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Fatigue-Related Gene Networks Identified in CD14⁺ Cells Isolated From HIV-Infected Patients—Part I: Research Findings

Joachim G. Voss, PhD, RN¹, Adrian Dobra, PhD^{1,2}, Caryn Morse, MD^{3,4}, Joseph A. Kovacs, MD^{3,4}, Robert L. Danner, MD^{4,5}, Peter J. Munson, PhD⁶, Carolea Logan⁵, Zoila Rangel⁶, Joseph W. Adelsberger⁷, Mary McLaughlin, RN³, Larry D. Adams, PhD¹, Raghavan Raju, PhD⁸, and Marinos C. Dalakas, MD⁹

¹Biobehavioral Nursing & Health Systems Department, University of Washington, School of Nursing, Seattle, WA, USA

²Department of Statistics, University of Washington, Seattle, WA, USA

³National Institute for Allergies and Infectious Diseases, Bethesda, MD, USA

⁴Critical Care Medicine Department, National Institutes of Health, Clinical Center, Bethesda, MD, USA

⁵Critical Care Medicine Department, National Institutes of Health, Clinical Center, Functional Genomics and Proteomics Facility, Bethesda, MD, USA

⁶National Institutes of Health, Center for Information Technology, Mathematical and Statistical Computing Laboratory, Bethesda, MD, USA

⁷National Institutes of Health, National Cancer Institute, AIDS Monitoring Laboratory, Frederick, MD, USA

⁸Department of Surgery, University of Alabama, Birmingham, AL, USA

⁹Imperial College, Clinical Neurosciences and Neuromuscular Diseases, London, UK

Abstract

Purpose—Human immunodeficiency virus (HIV)–related fatigue (HRF) is multicausal and potentially related to mitochondrial dysfunction caused by antiretroviral therapy with nucleoside reverse transcriptase inhibitors (NRTIs).

Methodology—The authors compared gene expression profiles of CD14⁺ cells of low versus high fatigued, NRTI-treated HIV patients to healthy controls ($n = 5/\text{group}$). The authors identified 32 genes predictive of low versus high fatigue and 33 genes predictive of healthy versus HIV infection. The authors constructed genetic networks to further elucidate the possible biological pathways in which these genes are involved.

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Corresponding Author: Joachim G. Voss, PhD, RN, University of Washington, School of Nursing, Box 357266, Seattle, WA 98195, USA. vossj@uw.edu.

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Relevance for nursing practice—Genes including the actin cytoskeletal regulatory proteins Prokineticin 2 and Cofilin 2 along with mitochondrial inner membrane proteins are involved in multiple pathways and were predictors of fatigue status. Previously identified inflammatory and signaling genes were predictive of HIV status, clearly confirming our results and suggesting a possible further connection between mitochondrial function and HIV. Isolated CD14⁺ cells are easily accessible cells that could be used for further study of the connection between fatigue and mitochondrial function of HIV patients.

Implication for Practice—The findings from this pilot study take us one step closer to identifying biomarker targets for fatigue status and mitochondrial dysfunction. Specific biomarkers will be pertinent to the development of methodologies to diagnosis, monitor, and treat fatigue and mitochondrial dysfunction.

Keywords

CD14; fatigue; cofilin 2; Bayesian inference; prokineticin 2; HIV

Human immunodeficiency virus (HIV)–related fatigue (HRF) is the most prevalent symptom of HIV, with 80–100% of persons living with the disease complaining of ongoing lack of energy and exhaustion that lasts longer than 4 weeks (Cupler et al., 1995; Davis, 2004). HRF has been associated with deteriorating physical and mental health and quality of life and is predictive of diminished physical function (Ferrando et al., 1998). Hallmarked by chronic lack of energy and weakness, HRF contributes to loss of social function, lower quality of work, increased absenteeism and, ultimately, loss of employment and decreased adherence to antiretroviral therapy (ART; Darko et al., 1995; Darko, McCutchan, Kripke, Gillin, & Golshan, 1992; Ostrop, Hallett, & Gill, 2000). HIV infection and treatment with antiretroviral nucleoside analogues (nucleoside reverse transcriptase inhibitors, or NRTIs) affect mitochondrial deoxyribonucleic acid (DNA) content and function (Brinkman, ter Hofstede, Burger, Smeitink, & Koopmans, 1998). A number of important clinical syndromes observed in HIV-infected persons relate to mitochondrial dysfunction, including lactic acidosis, myopathy, cardiomyopathy, pancreatitis, peripheral neuropathy, and possibly lipodystrophy (Brinkman et al., 1998; Lewis & Dalakas, 1995). HRF could be one of the results of mitochondrial dysfunction, but this link has not been clearly established yet.

Multiple subjective measures with established reliability and validity exist for the evaluation of HRF, yet attempts to identify reliable biomarkers for HRF have been unsuccessful (Aaronson et al., 1999; Dalakas et al., 1990; Piper et al., 1998). For example, efforts to establish a correlation between immune suppression as measured by CD4⁺ cell counts and HRF have resulted in conflicting results (Barroso & Lynn, 2002; Ferrando et al., 1998; Phillips et al., 2004; Sullivan & Dworkin, 2003). Similar attempts to correlate HIV viral load, tumor necrosis factor α levels, hemoglobin, testosterone, or hepatic function with HRF have also failed to show an association (Barroso, Carlson, & Meynell, 2003; Darko et al., 1995; Moyle, 2002; Rabkin, Wagner, & Rabkin, 1999).

The availability of minimally invasive tests to assess for mitochondrial toxicity would greatly facilitate understanding of the contribution of mitochondrial dysfunction to clinical syndromes. Mitochondrial dysfunction in the liver and muscle ultimately results in the

development of lactic acidosis; however, venous lactate measurements are neither adequately sensitive nor specific enough for identification of early mitochondrial dysfunction (Blanco, Garcia-Benayas, Jose de la Cruz, Gonzalez-Lahoz, & Soriano, 2003; M. John et al., 2001). Tissue biopsies are currently the gold standards for the evaluation and diagnosis of mitochondrial toxicity in muscle and liver, but these invasive tests are risky, painful, and impractical for routine and repeated evaluations. An alternative nonsurgical toxicity assay would improve clinical diagnoses tremendously (Authier, Chariot, & Gherardi, 2005; Bongiovanni & Tordato, 2007).

We wanted to investigate whether peripheral CD14⁺ monocytes could serve as producers of surrogate fatigue biomarkers instead of using more difficult to obtain tissue biopsies and provide the necessary information for objective diagnosis of HRF from mitochondrial dysfunction. Even if other fatigue gene networks are predominant in muscle or other tissues, CD14⁺ cell gene expression patterns could be seen as a parallel metric of fatigue using these divergent markers, making development of a clinical assay for fatigue more attainable.

The purpose of this pilot study was to investigate the relationships between HRF and genomic expression markers of mitochondrial dysfunction in CD14⁺ cells to generate fatigue-associated candidate genes. For this project, we used a mitochondrially specific gene expression microarray (Voss et al., 2008) to assay mitochondrial and nuclear genes related to mitochondrial function in CD14⁺ cells of HIV/AIDS patients on ART regimens containing NTRIs with low and high fatigue compared to healthy controls.

