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Gene expression profiling in patients with polymyalgia rheumatica before and after symptom-abolishing glucocorticoid treatment

Frederik Flindt Kreiner¹, Rehannah Borup², Finn Cilius Nielsen², Peter Schjerling³ and Henrik Galbo¹*

Abstract

Background: The pathophysiology, including the impact of gene expression, of polymyalgia rheumatica (PMR) remains elusive. We profiled the gene expression in muscle tissue in PMR patients before and after glucocorticoid treatment.

Methods: Gene expression was measured using Affymetrix Human Genome U133 Plus 2.0 arrays in muscle biopsies from 8 glucocorticoid-naive patients with PMR and 10 controls before and after prednisolone-treatment for 14 days. For 14 genes, quantitative real-time PCR (qRT-PCR, n = 9 in both groups) was used to validate the microarray findings and to further investigate the expression of genes of particular interest.

Results: Prednisolone normalized erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in PMR patients. A total of 165 putatively clinically relevant, differentially expressed genes were identified (cut-off: fold difference > ±1.2, difference of mean > 30, and p < 0.05); of these, 78 genes differed between patients and controls before treatment, 131 genes responded to treatment in a given direction only in patients, and 44 fulfilled both these criteria. In 43 of the 44 genes, treatment counteracted the initial difference. Functional clustering identified themes of biological function, including regulation of protein biosynthesis, and regulation of transcription and of extracellular matrix processes. Overall, qRT-PCR confirmed the microarray findings: Microarray-detected group differences were confirmed for 9 genes in 17 of 18 comparisons (same magnitude and direction of change); lack of group differences in microarray testing was confirmed for 5 genes in 8 of 10 comparisons. Before treatment, using qRT-PCR, expression of interleukin 6 (IL-6) was found to be 4-fold higher in patients (p < 0.05).

Conclusions: This study identifies genes in muscle, the expression of which may impact the pathophysiology of PMR. Moreover, the study adds further evidence of the importance of IL-6 in the disease. Follow-up studies are needed to establish the exact pathophysiological relevance of the identified genes. The study was retrospectively listed on the ISRCTN registry with study ID ISRCTN69503018 and date of registration the 26th of July 2017.

Keywords: Polymyalgia rheumatica, DNA microarray, Muscle, Gene expression, Prednisolone, Interleukin 6
Background
Polymyalgia rheumatica (PMR) affects men and women above the age of 50 and is recognized as the most common chronic inflammatory, rheumatic disease in this age group [1–3]. Clinically, PMR is associated with prominent muscle complaints, including aching and tender and stiff proximal muscles [1]. Paraclinically, erythrocyte sedimentation rate (ESR) and blood levels of C-reactive protein (CRP) are markedly elevated [1]. Furthermore, concentrations of proinflammatory cytokines, including also interleukin (IL) 6 [4, 5], are elevated systemically as well as locally in muscle tissue [5]. Yet, the prevailing view is that PMR reflects inflammation in the synovia of bursae, joints and tendon sheaths [6]. Overall, however, the current understanding of the etiology, pathogenesis and pathophysiology of PMR is modest. Treatment with glucocorticoids (GCs) is rapidly effective [7, 8], and the majority of patients maintains remission, but many experience at least one GC-related serious adverse event [9].

The genetics of PMR remain elusive; however, the higher incidence in Caucasians [10] and the higher susceptibility in people carrying the HLA-DRB1*04 allele [11] suggest that genetic factors may in fact impact the pathophysiology of the disease. Studies have found associations between polymorphisms in the genes encoding e.g. IL-6 and tumor necrosis factor alpha (TNF-α) and the susceptibility to and severity of PMR [12], but generally findings have been inconclusive [13, 14].

In the present study, to extend the understanding of the pathophysiology of PMR, we profiled the gene expression in muscle tissue from GC-naive patients with PMR and matched non-PMR control subjects before and after symptom-eliminating treatment with prednisolone.

Methods
Subjects
Nine GC-naive patients with newly diagnosed, untreated PMR and 10 matched (age, sex, and BMI) non-PMR control subjects were studied in the fasting state in the morning before and after 14 days of prednisolone treatment (20 mg/day taken in the morning, also 1–2 h before the second biopsy) in a comprehensive clinical experimental research program, some of the results of which we recently reported [5, 15]. Before the first experiment, non-PMR experimental research program, some of the results of which we recently reported [5, 15]. Before the first experiment, non-PMR control subjects before and after matched non-PMR control subjects before and after matched non-PMR control subjects before and after treatment with prednisolone; in the study after a standard medical examination and a comprehensive blood and urine screening. Both groups did not meet the exclusion criteria described by Kreiner and colleagues [5]. Controlled chronic comorbidities were accepted in both groups. Diminishing the possibility of occult malignant disease, all subjects had normal thorax X-ray and abdominal ultrasound examination, and negative test for blood in the stools and urine. In addition, all subjects had comprehensive blood screening performed. In patients, only ESR and CRP were different from normal values; no blood values in control subjects were abnormal.

Some subjects received concurrent medication as previously detailed [5]. Before the first experiment, non-steroidal anti-inflammatory drug treatment was not allowed, and use of analgesics was limited to the centrally-acting opioid-like drug tramadol (Mandolgin, Mandoz A/S, Odense, Denmark); none of the subjects had taken tramadol in the morning before any of the two experiments.

Experiments and interventions
From all subjects, biopsies were obtained from trapezius muscles before and after treatment with prednisolone; in all patients, the trapezius muscle exhibited the symptoms characteristic of PMR, i.e. aching, tenderness and stiffness. Following local anesthesia of the skin and subcutis with Lidocaine (20 mg/mL), muscle tissue was sampled through a small incision in the cutis, subcutis and muscle fascia using a 5 mm Bergström needle with suction [18]. Muscle samples were snap-frozen in liquid nitrogen, weighed (wt weight ranged from 35 to 100 mg per sample), and stored at −80 °C until RNA extraction.

Total RNA extraction
Total RNA was extracted from 20 to 30 mg muscle sample by tissue homogenization in TriReagent (Molecular
Research Center, Cincinnati, Ohio, US) using a bead-mixer (FastPrep®-24 instrument, MP Biomedicals, Illkirch, France) with five inert 2.3 mm steel beads (BioSpec Products, Bartlesville, OK, US) and one silicon carbide crystal followed by addition of bromo-chloropropene to separate the homogenate into aqueous and organic phases. To precipitate RNA, isopropanol was added to the isolated aqueous phase. The precipitated total RNA was washed repeatedly in 75% ethanol and dissolved in RNase-free water before storing at −80 °C until further analysis. Total RNA concentrations were determined by spectroscopy; yields averaged 0.4 μg total RNA/mg muscle tissue.

DNA microarray analysis
Sample preparation and hybridization, and detection and quantification of signals

Total RNA was further purified using RNeasy Mini Kits (Qiagen, Valencia, CA, US), and the integrity and purity of the RNA was verified using an Agilent Bioanalyzer (Agilent, Palo Alto, CA, US) as previously described [19]. Based on the quality of the RNA, 8 patient samples and 10 control subject samples were selected for microarray assessment. ds-cDNA was synthesized from 2 μg total RNA using an oligo-dT primer containing a T7 RNA polymerase promoter, and labeled in an T7 promoter-driven in vitro transcription reaction producing biotin-labeled cRNA from the cDNA according to the manufacturer’s (Affymetrix, Santa Clara, CA, US) guidelines. Next, the hybridization mixture was prepared from the fragmented target cRNA as well as probe array controls, bovine serum albumin, and herring sperm DNA.

