour post insertion, which corresponded to the third night for all the patients, to minimize variations caused by patient movements or circadian rhythm. To validate the stability of values at steady state, we also analyzed samples collected at a second time point around the 85th hour post insertion, corresponding to the fourth night, and compared them with samples at 56 h. This allowed the analysis of 26 samples at 56 h and 22 samples at 85 h.

As a result, we found that three cytokines were significantly dysregulated between STIR+, STIR-, and control muscles: CXCL13, which was upregulated in STIR+ muscles compared with controls (median concentration 116.9 pg/ml [445.87] vs 18.06 [28.95], $p < 0.01$); CXCL5, which was downregulated in STIR+ compared with STIR- (832.9 pg/ml [425] vs 2659 [2166], $p < 0.05$); and G-CSF, which was downregulated in STIR+ compared with controls (5.33 pg/ml [10.95] vs 24.34 [29.84], $p < 0.05$). CXCL13 was also upregulated in STIR+ compared to STIR- muscle although not significantly (116.9 pg/ml [445.87] vs 34.59 [25.94], $p > 0.05$) and had a higher value in the STIR+ muscle compared with the contralateral STIR- muscle of the same patient ($p < 0.01$) (Fig. 3). The concentrations of these cytokines in the non-penetrant gene carrier were closer to the control and

