

Changes in subcutaneous adipose tissue microRNA expression in HIV-infected patients

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Objectives: We evaluated the possibility that a pattern of abnormal microRNA (miRNA) expression could be fuelling the mechanisms causing HIV-associated lipodystrophy (HAL).

Methods: In this case-control study, samples of subcutaneous adipose tissue from eight consecutive HIV-infected patients on combination antiretroviral therapy with HAL (cases) were compared with those of eight HIV-negative subjects (controls). Human miRNA microarrays were used to probe the transcriptomes of the samples. Analysis of differentially expressed miRNAs was performed using DataAssist v2.0 software, applying a paired Student's *t*-test.

Results: Data showed that 21 miRNAs out of 754 were overexpressed in the patient group. Ten of these (i.e. miR-186, miR-199a-3p, miR-214, miR-374a, miR-487b, miR-532-5p, miR-628-5p, miR-874, miR-125-b-1* and miR-374b*) were up-regulated to a significant degree (fold change >2.5; *P* < 0.01). Eleven other miRNAs (i.e. miR-let-7d, miR-24, miR-30c, miR-125a-3p, miR-149, miR-191, miR-196-b, miR-218, miR-342-3p, miR-452 and miR-454*) were 2- to 2.5-fold more expressed in HIV+ samples than in controls. Levels of mRNA for lipin 1, the target of miR-218, were significantly lower in subcutaneous adipose tissue from HIV patients.

Conclusions: In adipocytes of HIV-infected patients, the up-regulation of specific miRNAs could lead to an increased 'activation' that might contribute to the pathogenesis of HAL by increasing cell turnover and/or promotion of apoptosis.

Keywords: HIV-associated lipodystrophy, adipose tissue abnormalities, miRNAs

Introduction

Lipodystrophy syndrome (LS) is an adipose tissue redistribution reported in subjects with HIV infection who have been treated with combination antiretroviral therapy (cART).¹ It is characterized by morphological alterations including peripheral lipoatrophy (loss of subcutaneous fat in the cheeks, extremities and buttocks), fat accumulation (intra-abdominal region, trunk, neck and subcutaneous lipomas) and mixed forms and metabolic alterations such as dyslipidaemia and altered glucose metabolism.²⁻⁴ The pathogenesis of LS is multifactorial.⁵ Various drug classes have been associated with LS; in particular, lipoatrophy has been associated with mitochondrial toxicity induced by treatment with thymidine analogues, whereas visceral fat accumulation has been partially correlated with protease inhibitor use.² The clinical relevance of this condition is characterized by metabolic derangements and increased risk of severe cardiovascular diseases; moreover, the

patient has to deal with the highly negative perception of this disfiguring and stigmatizing condition, which can lead to erosion of body image and involuntary disclosure of HIV status.⁶ Few studies have focused on the role of abnormalities in adipose tissue differentiation in LS. Adipocytes derive from mesenchymal stromal progenitor/stem cells (MSCs), a rare population of non-haematopoietic stromal cells.⁷ MSCs are capable of differentiation into mesenchymal tissues such as bone, cartilage, adipose tissue and muscle.⁸ It has been proposed that HIV can interact directly with MSCs, resulting in alteration of their differentiation potential towards the adipocyte lineage.⁹

MicroRNAs (miRNAs) are short (~22 nt) non-coding RNA molecules that regulate gene expression at the post-transcriptional level through sequence alignment mechanisms.¹⁰ They have been implicated in muscle, adipose tissue and osteogenic differentiation and in commitment of MSCs towards the adipocyte lineage.¹¹⁻¹³

In this study, we investigated whether the subcutaneous adipose tissue of HIV+ patients treated with cART is characterized by an miRNA expression profile different from that of HIV- subjects. Our working hypothesis was that specific miRNAs could play a key role in the pathogenesis of LS by altering the differentiation of adipose tissue progenitor cells.