Affymetrix GeneChip Human Genome U133 Plus 2.0 (Santa Clara, CA, US) arrays, which comprise 54,675 probe sets, were used. Following hybridization, the probe arrays were washed and stained with phycocyanin streptavidin (SAPE) using the Affymetrix Fluidics Station 450 and scanned using an Affymetrix GeneArray 3000 7G scanner 488 nm to generate fluorescent images as described in the Affymetrix GeneChip protocol. The amount of bound target at each location of the probe array is proportional to the amount of bound light emitted at 570 nm. Scanned data were stored as image files in cel-format.

Data analysis
Cel-files were imported into the statistical software package R v. 2.7.2 using BioConductor v. 2.8 [20], and gcRMA modeled using quantiles normalization and median polish summarization [21]. The modeled log-intensity of approximately 54,600 probe sets was used for selecting differentially expressed genes. The microarray data were submitted to the gene expression repository at Array Express (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-3671. Differentially expressed genes were selected based on an initial two-way ANOVA analysis including the parameters disease (PMR versus control) and treatment (before versus after treatment) with a p-value <0.05 and mutual fold change cut-off of 1.2 and reflecting either main effect or intervention. The resulting 565 selected probe sets were further analyzed. Pairwise differentially expressed transcripts were depicted by a univariate two-sample t-test with equal variance. Multiple testing corrections were performed using the multtest package in Bioconductor v. 2.7.2. Control of Type I error rate was performed by computing adjusted p-values for simple multiple testing procedures from a vector of raw (unadjusted) p-values by applying the Benjamini & Hochberg FDR analysis [22]. Only transcripts exhibiting a fold change larger than 1.2 and a difference of means larger than 30 (real unlogged values) between (mutual) classes were considered.

Gene grouping criteria
Predefined criteria were applied to identify genes of potential pathophysiological impact. The criteria were: 1. difference in expression level between untreated patients and untreated controls (Table 2), and 2. response to prednisolone treatment of expression levels in a given direction in patients only (Table 3). Those genes that differed between untreated patients and controls and that also responded to prednisolone treatment in patients, i.e. the aggregate of criteria 1 and 2, were also identified (criterion 3) (Table 4).

Assessment of biological function
For genes in all three criteria sets, biological functions were assessed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool [23] with default options and annotations current as of February 2013. Functional annotation clustering was performed; this process associates individual genes in a large gene list with biological terms and group sets of genes according to functionally similar terms. Moreover, the importance of each cluster is ranked using enrichment scores, which are the geometric means of the enrichment P values (EASE score [24]) for each annotation term in the cluster. While enrichment scores above 1.3 are considered particularly interesting, clusters with scores below 1.3 could also be of central importance (e.g. short gene lists do not generally get very high enrichment scores, illustrating that categories with lower scores may still be biologically relevant) [23]. In the presentation of the results, clusters with the highest enrichment scores will be presented.

Quantitative RT-PCR
To confirm mRNA level fold differences and fold changes found using the microarrays, mRNA levels for a selection (Tables 5 and 6) of the filtered genes were
| Gene symbol | Gene name                                                                 | Probe set(s) | FD* | p         |
|-------------|---------------------------------------------------------------------------|--------------|-----|-----------|
| BDNF        | brain-derived neurotrophic factor                                         | 244503_at    | +1.8| 0.016     |
| ETS2        | v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)              | 201328_at    | +1.8| 0.007     |
| SVIP        | small VCP/p97-interacting protein                                        | 230285_at    | +1.7| 0.002     |
| SH3RF2      | SH3 domain containing ring finger 2                                       | 228892_at    | +1.6| 0.004     |
| TM4SF18     | transmembrane 4 L six family member 18                                    | 230061_at    | +1.5| 0.007     |
| TMTC1       | transmembrane and tetratricopeptide repeat containing 1                  |              |     |           |
| TMEM18      | transmembrane protein 18                                                  | 225489_at    | +1.5| 0.008     |
| N4BP2L1     | NEDD4 binding protein 2-like 1                                            | 213375_s_at  |     |           |
| FMO2        | flavin containing monoxygenase 2 (non-functional)                         | 228268_at    | +1.5| 0.002     |
| RPL37       | ribosomal protein L37                                                     | 224763_at    | +1.5| <0.001    |
| TMPO        | thymopoietin                                                              | 224944_at    | +1.3| 0.002     |
| RERE        | arginine-glutamic acid dipeptide (RE) repeats                             | 200940_s_at  | +1.3| 0.003     |
| TUBD1       | tubulin, Delta 1                                                          | 231853_at    | +1.3| 0.003     |
| MARK4       | MAP/microtubule affinity-regulating kinase 4                              | 55065_at     | +1.3| 0.005     |
| ZNF195      | zinc finger protein 195                                                   | 204234_s_at  | +1.3| 0.003     |
| PCF11       | PCF11, cleavage and polyadenylation factor subunit. Homolog (S. cerevisiae) | 203378_at    | +1.3| 0.007     |
| DFFA        | DNA fragmentation factor, 45 kDa. alpha polypeptide                        | 226116_at    | +1.3| 0.010     |
| PSPC1       | paraspeckle component 1                                                   | 218371_s_at  | +1.3| 0.007     |
| RBBP6       | retinoblastoma binding protein 6                                          | 212783_at    | +1.3| 0.004     |
| EIF4B       | eukaryotic translation initiation factor 4B                               | 211937_at    | +1.3| 0.017     |
| NPM1        | nucleophosmin (nuclear phosphoprotein B23, numatrin)                     | 221691_s_at  | +1.3| 0.011     |
| RSBN1       | round spermatid basic protein 1                                           | 213604_at    | +1.2| 0.003     |
| PSIP1       | PC4 and SFRS1 interacting protein 1                                       | 209337_at    | +1.2| 0.010     |
| EIF3G       | eukaryotic translation initiation factor 3. subunit G                     | 208887_at    | +1.2| 0.006     |
| COL4A3BP    | collagen. Type IV, alpha 3 (Goodpasture antigen) binding protein          | 219625_s_at  | +1.2| 0.003     |
| PCID2       | PCI domain containing 2                                                   | 219940_s_at  | +1.2| 0.003     |
| PXDC1       | PX domain containing 1                                                    | 212923_s_at  | +1.2| 0.042     |
| BCKDHA      | branched chain keto acid dehydrogenase E1, alpha polypeptide              | 202331_at    | +1.2| 0.024     |
| AKR7A2      | aldo-keto reductase family 7, member A2                                   | 202139_at    | +1.2| 0.010     |
| MRPS2       | mitochondrial ribosomal protein S2                                         | 218001_at    | +1.2| 0.018     |
| RORA        | RAR-related orphan receptor A                                             | 226682_at    | +1.2| 0.049     |
Table 2 Genes the expression levels of which differed between untreated patients and untreated controls (78 genes) (Continued)

