

TRANSCRIPTION PROFILE ANALYSIS OF *VASTUS LATERALIS* MUSCLE FROM PATIENTS WITH CHRONIC FATIGUE SYNDROME

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Chronic fatigue syndrome (CFS) is a disabling condition characterized by unexplained chronic fatigue that impairs normal activities. Many body systems are affected and etiology has not yet been identified. In addition to immunological and psychological aspects, skeletal muscle symptoms are prominent in CFS patients. In an effort to establish which pathways might be involved in the onset and development of muscle symptoms, we used global transcriptome analysis to identify genes that were differentially expressed in *vastus lateralis* muscle of female and male CFS patients. We found that the expression of genes that play key roles in mitochondrial function and oxidative balance, including superoxide dismutase 2, were altered, as were genes involved in energy production, muscular trophism and fiber phenotype determination. Importantly, the expression of a gene encoding a component of the nicotinic cholinergic receptor binding site was reduced, suggesting impaired neuromuscular transmission. We argue that these major biological processes could be involved in and/or responsible for the muscle symptoms of CFS.

Chronic fatigue syndrome (CFS) is characterized by severe disabling fatigue, but is also associated to other symptoms, such as musculoskeletal pain, sleep disturbance, headache, recurrent sore throat and neuro-cognitive alterations, including impaired concentration. CFS has a similar prevalence in people of different socioeconomic status and affects all ethnic groups. The only demographic risk factor is gender, with females being more prone to the illness than males.

There are two prominent definitions of CFS in current use, one from the United States Centers for

Disease Control and Prevention (CDCP) proposed in 1994 and implemented in 2003 by the International Chronic Fatigue Syndrome Study Group and a second based on Oxford Criteria (1). The most important difference between the two definitions is that the CDCP definition includes several physical symptoms that are secondary to immunological or infective pathologies, while the definition based on Oxford Criteria focuses on the presence of mental fatigue. Not surprisingly, skeletal muscles are the most investigated organ in studies designed to understand low tolerance to exercise. Oxidative

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stress, which other laboratories as well as our own have proposed as a possible contributor to the pathophysiology and clinical symptoms of CFS, has become a focus of this research (2-4). According to a recent study, reactive oxygen species (ROS) are not only responsible for molecular damage, but also act as important signaling molecules that modulate contractile function in both un-fatigued and fatigued skeletal muscle. Several biochemical and metabolic manifestations of CFS in muscle fibers have been investigated, but the conclusions reached have been controversial. CFS patients have been shown to have reduced serum concentrations of acylcarnitine, probably reflecting a reduction in muscle mitochondrial energy production. Mild exercise produces substantial muscular acidification and muscle adenylate and creatine kinase activities are defective in the muscles of CFS patients (5). Moreover, muscle pain in the absence of peripheral tissue damage and reduced oxygen saturation after exercise have been reported (6).

In view of the available, but controversial, evidence on muscle fiber involvement in CFS and considering the characterization of single muscle fibers, including a determination of contractile properties *in vitro* (7), we decided to use an approach not yet applied to study the skeletal muscles of CFS patients, the identification of changes in gene expression using a global microarray approach. Our objective was to determine whether consistent changes in gene expression could be identified in CFS patients. In this paper, we report the results of a global transcriptome analysis and progress towards identifying consistent gene expression alterations that might indicate pathophysiological mechanisms.

MATERIALS AND METHODS

Subjects

CFS patients were recruited at the National Reference Center for CFS Study of Chieti-Pescara University (diagnosis with CDCP criteria). Ten patients were recruited for this study: five women (mean age 44.8 ± 3.4) affected for 5.4 ± 0.7 years and five men (mean age 37.0 ± 3.2) affected for 7.8 ± 1.9 years, mean \pm SEM. Control subjects were volunteers of the medical and nursing staff at the same center. A further criterion for controls was no current or past history of diffuse musculoskeletal pain/fatigue lasting more than ten consecutive days. Four

healthy women (mean age 35.0 ± 5.0) and five healthy men (mean age 43.0 ± 8.0) were recruited as controls, mean \pm SEM (7).

To confirm the clinical CFS diagnosis and, in particular, to exclude patients affected by fibromyalgia we utilized also a direct test. In a previous study we demonstrated that oxidative damage in CFS altered fluidity and fatty acid composition of skeletal muscle membranes with a specific and peculiar pattern completely different from that obtained by patients suffering from fibromyalgia syndrome. In fact fibromyalgia patients, who generally exhibit symptoms very similar to CFS patients, display higher total membrane fluidity than CFS and control samples (2). For this reason, it has been proposed that this specific damage of skeletal muscle membrane is able to alter the excitation-contraction mechanism (3) and it could be useful to distinguish between the two pathological conditions. During the sample preparation for this study, tests to verify the membrane fluidity were randomly performed in some bioptic specimens as inclusion criterion and the results confirmed the clinical diagnosis.