Materials and methods

Samples and miRNA array analysis

Abdominal subcutaneous adipose tissue fragments were obtained from HIV+ patients (male, $n=8$) on cART and HIV- subjects (male, $n=8$) and stored at -20°C in RNeasy (Sigma-Aldrich, St Louis, MO, USA). The study protocol was approved by the Ethics Committee of the San Gerardo Hospital. All subjects gave written informed consent before being enrolled in the study.

For each subject, height, weight, waist circumference and systolic and diastolic blood pressure data were collected. The inclusion criteria were as follows: age 25–65 years; documented HIV infection (for HIV+ group); facial lipoatrophy with or without peripheral lipoatrophy (only for HIV+ group); body mass index (BMI) 18–30; fasting glucose values <126 mg/dL; and absence of hypolipidaemic therapy and steroid use. HIV+ patients were in therapy with the following cART: four patients were on a non-nucleoside reverse transcriptase inhibitor (NNRTI) regimen (efavirenz or nevirapine), three patients were on a protease inhibitor regimen (lopinavir/ritonavir or atazanavir/ritonavir) and one patient was on an NNRTI+ integrase inhibitor regimen (etravirine+ raltegravir). NNRTI and protease inhibitor regimens also included a combination of nucleoside reverse transcriptase inhibitors (tenofovir+ emtricitabine or abacavir+ lamivudine).

Total RNAs, including miRNAs, were isolated from subcutaneous adipose tissue using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), reverse transcribed, pre-amplified and subsequently amplified in TaqMan Array MicroRNA Card (A and B) v 3.0 using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900 HT sequence detector, following the manufacturers' instructions.

The results were analysed using RQ Manager 1.2 software (Applied Biosystems), applying the comparative Ct method ($\Delta\Delta\text{Ct}$) and using a 0.2 threshold. Ct (threshold cycle) is generally assumed as a relative measure of the concentration of target in the PCR reaction. miRNA expression levels were normalized to an internal control (MammU6) and plotted as fold changes on a \log_2 ratio scale; undetermined Ct or $\text{Ct} >35$ were not considered.

Lipin 1 mRNA expression analysis

Total RNA from subcutaneous adipose tissue was digested with DNase I (Fermentas, Glen Burnie, MD, USA), reverse transcribed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and amplified in triplicate using TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes were used for lipin 1, and 18S rRNA, taken as endogenous control (Hs00299515_m1 and Hs03928990_g1; Applied Biosystems). Lipin 1 gene expression was calculated using the $\Delta\Delta\text{Ct}$ method and expressed as the fold change (RQ).

Bioinformatic and statistical analysis

Analysis of differentially expressed miRNAs was performed using DataAssist v2.0 software (Applied Biosystems), applying a paired Student's *t*-test. Functional analysis of miRNAs and predicted target genes was determined using myMIRsite (www.itb.cnr.it/micro/index.html). The predicted miRNA target genes were then analysed using GeneCodis2.0 software.^{14,15}

Analysis of lipin 1 expression was performed using RQ Manager 1.2 software (Applied Biosystems), applying a paired Student's *t*-test.

Unless otherwise specified, data are expressed as mean \pm SD. Values of $P < 0.05$ were considered to be statistically significant.

Results

Clinical and metabolic data

All clinical, metabolic and anthropometric parameters were almost superimposable between HIV+ patients and HIV- subjects (Table 1). In particular, the subjects did not differ in fasting glucose levels (hyperglycaemia was among the exclusion criteria), BMI and waist circumference. In the HIV+ group, the patients were treated with the following cART: four patients (50%) were on an NNRTI regimen, three patients (37%) were on a protease inhibitor regimen and one patient (13%) was on an NNRTI+ integrase inhibitor regimen (NNRTI+ raltegravir). The CD4 cell count was >400 cells/ mm^3 for all patients and HIV-RNA was undetectable (<50 copies/mL).

