Fatigue-related Gene-Networks identified in $CD14^+$ cells isolated from HIV infected Patients - Part I: Research Findings

Key Words: CD14, Fatigue, Cofillin 2, Bayesian Inference, Prokineticin 2, HIV

Abstract

Purpose: HIV-related fatigue (HRF) is multi-causal and potentially related to mitochondrial dysfunction caused by antiretroviral therapy with nucleoside reverse transcriptase inhibitors (NRTIs).

Methodology: We compared gene expression profiles of $CD14^+$ cells of low versus high fatigued, NRTI-treated HIV patients to healthy controls (n=5 each). We identified 32 genes predictive of low versus high fatigue, and 33 genes predictive of healthy versus HIV infection. We constructed genetic networks to further elucidate the possible biological pathways in which these genes are involved.

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 could be easily accessible cells to further study 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 testing methodologies to diagnosis, monitor and treat fatigue and mitochondrial dysfunction.

HIV-related fatigue (HRF) is the most prevalent symptom and between 80-100% of persons living with HIV disease complaint of ongoing lack of energy and exhaustion that lasts longer than four weeks (Cupler et al., 1995; Davis, 2004). HRF has been associated with deteriorating physical and mental health, quality of life and is predictive of diminished physical function (Ferrando et al., 1998). Hallmarked by chronic lack of energy and weakness, HRF is known to contribute to loss of social function, lower quality of work, increased absenteeism, and, ultimately, loss of employment and decreased adherence to antiretroviral therapy (ART) (Darko, McCutchan, Kripke, Gillin, & Golshan, 1992; Darko et al., 1995; Ostrop, Hallett, & Gill, 2000). Human Immunodeficiency Virus (HIV) infection and treatment with antiretroviral nucleoside analogues (nucleoside reverse transcriptase inhibitors or NRTIs) affect mitochondrial 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, 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 alpha 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; J.G. 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 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 considered to be 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 and an alternative non-surgical toxicity assay would improve clinical diagnoses tremendously (Authier, Chariot, & Gherardi, 2005; Bongiovanni & Tordato, 2007).

We wanted to investigate if peripheral $CD14^+$ monocytes could serve as a fatigue surrogate tissue 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 parallel metric of fatigue using these divergent markers. This would make 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 to generate hypotheses by identifying fatigue-associated candidate genes in the context of HIV disease. For this project, a mitochondrially-specific gene expression microarray (Voss et al., 2008) was used 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.

Methods

The methodologies utilized are described in Part 1 of this manuscript, while the statistical data analysis are described in much greater detail in Part 2. We will describe the statistical methods here in an abbreviated version and refer from here on to Part II as the statistical analysis section of this research. Gene expression technology has been used to assay patients with chronic fatigue syndrome from a mix of peripheral blood cells, purified cell fractions, and cerebrospinal fluid (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 a non-invasive assessment of mitochondrial toxicity (Voss, et al., 2008).

$CD14^+$ monocytes consist of two major subsets: a major non-activated 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 non-activated $CD14^+$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. This approach, using a purified cell type was selected 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 a 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 HIV-

related fatigue and mitochondrial toxicity, we utilized samples from 10 HIV positive patients on NRTI-containing and protease-inhibitor sparing ART regimens and 5 healthy controls. Peripheral blood cells were collected via aphaeresis for each patient. HIV patients were evaluated using the revised 26-item Piper Fatigue Scale with fatigue scores varying between 0-10: where (0-3) was considered no fatigue, (4-7) as moderate fatigue, and (8-10) as severe fatigue (Piper, et al., 1998). For the purposes of this pilot study, we combined patients with moderate and severe fatigue scores 4-10 into a high fatigue group and considered scores 0-3 as the low fatigue group. In this substudy, three categories of $CD14^+$ cell samples were compared: cells from HIV patients with high fatigue (n=5), HIV patients with low fatigue (n=5), and healthy controls (n=5). There were no significant differences between healthy controls and both HIV patient groups (see Table 1).

Insert 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±0.61% and average yield was 5.5±0.5 million cells. Cells were transferred to a QIAShredder MiniSpin Column (Qiagen, Valencia, CA) to sheer DNA, then spun and cell extracts were frozen and stored at -70°C until RNA extraction. RNA was extracted as previously described using the Qiagen RNeasy method (Qiagen) (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 4 volumes 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 RIPA buffer (Sigma-Aldrich, St. Louis, MO) and concentrations were determined by BCA assay (Pierce, Rockford, IL).

