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Comparative Transcriptional Profiling in HIV-Infected Patients Using Human Stress Arrays: Clues to Metabolic Syndrome

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Abstract. *Highly active antiretroviral therapy (HAART therapy) for HIV-1 infection has significantly increased the survival and quality of life of patients with this disease. However, in several epidemiological studies the onset of metabolic syndrome is a phenomenon reported to be extremely frequent. In the present study, genes involved in the molecular cascade responsible for the alteration of fat tissue and of lipid and glucose metabolism in patients with HIV-1 infection treated with antiretroviral therapy were identified. Towards this goal, hybridization using Atlas cDNA Expression Arrays allowed simultaneous monitoring of the expression levels of approximately 250 genes and identification of a panel of changes in relation to different therapeutic groups and in the presence of metabolic syndrome, with some genes being up-regulated, while others are down-regulated in the different subgroups of patients. The results of this analysis have shown a panel of transcriptional changes associated with oxidative stress mechanisms that provide a basis for further studies on understanding of mechanisms that, in vivo, are the foundation the metabolic disorders in patients with HIV infection.*

Highly active antiretroviral therapy (HAART therapy) for HIV-1 infection has significantly increased the survival and quality of life of patients with this disease. However, some side effects are so relevant that they can influence the health and social life of patients (1, 2). Among these side-effects there is a syndrome characterized by fat redistribution (lipodystrophy), and an abnormal lipid and glucose profile. This phenomenon is generally defined as HIV-related metabolic syndrome, even in the absence of a universally accepted definition and well-defined clinical and laboratory parameters (3, 4). Lipodystrophy is commonly meant as a condition characterized by reduction of peripheral fat tissue (arms, legs, face *etc.*), compared with a central type of fat accumulation ("buffalo's back", abdomen), and it is frequently associated with lipid (cholesterol and triglycerides) and glucose (low glucose tolerance, hyperglycemia, diabetes) alterations (5, 6).

In several epidemiological studies the onset of metabolic syndrome is a phenomenon reported to be extremely frequent, with an incidence reaching, in some cases, even 83% of patients treated with HAART (7, 8).

Several studies have shown that two classes of transcription factors are involved in lipogenesis: CCAAT/enhancer-binding proteins (C/EBPs) (9) and peroxisome proliferator-activated receptors (PPAR) (10), which belong to the classes of transcription factors leucine zipper and nuclear hormone receptors respectively. (11, 12). Following the stimulus to differentiate, these two classes of transcription factors activate a cascade of genes that initially induce a mitotic stimulus followed by the activation of several genes specific for adipogenesis, which contribute mainly to the acquisition of the mature adipocytic phenotype. It has also been demonstrated that in patients treated with HAART, there was an alteration in the differentiation from preadipocytes to adipocytes mediated by adipogenetic transcription factors such as C/EBPα and PPARγ (13). This phenomenon could be the consequence of an enhanced antagonist effect of C/EBPβ and could have as a consequence an increase of the apoptosis of adipocytes that could interfere with preadipocyte differentiation.

Drawing from this background, we decided to identify genes involved in the molecular cascade responsible for the alteration of fat tissue and of lipid and glucose metabolism in patients with HIV-1 infection treated with antiretroviral therapy.

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Materials and Methods

Selection of the study population and creation of a database. Only patients that were not affected by endocrines metabolic disorders who had no family history significant for these diseases were enrolled in this study. Patients were then divided into two groups: the first group included patients without metabolic syndrome. The first group was further divided into two subgroups: naive patients (subgroup 1) and those treated with HAART therapy (subgroup 2). The second group included patients on HAART treatment affected by metabolic syndrome with clinical and laboratory diagnosis. This second group was then separated into two subpopulations including patients treated without protease inhibithors (PIs) (subgroup 3) and those treated with PIs (subgroup 4).