Biopsy specimens of the *vastus lateralis* muscle were taken under local anesthesia (1% lidocaine), using a 5-mm muscle biopsy needle with applied suction at the level corresponding to one-third of the distance from the upper margin of the patella to the anterior superior iliac. Each biopsy was divided. The first portion was treated as described in Pietrangelo et al. (7) and the second portion of each sample was stored in 2X Lysis Buffer (Applied Biosystems, n 4305895) and frozen at -80°C for RNA extraction for transcriptional profile (the present work).

The full CFS muscle analysis project has been designed as translational study, in fact it involved different sets of experiments on each biopsy sample: the transcription profile analysis and measurement of tension development and fiber-type characterization (7). These experimental sets involved both molecular and cellular level of investigation on the same CFS patient. The study protocol was approved by the Ethics Review Committee of the University "G. d'Annunzio" of Chieti-Pescara.

Microarray

Whole muscle biopsies used as a source of aaRNA for this study are likely contaminated by non-skeletal muscle cell types, including smooth muscle, endothelial cells, end plates and fibroblasts that might contribute to differentials in gene expression. Accordingly, a general oligonucleotide microarray rather than a muscle-specific library was used for these studies.

The human oligonucleotide set used in this study consists of 21,329 70-mer oligonucleotides (Operon version 2.0) designed based on Human Unigene clusters

and corresponding primarily to the 3'-terminal region of each gene. Each oligonucleotide is spotted by a robotic station Biorobotics Microgrid II in two replicates on a MICROMAX glass slide SuperChip I (Cat No MPS696) provided by PerkinElmer Life Sciences Inc. Details of the slide design and sequence selection can be found at the manufacturer's website (<http://microcribi.cribi.unipd.it/e-index.htm>).

Gene expression profile analysis was carried out on four patients (two women both 48 years old affected for 4 and 6 years, respectively, and two men 27 and 36 years old affected for 7 and 12 years, respectively) as only the biopsy samples from these four patients were eligible for transcriptional profile analysis. Gender-specific control RNA samples, prepared by pooling individual samples (four women and five men) were processed collectively, in order to have the same baseline of transcript control for each individual CFS transcriptional profile. RNA samples from CFS patients were processed individually.

RNA isolation, amplification and labeling

Frozen biopsy samples, stored in 2X Lysis Buffer (Applied Biosystems, cod. no. 4305895), were homogenized for 3 to 5 min in 1 ml TRIZOL/50 mg tissue (Invitrogen/Life Technologies) using an ultra-turrax-T8 blender (IKA-Werke, Staufen, Germany). Total RNA was purified following the standard TRIZOL protocol. A small aliquot of RNA was then used for quantification and quality control using the RNA 6000 LabChip kit and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). High quality RNA was processed. Aliquots of 2 µg of each RNA sample were reverse transcribed and labeled using the Amino Allyl MessageAmp™ aRNA amplification and labeling kit (Catalog #1752, Ambion).

Microarray co-hybridization

Labeled aaRNA of pooled control (Cy3 fluorochrome) and individual CFS sample (Cy5 fluorochrome) were dissolved in 120 µl of hybridization buffer and 3 µl of water, denatured at 90°C for 2 min and both were applied directly to the slides. Microarray hybridization was carried out in the hybridization station ArrayBooster™ (Advalitix, Brunnthal, Germany) humidified with 250 µl of hybridization buffer. Microarrays were placed in the ArrayBooster™ and covered with an Advacards™ containing three agitation chips and hybridized overnight at 48°C. Post-hybridization washing was performed in the dark at room temperature using 1x SSC and 0.2% SDS solution for 4 min, 0.1 x SSC and 0.2% SDS solution for 4 min, 0.2 x SSC solution for 4 min and 0.1 x SSC solution for 4 min. Experiments were performed in duplicate using different microarray slides in which we crossed the fluorochrome (dye swap).