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 delta Ct relative quantification (Applied Biosystems [AB], Foster City, CA). After thermal cycling the amplification data was extracted from the AB 7300 data files for each 96 well plate and exported into Excel (Microsoft, Redmond WA) for further processing. Briefly, for delta Ct calculation per patient cDNA sample, the delta Cts were calculated by subtracting from the gene-specific Ct triplicate average from the corresponding triplicate Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control Ct average producing the normalized delta Ct value. For each gene per patient group, delta Ct averages were calculated with standard errors. Relative quotient (RQ) values between patient groups and control subjects were determined by the equation $RQ= 2^{-(\text{deltaCt patient CD14+ - delta Ct control CD14+})}$ as described by the manufacturer (AB). The Applied Biosystems 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

Twenty micrograms $CD14^+$ cell total protein per well from fatigued and non fatigued HIV patients and negative controls were loaded onto Nupage 4-12% Bis-Tris SDS-PAGE denaturing non-reducing polyacrylamide gels in 1X MES running buffer and separated 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 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 anti-mouse 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 anti-human 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 microgram per ml (Invitrogen) Loading was quantified using mouse monoclonal beta 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 pair wise 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 non-linear.

(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 CEL files were preprocessed (i.e., background corrected and normalized) using Guanine Cytosine Robust Multi-array Average (Z Wu, Irizarry, Gentleman, Murillo, & Spencer, 2004; Z. Wu & Irizarry, 2005). After the removal of the 112 control probes included on the chip, the resulting dataset comprised 4712 probes. The data were confirmed with realtime PCR experiments for several genes.

Results

qRT-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 quantitative 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 tau 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) (Table 4a and 4b). All abbreviations used herein are official NCBI database gene names.

We were able to detect all of the selected genes by qRT-PCR (Table 4). 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 Methods 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 between microarray intensity and qRT-PCR RQ data between patient subsets at the lowest mRNA expressions level including SULT2B1, IL21R and ADCY7 (Table 4A). We confirmed the increased expression of CFL2 by Western Blot and found a linear increase in expression of the protein from HIV negative to HIV+ low fatigued, to HIV+ patients with high fatigue (Figure 1).

Insert Figure 1

Genes associated with HIV-related fatigue

We grouped HRF genes into broad classes by protein function or cellular location, some with overlapping constituents, using information from the publically 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 & DNA binding (CHD1L, HLCS, GSPT1, *DDX19B* and *SBNO1*); mitochondrial function (CHD1L*,TIMM17B*, *GSR*, ALDOB, *IMMT*, *SLC25A26*); cell migration and activation (*CFL2*, MYH10, PAG1); cytokine signaling (IL21R, CCL28*, IL7R*, *B2M*); cell cycle & growth (GSPT1, SRP14, *UGCG*); hormone metabolism (*SULT2B1*, HSD17B3, PROK2); apoptosis (AGTR2); G protein signaling (AGTR2, ADCY2); lipid/cholesterol metabolism (*ATP10A*, *ELA3B*, FABP4); and ER protein transport (VPS13A, *DPM1*). Several of the significant genes were confirmed 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 gonadal and adrenal androgen deficiencies have been identified in people living with HIV disease (Croxson et al., 1989; Honour, Schneider, & Miller, 1995). Replacement therapies with testosterone have shown to improve fatigue in men living with HIV (J. G. Rabkin, Wagner, McElhiney, Rabkin, & Lin, 2004). PROK2 is a small 88 amino acid secreted peptide and 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). Down-regulation 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 and translocates 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). TIMM17 is part of a protein complex that translocates proteins into the mitochondrial matrix and is necessary for cellular viability (Schulke, et al., 1999). Immune genes such as IL21R, CCL28, IL7R, and B2M are activated in HIV patients with HRF compared to patients without, suggesting for a tight link between immune activation and fatigue processes.