Differentially expressed miRNA in the subcutaneous adipose tissue of HIV+ patients on cART therapy

The complete profiling of 754 human mature miRNAs in subcutaneous adipose tissue samples was obtained. Gene expression analysis performed with microfluidic cards A and B revealed that 309 miRNAs (41%) were not detectable both in HIV+ patients and HIV- subjects. It is likely that these miRNA species are not expressed in adipose tissue or were not detectable in our samples due to their relatively low expression (data not shown). The box plot analysis displays that the Ct value distributions are homogeneous in both card A (Figure 1a) and card B (Figure 1b) for HIV+ patients and HIV- subjects, indicating no significant intra- and inter-variation among biological replicates.

Among the detectable 445 miRNAs (59%), we observed a different expression for 21 of them (4.7% of the measurable miRNAs), with all being significantly up-regulated (fold change ≥ 2 ; $P < 0.01$) in HIV+ patients compared with controls. Volcano plots showed that most of the miRNA whose levels were higher in the HIV+ group are found on card A (Figure 1c), whereas few of them from card B where highly expressed (Figure 1d).

Ten of these (i.e. miR-186, miR-199a-3p, miR-214, miR-374a, miR-487b, miR-532-5p, miR-628-5p, miR-874, miR-125-b-1* and miR-374b*) were up-regulated to a statistically significant degree

Table 1. Clinical, metabolic and anthropometric parameters of the HIV+ patients and HIV- subjects

Variables	HIV- ($n=8$), mean \pm SD	HIV+ ($n=8$), mean \pm SD
Age (years)	44.75 \pm 11.658	52.875 \pm 9.062
Body weight (kg)	71.25 \pm 8.464	74.75 \pm 11.78
Height (cm)	169.75 \pm 10.511	176.5 \pm 8.701
BMI (kg/m^2)	24.7125 \pm 0.601	23.912 \pm 2.761
Waist circumference (cm)	89.125 \pm 2.695	91.5 \pm 10.350
Systolic blood pressure (mmHg)	107.5 \pm 13.887	116.875 \pm 15.569
Diastolic blood pressure (mmHg)	81.25 \pm 6.943	81.25 \pm 8.345
Blood glucose level (mg/dL)	89 \pm 10.198	84.875 \pm 6.311

(fold change >2.5 ; $P < 0.01$). Eleven other miRNAs (i.e. miR-let-7d, miR-24, miR-30c, miR-125a-3p, miR-149, miR-191, miR-196-b, miR-218, miR-342-3p, miR-452 and miR-454*) were 2- to 2.5-fold more expressed in HIV+ samples than in controls (Figure 1e).

Since diabetes and obesity were among the exclusion criteria, no significant correlations were observed between differentially expressed miRNAs and BMI or fasting glucose levels in the two groups (data not shown).

The heat map of the miRNA expression categorized two well-defined clusters that correspond to samples from HIV+ patients and HIV- subjects (Figure 2).

To identify downstream genes dysregulated as a consequence of miRNA alterations, the 21 differentially expressed miRNAs were analysed using the myMIRsite program, a tool that provides meta-predictions based on integration, filtering and reranking of outputs by using several other available software, such as TargetScan,