Method

We describe the methodologies that we used in this study here, in Part 1 of this article. For an in-depth discussion of the statistical data analysis, see Part 2, “Statistical Analysis.”

Investigators have used gene expression technology, which we relied upon for this study, to study samples including a mix of peripheral blood cells, purified cell fractions, and cerebrospinal fluid from patients with chronic fatigue syndrome (Cherry et al., 2002; Nolan et al., 2003). Our recent development of a mitochondrial gene expression microarray has allowed us to investigate genes involved in fatigue-related symptom perceptions and to conduct a noninvasive assessment of mitochondrial involvement (Voss et al., 2008).

CD14⁺ monocytes consist of two major subsets: A major nonactivated CD14^{hi}/CD16^{lo} population and a minor activated CD14^{lo}/CD16^{hi} population (Crowe, Zhu, & Muller, 2003; Ziegler-Heitbrock, 2007). We chose to characterize the major nonactivated CD14⁺ cells that are CD14^{hi}/CD16^{lo}, which we will call CD14⁺ cells, obtained from peripheral blood to assay for mitochondrial dysfunction markers in HIV treatment-induced fatigue to avoid an obvious inflammatory signal. We selected this approach, using a purified cell type, so that variations in peripheral blood mononuclear cell fractions (PBMCs) between patients would not confound the expression data analysis. CD14⁺ monocytes are easily obtained in high numbers from peripheral blood, are susceptible to HIV infection (Lambotte et al., 2000; Zhu et al., 2002), and are thought to be a major reservoir for viral persistence during ART treatment (Alexaki, Liu, & Wigdahl, 2008; Crowe et al., 2003).

During an NIH intramural natural history study, “Assessing the Relationship Between Fatigue and Mitochondrial Toxicity in Patients with HIV/AIDS” (05-CC-0127), with a focus on HRF and mitochondrial toxicity, we utilized samples from 10 HIV positive patients on NRTI-containing and protease-inhibitor-sparing ART regimens and 5 healthy controls. We collected peripheral blood cells via aphaeresis for each patient. We evaluated fatigue in HIV patients using the revised 26-Item Piper Fatigue Scale, with fatigue scores varying between 0 and 10, where 0–3 was considered *no fatigue*, 4–7 *moderate fatigue*, and 8–10 *severe fatigue* (Piper et al., 1998). For the purposes of this pilot study, we combined patients with moderate and severe fatigue scores of 4–10 into a high-fatigue group and considered patients with scores in the range 0–3 to be the low-fatigue group. In this substudy, we compared three categories of CD14⁺ cell samples: Cells from HIV patients with high fatigue ($n = 5$), from HIV patients with low fatigue ($n = 5$), and from healthy controls ($n = 5$). There were no significant differences in demographic characteristics or comorbidities between healthy controls and participants in the HIV patient groups (see Table 1).

CD14⁺ Cell Isolation, RNA, and Protein Extraction

CD14⁺ cells were isolated from total PBMCs after aphaeresis with a negative CD14⁺ isolation procedure, followed by CD14⁺ positive cell sorting, according to the manufacturer's instructions. Average purity was $97.8 \pm 0.61\%$ and average yield was 5.5 ± 0.5 million cells. Cells were transferred to a QIAshredder MiniSpin Column (Qiagen, Valencia, CA) to shear DNA then spun, and cell extracts were frozen and stored at -70°C until ribonucleic acid (RNA) extraction. RNA was extracted as previously described using the Qiagen RNeasy method (Voss et al., 2008). The protein fraction was purified from the initial RNeasy column flow-through as specified by the manufacturer. Protein was precipitated out of the flow-through by addition of four volumes of ice-cold acetone. Proteins were pelleted by centrifugation and briefly air dried, washed with ice-cold absolute ethanol, and briefly air dried again. Pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO) and concentrations were determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed using the Promega Reverse Transcription Reaction System (Madison, WI) as specified by the manufacturer. PCR reactions were set up as per the manufacturer's instructions for Ct relative quantification (Applied Biosystems [AB], Foster City, CA). After thermal cycling, the amplification data were extracted from the AB 7300 data files for each 96-well plate and exported into Excel (Microsoft, Redmond, WA) for further processing. Briefly, for Ct calculation per patient cDNA sample, the Cts were calculated by subtracting the gene-specific Ct triplicate average from the corresponding triplicate glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control Ct average producing the normalized Ct value. For each gene per patient group, Ct averages were calculated with standard errors. Relative quotient (RQ) values between patient groups and control subjects were determined by the equation $RQ = 2^{-(Ct_{\text{patient CD14}^+} - Ct_{\text{control CD14}^+})}$, as described by the manufacturer (AB). The AB primer/probe assays used were AGTR2 Hs02621316_s1, SULT2B1 Hs01105284_m1, ADCY7 Hs00936808_m1, CCL28 Hs00219797_m1, CFL2 Hs00368395_g1, PROK2

Hs00363716_m1, PAG1 Hs00179693_m1, IL21R Hs00222310_m1, and the GAPDH as endogenous control assay. Additionally, we first tried assays that did not amplify detectable AGTR2 (s1Hs00169126_m1) and SULT2B1 (Hs00190268_m1) message; the 5' end of these assays' amplicons were located more to the 5' direction in the messages of interest than the assays used above for these genes and therefore may have been too far from the poly A tail to amplify well from our oligo dT primed cDNA template mixtures.

Western Blotting

We loaded 20 µg CD14⁺ cell total protein per well from fatigued and nonfatigued HIV patients and negative controls onto Nupage 4–12% Bis-Tris SDS-PAGE denaturing nonreducing polyacrylamide gels in 1 × 2 -(N-morpholino)ethanesulfonic acid (MES) running buffer and separated them by size next to Novex Sharp Standard molecular weights using the Xcel Mini-Cell (Invitrogen, Carlsbad, CA). After running, proteins were transferred from gels onto polyvinylidene fluoride (PVDF) membranes using the Xcel blotter device as specified (Invitrogen). After transfer, filters were blocked, probed, washed, and developed using the WesternBreeze chromogenic immunodetection kit for mouse primary antibody (Invitrogen). Secondary antimouse antibody was visualized by the conjugated alkaline phosphatase catalysis of the BCPIP/NBT substrate to a chromogen product. Developed blots were photographed and quantified using a Gel Logic 200 imaging system and software (Kodak, Rochester, NY). The specificity of mouse monoclonal antihuman CFL2 primary antibody 6G9 (Sigma) was confirmed by Western blot probing against CFL2 recombinant protein H00001073-P01 (Novus Biologicals, Littleton, CO) and CFL2 transient overexpression lysate and negative control empty vector lysate (Origene, Rockville, MA) using primary antibody at 1 µg per ml (Invitrogen). Loading was quantified using mouse monoclonal β-actin antibody AC-74 (Sigma).