| Gene Symbol | Description | Probe Set(s) | Fold Difference | p-Value |
|-------------|-------------|--------------|----------------|---------|
| RPL36AL     | ribosomal protein L36a-like | 207585_s_at | +1.2 | 0.011 |
| TFRC        | transferrin receptor (p90, CD71) | 208691_at | −3.0 | 0.004 |
| SFRP4       | secreted frizzled-related protein 4 | 204051_s_at, 204052_s_at | −2.9 | 0.001 |
| NOV         | nephroblastoma overexpressed | 214321_at | −2.0 | 0.037 |
| PAQR9       | progestin and adipocytokine receptor family member IX | 1558322_a_at | −2.0 | <0.001 |
| C2orf88     | chromosome 2 open reading frame 88 | 228195_at | −1.9 | 0.011 |
| FAM69A      | family with sequence similarity 69, member A | 213689_x_at | −1.8 | 0.001 |
| TP53N2       | tumor protein p53 inducible nuclear protein 2 | 224836_at | −1.8 | <0.001 |
| SH3BP1      | SH3-domain kinase binding protein 1 | 1554168_a_at, 223082_at | −1.8 | 0.002 |
| NINJ2       | ninjurin 2 | 219594_at | −1.7 | 0.039 |
| MEST        | mesoderm specific transcript homolog (mouse) | 202016_at | −1.7 | 0.010 |
| ITGB1BP2    | integrin beta 1 binding protein (melusin) 2 | 219829_at | −1.6 | <0.001 |
| PLXDC1      | plexin domain containing 1 | 219700_at | −1.5 | 0.006 |
| BPGM        | 2,3-bisphosphoglycerate mutase | 203502_at | −1.5 | <0.001 |
| MTF1        | mitochondrial fission process 1 | 223172_s_at | −1.5 | 0.004 |
| MAP2K3      | mitogen-activated protein kinase kinase 3 | 215499_at | −1.5 | 0.003 |
| LRRN4C      | LRRN4 C-terminal like | 1556427_s_at | −1.4 | 0.042 |
| FBOX9       | F-box protein 9 | 210638_s_at, 212987_at | −1.4 | <0.001 |
| HERC1       | HECT and RLD domain containing E3 ubiquitin protein ligase family member 1 | 218306_s_at | −1.4 | <0.001 |
| JARID2      | jumonji, AT rich interactive domain 2 | 203297_s_at | −1.4 | <0.001 |
| TRAK1       | trafficking protein, kinesin binding 1 | 202079_s_at | −1.4 | 0.004 |
| ZNF252P     | zinc finger protein 252, pseudogene | 228200_at | −1.4 | <0.001 |
| PRSS23      | protease, serine, 23 | 202458_at | −1.4 | 0.030 |
| OLML2B      | olfactomedin-like 2B | 213125_at | −1.4 | 0.049 |
| MSANTD4     | Myb/SANT-like DNA-binding domain containing 4 with coiled-coils | 227418_at | −1.3 | 0.043 |
| ZDHHC7      | zinc finger, DHHC-type containing 7 | 218606_at | −1.3 | <0.001 |
| RAP2A       | RAP2A, member of RAS oncogene family | 225585_at | −1.3 | 0.016 |
| LRP12       | low density lipoprotein receptor-related protein 12 | 219631_at | −1.3 | 0.050 |
| BMP1R1A     | bone morphogenetic protein receptor, type IA | 213578_at | −1.3 | 0.001 |
| RNF10       | ring finger protein 10 | 207801_s_at | −1.3 | <0.001 |
| COL5A1      | collagen, type V, alpha 1 | 203325_s_at | −1.3 | 0.007 |
| INSG1       | insulin induced gene 1 | 201626_at | −1.3 | 0.046 |
| SLC35E3     | solute carrier family 35, member E3 | 218988_at | −1.3 | 0.003 |
| MEMO1       | dpy-30 homolog (C. elegans) // mediator of cell motility 1 | 219065_s_at | −1.3 | 0.004 |
| MYL4        | myosin, light chain 4, alkali; atrial, embryonic | 210395_x_at | −1.2 | 0.002 |
| COX7A2      | cytochrome c oxidase subunit VIIa polypeptide 2 (liver) | 201597_at | −1.2 | 0.019 |
| MGAT4B      | mannosyl (alpha-1,3)-glucoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B | 224598_at | −1.2 | 0.003 |
| MRC2        | mannose receptor, C type 2 | 209280_at | −1.2 | 0.010 |

Fold difference. * Fold differences for genes with more than one probe set were calculated as the average of the individual values, which did not differ markedly.
Table 3: Genes the expression levels of which responded to prednisolone treatment in a given direction only in patients with polymyalgia rheumatica (131 genes)

| Gene symbol | Gene name                                      | Probe set(s)       | FC*  | p     |
|-------------|------------------------------------------------|--------------------|------|-------|
| COL1A1      | collagen, type I, alpha 1                       | 1556499_s_at       | +4.7 | 0.028 |
| CTGF        | connective tissue growth factor                 | 209101_at          | +2.9 | 0.012 |
| MEST        | mesoderm specific transcript homolog (mouse)   | 202016_at          | +2.7 | 0.049 |
| CDH11       | cadherin 11, type 2, OB-cadherin (osteoblast)  | 207173_x_at        | +2.6 | 0.012 |
| S1PR3       | sphingosine-1-phosphate receptor 3              | 228176_at          | +2.5 | 0.009 |
| CD248       | CD248 molecule, endosialin                      | 210025_at          | +2.5 | 0.019 |
| FBN1        | fibrillin 1                                     | 202766_s_at, 235318_at | +2.4 | 0.031 |
| NINJ2       | ninjurin 2                                      | 219594_at          | +2.3 | 0.002 |
| MFAP5       | microfibrillar associated protein 5             | 200975b_s_at, 213764_s_at, 213765_at | +2.7 | 0.038 |
| SH3PXD2B    | SH3 and PX domains 2B                          | 231823_s_at        | +2.2 | 0.011 |
| C1orf33     | chromosome 13 open reading frame 33            | 227058_at          | +2.2 | 0.044 |
| FOSL2       | FOS-like antigen 2                              | 218880_at          | +2.2 | 0.026 |
| BGN         | biglycan                                       | 201261_x_at        | +2.1 | 0.029 |
| NEDD9       | neural precursor cell expressed, developmentally down-regulated 9 | 233223_at          | +2.1 | 0.004 |
| COL5A2      | collagen, type V, alpha 2                       | 221730_at          | +2.0 | 0.049 |
| NTSE        | S'-nucleotidase, ecto (CD73)                    | 203939_at          | +2.0 | 0.044 |
| TUBB6       | tubulin, beta 6 class V                        | 209191_at          | +2.0 | 0.031 |
| SPARC       | secreted protein, acidic, cysteine-rich (osteonectin) | 200665_s_at       | +2.0 | 0.043 |
| FN1         | fibronectin 1                                   | 210495_x_at, 212464_s_at, 216442_x_at | +1.9 | 0.045 |
| GFPT2       | glutamine-fructose-6-phosphate transaminase 2   | 205100_at          | +1.9 | 0.034 |
| NFKBIZ      | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta | 2252717_s_at | +1.9 | 0.025 |
| DCLK1       | doublecortin-like kinase 1                     | 205399_at          | +1.9 | 0.034 |
| METRN1      | meteorein, glial cell differentiation regulator-like | 225955_at | +1.9 | 0.023 |
| COL1A2      | collagen, type I, alpha 2                       | 2229218_at         | +1.8 | 0.048 |
| LAMB1       | laminin, beta 1                                | 201505s_at         | +1.8 | 0.003 |
| LSP1P1      | lymphocyte-specific protein 1 pseudogene        | 214110_s_at        | +1.8 | 0.020 |
| COL6A3      | collagen, type VI, alpha 3                     | 201438_at          | +1.8 | 0.003 |
| GAS7        | growth arrest-specific 7                       | 202191_s_at, 202192_s_at | +1.8 | 0.028 |
| ARHGAP26    | Rho GTPase activating protein 26                | 206627_at          | +1.7 | 0.021 |
| OLFML2B     | olfactomedin-like 2B                            | 213125_at          | +1.7 | 0.031 |
| SPON2       | spondin 2, extracellular matrix protein         | 218638_s_at        | +1.7 | 0.002 |
| COL6A1      | collagen, type VI, alpha 1                     | 213428_s_at        | +1.7 | 0.006 |
| CILP        | cartilage intermediate layer protein, nucleotide pyrophosphohydrolase | 206627_at | +1.7 | 0.012 |
| OLFML3      | olfactomedin-like 3                             | 218162_at          | +1.7 | 0.026 |
| FAM69A      | family with sequence similarity 69, member A    | 213689_x_at        | +1.7 | <0.001 |
| CORO1C      | coronin, actin binding protein, 1C              | 222409_at          | +1.6 | 0.020 |
| MAP1B       | microtubule-associated protein 1B               | 226084_at          | +1.6 | 0.039 |
Table 3: Genes whose expression levels responded to prednisolone treatment in a given direction only in patients with polymyalgia rheumatica (131 genes) (Continued)