Array scanning and statistical analysis of expression data

Array scanning was carried out using a GSI Lumonics LITE dual confocal laser scanner with a ScanArray Microarray Analysis System (Perkin Elmer), and raw images were analyzed with QuantArray Analysis Software (GSI Lumonics, Ottawa, Canada). Normalization of expression levels of all spot replicates was performed with MIDAS (TIGR Microarray Data Analysis System). The LOWESS (Locfit) normalization was applied to expression data before performing any other statistical analyses. After normalization, values of spot replicates in all array experiments were averaged. Identification of differentially expressed genes was performed using Significance Analysis of Microarray (SAM). The statistic used in SAM is given as $d = (\mu_1 - \mu_2) / (s + s_0)$, where the numerator is the group mean difference, s is the standard error, and s_0 is a regularizing constant. Setting $s_0 = 0$ yields a t -statistic. The value of s_0 , called the fudge constant, is found by removing the trend in d as a function of s in moving windows across the data to reduce false positive results. As the statistic is not t -distributed, significance is computed using a permutation test. Genes with a computed statistic larger than the threshold, in our analysis established as 1000, were considered significant. The false discovery rate (FDR) associated with the given threshold was also calculated from the permutation data. The expression level of each gene was defined as the log base-2 of the ratio between the intensity of cyanine-coupled aaRNA from CFS samples (numerator) and the intensity of cyanine-coupled aaRNA from pooled control samples ($\log_2 I_{\text{CFS}} / I_{\text{Ctrl}}$). Two distinct SAM analyses were performed, one using the normalized array data from both female samples, and a second using the normalized array data from both male samples. These analyses identified 218 differentially expressed genes (96 up- and 122 down-regulated, $\Delta = 3.0$, FDR = 0) in the two female patients, and 453 genes (19 up- and 434 down-regulated, $\Delta = 3.2$, FDR = 0) in the two male patients.

For each patient, each gene was selected when four values of $\log_2 I_{\text{CFS}} / I_{\text{Ctrl}}$ were regulated in agreement (duplicate spots per array and two microarrays per patient). Thus, for the 2 patients of each gender, the mean and standard deviation for each differentially expressed gene were calculated from eight values of $\log_2 I_{\text{CFS}} / I_{\text{Ctrl}}$.

RESULTS

Transcription profile summary

Ten CFS patients (five female and five male) and nine age-matched healthy controls (four female and five male) were enrolled in the entire

Table I. List of differentially expressed genes that were up-regulated in each of the four biopsies of vastus lateralis muscle from two female and two male CFS patients.

Description	Gene Symbol	Mean Log ₂ $I_{\text{CFS}}/I_{\text{Ctrl}}$ Female \pm SD	Mean Log ₂ $I_{\text{CFS}}/I_{\text{Ctrl}}$ Male \pm SD
DNA repair			
Polymerase (DNA directed), beta	POLB	0.75 \pm 0.11	0.41 \pm 0.09
Energetic balance			
Very low density lipoprotein receptor	VLDLR	0.87 \pm 0.19	0.59 \pm 0.27

First column: descriptive gene name; second column: GeneBank ID; third column: UniGene ID; fourth column: identifying Gene Symbol; fifth column: log base-2 of the ratio between the intensity of cyanine-coupled aaRNA from CFS samples (numerator) and the intensity of cyanine-coupled aaRNA from pooled control samples ($\log_2 I_{\text{CFS}}/I_{\text{Ctrl}}$) (mean \pm standard deviation) for female CFS patients; sixth column: same as column 5, for male CFS patients.

study comprising the muscle transcriptional profile analysis. Biopsies of the vastus lateralis muscle were collected from each subject and divided into three fragments (see Methods). One fragment was used for transcription profile analysis. A second fragment was used to isolate single fibers for mechanical studies and for electrophoretic analysis (7).

Transcriptional analysis was used to monitor expression levels of thousands of genes simultaneously, with the specific goal of identifying transcripts and/or pathways involved in CFS muscle symptoms. Our experimental design compared the RNA of each CFS patient with a pool of control RNA derived from all control subjects of the same gender. Pooled control RNA was used to minimize the effects of inter-individual heterogeneity among controls and create a more homogenous baseline, thus providing greater confidence that up- or down-regulated transcripts identified in individual CFS patients are linked to the muscle symptoms of the syndrome.

Genes differentially expressed in both female and male CFS patients

The SAM analyses identified 218 differentially expressed genes (96 up- and 122 down-regulated)

in the two female patients, and 453 genes (19 up- and 434 down-regulated) in the two male patients. We selected and reported in Table I the genes up-regulated, in Table II the genes down-regulated in both female and male CFS patients and in Table III the genes up-regulated in the female but down-regulated in the male CFS patients.

