The Cytokine gene network as potential biomarkers for muscle weakness in OPMD

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Study highlights:

- We identified six cytokines (IL15, LTA, CCL7, CCL8, CCL25, CCL27) with lower expression levels in skeletal muscles of symptomatic patients with OPMD, but unchanged in pre-symptomatic expPABPN1 carriers.
- Five of six OPMD-dysregulated cytokine genes clustered tightly in controls. This clustering was found to be disrupted in OPMD.
- Down-regulated cytokine genes in OPMD patients were up-regulated in a mouse model of OPMD. In humans, the expression of these cytokines correlates with disease severity rather than age. In OPMD mice, their expression correlated also with age.
- The findings suggest that the cytokines with altered expression in OPMD patients are putative biomarkers for muscle weakness in OPMD.

Abstract

Cytokines are circulating immunogenic molecules, and their potential as biomarkers has been exploited in the last decade. Cytokines are expressed in many tissues, including muscles, but their application to monitoring muscle pathology in neuromuscular disorders is sparse. Oculopharyngeal muscular dystrophy (OPMD) is a dominantly inherited, late-onset myopathy, caused by an alanine-expansion mutation in the gene encoding for Poly(A) RNA binding protein 1 (*expPABPN1*). Biomarkers for OPMD disease severity and progression are not available. We identified candidate cytokines with altered expression levels in OPMD mice carrying *PABPN1* mutations. Down-regulation of six cytokines, IL15, LTA, CCL7, CCL8, CC25 and CCL27, was found in *vastus lateralis* muscle from OPMD patients, but not in expPABPN1 carriers at a pre-symptomatic stage. In contrast, these cytokines were up-regulated in the mouse model of OPMD. These six cytokines formed a distinguished cluster, which was dispersed in OPMD. Our findings indicate that this cytokine network is a potential biomarker for muscle weakness in OPMD.

Introduction

Ideal molecular biomarkers can quantitatively and accurately detect changes in medical conditions. Quantitative biomarkers can provide accurate clinical assessment of pathological conditions, moreover a patient's response to therapy. The advantage of molecular biomarkers is high sensitivity that can detect early stages of a disease, and efficacy of therapy (1, 2). Secreted molecules, such as cytokines and miRNA, have been explored as biomarkers, particularly when these molecules are believed to be secreted from the primarily affected tissue into the blood plasma. The advantages of cytokines levels are modulated by different pathological conditions and can by robustly monitored using multiplex quantitative assays, which further can be used in different prediction models like risk assessment and toxicity (3). Changes in cytokines networks in blood have been found in a wide range of disorders that are associated with the immune system, such as different cancers, rheumatoid arthritis, neuroinflammatory CNS disorders, and Systemic Lupus Erythematosus (1, 4-7). Cytokines as potential biomarkers were also reported in diverse conditions like stroke, heart faliure, mood disorders (8, 9) (9) (10), and in some muscular dystrophies like Duchenne muscular dystrophy (DMD) (11). In DMD, elevation in circulating cytokines is probably due to an increase in inflammation (11). Changes in cytokines levels were also found after change in muscle exercise (12-14) and in elderly (15, 16). These studies suggest that cytokines levels can monitor changes in muscle function in conditions without inflammation. So far, biomarkers for rare neuromuscular disorders, such as oculopharyngeal muscular dystrophy (OPMD), have not been identified.

OPMD is a dominantly inherited myopathy, characterized by weakness of ocular and pharyngeal muscles typically starting from the fifth decade (17). OPMD is caused by an alanine-expansion mutation in the gene encoding for PABPN1 (18). OPMD pathophysiology has been studied with the A17.1 mouse model, which was generated by stable overexpression of the expanded PABPN1 under a muscle-specific promoter (19). The hallmark of OPMD is formation of PABPN1 aggregates in myonuclei (20), and is considered as the histocytological mark for the disease. Indeed, animal models for OPMD were assessed for aggregates formation and muscle weakness in those models were correlated with aggregate formation and muscle pathology (19, 21, 22). Moreover, aggregates were used to assess the efficiency of small molecules as potential therapies for OPMD (19, 21, 23). Recently, we reported that in heterozygous carriers of expanded PABPN1, aggregates are found prior to muscle symptoms, aggregates abundance in *vastus lateralis* muscle was

similar to that in symptomatic OPMD patients (24). This calls for accurate biomarkers in OPMD that could assess pre-clinical signs of disease.