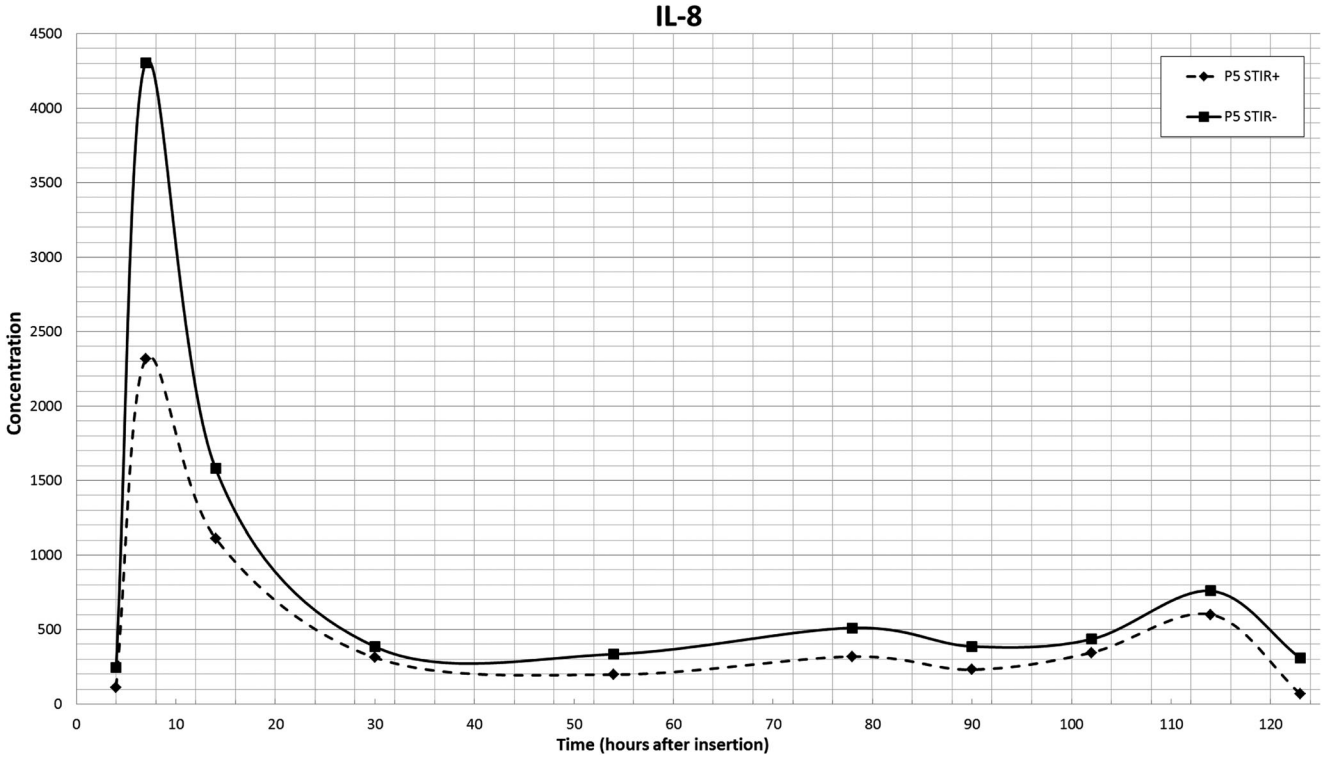

Fig. 2 Time course on sequential samples. The behavior of IL8 is shown as an example. The shape of the curve depended on the analyzed cytokine, but a steady state was reached after 48 h for most of the analyzed cytokines. The first point did not always correspond to the basal value (data not shown)

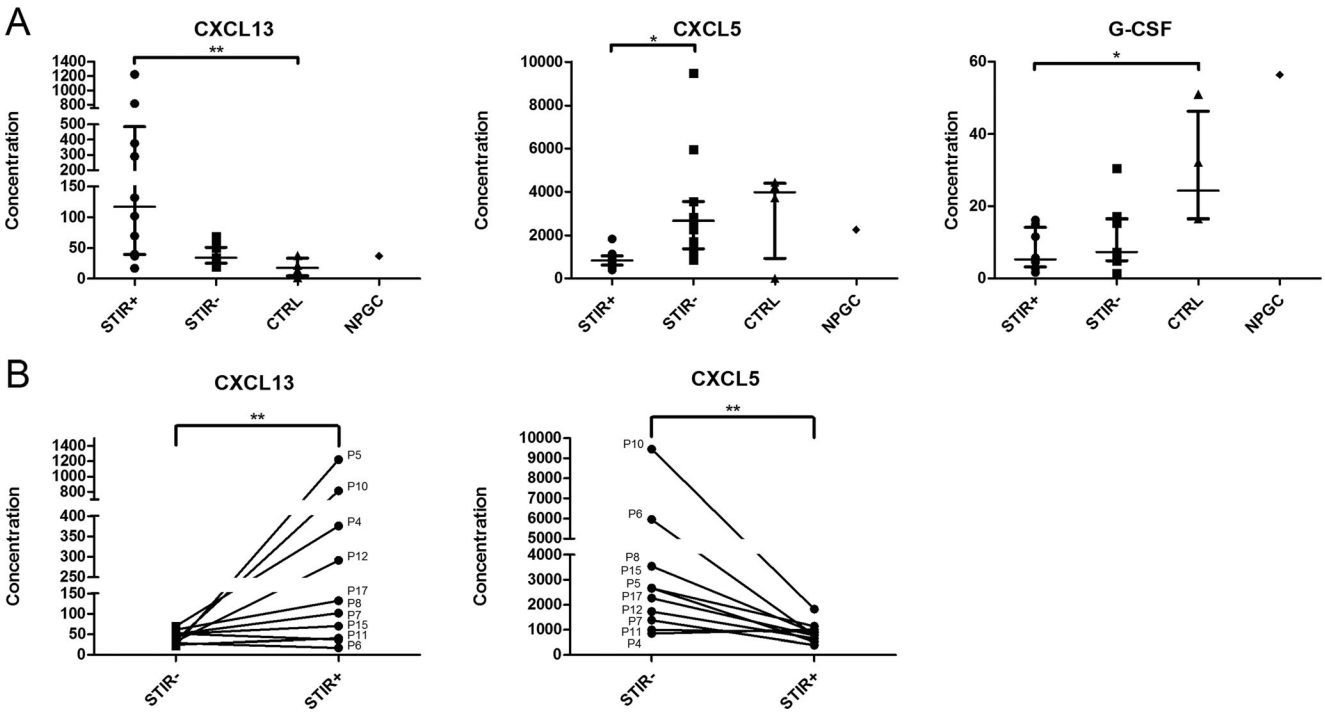

Fig. 3 Dysregulated cytokines. **a** Dot plots with median and interquartile range showing concentrations of CXCL13, CXCL5, and G-CSF in the four analyzed groups (STIR+ muscles, STIR- muscles, controls, and the non-penetrant gene carrier, NPGC). **b** Comparisons of CXCL13 and CXCL5 concentrations in paired STIR+ and contralateral STIR- muscles of the same patients. Concentrations are expressed in pg/ml; $*p < 0.05$, $**p < 0.01$. *NPGC* non-penetrant gene carrier

STIR- rather than to STIR+ muscles, although for the other cytokines the non-penetrant gene carrier often showed an outlier behavior. All the results are shown in Supplementary Figure 1. Few other chemokines, such as CCL19 and CXCL12, also showed elevated values in some STIR+ samples compared with STIR- and controls without reaching a significant difference (Supplementary Figures 1 and 2).