A total of 100 individuals were selected and divided into the four previously identified groups: 25 in subgroup 1, 26 in subgroup 2, 20 in subgroup 3 and 29 in subgroup 4. From all these patients, clinical information potentially useful for the study was collected.

Collection and treatment of peripheral blood for molecular analysis. For each patients approximately 20 ml of peripheral blood was collected in heparinized tubes. Total RNA of peripheral blood nucleated cells was extracted using commercial kits (Qiagen, Hilden, Germany). RNA thus obtained was then checked for integrity and for the presence of any contamination of genomic DNA both through spectrophotometric reading and through electrophoresis. All suitable samples were stored at –80˚C and used for analysis using cDNA arrays. In particular, we mixtures of total RNA were created for each group, taking exactly the same amount of total RNA (1 μg) from each patient.

Array hybridization and analysis of the data obtained. Hybridization using the Atlain cDNA Expression Array (Clontech Laboratories, Mountain View, Calif) in particular the Human Stress Arrays as previously described by Baldi *et al*. (14), including genes involved directly or indirectly in the phenomena of human stress, in the regulation of apoptosis, in the molecular mechanisms responsible for DNA repair, in the regulation of oxidative metabolism dependent on mitochondria and also in immunosuppression phenomena.

In order to identify the most significant changes, a cut-off of two was arbitrarily selected. In this way, we selected only genes whose expression varied among the different groups more than twofold. This arbitrary cut-off has already been successfully used to analyze the data obtained with the Clontech arrays.

Briefly the hybridization was performed according to the manufacturer's instructions. Arrays were scanned using a PhoshorImager and analysed by ImageQuant 5.0 software (Molecular dynamics, Sunnyvale, CA, USA). All those genes whose expression varied more than twofold, underwent further analysis to confirm the difference in gene expression with a different technology. The assay was performed in duplicate, utilizing two different preparations. The values were normalized using the expression levels of the housekeeping genes spotted on the arrays.

Results

Figure 1 is a representation of the phosphorImager output of the four Clontech Atlas TM Human Stress Array nylon filters after hybridization with ^{32}P-labeled cDNA derived from total RNA from the four subgroups of patients as described in the Material and Methods. Gene microarrays showed the expression of 234 key molecules of cellular stress response, including antioxidative enzymes, DNA repair proteins and chaperone molecules. Table I summarizes the differential expression of the genes identified in the different subgroups of patients.

In the analysis of genes differentially expressed in patients without metabolic syndrome comparison of HAART-naïve *versus* HAART-treated patients (subgroup 1 *vs*. subgroup 2), we identified four genes (Table I).

In the analysis of the genes differentially expressed in HAART-naive patients without metabolic syndrome *vs*. patients with metabolic syndrome on HAART treatment without PIs (subgroup 1 *vs*. subgroup 3), we identified five genes (Table I); in HAART-naive patients without metabolic syndrome *vs*. patients with metabolic syndrome on PIs therapy including HAART treatment (subgroup 1 *vs*. subgroup 4), we identified three genes (Table I); in patients without metabolic syndrome treated with HAART *vs*. patients with metabolic syndrome on HAART treatment without PIs (subgroup 2 *vs*. subgroup 3), we identified ten genes (Table I); in patients without metabolic syndrome but HAART-treated *vs*. patients with metabolic syndrome on HAART treatment including PIs (subgroup 2 *vs*. subgroup 4), we identified four genes (Table I); finally in the two subgroups of the population of patients affected by metabolic syndrome (subgroup 3 *vs*. subgroup 4), we identified five genes (Table I).

Discussion

The use of cDNA arrays allowed us to simultaneously monitor the expression levels of approximately 250 genes and to identify a panel of changes in relation to different therapeutic groups and in the presence of metabolic syndrome, with some genes that are up-regulated, while others are down-regulated in the different subgroups of patients. Some functional considerations regarding the genes identified are described below.