Microarray Analysis

We followed a microarray data analysis approach as previously described (Dobra, 2009; Dobra et al., 2004). In the first step of this approach, we identified reduced sets of genes from the microarray data that are relevant for the phenotypes of interest. The validity of this selection method was quantified by the patient phenotype (i.e., fatigue and HIV status) prediction performance of the Bayesian model averaging classifier. These genes distinguished the phenotypes of interest but were not necessarily differentially expressed individually because we did not perform any tests proving differential expression. They, in effect, differentiate based on their combinatorial power as a set, not in isolation individually. In the second step of our approach, we developed association networks involving the reduced set of genes from the first step with the phenotype of interest. We constructed two distinct types of networks:

- a. Association networks, in which the links between two genes or between a gene and the phenotype represent strong pairwise associations. Here, the word *association* substitutes for the word *correlation*. In statistics, *correlation* refers to a linear association, while *association* can be any type of dependence, linear or nonlinear.
- b. Liquid association networks, in which the association edges represent pairs of genes whose association changes with respect to the phenotype of interest.

The two types of networks complement each other. Two genes that are directly connected in an association network are likely to be functionally related (Butte, Tamayo, Slonim, Golub, & Kohane, 2000; Steuer, Kurths, Fiehn, & Weckwerth, 2003). On the other hand, two genes are linked in a liquid association network if their relationship is influenced by the phenotype with respect to which the network was constructed (Li et al., 2007).

This inferential approach is based on a new stochastic search algorithm called the bounded mode stochastic search (BMSS; Dobra, 2009). These Affymetrix Probe Result File (CEL) files were preprocessed (i.e., background corrected and normalized) using Guanine Cytosine Robust Multi-array Average (Z. Wu & Irizarry, 2005; Z. Wu, Irizarry, Gentleman, Murillo, & Spencer, 2004). After the removal of the 112 control probes included on the chip, the resulting data set comprised of 4,712 probes. The data were confirmed with real-time PCR experiments for several genes.

Results

Quantitative RT-PCR Replication of Array Results

Our microarray expression analysis demonstrated that there were gene association networks that differentiated between the patient subgroups (Table 2). To confirm the microarray data and expression patterns between patient groups, we performed real-time quantitative RT-PCR (qRT-PCR) on the same RNA samples used for our microarray analyses. We chose eight genes from the fatigue association list (Table 3) with higher Kendall τ values differentiating HIV+ high fatigue versus HIV+ low-fatigue patients for this analysis. These fatigue liquid association-identified genes were CFL2, SULT2B1, PAG1, AGTR2 (high positive association with fatigue), and CCL28, PROK2, IL21R, ADCY7 (high negative association with fatigue; Tables 4 and 5). All abbreviations used herein are official NCBI database gene names.

We were able to detect all of the selected genes by qRT-PCR (Tables 4 and 5). We needed to utilize an additional second set of primer/probes to detect target genes AGTR2 and SULT2B1. The amplicons of the second primer/probe sets were located more to the 3' ends for these genes' messages compared to the more 5' region of the previous primer/probe sets we had tried (see Method section for primer codes). Moderate expression genes such as CFL2, PROK2, and PAG1 correlated between microarray raw expression intensity and qRT-PCR. We were unable to establish a correlation for several liquid-association genes, including SULT2B1, IL21R, and ADCY7, between microarray intensity and qRT-PCR RQ data between patient subsets at the lowest mRNA expressions level (Table 4). We confirmed the increased expression of CFL2 by Western blot and found a linear increase in expression of the protein from HIV- to HIV+ low fatigued to HIV+ patients with high fatigue (Figure 1).

Genes Associated With HRF

We grouped HRF genes into broad classes by protein function or cellular location, some with overlapping constituents, using information from the publicly available protein function databases (OMIM, Genecards, and KEGG). Note that positive fatigue correlation is

indicated by italics while negative correlation is indicated by normal font type. Classes were: RNA and DNA binding (*CHD1L*, *HLCS*, *GSPT1*, *DDX19B*, and *SBN01*), mitochondrial function (*CHD1L*, *TIMM17B*, *GSR*, *ALDOB*, *IMMT*, *SLC25A26*), cell migration and activation (*CFL2*, *MYH10*, *PAG1*), cytokine signaling (*IL21R*, *CCL28*, *IL7R*, *B2M*), cell cycle and growth (*GSPT1*, *SRP14*, *UGCG*), hormone metabolism (*SULT2B1*, *HSD17B3*, *PROK2*), apoptosis (*AGTR2*), G protein signaling (*AGTR2*, *ADCY2*), lipid/cholesterol metabolism (*ATP10A*, *ELA3B*, *FABP4*); and endoplasmic reticulum (ER) protein transport (*VPS13A*, *DPM1*). We confirmed several of the significant genes with RT-PCR. Many of these genes have already been implicated in HIV pathology.

SULT2B1 is the gene most strongly associated with HRF status. Hallmarks of progressive HIV disease include symptoms such as persistent fatigue, loss of muscle mass, loss of sexual function, and many other testosterone driven disorders. *SULT2B1* is a key gene in androgen synthesis pathways, and investigators have identified gonadal and adrenal androgen deficiencies in people living with HIV disease (Croxxson et al., 1989; Honour, Schneider, & Miller, 1995). Testosterone-replacement therapies have improved fatigue in men living with HIV (Rabkin, Wagner, McElhiney, Rabkin, & Lin, 2004). *PROK2* is a small 88-amino acid secreted peptide and G-protein coupled receptor (GPCR) agonist that regulates a variety of metabolic pathways and is highly expressed in the monocyte lineage (Monnier & Samson, 2008). Most relevant to fatigue, gene-targeting experiments in mice show that *PROK2*, a downregulated gene, controls torpor, attenuates circadian rhythms, and disrupts normal sleep patterns (Gottlieb, O'Connor, & Wilk, 2007; Jethwa et al., 2008; Monnier & Samson, 2008).

Several mitochondrial genes, *TIMM17B* and *IMMT*/mitofilin, are mitochondrial membrane proteins involved in translocation of proteins into the mitochondrial matrix (Schulke et al., 1999). *IMMT*/mitofilin is an inner membrane protein and plays a critical role in the organization of mitochondrial cristae morphology (G. B. John et al., 2005). Downregulation by siRNA leads to decreased cellular proliferation and increased apoptosis, with increased reactive oxygen species production and membrane potential (G. B. John et al., 2005). *IMMT*/mitofilin also binds to PARP-1 and forms a complex inside the inner membrane, translocating PARP-1 into mitochondria; PARP-1 then is involved in mitochondrial DNA damage signaling and repair through a protein complex also containing DNA ligase III (Rossi et al., 2009). *TIMM17B* is part of a protein complex that translocates proteins into the mitochondrial matrix and is necessary for cellular viability (Schulke et al., 1999). In addition, we found immune genes such as *IL21R*, *CCL28*, *IL7R*, and *B2M* to be expressed in HIV patients with HRF compared to patients without, suggesting a connection between immune activation and HRF.