The correlation between the values obtained at 56 and 85 h was high for all the groups of samples: STIR+ (rho 0.94, $p < 0.0001$), STIR- (rho 0.96, $p < 0.0001$), and control muscles (rho 0.83, $p < 0.0001$).

CXCL13, CXCL9, CXCL10, CXCL11, CCL19, and CCL24 concentrations were also higher in all the three FSHD serum samples than control; on the contrary, CXCL5 and CXCL12 values were lower in 2/3 patients compared with control. The results of the serum analysis are displayed in Supplementary Table 1.

Discussion

In the last decades, the largest part of research efforts in FSHD has been focused on the genetic mechanisms underlying the disease. This brought the development of a unifying genetic model, which is based on the inappropriate transcription of the toxic isoform of the *DUX4* retrogene in adult skeletal muscle [14–17]. DUX4 is a transcription factor that regulates the expression of, among others, immunomodulatory genes such as *DEFB103*, which is a chemoattractant for inflammatory cells, and cancer testis antigens. A contribution of DUX4-mediated damage may therefore come from triggering a sustained expression of genes causing a multifocal, inapposite immune response [18]. This is also consistent with the hypothesis that the disease evolves through sequential bursts of muscle degeneration involving individual muscles in an asynchronous manner. Indeed, while inflammation was reported to be an inconstant finding in FSHD muscle samples [19–21], biopsies obtained from muscles selected according to their MRI pattern (i.e., STIR+ muscles) invariably disclosed the presence of perivascular inflammatory infiltrates, mainly constituted by CD4+ cells, and endomysial infiltrates mainly composed by CD8+ cells [5]. Despite these and other older evidences pointing towards a potential involvement of immune response in the disease [22–24], the immunological aspects of FSHD have not been mined enough and translational studies, merging information from clinical imaging with molecular data, are lacking in the field. Moreover, given the difficulties in developing a reliable animal model able to recapitulate all the features of this complex and perhaps unique disease regarding the causative mechanisms, the study of patients is of paramount importance.

The microdialysis technique was developed more than 30 years ago, and it is regularly used in the study of muscle metabolism for the analysis of small molecules like glucose, lactate, pyruvate, and glycerol, at rest or during exercise. It has recently found numerous applications in intensive care, e.g. for tissue ischemia monitoring, and neurosurgery, for the analysis of inflammatory markers in the brain of head injured patients with prognostic implications [9]. Latest technical developments led to the production of probes with high cut-off membranes, with pores that allow the transfer of molecules including cytokines up to 100 kDa [10, 25], that are currently used for clinical and research purposes.

With the implementation of a prolonged microdialysis protocol in skeletal muscle tissue, we obtained a continuous sampling of interstitial fluid from patients' muscles selected based on their MRI features that allowed the analysis of a broad set of cytokines, chemokines, and growth factors. All the tested inflammatory mediators have a molecular weight <100 kDa, thus theoretically allowing their free transport through the pores of the membrane. The actual permeability of the membrane for tested molecules was also confirmed by the detectable concentration of most of them in the collected fluids. It emerged that only a small number of inflammatory mediators, mostly belonging to the group of chemokines, are selectively modulated in STIR+ FSHD muscles. Chemokines are a group of small molecules that act as chemoattractants to drive immune cell migration. The CXC chemokines are defined by the arrangement of the first two of four invariant cysteine residues and play an essential role in regulating tumor-related immunity [26]. A recent gene expression microarray study confirmed that several chemokines and their receptors are induced by DUX4 upregulation in human immortalized myoblasts [27].