Flap endonuclease 1 (FEN1) is a nuclear enzyme involved in the phenomena of RNA transcription and DNA repair activities (15). It is an enzyme whose function is vital to cells. It is interesting to note that *in vitro* experimental evidence has shown that FEN1 is able to exert its enzymatic activity in the processes of replication of integrated HIV (16). Its increased expression in HAART-treated patients without metabolic syndrome (subgroup 2) compared to those with metabolic syndrome treated by HAART including PIs (subgroup 4) could be one of the mechanisms by which the patients' cells try to acquire a phenotype more resistant to the toxic effects of antiviral therapy, enhancing the mechanisms of DNA repair in mitochondria.

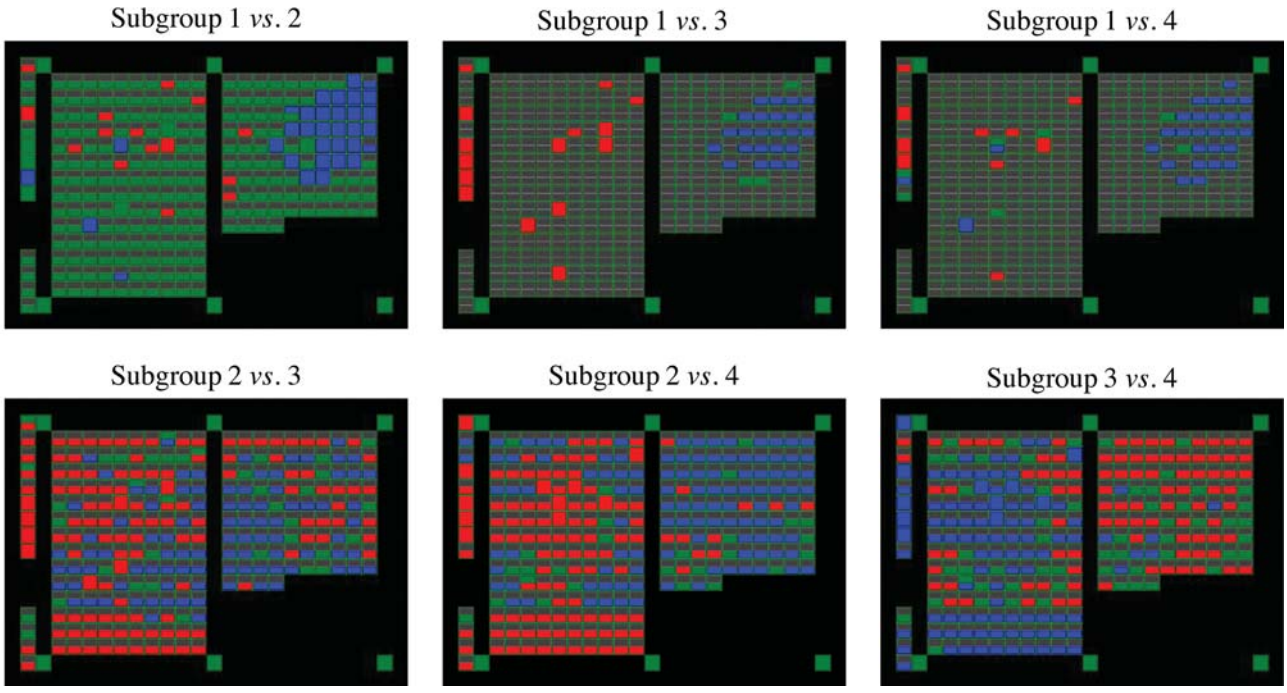

Figure 1. *Schematic comparison of the array data between the four subgroups of patients. Each box represents a gene on the array. Red, blue and green show the genes up regulated, down regulated and equally expressed respectively.*

Table I. *Differential expression of the genes identified in the different subgroups of patient.*