PABPN1 is a regulator of mRNA processing and changes in PABPN1 levels cause genome-wide changes in mRNA expression profiles (25), which we have previously reported in mRNA levels of OPMD muscles (26, 27). Amongst the dysregulated functional groups, we identified the cytokine activity group to be significantly affected in OPMD and mice models (27). We then hypothesized that changes in cytokine levels in affected OPMD muscles could mark the symptomatic state.

Results and discussion

mRNA expression profiles of the OPMD mouse model, A17.1, included up-regulation of the cytokine activity functional group (28). We reported that up-regulation in this mouse model is caused by alternative polyadenylation site usage in the 3'-UTR, and in this study we identified cytokines-related genes to be significantly affected (29). We studies cytokines levels in the vastus lateralis muscle as limb girdle muscles weakness is often amongst the initial muscle symptoms in our patients (24). mRNA expression levels of 15 cytokines-related genes were quantified using RT-qPCR, initially in a subset of 7 OPMD patients and 9 age-matched controls (Supplementary Fig. 1). A significant lower expression level in OPMD vs. controls was found for four genes (CCL7, CCL8, CCL25, CCl27; p-value < 0.05), and for three genes (IL-15, IRF1, LTA) fold changes were in the same direction without statistical significance (p-value = 0.06) (Supplementary Fig. 1). These seven genes were further analyzed in control N=15 and OPMD N=16 (Supplementary Table 1). Lower expression of CCL7, CCL8, CCL25, CCL27, IL15 and LTA was found in OPMD, while the expression of IRF1 did not differ between control and OPMD (Fig. 1A).

We then investigated whether a change in cytokines levels is associated with symptoms in expPABPN1 carriers. Uniquely to our OPMD dataset, we previously collected VL muscle biopsies from younger OPMD family members without muscle symptoms. The expPABPN1 carriers were identified using a genetic test, and defined as pre-symptomatic or OPMD patients based on symptoms (24, 26). Muscles from pre-symptomatics showed a normal muscle histology, however nuclear aggregates were found to the same extent as in symptomatic patients (24). In contrast to OPMD muscles, the expression levels of the six cytokines in the expPABPN1 carriers did not differ significantly from age-matched controls (Fig. 1B). We then assessed whether cytokines levels are affected by age. None of the six cytokines were found to be age-associated (Supplementary Table 2). Together, this indicates that expression of these six cytokines is primarily associated with symptoms in expPABPN1 carriers.

In the A17.1 mouse model for OPMD, the expPABPN1 gene is constitutively expressed under a muscle specific promoter and changes in mRNA expression profiles are found in 6-week old mice (28). Muscle weakness and muscle pathology in this mouse model is found at week 18 and onwards, predominantly in fast muscles, such as TA (28). A change in the six OPMD-dysregulated cytokines was indeed found in A17.1 TA muscles from 6 week-old mice (Fig. 1C). However, in contrast to OPMD, a positive fold change was found in the A17.1 TA

muscle (Fig. 1C). We found that in the A17.1 18 weeks old mice, only Il17, Ccl7 and Lta remained significantly elevated (Fig. 1D). We then distinguished between the contribution of age and genetics to cytokine expression levels using linear regression models stratified for genetics. We found that the expression of Il15, Lta, Ccl25 and Ccl27 was significantly reduced with age in A17.1 mice (Supplementary Table 2). The expression of Il15 and Lta was also reduced with age in the control mice (Supplementary Table 2). This suggests that reduced expression with age of Ccl25 and Ccl27 is predominantly affected by expPABPN1 whereas that of Il15 and Lta is less affected by expPABPN1.

Next, we assessed whether patterns of co-expression could be identified, as indicators of gene network. Co-expression between every two genes was assessed by Pearson correlations (Supplementary Table 3), and clustering of the expression profiles was determined. Two separate groups were identified, using all 15 cytokine-related genes in control (Fig. 2A). A strong correlation cluster encompassed LTA, CCL7, CCL8, CCL25, CCL27, and PPBP, and a weaker cluster included IRF1, IRL1, CMTM4, SPP1 and IRL2 (Fig. 3A and Supplementary Table 3A). The expression of the control gene, GUSB, did not have significant correlations with any of the cytokine genes (Fig. 2A). Remarkably, in the most highly correlated cluster, five out of the six genes were identified as having a lower expression in OPMD. In contrast to the pattern of association between genes in control, clustering was disrupted in OPMD (Fig. 3A). We then further assessed clustering of the OPMD dysregulated cytokines in the extended dataset. In control, the LTA, CCL7, CCL8, CCL25, and CCL27 cluster remained distinguished, but in OPMD correlations between those genes were weaker (Fig. 2B and Supplementary Table 3B). This analysis reveals that these cytokines co-expressed in muscles from controls creating an expression network, but this network is disrupted in OPMD.