Several genes identified in the comparison of high and low fatigue also play a role in HIV disease progression. Higher serum levels of *B2M*, the MHC-I β chain protein, have been associated with HIV disease progression (Mocroft et al., 1997). Similarly, investigators have found higher levels of *CCL28* in blood and other fluids from HIV-infected patients compared to healthy subjects (Piacentini, Fenizia, Naddeo, & Clerici, 2008). Chen et al. (2009) recently showed that *CHDL1* inhibits apoptosis by binding to the transcription factor Nur77 and inhibiting transport of Nur77 into the mitochondria. Nur77, itself, is implicated in apoptosis of thymocytes and T cells, indicating that *CHDL1* may regulate a complex

apoptotic pathway (Strasser, Puthalakath, O'Reilly, & Bouillet, 2008). The expression of two proteins, CFL2 and PAG1, indicates that the regulation of stability of the actin cytoskeleton is a feature in fatigue. In our study, CFL2 was positively associated with high fatigue and PAG1 with low fatigue. Cofilins belong to the actin-depolymerizing factor (ADF) family (Van Troys et al., 2008; Y. Wu et al., 2008). When in an activated state, ADF proteins destabilize the F-actin filament web under the cell membrane by converting F-actin to G-actin. Most studies on cofilins and HIV have focused on T-cell infection, during which the HIV coreceptor CXCR4 is activated by binding to the HIV protein gp120. This process initiates F-actin depolymerization by a signaling pathway downstream from CXCR4 that activates cofilin through dephosphorylation at a regulatory residue. This activation of cofilin allows HIV to penetrate through the now porous actin cortical web into the cytoplasm and eliminates a natural cellular cytoskeletal barrier to HIV infection (Yoder et al., 2008). Once infection is established however, the HIV protein NEF inhibits cofilin activation by enhancing Pak2 kinase activity and phosphorylating cofilin. This inhibition of cofilin enhances F-actin stability, diminishing cell motility (Stolp et al., 2009). The action of CFL2 in CD14⁺ cells is uncharacterized at this point, but similar mechanisms are suspected. PAG1 is a transmembrane protein with a long intracellular domain that was first identified in lipid rafts, where cell signaling proteins are concentrated, and is most highly expressed in the immune system (Svec, 2008). Interestingly, PAG1 is considered a scaffolding protein that serves as an indirect connector between cytoskeletal F-actin and the cell membrane and brings together signaling molecules in close proximity to each other (Itoh et al., 2002). PAG1 had the opposite expression pattern in our study to CFL2 and was higher in nonfatigued normal patients than in HIV⁺ patients. Overexpression experiments in T cells show that PAG1 inhibits cell activation and immune synapse formation. It also inhibits B and other immune cell activation (Horejsi, 2004; Svec, 2008). Investigators posit that activated PAG1 inhibits signaling and cell migration by stabilizing the attachment of lipid rafts to the F-actin web at the cell membrane, keeping the cytoskeleton in an inactive mode (Saibil, Deenick, & Ohashi, 2007).

Genes Associated With HIV Disease

This study confirms genes in CD14⁺ cells previously identified to be related to HIV disease. The HIV disease signature contains genes that can be grouped into two broad sets: Inflammation and signaling pathways. The most organized cluster has a hub around OSBPL7, with immune function genes HLA-DQA1, CXCL12 (chemokine agonist of HIV coreceptor CXCL4), GOT1, and the apoptotic gene SIVA1. These inflammatory regulator genes are associated with the key cell membrane signal integrating genes ADCY2 and PLCβ1. Other regulators of cell behavior are PPARα, involved in cell growth and differentiation; CASC4, which is associated with HER-2/neu proto-oncogene overexpression; HIPK2, a serine/threonine nuclear kinase that interacts with homeodomain transcription factors to inhibit cell growth and promote apoptosis; and GFRA2 (glial cell line-derived neurotrophic factor 2), a potent neurotrophic factor that plays a key role in the control of neuron survival and differentiation. NME6, though a metabolic enzyme with nucleoside diphosphate kinases activity involved in synthesis of nucleoside triphosphates other than ATP, is an inhibitor of P53-induced apoptosis. Histone deacetylase (HDAC) type 6 was positively associated with HIV infection in the present study, confirming previous

findings (Archin et al., 2009). CXCL12 is a potent inhibitor of HIV cellular infectivity through blocking adhesion of HIV to its coreceptor CXCR4 (Altenburg, Jin, Alkhatib, & Alkhatib, 2010).

We found several novel gene associations with HIV status from our array analyses that warrant further study. Some of these genes are involved in regulation of translation and transcription, such as TXLNB (TaxilinB), a member of a gene family that binds to syntaxins, and the NAC protein (nascent polypeptide-associated complex). NAC is part of a heterodimer complex that attaches to nascent polypeptide chains emerging from ribosomes and prevents polypeptide transfer to the ER. YAF2 is a zinc finger protein involved in negative regulation of muscle-restricted genes. MYC is a binding partner of YAF2 and also member of the E2F6.com-1 complex, a repressive complex that methylates “Lys-9” of histone H3, suggesting that it is involved in chromatin-remodeling. Another *de novo* gene in connection to HIV infection is GARS, a glycyl-tRNA synthetase shown to be a target of autoantibodies in human autoimmune diseases, including polymyositis and dermatomyositis.

Two of the HIV-associated genes are involved in fatty acid metabolism, the novel ACAD9 and the much better understood PPAR α . ACAD9 is a mitochondrial enzyme that catalyzes the initial rate-limiting step in the β oxidation of fatty acyl-CoA. PPAR α regulates lipid metabolism and transcription of genes regulating fatty acid β oxidation. Finally, the signaling pathway members ADCY2 and PLC β 1 are involved classically in the regulation of muscle contraction through G-protein coupled receptors. They are joined by other regulators of contraction here: TNNC1, which covers active sites for myosin on actin filaments and regulates actin/myosin interactions via calcium levels, and CHN1 (*n*-chimerin), a GTPase-activating protein for p21-rac and a phorbol ester receptor involved in ocular motor axon path finding (Zhu et al., 1993). How they interact with HIV is currently not understood.

Discussion

Our main goal in this study was to identify candidate genes associated with HRF. While it was a pilot study and lacked sufficient power for absolute statistical confidence for the identified genes, it nonetheless has identified several hypotheses to explore in future studies with higher sample numbers that more unequivocally identify HRF-associated genes.

The etiology of fatigue is of fundamental interest to HIV clinicians and patients alike. Current investigators in fatigue research are trying to determine whether mitochondrial dysfunction and mitochondrial toxicity occur first and induce the fatigue state or if other unknown processes such as HIV infection induce the fatigue state, which leads to increased dysfunction and toxicity in mitochondria. Since it is known that HIV disease itself can cause fatigue and that certain ART drugs, especially the NRTIs, can induce fatigue, we have started by asking a specific question: Can we identify gene expression patterns that characterize fatigue in HIV disease? Possible differences in the systemic immune activation or other systemic pathology, tissue-specific reactions to HIV disease, or differences in the complex pathways involved in regulating or modifying fatigue may be responsible for the development of fatigue. It would be useful for understanding the process leading to fatigue to know if the genes uncovered here in CD14⁺ cells are important players in HIV pathology

or simply reactive markers downstream of inflammatory and apoptotic processes. Future research should be pointed toward solving this question, as it is beyond the scope of current study.