| Gene          | Description                                          | Probe IDs               | Expression | Log2 Fold Change | p-Value |
|---------------|------------------------------------------------------|-------------------------|------------|------------------|---------|
| COL6A2        | collagen, type VI, alpha 2                           | 209156_s_at            | +1.6       | 0.020            |
| PRKCBP        | protein kinase C, delta binding protein              | 213010_at              | +1.6       | <0.001           |
| CLIC4         | chloride intracellular channel 4                    | 201560_at              | +1.6       | 0.010            |
| LRRN4CL       | LRRN4 C-terminal like                                | 1556427_s_at           | +1.5       | 0.006            |
| CD109         | CD109 molecule                                       | 226545_at              | +1.5       | 0.034            |
| DBN1          | drebrin 1                                            | 202806_at              | +1.5       | 0.020            |
| SFXN3         | sideroflexin 3                                       | 220974_x_at            | +1.5       | 0.016            |
| TNXA / TNXB   | tenascin XA (pseudogene) / tenascin XB              | 206093_x_at            | +1.5       | 0.030            |
| PRSS23        | protease, serine, 23                                 | 202458_at              | +1.5       | 0.022            |
| TUBA1A        | tubulin, alpha 1a                                    | 209118_s_at            | +1.5       | 0.038            |
| SAMHD1        | SAM domain and HD domain 1                          | 235529_x_at            | +1.5       | 0.024            |
| ITG1BP2       | integrin beta 1 binding protein (melusin) 2          | 219829_at              | +1.5       | 0.003            |
| ATP2C1        | ATPase, Ca++ transporting, type 2C, member 1         | 209934_s_at            | +1.5       | <0.001           |
| PXDC1         | PX domain containing 1                               | 212923_s_at            | +1.5       | 0.014            |
| PAQR9         | progestin and adipoQ receptor family member IX       | 1558322_a_at           | +1.4       | 0.027            |
| P4HA2         | prolyl 4-hydroxylase, alpha polypeptide II           | 202733_at              | +1.4       | 0.024            |
| ANXA2         | annexin A2                                           | 201590_x_at            | +1.4       | 0.025            |
| ACVRL1        | activin A receptor type II-like 1                   | 226950_at              | +1.4       | 0.009            |
| CHSY1         | chondroitin sulfate synthase 1                      | 203044_at              | +1.4       | 0.021            |
| C10orf54      | chromosome 10 open reading frame 54                 | 225373_at              | +1.4       | 0.016            |
| PLAG1         | pleiomorphic adenoma gene-like 1                    | 207943_s_at            | +1.4       | 0.012            |
| CTTNB2NL      | CTTNB2 N-terminal like                               | 226000_at              | +1.4       | 0.019            |
| SYNO2         | synaptopodin 2                                       | 225720_at              | +1.4       | 0.013            |
| ANXAP2        | annexin A2 pseudogene 2                              | 208816_x_at            | +1.4       | 0.042            |
| TGFB1I1       | transforming growth factor beta 1 induced transcript 1| 209651_at              | +1.4       | 0.043            |
| ACTB          | actin, beta                                          | 213867_x_at            | +1.4       | 0.048            |
| ADNP2         | ADNP homebox 2                                       | 203321_s_at            | +1.3       | 0.009            |
| MTFF1         | mitochondrial fission process 1                     | 223172_s_at            | +1.3       | 0.017            |
| TPS3INP2      | tumor protein p53 inducible nuclear protein 2        | 224836_at              | +1.3       | 0.017            |
| PDGFRB        | platelet-derived growth factor receptor, beta polypeptide| 202273_at          | +1.3       | 0.009            |
| FBXO9         | F-box protein 9                                      | 210638_s_at            | +1.3       | 0.002            |
| VAT1          | vesicle amine transport protein 1 homolog (T. californica)| 208626_s_at       | +1.3       | 0.043            |
| LTBP1         | latent transforming growth factor beta binding protein 1| 202729_s_at          | +1.3       | 0.026            |
| SH3KBP1       | SH3-domain kinase binding protein 1                 | 1554168_a_at           | +1.3       | 0.044            |

Kreiner et al. BMC Musculoskeletal Disorders (2017) 18:341
Table 3 Genes the expression levels of which responded to prednisolone treatment in a given direction only in patients with polymyalgia rheumatica (131 genes) (Continued)

| Gene               | Description                                      | Exp. Direction | Exp. Value | P-Value |
|--------------------|--------------------------------------------------|----------------|------------|---------|
| JARID2             | jumonji, AT rich interactive domain 2            | +1.3           | 0.027      |
| ACTG1              | actin, gamma 1                                   | +1.3           | 0.015      |
| BPGM               | 2,3-bisphosphoglycerate mutase                   | +1.2           | 0.036      |
| TUBB               | tubulin, beta class I                            | +1.2           | 0.039      |
| DDAH1              | dimethylarginine dimethylaminohydrolase 1        | +1.2           | 0.033      |
| BDNF               | brain-derived neurotrophic factor                | −3.1           | 0.001      |
| SLC25A34           | solute carrier family 25, member 34              | −1.9           | 0.009      |
| SVIP               | small VCP/p97-interacting protein                | −1.7           | 0.004      |
| VPS8               | vacuolar protein sorting 8 homolog (S. cerevisiae) | −1.6           | <0.001    |
| PIAS2              | protein inhibitor of activated STAT, 2           | −1.6           | 0.011      |
| LOC100507303       | uncharacterized LOC100507303                     | −1.6           | 0.004      |
| RPL37              | ribosomal protein L37                            | −1.5           | <0.001    |
| TMTC1              | transmembrane and tetratricopeptide repeat containing 1 | −1.4           | 0.006      |
| MLYCD              | malonyl-CoA decarboxylase                         | −1.5           | 0.004      |
| UCP3               | uncoupling protein 3 (mitochondrial, proton carrier) | −1.5           | 0.016      |
| TUBD1              | tubulin, delta 1                                 | −1.4           | 0.003      |
| BCKDHA             | branched chain keto dehydrogenase E1, alpha polypeptide | −1.4           | 0.004      |
| TRIM39             | tripartite motif containing 39                   | −1.4           | 0.002      |
| ZNF331             | zinc finger protein 331                          | −1.4           | 0.003      |
| NRRF2              | nuclear receptor binding factor 2                | −1.4           | 0.021      |
| GTF2H5             | general transcription factor IIH, polypeptide 5   | −1.4           | 0.007      |
| FMO2               | flavin containing monooxygenase 2 (non-functional) | −1.4           | 0.002      |
| TMEM18             | transmembrane protein 18                         | −1.4           | 0.028      |
| HSDL2              | Hydroxysteroid dehydrogenase like 2              | −1.4           | 0.006      |
| N4BP2L1            | NEDD4 binding protein 2-like 1                   | −1.4           | 0.033      |
| PEBP4              | phosphatidylethanolamine-binding protein 4       | −1.4           | 0.009      |
| RANBP9             | RAN binding protein 9                            | −1.4           | 0.002      |
| ST3GAL5            | ST3 beta-galactoside alpha-2,3-sialyltransferase 5 | −1.3           | 0.003      |
| ACADSB             | acyl-CoA dehydrogenase, short/branched chain     | −1.3           | 0.006      |
| RNF114             | ring finger protein 114                          | −1.3           | 0.020      |
| MRPS2              | mitochondrial ribosomal protein S2               | −1.3           | 0.006      |
| TMEM50B            | transmembrane protein 50B                        | −1.3           | 0.027      |
| EIF3G              | eukaryotic translation initiation factor 3, subunit G | −1.3           | 0.005      |
measured using quantitative real-time PCR (qRT-PCR). Moreover, mRNA levels for additional genes (Table 5) that did not differ using microarrays, but which were of particular interest in elucidating the PMR disease mechanisms, were included in the qRT-PCR analysis.