We also assessed co-expressions in the A17.1 mouse model. In contrast to the results in humans, in control FVB mouse Lta, Ccl7, Ccl8, Ccl25, and Ccl27 did not form a cluster (Fig. 3C). The co-expression correlation pattern differed between FVB and A17.1, but a recognized cluster was not formed, instead a small cluster including Lta, Ccl25 and Ccl27 was found in A17.1 (Fig. 3C). This indicates that the pattern of cytokines co-expression differs between OPMD patients and the OPMD mouse model.

To investigate whether these six cytokines could potentially be robust predictive biomarkers for OPMD, we applied a multivariable linear regression model adjusting for gender, age and batch effects. In humans, as expected, all six cytokines were found to be significantly associated with diagnosis, and CCL25 and CCL7 were found to have the strongest effect

(Table 1). This indicates that a decrease in these six cytokines levels in OPMD is robust and overcomes age and gender differences among patients. In mice, five cytokine genetics were found to associate with expression level, but the direction of the effect was opposite to that found in OPMD patients. Additionally, in mice, Ccl25, Il15, Lta and Ccl27 were found to associate with age (Table 1).

From these six cytokines, IL15 constitutive expression in muscles was previously shown, and elevated IL15 levels were found in conditions with increased muscle mass (30). Additionally, reduced IL15 levels were implicated in muscle wasting. Reduced Il15 were found to be causing muscle atrophy and muscle wasting in mice under stress conditions (31, 32) and was therefore proposed as a potential therapeutic target for muscle wasting (31). The expression of CCL genes in muscles correlates with muscle wasting, for examples due to chronic binge alcohol or Adeno virus administration (33, 34). Network analysis of chemokine expression in blood reveals consistent co-expression in control, but it was gradually lost with cancer disease progression (35).

Several studies demonstrated that circulating cytokines levels change after muscle exercise and in changing muscle physiology conditions (12-14). It was suggested that oxidative stress markers are secreted from muscles to blood after anaerobic exercise (36), and thus could be used as measurers for muscle fatigue and pathology. Here we found that the expression profile of six cytokines is specifically changed in affected OPMD muscles. We show that their mRNA levels in muscles are associated with the disease. Future studies should investigate levels of these cytokines in blood from OPMD. Additionally, future studies should examine whether cytokine levels can predict disease severity and progression. Biomarkers are employed to predict disease condition and to accurately report the efficacy of therapies (3). For pre-clinical assessment of therapies the same set of biomarkers should be employed in patients and in animal models. Our results here reveal that the expression of the OPMD-dysregulated cytokines in humans affected by OPMD is opposite to that found in the mouse model of OPMD. Moreover, we found that an age-associated cytokine profile differ between mouse and human. The basis of this distinct cytokine profile remains to be resolved, but may relate to pathological differences in disease progression (atrophic stage, fibrosis, inflammation, ect), or to differences between human VL and mouse TA muscles, or to differences in muscle aging biology between the two organisms. This study reveals molecular differences between OPMD muscles from heterozygous patients and the A17.1 mouse, and encourages the development of alternative models for OPMD, such as the one

recently reported(37). Since subsets of muscles are predominantly affected in OPMD and during aging(38), future studies should investigate the cluster of cytokines in different muscles and correlate with circulating cytokine profiles.

In conclusion, we identified six cytokines whose mRNA expression profile altered in OPMD muscles and is associated with OPMD symptoms. We found that changes in these six cytokines are opposite to that found in a mouse model of OPMD, however their age-associated changes where similar. We show that expression of these genes form a distinguished cluster in human. This cytokines cluster is significantly weakened in OPMD and the expression of these genes can predict the disease. We suggest that mRNA expression levels of these six cytokines can potentially be specific biomarkers for OPMD disease severity and progression.