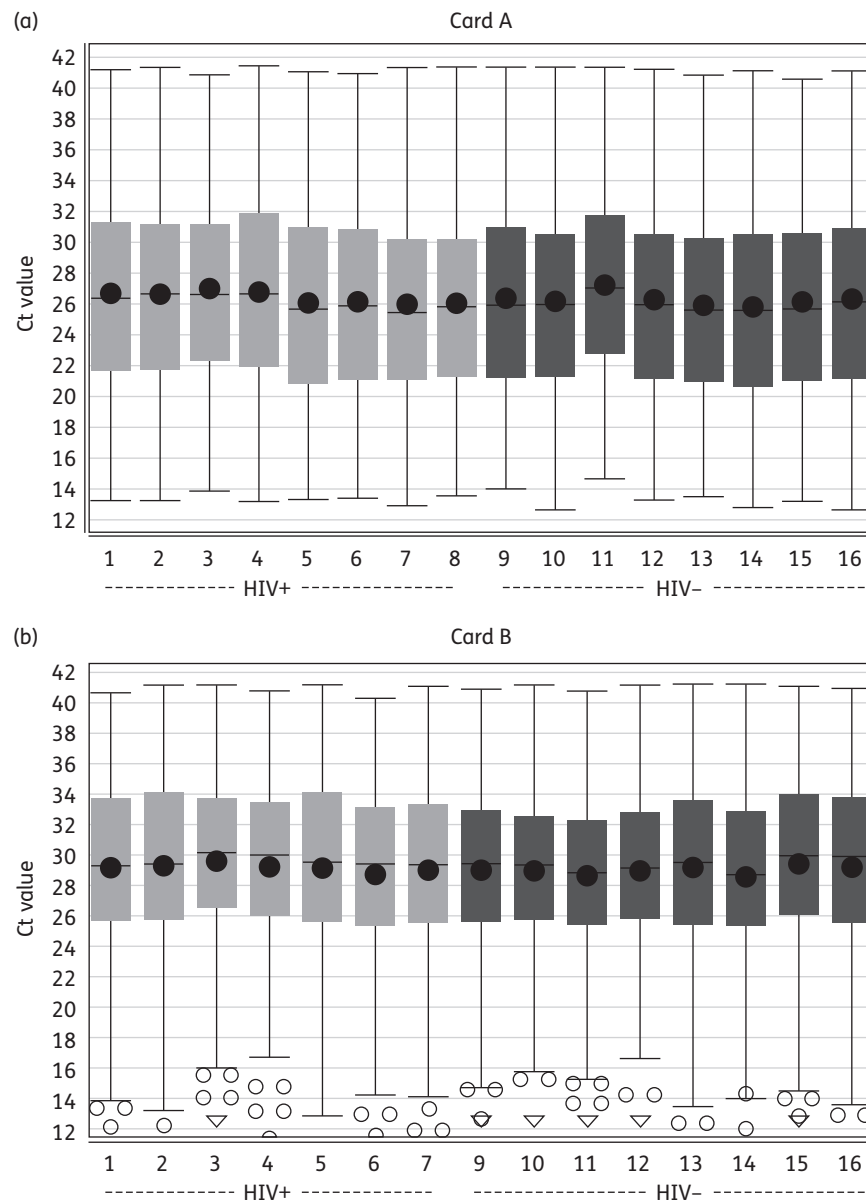


Figure 1. miRNA expression analysis. (a and b) Ct values were determined using RQ Manager 1.2 (Applied Biosystems) and then analysed using DataAssist software (Applied Biosystems). Box plots (DataAssist) show the overall range of Ct distribution, displayed by samples and sorted by group. (a) Data from card A (samples 1–8 are from HIV+ patients and samples 9–16 are from HIV- subjects). (b) Data from card B (samples 1–7 are from HIV+ patients and samples 9–16 are from HIV- subjects). Every box contains the middle 50% of the data (Ct values). The black horizontal line indicates the median Ct value and the black dot represents the mean Ct. The ends of the vertical lines indicate the minimum and the maximum Ct values. The points outside the ends of vertical lines are outliers. (c and d) Volcano plots (DataAssist) display P values versus fold change or groups based on input fold change boundary and P values. Fold change boundary of 2 (2-fold change) and a P value of 0.01 were used. (c) Data from card A. (d) Data from card B. (e) miRNA expression levels are normalized to an internal control (MammU6) and plotted as fold changes in terms of \log_2 (RQ) scale [\log_2 (RQ) = 1 means 2-fold up-regulation]. Data are expressed as mean \pm SD ($n=8$). All the represented miRNAs (21 of the 754 analysed miRNAs) showed P values <0.01 and fold changes over 2.