From 9 patient samples and 9 control subject samples, cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) from 500 ng total RNA (same pool as used in the microarray runs) in 20 μl. For each target mRNA, 0.25 μl cDNA was amplified in 25 μl Quantitect SYBR Green Master Mix (Qiagen) with corresponding primers (100 nM of both antisense and sense primers, Table 6) on a Stratagene MX3000P RT-PCR instrument (Stratagene, La Jolla, CA, US).

The applied thermal profile was as follows: 95°Celsius, 10 min-(95 °C, 15 s-58 °C, 30s-63°C, 90s)x50–95 °C, 60s-55°C, 30s-95°C, 60s. Standard curves were made using dilution series of a cDNA pool and related to the threshold cycles (Ct) at the 63 °C step at which the signal intensity was acquired. To ensure specificity, melting curves were analyzed post amplification (at the 55 °C to 95 °C step). The Ct values for the samples were converted to relative values using the standard curves and normalized to the internal “housekeeping” control, ribosomal protein P0 (RPLP0). Microarray analysis confirmed that the RPLP0 mRNA level is stable under the current conditions and therefore suitable as the normalizer.

**Statistics**

Statistical methods used in the evaluation of the microarray data are described above. Data are reported in compliance with the guidelines for minimum information about a microarray experiment (MIAME).

Statistical analyses of qRT-PCR and anthropometric data as well as of ESR and CRP levels were performed using SPSS software version 20.0 for Macintosh. qRT-PCR data were log-transformed. Statistically significant differences were detected using Student’s t tests, paired or unpaired as applicable. Identical conclusions were achieved with standard non-parametric tests. P-values less than 0.05 were considered significant in two-tailed testing.

**Results**

Clinical characteristics for all participants are given in Table 1. In all of the PMR patients, treatment with prednisolone abolished symptoms within a few days, supporting the PMR diagnosis; at day 15, ESR and CRP levels were markedly reduced in the patients and did no longer differ significantly from values in controls (Table 1).
| Gene symbol | Gene name | FD* | p   | FC* | p   |
|-------------|-----------|-----|-----|-----|-----|
| BDNF        | brain-derived neurotrophic factor | +1.8 | 0.016 | −3.1 | 0.001 |
| SVIP        | small VCP/p97-interacting protein | +1.7 | 0.002 | −1.7 | 0.004 |
| TM4SF18     | transmembrane 4 L six family member 18 | +1.5 | 0.007 | −1.3 | 0.033 |
| TMTC1       | transmembrane and tetratricopeptide repeat containing 1 | +1.5 | 0.001 | −1.5 | 0.003 |
| TMEM18      | transmembrane protein 18 | +1.5 | 0.008 | −1.4 | 0.028 |
| NFIP2L1     | NEDD4 binding protein 2-like 1 | +1.5 | 0.019 | −1.4 | 0.033 |
| FMO2        | flavin containing monoxygenase 2 (non-functional) | +1.5 | 0.002 | −1.4 | 0.012 |
| RPL37       | ribosomal protein L37 | +1.5 | <0.001 | −1.5 | <0.001 |
| FAM184B     | family with sequence similarity 184, member B | +1.4 | 0.013 | −1.3 | 0.042 |
| LOC100507303| uncharacterized LOC100507303 | +1.4 | 0.019 | −1.6 | 0.004 |
| RF114       | ring finger protein 114 | +1.3 | 0.016 | −1.3 | 0.030 |
| RERE        | arginine-glutamic acid dipeptide (RE) repeats | +1.3 | 0.003 | −1.3 | 0.008 |
| TUBD1       | tubulin, delta 1 | +1.3 | 0.003 | −1.4 | 0.003 |
| ZNF195      | zinc finger protein 195 | +1.3 | 0.003 | −1.3 | 0.002 |
| DFFA        | DNA fragmentation factor, 45 kDa, alpha polypeptide | +1.3 | 0.010 | −1.2 | 0.016 |
| RBBP6       | retinoblastoma binding protein 6 | +1.3 | 0.004 | −1.2 | 0.025 |
| NPM1        | nucleophosmin (nucleolar phosphoprotein B23, numatrin) | +1.3 | 0.011 | −1.2 | 0.022 |
| EIF4B       | eukaryotic translation initiation factor 4B | +1.3 | 0.017 | −1.3 | 0.015 |
| RSBN1       | round spermatid basic protein 1 | +1.2 | 0.003 | −1.2 | 0.016 |
| PSBP1       | PC4 and SFRS1 interacting protein 1 | +1.2 | 0.010 | −1.3 | 0.007 |
| EIF3G       | eukaryotic translation initiation factor 3, subunit G | +1.2 | 0.006 | −1.3 | 0.005 |
| PXDC1       | PX domain containing 1 | +1.2 | 0.042 | +1.5 | 0.014 |
| BCKDHA      | branched chain keto acid dehydrogenase E1, alpha polypeptide | +1.2 | 0.024 | −1.4 | 0.004 |
| AKR7A2      | aldo-keto reductase family 7, member A2 | +1.2 | 0.010 | −1.2 | 0.002 |
| MRPS2       | mitochondrial ribosomal protein S2 | +1.2 | 0.018 | −1.3 | 0.006 |
| RORA        | RAR-related orphan receptor A | +1.2 | 0.049 | −1.2 | 0.044 |
| RPL36AL     | ribosomal protein L36a-like | +1.2 | 0.011 | −1.2 | 0.008 |
| PAQR9       | progestin and adipok receptor family member IX | −2.0 | <0.001 | +1.4 | 0.027 |
| FAM69A      | family with sequence similarity 69, member A | −1.8 | 0.001 | +1.7 | <0.001 |
| TPS3BP2     | tumor protein p53 inducible nuclear protein 2 | −1.8 | <0.001 | +1.3 | 0.017 |
| SH3KBP1     | SH3-domain kinase binding protein 1 | −1.8 | 0.002 | +1.3 | 0.035 |
| NINJ2       | ninjurin 2 | −1.7 | 0.039 | +2.3 | 0.002 |
| MEST        | mesoderm specific transcript homolog (mouse) | −1.7 | 0.010 | +2.7 | 0.049 |
| ITGB1BP2    | integrin beta 1 binding protein (melusin) 2 | −1.6 | <0.001 | +1.5 | 0.003 |
| BPGM        | 2,3-bisphosphoglycerate mutase | −1.5 | <0.001 | +1.2 | 0.036 |
| MTP1        | mitochondrial fission process 1 | −1.5 | 0.004 | +1.3 | 0.017 |
| MAP2K3      | mitogen-activated protein kinase kinase 3 | −1.5 | 0.003 | +1.3 | 0.021 |
| LRRN4CL     | LRRN4 C-terminal like | −1.4 | 0.042 | +1.5 | 0.006 |
| FBXO9       | F-box protein 9 | −1.4 | <0.001 | +1.3 | 0.001 |
| JARID2      | jumonji, AT rich interactive domain 2 | −1.4 | <0.001 | +1.3 | 0.007 |
| PRS323      | protease, serine, 23 | −1.4 | 0.030 | +1.5 | 0.022 |
| OLFML2B     | olfactomedin-like 2B | −1.4 | 0.049 | +1.7 | 0.031 |
| MEMO1       | mediator of cell motility 1 | −1.3 | 0.004 | +1.3 | 0.012 |