Methods

OPMD patients, pre-symptomatic carriers, controls and clinical features

Fifteen genetically confirmed OPMD patients and nine expPABPN1 carriers at a pre-symptomatic stage were included in this study. Subjects' characteristics (age, gender and diagnosis) are listed in Supplementary Table 1. Age distribution between expPABPN1 carriers and controls did not differ (Supplementary Figure 1). Cytokines levels were determined in vastus lateralis, as in the Dutch and Danish patients Initial muscle symptoms also include the limb griddle muscles (24, 39, 40). A subset of the subjects (control N=9; OPMD N=7) was first analysed for 15 cytokines. Additional subjects (control N=7; OPMD N=8) were included for analysis of a subset of 7 differentially expressed cytokines. Results are presented for the combined data, and in models correction for possible technical variations between the two groups (batch effect) is included in the multivariate models. The pre-symptomatic subjects were identified amongst the OPMD family members. Control biopsies were collected from knee operations from subjects without OPMD. All subjects were described in previous studies (24, 26). Muscle biopsies collection and RNA isolation were carried out as described in (26). The study was approved by the local Radboud, Nijmegen Ethical Committee (CMO nr. 2005/189) and written informed consent for RNA investigation was obtained from all patients and controls.

cDNA synthesis and quantitative RT-PCR

RNA samples from A17.1 and FVB control at 6 and 18 weeks old animals were described in (28). 1 µg total RNA was converted to cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, MA USA). Quantitative RT-PCR was performed using Syber-Green master mixture (Bio-RAD Laboratories, USA) on LightCycler® 480 (Roche Diagnostics, Basel, Switzerland). mRNA expression levels were determined after normalizing to HPRT housekeeping gene using the dCT calculation; fold changes were calculated after normalization to the average of control using the ddCT calculation.

Statistical analysis

Statistical significance of fold change was assessed in human samples with the non-parametric Mann-Whitney t-test. Analysis was carried out in Graphpad Prism 6.

Further analyses of data from the human OPMD patients and age-matched controls were carried out after transformation to the natural logarithm due to skewness of the expression data. Multivariable linear regression models were used to assess age effects on the expression levels (as a dependent variable). For the human samples, models included diagnosis (0: control; 1: OPMD), age, gender (0: female; 1:male) and batch (1, 2) as independent variables. For the mouse samples (all are male and all isolations were performed in one batch), included genetics (0: FVB; 1: A17.1) and age (0: 6 weeks; 1: 18 weeks). Pearson correlation tests were employed to assess correlations in expression levels between genes. Statistical analyses were performed with IBM SPSS Statistics (version 20) and. P-values <0.05 were considered as statistically significant. Correlation heatmaps were generated in RStudio (version 0.99.484) using the '*corrgram*' package (version 1.8) with Pearson correlations. Genes were ordered with PCA-based re-ordering on the control condition. All other charts were generated in Graphpad Prism 6.

Author contributions

MR performed experiments; YR carried out all statistical analysis; Human biopssies were collected by JV, BvE and BvdS. Mouse samples were provided by GD. VR designed the study. All Authors read and commented on the MS. MS was written by VR, MR and YR.

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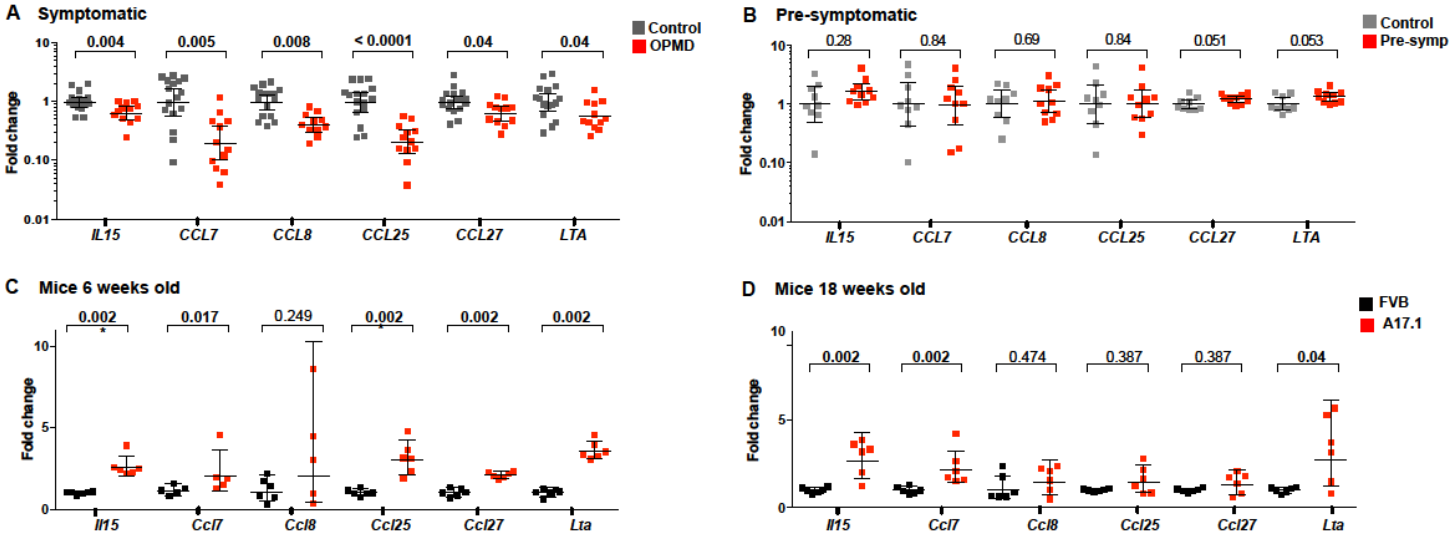
Table 1: Age-association of differentially expressed cytokines in human and mouse.