We based the selection of our samples for the current analysis on either a high- or low-fatigue score in HRF. In future studies, we need to determine if the degree of expression of associated markers is related to the severity of symptom experience to establish a critical clinical measure for patient evaluation. In addition, analysis of gene expression in other diseases that induce fatigue would allow us to determine if any of the HRF-associated genes are specific to HIV disease or are more general markers of fatigue status.

While comparison of our microarray data to independent qRT-PCR quantifications matched for the higher expression genes, several very low expression genes were not validated. The low expression array data should be interpreted with caution. Published data show that low expression genes have the least reproducibility between oligonucleotide arrays and qRT-PCR, while moderate expression genes have the highest reproducibility between these platforms. Moreover, we do not necessarily expect Kendal's τ s calculated from liquid association analysis between gene expression and a phenotype to directly correspond to microarray raw intensity levels or to qRT-PCR RQ differences between patient groupings. Liquid association relationships are developed using Bayesian statistical regressions that are not equivalent to more traditional t test statistical comparisons. The low expression genes, such as SULT2B1 and AGTR2, need to be further analyzed between additional sets of HRF patients to verify positive association with fatigue.

We have several questions that we want to explore in future research. HIV virus infects CD14⁺ monocytes, and they provide a cell reservoir for HIV persistence even during successful HIV treatment with ART (Crowe et al., 2003). Are the effects of HIV disease and ART additive in relationship to fatigue, and can these additive effects be observed in the molecular signatures? Are some of the mitochondrial genes identified in CD14⁺ cells of HRF patients similarly expressed in other tissue types of HIV patients, particularly in skeletal muscle? While skeletal muscle, itself, is not infected with HIV, do ART-related and immunological effects from HIV disease play an important causative role in the development of fatigue symptoms? (Appay & Sauce, 2008; Fantuzzi, Belardelli, & Gessani, 2003) Certainly tissue macrophages can secrete inflammatory cytokines or apoptotic factors to surrounding tissue that would affect physiological processes in that tissue, or as circulating CD14⁺ monocytes while in the blood stream, they could also produce similar factors systemically. In fact, a major paradigm of HIV disease progression is long-term systemic immune activation, which is consistent with several genes identified in our HIV-associated gene set (Appay & Sauce, 2008; Centlivre, Sala, Wain-Hobson, & Berkhout, 2007; Douek, 2007).

The identification of disease-specific and general pathways regulating the etiology of fatigue could be clinically useful in patient treatment. It might be pertinent to the question of what effect hepatitis virus infection and clearance has in HRF. A number of HIV patients examined in this study had been exposed to either or both hepatitis B and C (HBV and HCV) viruses as evidenced from antibodies reactive against hepatitis proteins in the blood

work. The scope of this study was not large enough to examine the effects of previous hepatitis infection on fatigue levels in HCF disease, but investigators could address this question in future research using a larger data set designed to examine the issue.

Conclusions

We have identified for the first time a potential network of genes in CD14⁺ cells of HRF in ART-treated HIV patients compared to healthy controls. Furthermore, these genes may represent potential candidate biomarkers that could be useful diagnostically, as they are measurable in an easily accessible cell type, the CD14⁺ cell. We accomplished our study aims by generating a number of hypotheses related to genes involved in HRF to follow up with larger samples sizes, comparisons between CD14 and other PBMC populations and an animal model system of HRF or at least with ART-induced mitochondrial dysfunction.

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Figure 1.

CFL-2 protein expression by Western blot of human immunodeficiency virus (HIV)+ high and low fatigue compared to HIV- controls. Patient samples ($n = 4$ all groups) were run on SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) polyacrylamide gels and stained for CFL-2 then β -actin for normalization. Bands were quantified with the Gel logic software and expression was normalized to β -actin loading for each lane. The average expression per group was calculated as a ratio to the HIV- control sample average.

Demographic Characteristics of (1) Patients With HIV and (2) Normal Subjects.

Table 1

Gender	Age (years)	Duration of HIV Infection at Biopsy (years)	CD4 Count (Cells/mm ³)	Viral Load (copies/mL)	Fatigue Level ^a	Current ART	IV Drug Use	Chronic Hepatitis?	Hepatitis Serologies	Hepatitis Viral Load (Where Applicable/Available)
Female ¹	39.5	8.5	695	<50	0–3	TDF/FTC/NVP	Drug use (not iv)	Chronic hepatitis c	HBsAg –; HBsAb+; HBcAb+; HCVAb+	HCV bDNA 55,397 (04/27/05)
Male ¹	43.1	10.8	534	<50	0–3	TDF/ABC/EFV	No	Chronic hepatitis b	HBsAg+; HBsAb–; HBcAb+; HCVAb–	
Female ¹	39.9	8.6	1192	<50	0–3	TDF/3TC/NVP	Drug use (not iv)	Chronic hepatitis c	HBsAg–; HBsAb+; HBcAb–; HCVAb+	HCV bDNA 5,904,170 (08/19/05)
Male ¹	35.3	8.7	962	507	4–7	DDI/3TC/EFV	No	No	HBsAg–; HBsAb+; HBcAb+; HCVAb–	
Male ¹	46.0	6.9	360	<50	4–7	TDF/AZT/3TC/NVP	No	No	HBsAg–; HBsAb+; HBcAb+; HCVAb–	
Male ¹	42.2	14.6	773	<50	8–10	TDF/FTC/NVP	No	No	HBsAg –; HBsAb+; HBcAb+; HCVAb–	
Male ¹	52.2	2.7	302	<50	8–10	TDF/FTC/EFV	No	No	HBsAg–; HBsAb+; HBcAb+; HCVAb–	
Male ¹	47.1	8.6	293	<50	0–3	AZT/ABC/EFV	No	No	HBsAg–; HBsAB+; HBcAb+; HCAb–	
Male ¹	46.6	22.5	348	<50	4–7	TDF/3TC/EFV	Yes	Cleared hev without therapy	HBsAg–; HBsAb+; HBcAb+; HCAb+	HCV bDNA < 615 (4/24/03)
Male ¹	38.5	13.5	454	<50	0–3	TDF/FTC/EFV	No	Chronic hepatitis b	HBsAg+; HBsAb–; HBcAb+; HCAb–	HBV DNA < 200 (02/15/01)
Female ²	38.3	N/A	297	N/A	0–3	N/A	No	No	HBsAg–; HBsAb–; HBcAb–; HCVAb–	
Male ²	48.2	N/A	524	N/A	0–3	N/A	No	No	HBsAg–; HBsAb–; HBcAb–; HCVAb–	
Male ²	23.8	N/A	1710	N/A	0–3	N/A	No	No	HBsAg–; HBsAb+; HBcAb–; HCVAb–	
Male ²	51.8	N/A	884	N/A	0–3	N/A	No	No	HBsAg–; HBsAb+; HBcAb–; HCVAb–	
Female ²	22.8	N/A	883	N/A	0–3	N/A	No	No	HBsAg–; HBsAb–; HBcAb–; HCVAb–	

Note: 3TC = lamivudine; ABC = abacavir; ART = antiretroviral therapy; AZT = zidovudine; bDNA = branched DNA testing; DDI = didanosine; DNA = deoxyribonucleic acid; EFV = efavirenz; FTC = entricitabine; HBcAb– = hepatitis B core antibody; HBsAg–/+ = hepatitis B surface antigen negative/positive; HBsAb– = hepatitis B surface antibody negative; HBV = hepatitis B virus; HIV = hepatitis C virus; HCV = hepatitis B virus; NVP = nevirapine; TDF = tenovovir.