	Subgroup 1 *vs*. 2	Subgroup 1 *vs*. 3	Subgroup 1 *vs*. 4	Subgroup 2 *vs*. 3	Subgroup 2 *vs*. 4	Subgroup 3 *vs*. 4
Up-regulated	*FKBP51* *HSP27* *TCP1-eta*		*FAP48* *HSP27* *NEDD8*	*FKBP51* *HSP90* *SOD2* *EC-SOD* *NEDD8*	*FEN1* *XRCC1* *HSP27*	*FAP48* *HSP27* *RAG-1* *MDR3*
Down-regulated	*HSP90*	*FAP48* *FKBP13* *HSP27* *SOD2* *NEDD8*		*HSP27* *RAG-1* *MDR3* *MRP1* *SEH*	*FAP48*	*SOD2*

Subgroup 1, Patients without metabolic syndrome, HAART-naïve; subgroup 2, HAART-treated patients without metabolic syndrome; subgroup 3, patients with metabolic syndrome on HAART treatment without protease inhibitors; subgroup 4, patients with metabolic syndrome on HAART treatment including protease inhibitors.

The X-ray repair cross complementing protein 1(XRCC1) is a protein that participates in DNA repair phenomena and is essential to maintain the genetic stability of cells under various endogenous and exogenous stresses induced on DNA (17). *XRCC1* polymorphisms were also identified in association with human tumors (18, 19). Its increased expression in patients of subgroup 2 with respect to subgroup 4 could be explained by the same rationale as that for FEN1.

48-kDa (FKBP)-associated protein (FAP48) belongs to the family of FK506-binding proteins, a group of immunophilins that can bind immunosuppressive drugs FK506 and rapamicine (20). This gene was found to be overexpressed in subgroup 4 compared to subgroup 2 patients. The overexpression of FAP48 in lymphoid T Jurkat cells causes inhibition of cell proliferation. This antiproliferative stimulus applied to the population of HIV^+ lipodystrophic patients

could be interpreted as an attempt to inhibit differentiation of pre adipocytes, with an increase of the apoptotic phenomena (21).

In addition, the overexpression of FAP48 increases the production of interleukin-2 (IL-2). In light of previous reports, this suggests that an altered balance between lipogenesis and lipolysis may be a consequence of altered expression of IL-2 that would cause abnormalities of the balance between cortisol and dehydroepiandrosterone in patients with lipodystrophy (22). This effect on the production of IL-2 is comparable to that resulting from the action of leptin, a cytokine with a chimerical function, immunomodulatory on one hand, regulator of adipocytic differentiation and accumulation of fat on the other hand, and may have a key role in energy balance and an development of fat mass (23, 24). Our study also showed the direct involvement of FAP48 in adipogenesis, in fact pre adipocyte FAP48 stable clones are able to differentiate into mature adipocytes more rapidly than pre adipocyte empty vector stable clone (25 Esposito et al submitted).

FK506-binding protein 13 precursor (FKBP13) and 51-kDa FK506-binding protein (FKBP51) are proteins also belonging to the same family of FK506-binding proteins, as FAP48, with which they share and integrate functions (26). Interestingly, this group of genes is the most represented among those identified.

27-kDa Heat-shock protein (HSP27) is one of the members of the heat-shock protein family, that is induced by a variety of physiological and pathological insults. HSP27, in particular, is induced by cytotoxic stimuli and its function is to prevent apoptosis. It exerts its antiapoptotic action by acting as a chaperone molecule binding the different components of the apoptotic pathway, especially those involved in the activation of caspases (27). Its overexpression in patients without metabolic syndrome in subgroup 2 in comparison to those with metabolic syndrome in subgroup 4 could represent another defence mechanism by cells to counteract the onset of lipodystrophy phenomena that could liberate pre-adipocytes by proliferation constraints imposed by the control mechanisms of apoptosis by promoting theyr differentiation into adipocytes.