	Human				Mouse			
	Diagnosis		Age		Genetics		Age	
	B (SE)	p-value	B (SE)	p-value	B (SE)	p-value	B (SE)	p-value
CCL25	**-1.62 (0.39)**	**< 0.0001**	-0.02 (0.02)	0.392	**0.73 (0.15)**	**< 0.0001**	**- 0.48 (0.15)**	**0.004**
CCL7	**-1.71 (0.53)**	**0.004**	-0.03 (0.03)	0.217	**0.55 (0.24)**	**0.038**	0.09 (0.24)	0.723
IL15	**-0.40 (0.17)**	**0.028**	0.001 (0.01)	0.926	**0.96 (0.10)**	**< 0.0001**	**0.58 (0.1)**	**< 0.0001**
LTA	**-0.64 (0.29)**	**0.035**	0.02 (0.01)	0.116	**1.14 (0.17)**	**< 0.0001**	**- 0.57 (0.17)**	**0.003**
CCL8	**-0.75 (0.34)**	**0.035**	-0.004 (0.02)	0.796	0.74 (0.38)	0.067	- 0.92 (0.38)	0.026
CCL27	**-0.41 (0.19)**	**0.047**	-0.01 (0.01)	0.253	**0.49 (0.13)**	**0.001**	**- 0.45 (0.13)**	**0.002**

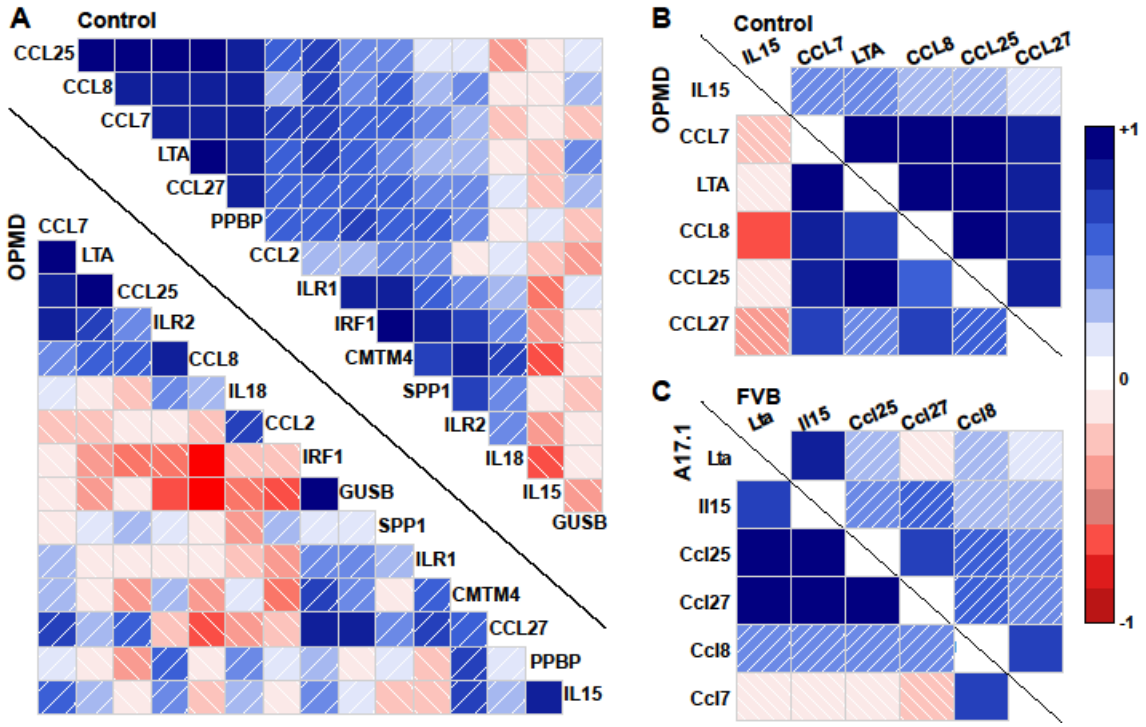
Betas (B) and standard errors (SE) of effects of diagnosis and age on cytokine expression levels are provided. Multivariable linear regression models were employed. For human samples models included diagnosis (0: controls, 1: OPMD), age (in years), gender (0: female, 1: male) and PCR experiment as independent variables. For mouse samples models included genetics (0: FVB, 1: A17.1) and age (0: 6 weeks, 1: 18 weeks) as independent variables. P-values <0.05 were considered statistically significant and are marked in bold.