**FD** fold difference, **FC** fold change

* + and −; expression levels were higher and lower, respectively, in patients with polymyalgia rheumatica than in controls before treatment with prednisolone

* + and −; expression levels increased and decreased, respectively, in patients with polymyalgia rheumatica after treatment with prednisolone

* Entry in bold indicates that the gene also responded significantly to prednisolone in controls. The response in controls for the RORA gene was of the same magnitude as in patients but in the opposite direction.
Control subjects had normal ESR and CRP values both before and after treatment (Table 1).

**Differential expression of genes in untreated PMR patients vs controls and also responding to prednisolone in patients**

Among all 165 differentially expressed genes were 44 genes, the expression levels of which differed between untreated patients and controls and which in patients only also responded to prednisolone treatment in a given direction (Fig. 1 and Table 4). Of these 44 genes, the expression levels of 28 genes were higher in untreated patients than in untreated controls (mean fold difference: 1.4; range: 1.2–1.8); the expression levels of 16 genes were lower (mean fold difference: 1.4; range: 1.2–2.0). Upon prednisolone treatment, the expression levels of 27 were down-regulated in patients (mean fold change: 1.4; range: 1.2–3.1), whereas 17 genes were up-regulated (mean fold change: 1.5; range: 1.2–3.0).

In this subset, the biological function (Fig. 2) of the 78 genes as identified by the DAVID functional annotation clusters (19 clusters in total) included translation/protein biosynthesis (2 clusters, enrichment scores 0.8 and 0.62 [data not shown], transcription/regulation of transcription (2 clusters, enrichment score 0.69 and 0.4 [data not shown], nuclear transport and protein transport (enrichment score 0.83), and SH3 domain binding properties (enrichment score 1.15 [data not shown]).

### Genes responding to prednisolone in PMR patients

Expression of 131 of the total 165 genes responded to prednisolone treatment in patients (Fig. 1 and Table 3); of these genes, two responded significantly to treatment in controls, however in the opposite direction to that seen in patients. Of the 131 genes, the expression of 84 genes was up-regulated upon treatment (mean fold change: 1.7; range: 1.2–4.7); 47 genes were down-regulated (mean fold difference: 1.4; range: 1.2–3.1). In this subset, out of a total of 62 DAVID-identified clusters, the clusters of interesting biological function and high enrichment scores (Fig. 3) included extracellular matrix organization and cell adhesion (2 highly enriched clusters, enrichment scores 5.58 and 4.11 [not shown in Fig. 3]), cytoskeleton/microtubule organization (2 clusters, enrichment scores 2.38 and 1.62 [not shown in Fig. 3]), and actin filament/cytoskeleton associated processes (1 cluster, enrichment score 1.57).

### Genes differentially expressed in untreated PMR patients with prednisolone than in controls before treatment

565 transcripts were differentially expressed between patients and controls or before vs after treatment with prednisolone, reflecting either main effect or interaction. Among these transcripts, 165 genes fulfilled at least one of the 2 criteria (Methods) that define the potentially clinically relevant genes.

Of the 165 genes, expression levels of 78 genes differed between patients and controls before treatment (Fig. 1, Table 2). Among these genes, 41 genes were up-regulated in the patients (mean fold difference: 1.4; range: 1.2–1.8), while 37 were downregulated (mean fold difference: 1.5; range: 1.2 – 3.0).

| Gene symbol | Fold differences | Fold changes |
|-------------|------------------|--------------|
| (probe name) | qRT-PCR | Microarray | qRT-PCR | Microarray |
| BDNF | +1.90* | +1.80* | −1.58** | −3.1** |
| COL5A1 | −1.33 ns | −1.30** | +1.73 ns | +2.30 ns |
| EIF4B | +1.63 p = 0.0504 | +1.30* | −1.23* | −1.30* |
| MARK4 | +1.32 ns | +1.30** | −1.24* | −1.15 ns |
| MTFP1 | +1.00 ns | −1.50** | +1.33* | +1.30* |
| NPM1 | +1.38** | +1.30* | −1.09 ns | −1.22* |
| PRSS23 | −1.21 ns | −1.40* | +1.27 ns | +1.51* |
| TFRC | −1.63 ns | −3.00* | +1.17 ns | +1.76 ns |
| TUBD1 | +1.26** | +1.30** | −1.08 ns | −1.40** |

| Genes that did not differ in microarray testing |
|-----------------------------------------------|
| ACTA1 (203872_at) | −1.03 ns | −1.02 ns | 1.06 ns | +1.00 ns |
| DES (216947_at) | +1.16 ns | +1.00 ns | −1.07 ns | +1.00 ns |
| IL6 (205207_at) | +4.54 * | +1.02 ns | −3.25 * | +1.02 ns |
| TNFA (207113_s_at) | +1.31 ns | +1.00 ns | −1.31 ns | −1.00 ns |
| TUBB8 (220069_at) | −1.02 ns | −1.02 ns | +1.10 ns | +1.00 ns |

qRT-PCR quantitative real-time PCR  
* p < 0.05  
** p < 0.01  
ns, not statistically significant. Data are geometric means  
a and b, expression levels were higher and lower, respectively, in patients with polymyalgia rheumatica than in controls before treatment with prednisolone  
c and d, expression levels increased and decreased, respectively, in patients with polymyalgia rheumatica after treatment with prednisolone  
**p < 0.05. *p < 0.01. ns, not statistically significant. Data are geometric means  
a Microarray numbers were calculated as the mean of the individual probe values  
b + and −, expression levels were higher and lower, respectively, in patients with polymyalgia rheumatica than in controls before treatment with prednisolone  
c + and −, expression levels increased and decreased, respectively, in patients with polymyalgia rheumatica after treatment with prednisolone

### Table 5 Quantitative RT-PCR fold differences between untreated patients with polymyalgia rheumatica (PMR) and non-PMR controls, and fold changes between treated and untreated PMR patients

| Gene symbol | Fold differences | Fold changes |
|-------------|------------------|--------------|
| (probe name) | qRT-PCR | Microarray | qRT-PCR | Microarray |
| BDNF | +1.90* | +1.80* | −1.58** | −3.1** |
| COL5A1 | −1.33 ns | −1.30** | +1.73 ns | +2.30 ns |
| EIF4B | +1.63 p = 0.0504 | +1.30* | −1.23* | −1.30* |
| MARK4 | +1.32 ns | +1.30** | −1.24* | −1.15 ns |
| MTFP1 | +1.00 ns | −1.50** | +1.33* | +1.30* |
| NPM1 | +1.38** | +1.30* | −1.09 ns | −1.22* |
| PRSS23 | −1.21 ns | −1.40* | +1.27 ns | +1.51* |
| TFRC | −1.63 ns | −3.00* | +1.17 ns | +1.76 ns |
| TUBD1 | +1.26** | +1.30** | −1.08 ns | −1.40** |