Whereas the expression of 218 genes was altered in the female patients and the expression of 453 genes in the male patients, the expression of only 47 genes was significantly altered in the biopsy samples of all CFS patients: two genes were up-regulated (Table I) and 38 genes were down-regulated (Table II) in biopsies from both male and female patients; seven genes were up-regulated in female but down-regulated in male patients (Table III). These differentially expressed genes in common were used as the basis of our attempt to identify signaling-pathway alterations in muscle that are associated with this syndrome.

Specific metabolic alterations

The differentially expressed genes point to the potential involvement of several signaling pathways and cellular processes in the development of CFS in skeletal muscles. These include: (a) control of

Table II. List of differentially expressed genes that were down-regulated in each of the four biopsies of vastus lateralis deriving from two female and two male CFS patients.

Description	Gene Symbol	Mean Log ₂ I _{CFS} /I _{Ctrl} Female±SD	Mean Log ₂ I _{CFS} /I _{Ctrl} Male±SD
Oxidative stress			
<i>Superoxide dismutase 2, mitochondrial</i>	<i>SOD2</i>	-1.03±0.09	-1.72±0.56
<i>Ferredoxin 1</i>	<i>FDX1</i>	-0.96±0.20	-1.11±0.27
<i>NAD(P)H dehydrogenase, quinone 1</i>	<i>NQO1</i>	-2.14±0.33	-0.49±0.21
<i>Homo sapiens inositol hexakisphosphate kinase 3 mRNA, complete cds</i>		-1.30±0.17	-0.51±0.26
Energetic balance			
<i>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3</i>	<i>PFKFB3</i>	-2.43±0.74	-1.39±0.23
<i>Pyruvate dehydrogenase kinase, isoenzyme 4</i>	<i>PDK4</i>	-2.90±0.79	-1.31±0.71
<i>ATP-binding cassette, sub-family A (ABC1), member 5</i>	<i>ABCA5</i>	-1.69±0.54	-1.10±0.48
<i>Adenosine monophosphate deaminase (isoform E)</i>	<i>AMPD3</i>	-1.48±0.30	-0.48±0.27
Neuromuscular transmission			
<i>Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)</i>	<i>CHRNA1</i>	-2.60±0.63	-1.79±0.45
Fiber phenotype			
<i>Myogenic factor 6 (herculin)</i>	<i>MYF6</i>	-1.69±0.34	-1.16±0.41
<i>Calmodulin 1 (phosphorylase kinase, delta)</i>	<i>CALM1</i>	-1.02±0.12	-0.38±0.18
<i>TGFB inducible early growth response</i>	<i>TIEG</i>	-1.72±0.21	-0.90±0.35

Column descriptions are as for Table I. The Table shows only the genes specifically involved in metabolisms related to muscle activity and/or phenotype.

Table III. List of differentially expressed genes that were up-regulated (positive value) in the biopsies of vastus lateralis from two female CFS patients, and down-regulated (negative value) in two male CFS patients.

Description	Gene Symbol	Mean Log ₂ I _{CFS} /I _{Ctrl} Female±SD	Mean Log ₂ I _{CFS} /I _{Ctrl} Male±SD
Fiber phenotype			
014L17_Calsequestrin 2 (cardiac muscle)	CASQ2	2.08±0.16	-0.43±0.25
DNA repair			
022G12_Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	DYRK2	2,24±0,38	-0.98±0.60
050J24_Nuclear receptor co- repressor/HDAC3 complex subunit	FLJ12894	0.71±0.31	-1.65±0.63
Growth/cytoskeleton regulation			
001I13_Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	PDE4B	2.05±0.38	-1.07±0.47
035J14_Thrombospondin	FLJ14440		
031O08_Thrombospondin 2	THBS2	2.02±0.60	-2.36±0.46
027N03_Thrombospondin 1	THBS1		-1.37±0.85

Column descriptions are as for Table I.

oxidative state, (b) DNA repair, (c) energy balance, (d, e) trophic/inflammatory processes, (f, g) growth/apoptosis pathways, (h) neuromuscular transmission, and (i) fiber phenotype.

a) *Oxidative stress.* At least three mitochondrial genes encoding proteins that might be directly or indirectly correlated with an imbalance of oxidative

state in the muscle were down-regulated in the vastus lateralis muscle of both female and male CFS patients: (i) superoxide dismutase 2 (SOD2), which is involved in superoxide anion metabolism and is a scavenger of mitochondrially generated ROS (8); (ii) ferredoxin 1 (FDX1), which is an iron-sulfur protein capable of transferring electrons