Fig.1: Differential expression of cytokines in OPMD patients and controls and mouse model



Plots show mRNA fold change in human (A-B) or mouse (C-D) muscles. A. expression levels in OPMD (N=15) vs. age-matched controls (N=16, average age 59.1 and 56.1, respectively). B. Pre-symptomatic (pre-symp) (N=10), vs. age-matched controls (N=9 (average age 37.9 and 36.7, respectively) **C-D** Expression levels in mouse A17.1 vs. FVB control at 6 (C) or 18 (D) weeks of age. N=6 for each group. P-values for human samples were determined with a non-parametric Mann-Whitney t-test. Significant changes are depicted in bold.

Fig.2: Co-expression clustering of differentially expressed genes in control and OPMD.



Correlation heatmaps of Pearson correlations show correlations between expression levels show patterns of gene clustering in human (**A-B**) or mouse (**C**). In (A) 15 cytokines related genes were included in the analysis, and in (B) and (C) only the OPMD dysregulated cytokines are included. Dataset in (A): control N=10, OPMD N=9; dataset in (B): control N=16; OPMD N=15. Positive Pearson correlation values are depicted in blue and in red negative values, according to the values scale. Genes are ordered using a PCA-based re-ordering in the control conditions. Significant correlations are depicted with filled boxes, while non-significant correlations are striped. The Pearson correlation values are found in Supplementary Table 3.

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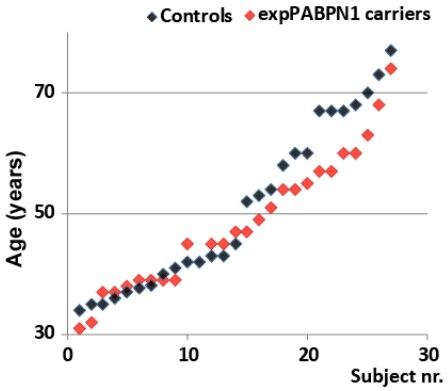
Supplementary material