**p < 0.05. *p < 0.01. ns, not statistically significant. Data are geometric means  
a Microarray numbers were calculated as the mean of the individual probe values  
b + and −, expression levels were higher and lower, respectively, in patients with polymyalgia rheumatica than in controls before treatment with prednisolone  
c + and −, expression levels increased and decreased, respectively, in patients with polymyalgia rheumatica after treatment with prednisolone

Genes that did not differ in microarray testing |
-----------------------------------------------|
ACTA1 (203872_at) | −1.03 ns | −1.02 ns | 1.06 ns | +1.00 ns |
DES (216947_at) | +1.16 ns | +1.00 ns | −1.07 ns | +1.00 ns |
IL6 (205207_at) | +4.54 * | +1.02 ns | −3.25 * | +1.02 ns |
TNFA (207113_s_at) | +1.31 ns | +1.00 ns | −1.31 ns | −1.00 ns |
TUBB8 (220069_at) | −1.02 ns | −1.02 ns | +1.10 ns | +1.00 ns |
Table 6 qRT-PCR primer sequences

| Gene  | Sense                      | Antisense                  |
|-------|----------------------------|----------------------------|
| ACTA1 | GCCGTGTCCCCTCCATCGT        | TTCAGGGTCAGGATACCTCTTTGCT |
| BDNF  | GAGGGGACCTGAGGGGTGTTG      | TTTTGTGCTGGCGGGTTACC       |
| COL5A1| CGCCGACCTCCCCAACTCTCCCT   | CTCAACTGACTCCCCCTCAA       |
| DES   | CATCCAGACTCTACCTGCCCTC    | TTGGATAGACCTGAGAACCCTTT    |
| EIF4B | CTGACGCTGAGGGCCCTACCAAAAA | GTCTCGAGGCCCTGCCCTCC       |
| IL6   | GAGGACCTGAGAAAACACACC     | CCTCAAACTCCAAAAAGACCATGTG |
| MARK4 | AGATCCGAGGCGGGGGAAG        | GGGTCATCATGTCAGGGAGAGTT    |
| TFP1  | AAGGGCAAGAAGGCTGAGGAGGTTG | ACGAGCGCTAGAGGCTGCCATACAAA|
| NPM1  | GTTTCCTTTGGGGGCTTTTG      | GCAGTTGAGCTGGAAACCACACTT  |
| PRSS23| CAGCGGTTCTGGGGCTATAG      | GCAATAATTTCCTGGCCTCCATTCT |
| TUBD1 | TGATTTGTGGAAAGGATGGA      | CAAACAATTGGCTTAAATGACGTTAAA|
| TFRC  | TGGGAATGCTGAGGAAAACAGACA  | TTTTGGAGATACGTAGGGGAGAGGAA|
| TNFA  | TTCCCCAGGGGCCTCTCCCTATCC  | GAGGGTGGCTGACAATGGGCTAC   |
| TUBA8 | GCCCAAGAGATGTGAATGTGCCTCT | GGTGCCGGGCGCTGTGAGTGATG    |
| RPLP0 | GAAGACTCTGGACCTGCCCTTCTCT | CCAGGGACTGTGTTGTACCCGTTG  |

qRT-PCR: quantitative real-time PCR

The primer set sequence for BDNF provided in Table 6 recognizes all BDNF isoforms; using this primer set, the results presented in Table 5 were obtained. The BDNF mRNA levels were also assessed with qRT-PCR using a BDNF primer set that specifically recognizes the BDNF isoform that is recognized by the probe on the used microarray; the results (fold difference +1.33, p < 0.1; fold change −2.4, p < 0.01) from this additional assessment were very similar to the results presented in Table 5.

![Venn diagram](image)

**Fig. 1** Venn-diagram showing 1. the number of genes that differed between untreated patients with polymyalgia rheumatica (PMR) and non-PMR controls (left circle, 34 + 44 genes) and 2. the number of genes that responded to treatment with prednisolone in a given direction in patients with PMR only (right circle, 44 + 87 genes). The overlap of the two circles includes the number of genes which fulfilled both criteria 1 and 2 (44).
To validate the levels found using microarrays, the expression of some of the genes were measured using qRT-PCR (Tables 5 and 6, and Fig. 5). Nine genes that fulfilled criterion 1 or criterion 2 according to microarray analysis were examined with qRT-PCR (Table 5 and Fig. 5b); 8 of the 9 genes were always regulated in the same direction as found using microarrays. However, for the comparison of patients and controls before treatment (criterion 1), the expression fold differences of 5 genes (COL5A1, MARK4, MTFP1, PRSS23, and TRFC), which were statistically significant in the microarray analysis, did not reach significance using qRT-PCR ($p > 0.05$). For the treated vs untreated patients comparison (criterion 2), the fold changes for NPM1, PRSS23 and TUBD1 were significant in the microarray but not in the qRT-PCR, whereas the fold change for MARK4 was significant only in qRT-PCR analysis. The fold changes for COL5A1 and TRFC were not statistically significant ($p > 0.05$) in the microarray nor in the qRT-PCR analysis.

Moreover, the expression levels of 5 genes (Table 5) of potential interest in PMR that did not differ in the microarray analysis were measured using qRT-PCR. Expression levels of IL-6 (Fig. 5a), which did not differ in the microarray experiments (FD and FC < 1.1), markedly differed both between untreated patients and controls (FD 4.54, $p < 0.05$) and between patients before and after treatment (FC $-3.25$, $p < 0.05$) using qRT-PCR (Table 5). The remaining four genes were found to differ neither between untreated patients and controls nor between patients before and after treatment with either method.
Discussion

In the present study, the gene expression in skeletal muscle was measured for the first time in patients with PMR and in non-PMR, matched controls before and after brief, symptom-relieving prednisolone treatment using DNA microarrays. Microarray findings were supplemented by testing of the expression levels of selected genes with qRT-PCR, which was also used to accurately measure expression levels of genes of particular interest. In all subjects, biopsies were obtained from the trapezius muscle. Before treatment, patients had marked clinical symptoms, including trapezius myalgia and tenderness, as well as elevated ESR and levels of CRP; upon treatment, paraclinical parameters had normalized and clinical symptoms had disappeared.

Subjects were studied in 2008; thus, we were not able to use the most recent PMR criteria, which were published in 2012 [17]. However, the latter criteria are still provisional and awaiting further validation, and, in the most recent reviews of PMR, the Chuang criteria are mentioned on par with the newer provisional criteria [2, 3, 8, 17]. The two criteria sets are very similar; however, the fact that the demand for a high ESR is stricter in the Chuang criteria implies that the patients in the present study would also be accepted with the new criteria.

A total of 565 genes were differentially expressed across all groups. In general, when measured by microarray, fold differences and fold changes in expression were modest, ranging from 1.2 (cut-off value) to 1.4 for most genes. Despite the relatively modest differences in gene expression levels, gene function analysis indicated that even these small differences may have a pathophysiological and phenotypic impact in PMR. A few genes were regulated more markedly, with fold differences and changes in the range of 2 to 4. In the microarray measurements, none of the genes that usually are associated with PMR [12], for example genes encoding proteins involved in inflammation, e.g. IL-6, were
Genes differentially expressed in untreated PMR patients vs controls

The applied study design allowed for 3 important comparisons. Firstly, by comparing expressions levels in untreated patients and control subjects, 78 genes of possible central importance for the phenotype of PMR were identified.