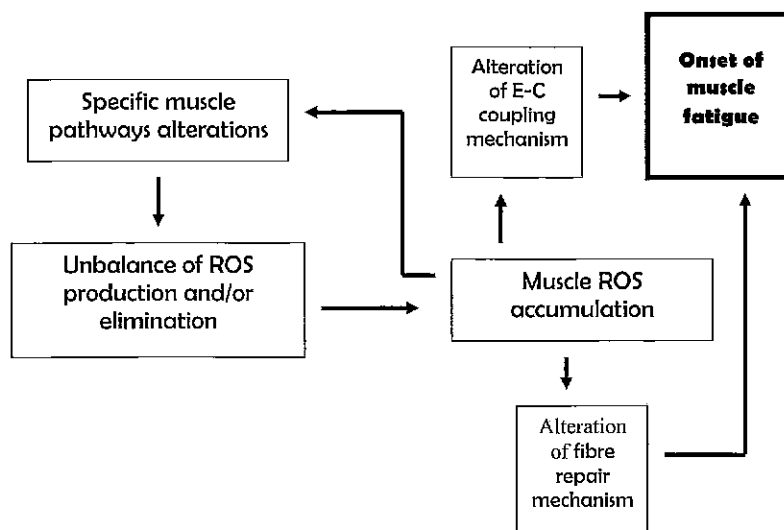


Fig. 1. Possible cellular mechanism of metabolic pathways alteration induced by ROS accumulation in skeletal muscles of CFS patients. ROS accumulation is able to modify both excitation-contraction (E-C) coupling than muscle repair.

from NADH to cytochrome p450 and is also involved in ROS generation (8), and (iii) NADPH dehydrogenase quinone 1 (NQO1), which encodes a cytosolic enzyme able to protect against toxic agents. Also down-regulated was the gene encoding inositol hexakisphosphate kinase 3 (IHPK3), which is important in the context of increased oxidative damage. This kinase, which also reduces the amount of inositol hexakisphosphate (IP_6), interacts with iron and inhibits the formation of hydroxyl radicals ($OH\cdot$) (9).

b) DNA repair and gene expression. One of the two genes up-regulated in CFS patients (Table I) was the gene coding for DNA-directed polymerase beta (POLB), an enzyme that repairs DNA double helices using the base excision repair (BER) mechanism. Importantly, we also found that the gene encoding Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain-2 (CITED2), a p300 protein that forms the starting complex for DNA polymerase beta acetylation, was down-regulated. Reduced acetylation reduces the activity of the dRP-lyase subunit, increasing the activity POLB (10). The gene, splicing factor proline/glutamine rich (SFPQ), which was also down-regulated, has been demonstrated to interact with RNA polymerase

II to regulate transcription (11). In addition to having a potential role in oxidative stress, IHPK3 down-regulation could result in deficiencies in IP_6 synthesis, altering DNA repair capability or impairing messenger RNA export. Also down-regulated was the gene for serine/arginine repetitive matrix 2 (SRRM2), which is a component of a nuclear matrix protein complex that functions as a coactivator of pre-mRNA splicing (12).

Down-regulation of the gene for the non-metastatic cells 1 (NM23A/NME1), encoding an endonuclease, could represent an attempted compensatory response designed to mitigate DNA damage.

It is more difficult to interpret the functional significance of genes found to be up-regulated in female but down-regulated in male patients (Table III). These genes include dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2, which regulates p53 to induce apoptosis in response to DNA damage (13), nuclear receptor co-repressor/HDAC3 complex subunit (TBL1XR1), which has been implicated in histone binding and cAMP-specific phosphodiesterase 4B (PDE4B), which is unlikely to play a role in CFS muscle.

c) Energy balance. One characteristic of the

CFS *vastus lateralis* gene profile was the depressed transcription of several genes implicated in the energy metabolism of skeletal muscle fibers. Specifically, we found that two allosteric enzymes, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (PFKFB3), were down-regulated, suggesting that glycolysis and/or gluconeogenesis was impaired. In fact, this allosteric enzyme is bifunctional and its specific activity depends on posttranscriptional phosphorylation. Also down-regulated was the gene for pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4), which phosphorylates pyruvate dehydrogenase, an enzyme important for the oxidative decarboxylation of pyruvate eventually used for oxidative phosphorylation. Moreover, glutamic-oxaloacetic transaminase 1 (*GOT1*) was down-regulated, suggesting an increase in gluconeogenesis without amino acid consumption, consistent with an effort of CFS *vastus lateralis* muscle to economize glucose. Genes involved in the phosphorylation of nucleoside diphosphates were also down-regulated, including adenosine monophosphate deaminase isoform E (*AMPD3*), a key enzyme in muscle purine degradation. The ATP-binding cassette member 5 was also found to be down-regulated, suggesting that efflux of cholesterol out of the fiber is inhibited.