^aFatigue level was measured with the Piper Fatigue Scale.

Table 2

Human Immunodeficiency Virus (HIV)–Associated Genes.

Gene Symbol	Gene Title	GO Biological Process Term	Affymetrix Probe ID	Kendall's τ
ADCY2 ^a	Adenylate Cyclase 2	ATP binding/adenylate cyclase activity/magnesium ion binding/nucleotide binding/activation of adenylate cyclase activity by G-protein signaling pathway/activation of protein kinase A activity/cAMP biosynthetic process/hormone-mediated signaling pathway/inhibition of adenylate cyclase activity by G-protein signaling pathway/natural killer cell activation	217688_at	0.63
NAT12 ^a	<i>N</i> -acetyltransferase 12 (GCN5-related, putative)	Metabolic process	228321_s_at	0.57
HLA-DQA1 ^a	Major histocompatibility complex, Class II, DQ α 1	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	203290_at	0.55
HDAC6 ^a	Histone deacetylase 6	Protein polyubiquitination/protein amino acid deacetylation/incompletely synthesized protein catabolic process/intracellular protein transport/negative regulation of microtubule depolymerization/cell cycle/multicellular organismal development/response to toxin/regulation of receptor activity/negative regulation of hydrogen peroxide metabolic process/positive regulation of receptor biosynthetic process/macrophagy/chromatin modification/histone deacetylation/lysosome localization/peptidyl-lysine deacetylation/positive regulation of apoptosis/Negative regulation of protein complex disassembly/regulation of transcription/negative regulation of proteolysis/negative regulation of oxidoreductase activity/response to misfolded protein/response to protein stimulus/regulation of microtubule-based movement/regulation of androgen receptor signaling pathway/cellular response to hydrogen peroxide/aggresome assembly/polyubiquitinated misfolded protein transport/Hsp90 deacetylation/response to growth factor stimulus/positive regulation of cellular chaperone-mediated protein complex assembly/tubulin deacetylation	211722_s_at	0.54
YAF2 ^a	YY1 associated factor 2	Negative regulation of transcription/positive regulation of transcription/metal ion binding/zinc ion binding	244783_at	0.54
TOMM20L	Translocase of outer mitochondrial membrane 20 homolog (yeast)-like	Protein targeting/mitochondrial outer membrane translocase complex	239080_at	0.53
ST3GAL5	ST3 β -galactoside α -2,3-sialyltransferase 5	Lactosylceramide α -2,3-sialyltransferase activity/sialyltransferase activity/protein amino acid glycosylation/integral to Golgi membrane	203217_s_at	0.52
GARS	Glycyl-tRNA synthetase	tRNA aminoacylation for protein translation/glycyl-tRNA aminoacylation/cell death/regulated secretory pathway	208693_s_at	0.51
HIPK2	Homeodomain interacting protein kinase 2	Negative regulation of transcription from RNA polymerase II promoter/protein amino acid phosphorylation/apoptosis/DNA damage response, signal transduction by p53 class mediator resulting in transcription of p21 class mediator/transforming growth factor β receptor signaling pathway/smoothed signaling pathway/positive regulation of cell proliferation/induction of apoptosis by intracellular signals/anterior/posterior pattern formation/virus–host interaction/positive regulation of transforming growth factor β receptor signaling pathway/negative regulation of BMP signaling pathway/DNA damage response, signal transduction by p53 class mediator resulting in induction of apoptosis/positive regulation of DNA binding/positive regulation of transcription, DNA-dependent/positive regulation of transcription from RNA polymerase II promoter/positive regulation of JNK cascade/SMAD protein signal transduction	224066_s_at	0.46

Gene Symbol	Gene Title	GO Biological Process Term	Affymetrix Probe ID	Kendall's τ
ALDH9A1 ^a	Aldehyde dehydrogenase 9 family, member A1	Cellular aldehyde metabolic process/carnitine metabolic process/neurotransmitter biosynthetic process/hormone metabolic process/oxidation reduction/oxidation reduction	201612_at	0.44
PIK3R3 ^a	Phosphoinositide-3-kinase, regulatory subunit 3 (γ)	1-phosphatidylinositol-3-kinase activity/protein binding	211580_s_at	0.42
ACAD9	Acyl-coenzyme A dehydrogenase family, member 9	Oxidation reduction	239976_at	0.20
TNNC1 ^a	Troponin C type 1 (slow)	Response to metal ion/regulation of muscle filament sliding speed/regulation of ATPase activity	209904_at	-0.36
LOC374491 ^a	TPTE and PTEN homologous inositol lipid phosphatase pseudogene	Unknown	233675_s_at	-0.38
ZFYVE16 ^a	Zinc finger, FYVE domain containing 16	Protein targeting to lysosome/signal transduction/vesicle organization/endosome transport/regulation of endocytosis/BMP signaling pathway	1555011_at	-0.40
TXLNB ^a	Taxilin β	Cytoplasm	227834_at	-0.40
NME6 ^a	Nonmetastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)	GTP biosynthetic process/UTP biosynthetic process/CTP biosynthetic process/apoptosis/antiapoptosis/nucleoside triphosphate biosynthetic process		-0.43
GOT1 ^a	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	oxaloacetate metabolic process/glycerol biosynthetic process/cellular amino acid metabolic process/aspartate biosynthetic process/glutamate catabolic process to aspartate/glutamate catabolic process to 2-oxoglutarate/cellular response to insulin stimulus/response to glucocorticoid stimulus/fatty acid homeostasis	208813_at	-0.43
SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	Sodium ion transport/calcium ion transport/cell communication/transmembrane transport		-0.44
GFRA2	GDNF family receptor α 2	Transmembrane receptor protein tyrosine kinase signaling pathway	205721_at	-0.44
PPAR α ^a	Peroxisome proliferator-activated receptor α	Response to hypoxia/transcription from RNA polymerase II promoter/fatty acid metabolic process/negative regulation of specific transcription from RNA polymerase II promoter/negative regulation of foam cell differentiation/negative regulation of receptor biosynthetic process/negative regulation of cholesterol storage/negative regulation of sequestering of triglyceride	206870_at	-0.44
AGXT	Alanine-glyoxylate aminotransferase	Protein targeting to peroxisome/response to hormone stimulus/glycine biosynthetic process, by transamination of glyoxylate/pyruvate biosynthetic process/glyoxylate metabolic/oxalic acid secretion/response to glucocorticoid stimulus/response to cAMP	210326_at	-0.44
ZAK ^a	Sterile α motif and leucine zipper containing kinase AZK	Cell cycle checkpoint/DNA damage checkpoint/activation of MAPKK activity/protein amino acid phosphorylation/response to stress/cytoskeleton organization/cell cycle arrest/protein kinase cascade/activation of JUN kinase activity/cell death/cell proliferation/cell differentiation/positive regulation of apoptosis	222757_s_at	-0.47
C7orf51	Chromosome 7 open reading frame 51	Hypothetical protein	1553288_a_at	-0.47
PC ^a	Pyruvate carboxylase	Gluconeogenesis/oxaloacetate metabolic process/lipid biosynthetic process	204476_s_at	-0.49
CXCL12 ^a	Chemokine (C-X-C motif) ligand 12	Chemokine activity/cytokine activity/growth factor activity T cell proliferation/chemotaxis/immune response/positive regulation of cell migration/CXCR4 (HIV coreceptor) agonist	209687_at	-0.49