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 beta chain protein, have been associated with HIV disease progression (Mocroft et al., 1997). Similarly, higher levels of CCL28 are found in blood and other fluids from HIV infected patients compared to healthy subjects (Piacentini, Fenizia, Naddeo, & Clerici, 2008). CHDL1 has recently been shown to inhibit apoptosis by binding to the transcription factor Nur77 and inhibiting transport of Nur77 into the

mitochondria (Chen et al., 2009). Nur77 itself is implicated in apoptosis of thymocytes and T-cells, indicating a complex apoptotic pathway may be regulated by CHDL1 (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 was associated with low fatigue. Cofilins belong to the actin-depolarizing factor (ADF) family (Van Troys et al., 2008; Y. Wu et al., 2008), when in an activated state ADF proteins de-stabilize 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 co-receptor CXCR4 is activated by binding to the HIV protein gp120. This process initiates F-actin depolymerization by a signaling pathway downstream from the HIV co-receptor CXCR4 that activates cofilin through de-phosporylation 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 enhances F-actin stability and cell motility is diminished (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 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 is higher in non fatigued normal patients. Overexpression experiments in T-cells show that PAG1

inhibits cell activation and immune synapse formation; PAG1 also inhibit B and other immune cell activation (Horejsi, 2004; Svec, 2008). Activated PAG1 is thought to inhibit 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

This study confirms genes in $CD14^{+}$ cells previously identified to be related to HIV disease. The HIV disease signature contains can be grouped into two broad gene 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 co-receptor CXCL4), GOT1 and the apoptotic gene SIVA1. These inflammatory regulator genes are associated with key cell membrane signal integrating genes ADCY2 and PLCβ1. Other regulators of cell behavior are PPAR α, 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 that inhibits cell growth and promotes apoptosis; and GFRA2 or glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factors that play key roles 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 our study (Archin et al., 2009). CXCL12 is a potent inhibitor of HIV cellular infectivity through blocking adhesion of HIV to its co-receptor CXCR4 (Altenburg, Jin, Alkhatib, & Alkhatib, 2010).

Several genes are de novo associations with HIV from our array analysis and need to be further studied for relevance to this disease. Some are involved in regulation of translation and transcription; TXLNB (TaxilinB) is a member of a family that binds to syntaxins and the NAC protein (nascent polypeptide-associated complex), that regulates protein translation on ribosomes and possible on transcription. YAF2 is a zinc finger protein involved in negative regulation of muscle-restricted genes. MYC is a binding partner and 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 and GARS a glycyl-tRNA synthetase shown to be a target of autoantibodies in the human autoimmune diseases, polymyositis or dermatomyositis. Two of these genes are involved in fatty acid metabolism: PPAR α and ACAD9. ACAD9 is a mitochondrial enzyme that catalyzes the initial rate-limiting step in the beta-oxidation of fatty acyl-CoA and PPAR α regulates lipid metabolism and transcription of genes regulating fatty acid beta-oxidation. The signaling pathway members ADCY2 and PLCβ1 are involved classically in the regulation of muscle contraction through G-Protein Coupled Receptors. It is worth noting that these 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).

Discussion

Our main goal in this study has been to identify candidate genes that associate with HIV related fatigue. While this is a pilot study and lacks sufficient power for absolute statistical confidence for the identified genes, it nonetheless has identified several hypotheses for future studies with higher sample numbers that more unequivocally identify HRF associated genes. The

etiology of how fatigue occurs biologically is of fundamental interest to HIV clinicians and patients alike. Current investigators in fatigue research try 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 in 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 towards solving this issue but is beyond the current scope of this study. The selection of our samples for the current analysis was based on either a high or a 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. 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 more general makers of fatigue status.

While comparison or 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 and matches published data showing that low expression genes have the least reproducibility between oligonucletide arrays and qRT-PCR

while moderate expression genes have the highest reproducibility between these platforms. Moreover we do not necessarily expect Kendal's Taus 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. There 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 hypotheses that we want to follow. 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 there effects of HIV disease and ART additive in relationship to fatigue and can 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? Another question is, while skeletal muscle by 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 (Appay & Sauce, 2008; Fantuzzi, et al., 2003). In fact, a major paradigm of HIV disease progression is long term systemic immune activation, which is consistent with several messages 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. This 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 could be answered with a larger dataset designed to examine this issue in the future.

Conclusions

We have identified for the first time a potential network of genes in CD14 cells of HIV-related fatigue in ART treated HIV patients compared to healthy controls. Furthermore these genes may represent potential candidate biomarkers that may be useful diagnostically as they are measurable in an easily accessible cell type, the $CD14^{+}$ cell. In conclusion, the study accomplished its aims by generating a number of hypotheses related to genes involved in HRF to be 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.

Figures

Figure 1. CFL-2 protein expression by western blot of HIV+ high and low fatigue compared to HIV- controls. Patient samples (n=4 all groups) were run on SDS page polyacrilamide gels and stained for CFL-2 then beta actin for normalization. Bands were quantified with the Gel logic software and expression was normalized to beta-actin loading for each lane and then the average expression per group was calculated as a ratio to the HIV- control sample average.

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