Only one gene of possible relevance for energy balance, very low density lipoprotein receptor (*VLDLR*), was found to be up-regulated in both female and male patients. This gene encodes the receptor responsible for VLDL uptake into the fiber and is involved in the primary pathway of fatty acid transport in skeletal muscle (14).

d) Atrophic process. Among the down-regulated transcripts for genes involved in the atrophic process there was forkhead box O3A (*FOXO3A*). Foxo family proteins are transcription factors that regulate the expression of several genes that were found to be down-regulated in our screenings, including *PDK4*, *SOD2*, *GADD45*, which are important in cellular responses, such as glucose metabolism, stress response, cell cycle regulation, and apoptosis (15). It is worth noting that another gene involved in the FOXO pathway, H1 histone family member X (*H1FX*), encoding a nucleosomal protein that links DNA to the nucleosome, was also down-regulated.

Importantly in the context of atrophic processes, ubiquitin-dependent catabolism was also likely depressed, as suggested by the down-regulation of ubiquitination factor E4A (*UBE4A*), which encodes the additional conjugation factor, E4. This factor, which is widely expressed in skeletal muscle, replaces the E3 enzyme and is able to transfer ubiquitin to its target. The depression of ubiquitin-dependent processes in CFS muscle is further confirmed by the down-regulation in female patients of proteasome (prosome, macropain) 26S subunit non-ATPase 3 (*PSMD3*), encoding a non-ATPase subunit of the proteasome; in male patients, the down-regulation of proteasome (prosome, macropain) subunit beta type 2 (*PSMB2*) encoding a beta subunit of the 20S proteasome was observed (see Table II).

e) Inflammatory process. The following genes were down-regulated in all patients, suggesting a silencing of inflammatory response in CFS muscles (16): i) secretory leukocyte protease inhibitor (antileukoproteinase) (*SLPI*), a gene encoding a serine protease with multiple functions in innate host defense, inflammation and infection; ii) *GRO2* oncogene (*GRO2*), a gene encoding chemokine C-X-C motif ligand 2/growth-related oncogene beta (*CXCL2/GRO2*), which is a potent chemotactic agent for polymorphonuclear leukocytes; iii) interferon-inducible guanylate binding protein 2, encoding a protein that antagonizes the proliferation and angiogenic response of endothelial cells to inflammation and also provides resistance against vesicular stomatitis virus and encephalomyocarditis virus proliferation; iv) delta sleep-inducing peptide immunoreactor (*TSC22D3/GILZ*), a gene encoding a mediator of glucocorticoid-induced immunosuppression, which interferes with AP-1 by interacting with Fos and Jun *in vitro* to inhibit binding of active AP-1 to its target DNA.

f, g) Growth/apoptosis and cytoskeletal regulation. Several down-regulated genes common to male and female CFS patients are implicated in cellular growth and apoptosis pathways, suggesting a relative silencing of these interconnecting pathways. We found that V-fos FBJ murine osteosarcoma viral oncogene homolog (*FOS*) and v-myc myelocytomatosis viral oncogene homolog (*MYC*) were down-regulated (Table II). *FOS* and *MYC* are transcription factors involved in proliferation and cell

cycle regulation through the induction of CDK4, but also induce apoptosis in sensitive cells. FOS down-regulation is consistent with the down-regulation of the FOS-interacting protein, TSC22/GILZ, with which it shares a common signal transduction pathway. SOX17 is an SRY-related HMG-box transcription factor and a DNA-binding protein that is important in self-renewal signaling (17). Also down-regulated were apoptosis antagonizing transcription factor (AATF), a transcriptional factor that stimulates EF2 transcription and promotes cell cycle progression, and *nuclear* factor NF-IL6-beta (CEBPD), which has been linked to cell growth and apoptosis in skeletal muscle (18).