Supplementry Table 1: Subject characteristics

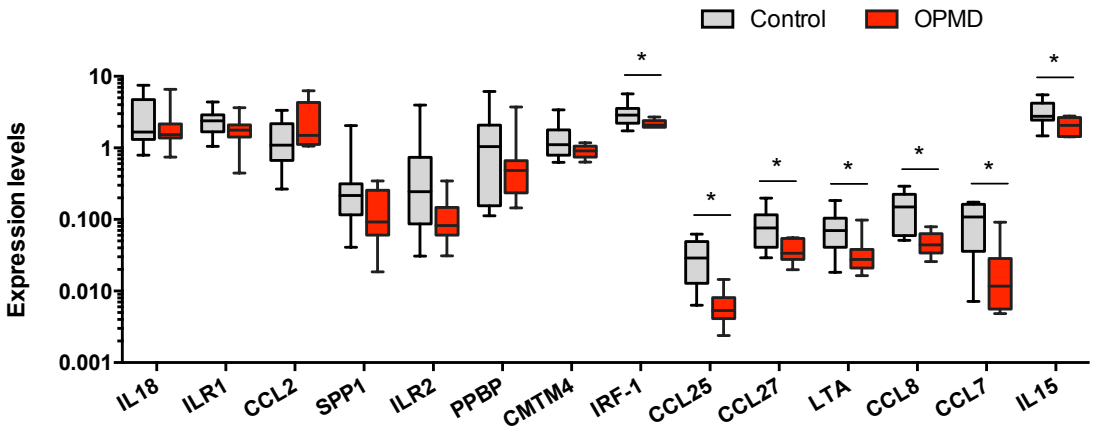
	Controls			expPABPN1 carriers	
	Age	Gender		Age	Gender
	Mean(SD) 37.9 (2.8)	50.0%		31-39	Females: 77.8%
Age-matched group	34	F	Pre-symptomatic	NA	F
	35	F		NA	M
	35	F		NA	F
	36	F		NA	F
	38	F		NA	M
	39	M		NA	F
	39	M		NA	F
	40	M		NA	F
	41	M		NA	F
	42	M			
	Mean(SD) 59.8 (10.7)	Females: 62.5%		Mean(SD) 56.1 (8.1)	Females: 86.7%
Age-matched group	43	F	Symptomatic	45	F
	43	M		47	F
	45	F		47	F
	52	F		49	F
	53	M		51	F
	54	F		54	F
	58	F		54	F
	60	F		55	F
	60	M		57	F
	67	F		57	M
	67	F		60	F
	67	F		60	F
	68	M		63	F
	70	F		68	F
	73	M		74	M
	77	M			

Supplementry Figure 1: Age-distribution of subjects in this study.
NA=not aviable. The age of presymptomatic carriers is not attributed, and therefore only the age-range is indicated.



Scatter plot shows the distribution of subject across age in controls (gray) and expPABPN1 carriers (red). Significant age-distribution between the two groups was not found.

Supplementry to Figure 1: Expression levels of 15 cytokine related genes in control and OPMD muscles.



Expression levels are calculated by the dCT after normalization to HPRT levels. HPRT levels were consistent across samples. Control N=10; OPMD N=9. Significant change was assessed with unpaired nonparametric T-test.

Supplementry Table 2: Age or diagnosis association in human and mouse.

	Human				Mouse			
	Control		OPMD		FVB		A17.1	
	B (SE)	pvalue	B (SE)	pvalue	B (SE)	pvalue	B (SE)	pvalue
IL15	0.02 (0.01)	0.123	- 0.03 (0.01)	0.087	**- 0.60 (0.08)**	**<0.001**	**- 0.56 (0.20)**	**0.02**
LTA	0.02 (0.01)	0.147	- 0.13 (0.03)	0.677	**- 0.43 (0.12)**	**0.006**	**- 0.70 (0.32)**	**0.05**
CCL25	- 0.03 (0.02)	0.140	0.00 (0.04)	0.961	- 0.11 (0.09)	0.22	**- 0.84 (0.24)**	**0.006**
CCL27	- 0.01 (0.01)	0.786	- 0.02 (0.15)	0.184	- 0.20 (0.11)	0.100	**- 0.70 (0.21)**	**0.007**
CCL7	- 0.04 (0.02)	0.131	- 0.02 (0.06)	0.764	- 0.14 (0.18)	0.43	0.32 (0.45)	0.50
CCL8	- 0.02 (0.01)	0.281	0.03 (0.04)	0.380	- 0.53 (0.37)	0.18	-1.32 (0.67)	0.08

Multivariable linear regression models on ln-transformed expression levels stratified for diagnosis (human) or genetics (mouse) were used to assess age effects on the expression levels (as a dependent variable). Independent variables in the human dataset included age, gender (0: female; 1:male) and batch (1, 2). Independent variables in the mouse dataset included age. Betas for age (standard errors) are provided. Models for the human samples are adjusted for possible gender and batch effects. In human OPMD and age-matched group are included (Supplementary Table 1, age is a contentious variable. In mouse, age is an ordinal covariate (0=6 weeks; 1=18 weeks).

Supplementry Table 3: Pearson correlations in controls and OPMD.

A. Correlations in human control and OPMD amongst 15 cytokines-related genes.

Figures/pearson correlation.xlsx

B. Correlations amongst the six OPMD-significant cytokines in human.

Figures/pearson correlation.xlsx

C. Correlations amongst the six OPMD-significant cytokines in mouse.

Figures/pearson correlation.xlsx