HSP90 is a protein with chaperone function, which is responsible for maintaining the correct folding, stability and function of target proteins. In particular, HSP90 exerts its chaperone action on proteins involved in the molecular mechanisms that regulate growth, survival and apoptotic cell death (28-30).

Mn^{2+} Superoxide dismutase precursor (MnSOD2). The enzymes belonging to the SOD family of are the first line of defence made by cells against the toxic effects of superoxide anions. MnSOD is one of the family members which is localized in mitochondria. Experimental evidence has shown that increased cellular levels of MnSOD exert a cytoprotective effect against several stressors: oxidative, inflammatory, and the stress caused by TNF-α, IL-1β, ionizing radiation and neurotoxins (31). This is also confirmed by studies of transgenic mice (32). The overexpression of this gene in PI-naive patients (subgroup 3) in comparison to HAART-naïve patients (subgroup 1) could explain in part the absence of toxic effects on adipose tissue caused by the protective effect of MnSOD. From this perspective, the action of SOD2 on TNF-α is relevant. As matter of fact, it has been demonstrated that during HAART therapy, there is an increase of T-cells producing TNF-α and IL-2, with a direct correlation between increased levels of TNF-α and development of lipodystrophy, probably through induction of production of leptin and subsequent depletion of fat from fat cells for down-regulation of lipogenic enzymes (lipoatrophy) and apoptosis of adipocytes (33).

Extracellular superoxide dismutase precursor (EC-SOD) shares the antioxidant activities of MnSOD but has mainly an extracellular localization (34).

Ubiquitin-like protein (NEDD8). The ubiquitin-like proteins (UBLs) are a group of small proteins that have a essential role in the regulation of protein degradation pathways, cell cycle regulation and differentiation, nuclear transport, and autophagy (35-37). The overexpression of this gene in subgroup 3 in comparison to subgroup 1 could be part of the arsenal of the molecular mechanisms induced in cells to defend themselves against the toxic effects of antiviral therapies. We also demonstrated an involvement of the NEDD8 in the induced toxicity of HAART therapy. NEDD8 overexpression is able to perturb key markers of adipogenic expression such as C/EBPα and PPARγ, and moreover, stimulated the degradation of p27 and β-catenin (38).

T-Complex protein 1 beta subunit (TCP1-age) belongs to the family of chaperonin containing T-complex polypeptide (TCP-1) as one of the subunits and folds various proteins, including actin and tubulin (39).

V(D)J recombination activating protein 1 (RAG1). Together with RAG2, RAG1 forms the nuclease that cleaves DNA at the end of the signal sequence and thus contributes to the recombination mechanism that generates diversity among T-cell receptors. Defect in RAG1 pathway are a cause of severe combined cellular and humural immune disorder (40-42).

Multidrug-resistance protein 3 and 1 (MDR1 and MDR3) belong to a family of transport proteins that makes the cells in which they are produced resistant to cytotoxic substances and may be a defence against the cell damage induced by HAART therapy (43). Their greatest expression in subgroup 3 compared to subgroup 4 could be related to an attempt to counteract the cellular toxicity at the base of lipoatrophy in patients treated with HAART, but without IP.

Soluble epoxide hydrolase (SEH) is a ubiquitous enzyme that forms dioic groups adding water to epoxides. It is

involved in lipid metabolism and is therefore to be considered a potential target for therapeutic interventions (44, 45).

The results of this analysis, although preliminary, have shown a panel of transcriptional changes associated with oxidative stress mechanisms that provide a basis for further studies on understanding of mechanisms that *in vivo* are foundation of metabolic disorders in patients with HIV infection. In fact, the identification of genes whose expression counteracts the phenomenon of fat redistribution in lipodystrophy and the metabolic alterations underlying dyslipidemia and diabetes may form the basis for intervention cause these phenomena.

These data may provide the background for the creation of gene therapy protocols aimed at treating the phenomena of fat redistribution and correlated metabolic abnormalities.

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