Many genes involved in focal adhesion control and cytoskeletal and/or extracellular matrix regulation were also found to be down-regulated in CFS patients. Among these were i) proline/arginine-rich end leucine-rich repeat protein (PRELP), encoding a protein linking type-I collagen to heparan sulfate basal membranes (19), collagen type-V alpha 3 (COL5A3), ii) LIM domain kinase 1 (LIMK1), encoding a protein that links proteins involved in actin cytoskeleton organization (20), and iii) spermidine/spermine N1-acetyltransferase-1 (SAT1), encoding a protein involved in the regulation of apoptosis, cellular proliferation, and cell cycle progression (21). It is also worth noting the down-regulation of two additional proteins: 1) protein tyrosine kinase 2 (PTK2/FAK), encoding a cytoplasmic protein localized to focal adhesions between growing cells that is important for transduction of external signals (22), and 2) prion protein (PRNP), also known as Creutzfeldt-Jakob disease Gerstmann-Strausler-Scheinker syndrome fatal familial insomnia, encoding a glycoprotein attached to the cell membrane via a glycosyl-phosphatidylinositol anchor, characteristics that would be consistent with roles in cell adhesion and transmembrane signaling. Another altered gene in CFS muscle, an anti-angiogenic factor with a role in the focal adhesion is thrombospondin (23) that results up-regulated in female but down-regulated in male patients (Table III).

h) Neuromuscular transmission. The gene, CHRNA1, encoding a component of the acetylcholine binding site of the nicotinic receptor (cholinergic receptor, nicotin, alpha polypeptide 1 [muscle]), was found to be down-regulated. The absence of this

gene's product might imply an impaired ability to respond to motor neuron firing at the neuromuscular junction. Motor neuron firing is important for slow-fiber-type specification (24).

h) Muscle fiber phenotype. The transcription factor, myogenic factor 6 (MYF6/MRF4/herculin), was found to be down-regulated in CFS patients. In the mouse, MFR4 accumulates in slow fibers, and there is evidence indicating that MFR4 regulates the shift of fiber type towards the slow phenotype (25). This suggests that muscle remodeling is directed towards the fast phenotype in CFS patients, an interpretation that is consistent with results from fiber typing at the protein level (7). Down-regulation of the following genes identified in the present transcriptome analysis also supports this view: (i) calmodulin 1 (CALM1/phosphorylase kinase delta), encoding an important calcium calmodulin-dependent kinase that is involved in decoding intracellular calcium oscillations during myogenesis and the differentiation of the slow-fiber phenotype (26), and (ii) TGFB inducible early growth response (Egralpha3), encoding a transcription factor that is highly expressed in developing muscle spindles (27). Since muscle spindles are more abundant in those parts of the muscle where type-I fibers are concentrated, down-regulation of this gene would appear to be consistent with muscle remodeling toward an increase in the fast-fiber type. Other observations consistent with the view that gene expression changes in CFS reflect a slow-to-fast fiber type shift include: i) up-regulation (restricted to the female patients, see Supplemental Material) of myosin light chain kinase 2 (MYLK2), encoding a calcium calmodulin-dependent kinase highly expressed in regenerating and adult fast fiber, and ii) alteration (down-regulation in males, up-regulation in females; Table III) of calsequestrin 2 (CASQ2), encoding a protein characteristic of slow muscle fibers and important for sarcoplasmic reticulum (SR) calcium storage and regulation of the ryanodine receptor.

DISCUSSION

In this study, we compared the gene expression profiles of *vastus lateralis* skeletal muscle in CFS patients and healthy subjects. In this context, the

aim of our analysis is to determine whether there are consistent gene expression changes in CFS patients that might directly implicate skeletal muscles in the manifestations of this disease and provide an indication of which cellular processes were involved. Previous studies by our group have demonstrated specific oxidative alterations in DNA and lipids in *vastus lateralis* samples from CFS patients (2-3). It is worth noting that Mn-SOD deficiency is associated with severe lipid peroxidation, and other down-regulated genes, such as FDX1, NQO1 and IHPK3, might contribute to the impairment of antioxidant mechanisms. In addition, we argued that oxidative damage could derive from mitochondrial dysfunction also considering the documented structural alterations of mitochondrial cristae of the muscle CFS mitochondria, as previously observed (28). One of the two genes up-regulated in muscles of both male and female CFS patients is the specialized DNA polymerase (most important for its base excision repair activity), POLB, which may be involved in responding to increased oxidative damage. In CFS muscles, POLB activity may be increased post-transcriptionally by a reduction in POLB acetylation, which would be an expected consequence of CITED2 down-regulation. We might also speculate that the cyclic recurrence and variability of muscle symptoms in this syndrome could reflect the tissue-specific ability of POLB to repair ROS-induced damage. In a previous study, CFS muscles were shown to have increased antioxidant activity due to increased activity of peroxidase, transferase and catalase (2). This outcome appears to represent an attempt on the part of CFS muscles to protect themselves against oxidative stress using antioxidant enzymes that work against ROS in the cytoplasm, and POLB, which works in the nucleus to repair DNA mutations. This positive feedback loop agrees with the hypothesis already proposed of elevated peroxynitrite in CFS muscle (6). In fact, nitric oxide could react with ROS and generate the potent oxidant peroxynitrite that in turn could decrease the level of Mn-SOD.