Gene Symbol	Gene Title	GO Biological Process Term	Affymetrix Probe ID	Kendall's τ
ACADL	Acyl-coenzyme A dehydrogenase, long chain	Fatty acid metabolic process/metabolic process/oxidation reduction	206069_s_at	-0.51
PLCB1 ^a	Phospholipase C, β 1 (phosphoinositide-specific)	Calcium ion binding/enzyme binding/hydrolase activity/phosphoinositide phospholipase C activity/signal transducer activity/intracellular signaling cascade/lipid catabolic process/oxygen and reactive oxygen species metabolic process/phosphoinositide metabolic process	213222_at	-0.51
SIVA1	SIVA1, apoptosis-inducing factor	Induction of apoptosis/positive regulation of apoptosis/interspecies interaction between organisms	222030_at	-0.51
OSBPL7 ^a	Oxysterol binding protein-like 7	Lipid transport/steroid metabolic process	208163_s_at	-0.52
CASC4 ^a	Cancer susceptibility candidate 4	Integral to membrane/HER-2/neu proto-oncogene overexpression	1559635_at	-0.53
CHN1 ^a	Chimerin (chimaerin) 1	GTPase activator activity/SH3/SH2 adaptor activity/metal ion binding/protein binding/zinc ion binding	212624_s_at	-0.54
ZNF347 ^a	Zinc finger protein 347	Regulation of transcription, DNA-dependent	237061_at	-0.61

Note. Gene symbols and titles from Unigene (<http://www.ncbi.nlm.nih.gov/UniGene>), biological processes from Gene Ontology (GO; <http://www.geneontology.org/>), probe ID from Affymetrix (<http://www.affymetrix.com>).

ATP = adenosine triphosphate; BMP = bone morphogenetic protein; CTP = cytidine-5'-triphosphate; DNA = deoxyribonucleic acid; GTP = guanosine-5'-triphosphate; tRNA = transfer ribonucleic acid; UTP = uridine-5'-triphosphate.

^a Twenty-two genes with direct association with HIV status.

Table 3

Fatigue-Associated Genes.

Gene Symbol	Gene Title	GO Biological Process Term	Affymetrix Probe ID	Kendall's τ
SULT2B1 ^a	Sulfotransferase family, cytosolic, 2B, member 1	Lipid metabolic process/steroid metabolic process	205759_s_at	0.74
CFL2-b ^{a,b}	Cofilin 2 (muscle)	Immune response/signal transduction/intracellular signaling cascade/regulation of T cell activation/negative regulation of T cell activation	224663_s_at	0.74
ELA3B ^a	Chymotrypsin-like elastase family, member 3B	Proteolysis/cholesterol metabolic process	206151_x_at	0.73
DDX19B ^a	DEAD (Asp-Glu-Ala-As) box polypeptide 19B	Positive regulation of T cell-mediated cytotoxicity/response to molecule of bacterial origin/antigen processing and presentation of peptide antigen via MHC Class I/antigen processing and presentation of exogenous protein antigen via MHC Class Ib, TAP-dependent/immune response/response to drug	230974_at	0.70
SBNO1	Strawberry notch homolog 1 (Drosophila)	ATP binding/DNA binding/hydrolase activity	216161_at	0.66
FAM120AOS ^a	Family with sequence similarity 120A opposite strand	Unknown	1558761_a_at	0.62
TIMM17B ^a	Translocase of inner mitochondrial membrane 17 homolog B (yeast)	Protein targeting to mitochondrion/transport/intracellular protein transport/intracellular protein transmembrane transport	1559909_a_at	0.62
ATP10A ^a	ATPase, class V, type 10A	ATP biosynthetic process/regulation of cell shape/phospholipid transport	1568743_at	0.62
GSR ^a	Glutathione reductase	Glutathione metabolic process/spermatogenesis/cell redox homeostasis/oxidation reduction	205770_at	0.62
SLC25A26 ^a	Solute carrier family 25, member 26	Regulation of DNA recombination/immune response/cell surface receptor linked signal transduction	225862_at	0.62
IL7R ^a	Interleukin 7 receptor	Golgi-to-endosome transport/locomotory behavior/protein localization/social behavior	226218_at	0.62
LOC100132884 ^a	Hypothetical protein LOC100132884	mRNA export from nucleus/transport/induction of apoptosis/response to zinc ion/protein transport/intracellular protein transmembrane transport	228899_at	0.62
B2M	β -2-microglobulin	Antiapoptosis/chemotaxis/inflammatory response/G-protein-coupled receptor protein signaling pathway/elevation of cytosolic calcium ion concentration/circadian rhythm/cell proliferation/sensory perception of pain	232311_at	0.62
UGCG	UDP-glucose ceramide glucosyltransferase	Lipid metabolic process/sphingolipid metabolic process/glucosylceramide biosynthetic process/glycosphingolipid biosynthetic process	204881_s_at	0.57
IMMT	Inner membrane protein, mitochondrial (mitofilin)	Molecular function/protein binding/cristae formation in mitochondria/mitochondrial inner membrane	242361_at	0.57
CFL2-a ^{a,b}	Cofilin 2 (muscle)	Immune response/signal transduction/intracellular signaling cascade/regulation of T cell activation/negative regulation of T cell activation	224352_s_at	0.53
DPM1	Dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	N-Glycan biosynthesis and metabolism	237213_at	0.41
CHD1L ^a	Chromodomain helicase DNA binding protein 1-like	ATP binding/ATP-dependent helicase binding activity/ATPase activity/protein binding	1557597_at	-0.49
CCL28 ^a	Chemokine (C-C motif) ligand 28	Chemokine activity/immune response/chemotaxis/elevation of cytosolic calcium ion concentration	224240_s_at	-0.49
IL21R ^a	Interleukin 21 receptor	Chemotaxis/immune response/elevation of cytosolic calcium ion concentration	219971_at	-0.53