CXCL13 is known as a B cell chemoattractant, required for the development of B cell follicles and secondary lymphoid structures, such as spleen and lymph nodes [28]. It has also been documented in muscle of inflammatory myopathies, particularly prominent in larger perimysial infiltrates in dermatomyositis, and therefore hypothesized to play a role in lymphoneogenesis in this disease [29]. Moreover, it has been found to be upregulated in different biological fluids in several inflammatory conditions. For instance, it is elevated in serum and cerebrospinal fluid during active multiple sclerosis [30], viral encephalitis, and neuroborreliosis [31]. It is also involved in the pathophysiology and proposed as a biomarker in systemic lupus erythematosus [32], rheumatoid arthritis [33], Sjogren's syndrome [34], and ANCA-associated vasculitis [35]. Interestingly, it has also been lately implicated as a mediator of neuropathic pain [36, 37], and it is functionally linked with CCL19, which showed a non-significant trend of increase in STIR+ muscles. CCL19 together with CXCL13 has been implicated in B and T cell recruitment in lymphoneogenesis [38].

CXCL5 and G-CSF are reduced in the extracellular fluid of STIR+ muscles. CXCL5 is also identified as epithelial neutrophil activating peptide-78 (ENA-78) due to its better known

function of attracting neutrophils. Remarkably, it is upregulated in phases of remissions of neuromyelitis optica [39] and acts as an attractant for adipose tissue derived stem cells [40]. G-CSF (granulocyte colony stimulating factor) is also involved in skeletal muscle progenitor cells proliferation and recruitment in diseased muscles [41]. Its decrease in FSHD muscles may be a contributor to defective muscle regeneration [42].

Even though systematically exploring dysregulated cytokines in the serum was not a specific objective of this study, we decided to acquire preliminary data on the comparison between microdialysates and the serum in a small number of patients. In this subset of samples, we found a concordant upregulation of CXCL13 in the serum of patients with STIR+ muscles compared with a control, which may support its role as a putative circulating biomarker as well.

Our study has some limitations. First of all, it is a pilot, exploratory study on a relatively small cohort. Second, although we tried to limit the possibility of having different cytokine recovery rates introducing catheters in contralateral muscles in the same patients, at the same time and under the same experimental conditions (flow rate, perfusate composition, microvial exchange times, etc.), we cannot completely rule out such an issue due for instance to the different tissue compositions of STIR+ and STIR- muscles, e.g., an excess of fibrous tissue not assessed by MRI could partially impair the recovery in STIR+ muscles. These factors might have contributed to the absence of significant differences between the three groups for most of the analyzed cytokines and chemokines. Therefore, we cannot exclude that non-significant differences are real. The fact that even dysregulated cytokines do not show the same behavior in all the pathological samples may also suggest that different processes can underlie STIR hyperintensities.

In conclusion, the innovative combination of continuous muscle sampling through microdialysis with the non-invasive MRI approach confirms the presence of an active and peculiar inflammatory phase in the microenvironment surrounding muscle fibers in STIR+ FSHD muscles. To the best of our knowledge, our study puts a new focus on chemokines whose role and potential as circulating biomarkers has not been particularly investigated yet in this disease [43], although a set of chemokines including CXCL9, 10, 12, 14, and 16 were found to be upregulated in STIR+ versus STIR- muscles in a gene expression microarray study [7]. CXCL13 was not found significantly upregulated in that study. Technical limitations intrinsic to the microarray technology, including low sensitivity and specificity of the CXCL13-specific probe, might explain this discrepancy. The full characterization of the interstitial milieu even with other techniques could be extremely useful to design early and targeted therapeutic strategies, helping to identify drugs to counteract dysregulated pathways, as well as defining new potential tissue biomarkers. Studies are needed to explore the serum levels of these chemokines in larger cohorts of patients and to confirm current results with other methods. Finally, the development of a muscle microdialysis protocol could be instrumental in the view of a selective drug delivery, since this technique gives the theoretical opportunity to deliver the drug locally and at the same time continuously monitor its therapeutic effects.

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Compliance with Ethical Standards This protocol is in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of our Institution. All involved subjects gave their written informed consent.

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Conflict of Interest Pursuant to the terms of a Master Academic Services Agreement with the Catholic University of the Sacred Heart, M. Monforte and E. Ricci have provided central reading services for MRI scans generated in aTyr's clinical trials of Resolaris (ATYR1940). E. Ricci is the site principal investigator for some of such trials. The other authors report no disclosures.

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