Although the enrichment scores, which are proportional to the extent to which the cluster is represented in the gene set (here 78 genes), were modest within this subset of genes, functional clustering analysis identified several clusters of genes, many of which were associated with protein translation and biosynthesis. Other identified clusters included regulation of transcription, cellular and nuclear protein transport, and rearrangement of the cytoskeleton; the latter process was also represented in a gene cluster that involved SH-3-domain-binding properties, which are associated with cytoskeletal elements and signaling proteins.

The identification of clusters associated with protein translation, biosynthesis and transport may suggest that PMR is associated with abnormal protein metabolism in muscle. It might be speculated that inflammation...
and immobilization, which induce negative protein balance in many chronic diseases, accounted for these findings. However, in the protein translation and biosynthesis clusters, more genes were up-regulated rather than down-regulated in patients versus controls in the present cohort. Furthermore, indicating a minor role of inactivity in the present study, the number of genes in muscle influenced by PMR was small compared to findings in response to inactivity per se [27].

Another finding that may possibly contribute to the muscle complaints, primarily the muscle stiffness, experienced by PMR patients [28] is that proteins involved in organizing the cytoskeleton, including tubulin delta 1 (TUBD1; similar findings with microarrays and with qRT-PCR) and microtubule affinity-regulating kinase 4 (MARK4; similar differences in microarray and qRT-PCR, but only significant in the former), were up-regulated in patients before prednisolone treatment (Tables 2 and 5) [28].

Another interesting gene in this subset was the gene encoding brain-derived neurotrophic growth factor (BDNF). This neurotrophic growth factor was markedly upregulated in patients before treatment as determined by both microarray and qRT-PCR (Tables 2 and 5, Fig. 5). While BDNF traditionally is associated with diseases such as Alzheimer’s and mood disorders [29], studies have shown that BDNF is also expressed in satellite cells surrounding...
skeletal muscle cells, and, based on studies in rats, a role for BDNF in maintaining the satellite cell population has been suggested [30]. We have previously shown that PMR is associated with high intramuscular levels of proinflammatory cytokines [5], and it might be speculated that in untreated PMR, BDNF is upregulated to counter the muscle damage resulting from the inflammatory processes as well as the muscle degeneration resulting from the reduced physical activity level of PMR patients.

Finally, the transferrin receptor/CD71 (TFRC) gene was down-regulated 3 fold in patients before treatment. The transferrin receptor protein is involved in the transport of iron into cells, it is required for erythrocyte development, and it is associated with diseases such as iron deficiency, anemia, and chronic disease in general. It has been suggested that low levels of soluble transferrin receptors reflect adaptation to iron deficiency and/or inhibition of iron resorption [31]. It is conceivable that in this group of patients, TFRC is down-regulated due to the chronic inflammatory disease burden associated with PMR. While intramyocellular iron deficiency may ensue, it is not likely that the muscular down regulation of TFRC was secondary to systemic iron deficiency. This is so because none of the subjects exhibited anemia. Other studies have identified that PMR is associated with antibodies against ferritin [32, 33]. Taken together, this suggests that iron metabolism and the function of proteins that rely on iron-binding may be influenced in PMR.

Genes responding to prednisolone in PMR patients
The phenotype of PMR in this and other studies [1, 5, 15, 34] profoundly responds to treatment with glucocorticoids, indicating that important information about the pathophysiology of the disease can be achieved by studying the gene expression before and after prednisolone treatment. Moreover, if studying only untreated subjects, it is conceivable that, due to sampling errors, including unrecognized impacts of e.g. diurnal gene expression variations between patients and controls, discovery of all genes relevant to the pathophysiology of PMR would not be achieved. For these reasons, comparison of expression levels before and after symptom eliminating prednisolone treatment in patients was also used for the identification of genes with importance for PMR. The number of genes that responded to treatment in a given direction only in patients was 131. Indicating that these genes were, in fact, involved in the pathophysiology of PMR, of the 131 mentioned genes that responded to treatment in patients, only 2 also responded in controls subjects, and they did so in the direction opposite to that seen in the patients. Genes responding in the same direction to prednisolone in both patients and controls were not emphasized, because it is likely that the response reflected a general effect of glucocorticoids of no importance for the pathophysiology of PMR.

The functional clusters in this subset of genes included genes involved in the organization of the cytoskeleton and genes relevant for the extracellular matrix. In this context, it is of note that both TUBD1 and MARK4 were down-regulated by prednisolone, the fold changes being significant in microarray and qRT-PCR, respectively (Table 5). The fact that such genes respond to prednisolone treatment in patients with PMR is in line with the hypothesis that muscle stiffness may be due to abnormal expression of cytoskeleton-related genes. Correspondingly, clinical remission, including abolishment of muscle stiffness, happened in parallel with or due to normalization of expression of such genes.

Genes differentially expressed in untreated PMR patients vs controls and also responding to prednisolone in patients
The strongest evidence in favor of a pathogenic role of a given gene would be that its expression differed between untreated patients and controls, and, furthermore, changed with prednisolone treatment in the former. The number of such genes was 44 in the present cohort. Strongly indicating that these genes do in fact play a role in PMR, the response to prednisolone of all but one of the 44 genes counteracted the difference in gene expression between untreated patients and controls. In this group of genes, the predominant biological functions appeared to be regulation of transcription as well as protein translation/biosynthesis.

The finding that the expression of some genes differed between untreated patients and controls while not responding to prednisolone treatment in patients may indicate that clinical remission may be achieved even though the underlying disease mechanisms are not completely resolved or that not all differences in gene expression may be of importance for clinical symptoms. As a limitation of the present study, it should be noted, however, that while all patients achieved clinical remission during the relatively brief 14-day treatment period, some genes might respond to long-term treatment only. Conversely, it is also interesting to note that in the untreated patients, some genes, the expression of which did not differ from that of controls, were, nevertheless, selectively influenced by prednisolone. It may be that in the patients the processes regulated by these genes were impaired by other, non-genetic factors that possibly also resulted in increased sensitivity to prednisolone. If so, the condition would be ameliorated by a prednisolone-induced effect on these genes.
Conclusions
This study is the first to demonstrate changes in the gene expression in skeletal muscle in PMR. The study has identified a number of genes that may play a role in the pathophysiology of PMR. Moreover, we show that the expression of the IL6 gene is upregulated in muscle in PMR, a finding that adds to the substantial body of evidence that this cytokine is central to the disease. Follow-up studies are needed to elucidate the exact pathophysiological relevance of the identified genes; however, it appears that many of the genes are involved in the regulation of protein biosynthesis, which may suggest that abnormal protein metabolism is a disease mechanism in PMR. Effects of prednisolone on genes involved in the organization of the cytoskeleton and the intracellular matrix in PMR patients may contribute to the amelioration, seen in response to treatment, of the muscle stiffness.

Abbreviations
CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; GC: Glucocorticoid; IL: Interleukin; PMR: Polymyalgia rheumatica; qRT-PCR: Quantitative real-time polymerase chain reaction

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Availability of data and material
The microarray data were submitted to the gene expression repository at Array Express (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-3671.

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Authors’ contributions
HG conceived of the study and, together with FFK, planned its design, recruited and examined the subjects and carried out the experiments. RB, HG conceived of the study and, together with FFK, planned its design, recruited and examined the subjects and carried out the experiments. RB, FCN, FFK and PS carried out the biochemical analyses, while all authors participated in the analysis of the data and the writing of the manuscript.

Ethics approval and consent to participate
The authors declare that they have no financial or nonfinancial competing interests.

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