Gene Symbol	Gene Title	GO Biological Process Term	Affymetrix Probe ID	Kendall's τ
HLCS ^a	Holocarboxylase synthetase (biotin-(propionyl-Coenzyme A-carboxylase (ATP-hydrolysing ligase)	Protein modification process	209399_at	-0.57
GSPT1 ^a	G1 to S phase transition 1	GTP binding/GTPase activity/nucleotide binding/protein binding	217595_at	-0.57
MYH10 ^a	Myosin, heavy chain 10, nonmuscle	Cytokinesis after mitosis/plasma membrane repair/actin cytoskeleton organization	213067_at	-0.62
ALDOB ^a	Aldolase B, fructose-bisphosphate	Fructose metabolic process/gluconeogenesis/glycolysis/NADH oxidation/response to carbohydrate stimulus/response to zinc ion/positive regulation of ATPase activity/response to chemical stimulus/response to peptide hormone stimulus	214424_s_at	-0.62
PROK2 ^a	Prokineticin 2	Cytokine production/negative regulation of protein kinase activity/fatty acid metabolic process/transport/positive regulation of cell proliferation/negative regulation of transcription/response to drug/cholesterol homeostasis/positive regulation of inflammatory response/response to glucocorticoid stimulus	232629_at	-0.62
FABP4 ^a	Fatty acid binding protein 4, adipocyte	Fatty acid binding/protein binding/transcription repressor activity/transport activity	235978_at	-0.62
VPS13A ^a	Vacuolar protein sorting 13 homolog A (S. cerevisiae)	Protein binding/golgi-to-endosome transport/protein localization	227988_s_at	-0.64
SRP14	Signal recognition particle 14kDa (homologous Alu RNA binding protein)	Cotranslational protein targeting to membrane/SRP-dependent cotranslational protein targeting to membrane/response to drug/protein targeting to ER/negative regulation of translational elongation	200007_at	-0.66
HSD17B3 ^a	Hydroxysteroid (17- β) dehydrogenase 3	Steroid biosynthetic process/lipid biosynthetic process/oxidation reduction	206985_at	-0.66
AGTR2 ^a	Angiotensin II receptor, type 2	G-protein-coupled receptor protein signaling pathway/positive regulation of apoptosis/positive regulation of nitric oxide biosynthetic process	207294_at	-0.66
GPM6A ^a	Glycoprotein M6A	Cell surface/integral to membrane	209470_s_at	-0.70
PAG1 ^a	Phosphoprotein associated with glycosphingolipid microdomains 1	S-adenosylmethionine transport/transmembrane transport	225622_at	-0.70
ADCY2 ^a	Adenylate cyclase 2	ATP binding/adenylate cyclase activity/magnesium ion binding/nucleotide binding/activation of adenylate cyclase activity by G-protein signaling pathway/activation of protein kinase A activity/cyclic adenosine monophosphate biosynthetic process/hormone-mediated signaling pathway/inhibition of adenylate cyclase activity by G-protein signaling pathway/natural killer cell activation	217688_at	-0.74

Note. Gene symbol and title from Unigene (<http://www.ncbi.nlm.nih.gov/UniGene>), biological processes from Gene Ontology (GO; <http://www.geneontology.org/>), Probe ID from Affymetrix (<http://www.affymetrix.com>).

ATP = adenosine triphosphate; DNA = deoxyribonucleic acid; ER = endoplasmic reticulum; GTP = guanosine-5'-triphosphate; MHC = major histocompatibility complex; mRNA = messenger ribonucleic acid; NADH = nicotinamide adenine dinucleotide; TAP = transporter of antigen presentation.

^a Twenty-seven genes with direct association with HIV status.

^b CFL2 a and b are two independent oligonucleotide sets for the CFL gene.

Table 4

RT-QPCR values and fold changes for Fatigued and Nonfatigued HIV Positive Patients.

Gene	Ct		RQ Folds	Array Intensity	
	Low Fatigue	High Fatigue		Low Fatigue	High Fatigue
PROK2	5.73	6.76	-2.05	520.45	186.26
PAG1	6.2	6.34	-1.1	114.93	58.21
IL21R ^a	7.66	7.84	-1.14	2.1	3.46
CCL28 ^b	9.1	8.34	1.69	15.16	7.32
CFL2a ^c	8.53	7.1	2.7	25.57	35.07
CFL2b ^c				6.76	15.11
ADCY7	3.49	4.26	-1.7	1.83	2.21
SULT2B1 ^{d,f}	10.89	11.53	-1.56	0.89	1.07
AGTR2 ^{e,f}	19.96	20.42	-1.38	2.35	2.02

Note. Both Ct values and fold ratios between samples are given for each gene. The GAPDH Ct values were used to normalize the gene data using the Ct method. The higher the Ct value, the lower the expression of a gene compared to the normalizing gene GAPDH. Genes with positive Q-PCR folds are higher in high fatigue, those with negative folds are higher in low fatigue. Array fold differences are given for comparison for each gene, from the raw microarray CEL data files.

^a IL21R was not amplified from two high- and two low-fatigue patient samples.

^b CCL28 was not amplified from one high- and one low-fatigue patient sample.

^c Both CLF2 array probe set intensities are presented.

^d SULT2B1 was not amplified from one high-fatigue patient.

^e AGTR2 was not amplified from one high- and one low-fatigue patient.

^f One high- and one low-fatigue RNA sample were exhausted and therefore not amplified for SULT2B1 and AGTR2 measurements. Positive control U937 cells amplified AGTR2 in the mid 30 Ct range (data not shown), while most patient samples Cts for SULT2B and AGTR2 were above 40.

Table 5

RT-QPCR values and fold changes for HIV Positive Patients and Healthy Subjects.

Genes	Ct		Q-PCR RQ Folds
	Control HIV ⁻	HIV ⁺	
PROK2 ^a	6.66	6.25	1.33
PAG1 ^a	5.66	6.27	-1.52
IL21R ^b	9.69	7.75	3.83
CCL28 ^c	9.36	8.72	1.56
CFL2	7.78	7.74	1.03
ADCY7	2.81	3.87	-2.10
SULT2B1 ^d	9.72	11.16	-2.73
AGTR2 ^d	16.51	20.17	-12.70

Note. Both Ct values and fold ratios between samples are given for each gene. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ct values were used to normalize the gene data using the Ct method. The delta-delta Ct values shown here are for HIV⁺ patients versus healthy controls. Genes with positive Q-PCR RQ folds are higher in HIV⁺ patients, those with negative folds are higher in HIV⁻ controls.

^aPROK2 and PAG 1 were not amplified from one normal control each.

^bIL21R was not amplified from four HIV patient samples.

^cCCL28 was not amplified from two HIV patient samples.

^dControl U937 cells were positive for AGTR2 in the mid 30 Ct range (data not shown), however, all patient samples Cts for SULT2B and AGTR2 were above 40.