The down-regulation of phosphofructokinase transcripts in all our tested patients suggests the hypothesis that CFS syndrome could be comparable, in some respects, to Type-VII glycogenosis or Tarui disease (29), a rare hereditary disease caused by

muscle phosphofructokinase (PFKM) deficiency. Deficiency of this enzyme results in glycogen accumulation in tissues, causing symptoms that include exercise intolerance or premature fatigue, weakness and stiffness with exercise, and painful muscle cramps. In many cases, this defect has systemic consequences, but in other cases, compensatory mechanisms limit the effects to specific tissues. In some patients with Tarui disease, the AMP deaminase activity of erythrocytes was increased by calmodulin-dependent intracellular calcium increase (30). In CFS muscle, we found that both AMP deaminase and calmodulin phosphorylase kinase were down-regulated. This represents direct evidence that CFS and Tarui disease are different, as both pathological forms cause clinically significant muscle weakness, but only in CFS are patients unable to resolve their weakened status with rest.

The observed down-regulation in both male and female patients of the gene encoding AMP deaminase deserves additional comment. AMP deaminase is an enzyme that is key in sustaining the availability of ATP during exercise. Previous studies have provided evidence of defective muscle adenylate activity in CFS patients after moderate exercise (2). This alteration could be linked to the high fatigability of CFS patients during exhausting work. The impaired degradation of muscle purine nucleotides has been observed in other illnesses, such as glycolytic defective illness, and also in mitochondrial diseases in which lipid and carbohydrate oxidation are affected.

The massive use of anaerobic glycolysis for ATP necessity during mild exercise and the increased number of glycolytic fibers (7) implies lactate accumulation, as shown previously (7). We might suggest that, in CFS muscles, there is a convergence of metabolic failure in muscle that involves aerobic energy production impairment, purine degradation depression and increased lipid utilization. All muscle samples from CFS patients showed down-regulation of the transcript for FoxO3a, encoding a protein of the atrogene group that is one of the principal regulators of ubiquitin-ligases (25). Setting aside the possible role of phosphorylation in FoxO inactivation, which our studies did not address, we found that several ubiquitin-protein ligase-related genes that are molecular targets of FOXO activity

(15) were down-regulated. Collectively, these data point to a depression of atrophy signals in CFS muscles. Immune dysfunction in some CFS patients has been documented, in particular a deregulation of the 2',5'-oligoadenylate-dependent ribonuclease L (RNase L) in immune cells, linked to reduced exercise capacity and consistent with oxidative stress in CFS patients. In our analysis we did not find any indication of altered immunity nor any indication of inflammatory response. In contrast, some inflammatory response genes were found to be down-regulated. Moreover, one of the most striking changes shown by the present transcriptome analysis of CFS patients is the pronounced down-regulation of *CHRNA1* encoding the nicotinic cholinergic receptor alpha 1 subunit, which is a component of the acetylcholine binding site. The fatigue experienced by CFS patients could be either central or peripheral in origin. The former depends on firing frequency and the latter on neuromuscular transmission and/or excitation-contraction coupling. Previous reports suggest that the symptoms of fatigue in CFS syndrome could derive from failure to follow firing frequency of motoneurons and not from a peripheral defect in muscle contractile function. Our results provide strong support for this view, suggesting that reduced transcript levels lead to insufficiency in this important cholinergic receptor component that could contribute to impaired neuromuscular transmission. A number of changes in gene expression reported here consistently point to a shift from slow- to fast-fiber phenotype already demonstrated (7). Down-regulation of *MRF4* and calmodulin kinase suggests that regulatory mechanisms supporting the slow-fiber phenotype are depressed. The down-regulation of a gene such as *CASQ2*, typical of the slow phenotype, and up-regulation of *MYLK2*, although restricted to a subset of patients, are fully consistent with this interpretation.

Despite the presence of individual and gender-specific variations, there are significant and consistent changes in the muscle transcription profiles of female and male CFS patients, in particular the energy production, oxidative damage management, muscular trophism, neuromuscular transmission and fiber phenotype determination are the major biological processes implicated. It is worth mentioning that many processes suggested

by transcription profile analysis find independent support in previously reported studies, as is the case with altered oxidative state and fiber phenotype transition. Thus, this study strongly supports the view that muscle fibers are directly involved in the functional and structural changes that underlie the pathogenic mechanisms of the disease and we can infer on muscle involvement at molecular and cellular level on CFS patients.

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