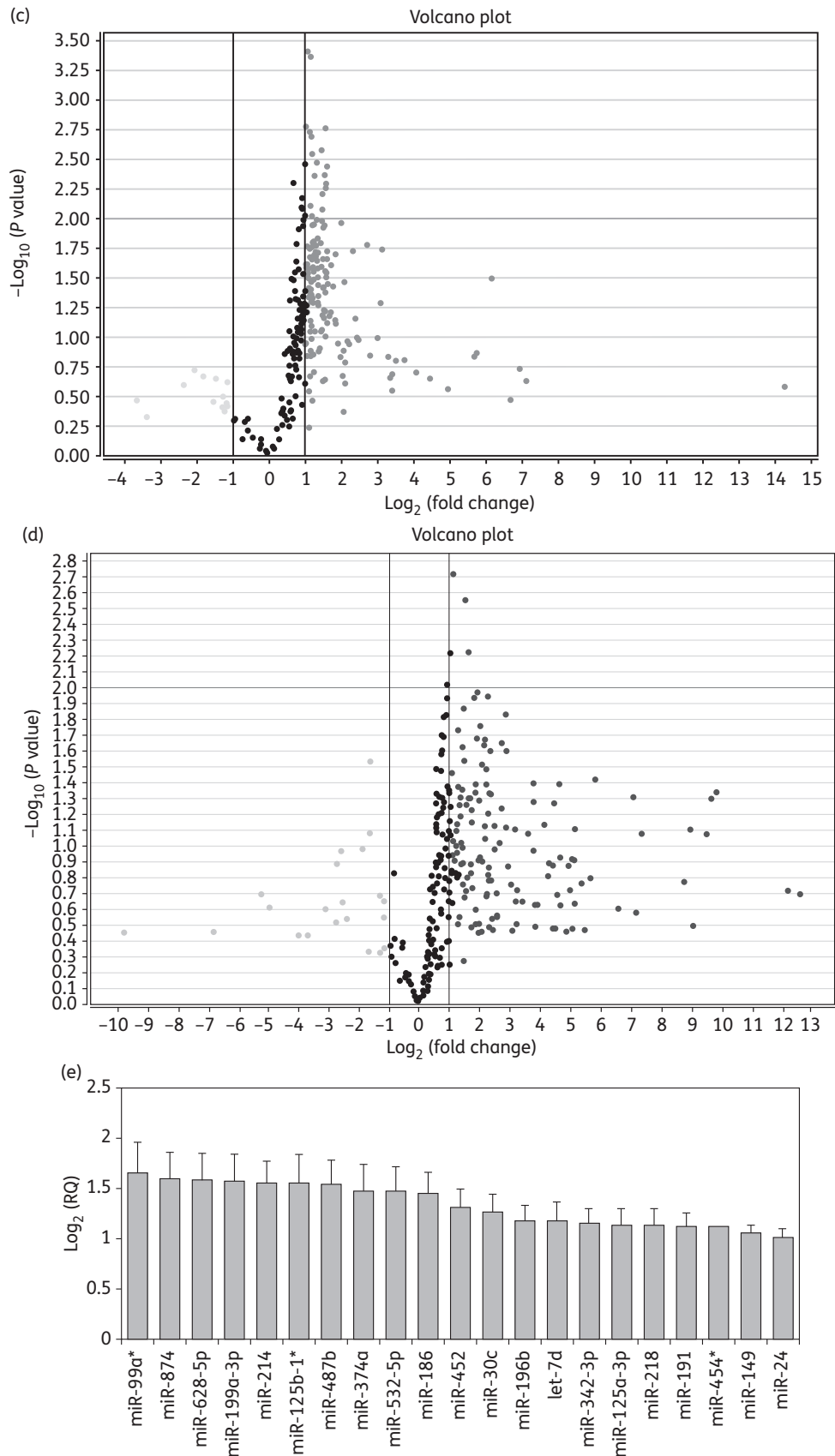


Figure 1. Continued

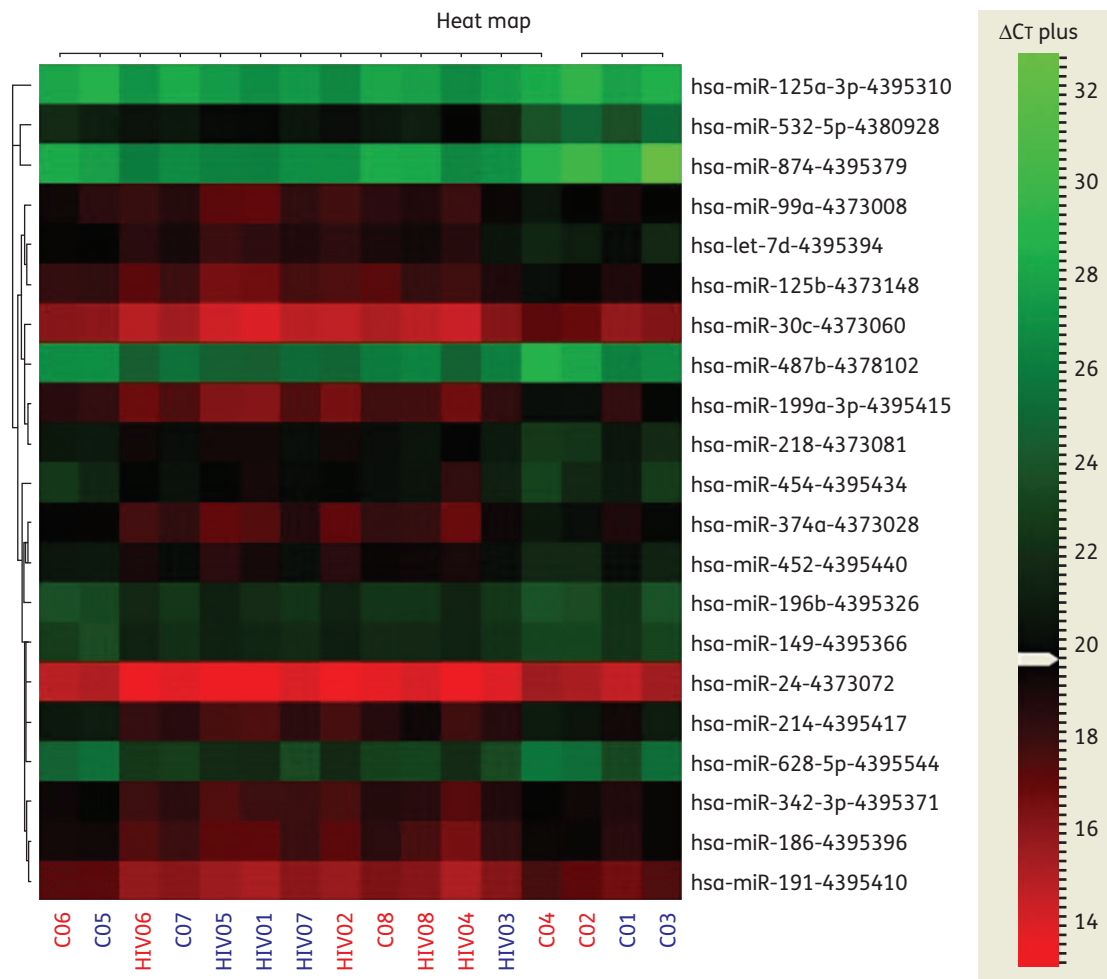


Figure 2. Unsupervised hierarchical clustering and heat maps of the miRNA microarray data. The miRNA microarray cluster of HIV+ patients and HIV– subjects clustered into two distinct sets based on signal strength. Sample names are indicated at the bottom. The first eight samples were obtained from HIV+ patients and the remaining eight samples were obtained from HIV– subjects. The colour key for the frequency scale is on the right. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

RNAHybrid, miRanda, Pictar, DIANA-microT and PITA. The outputs of the predicted target genes were subsequently uploaded to the GeneCodis2.0 software,¹⁶ in order to better understand the functional role of the predicted genes, the pathways and the processes in which they are involved (Table 2). GeneCodis analysis showed that several gene targets of altered miRNAs are involved in transport, gene expression and regulation of metabolic processes (Table 2).

In particular, the signalling pathways of MAPK, Wnt, JAK-STAT and calcium have been found to be a target of abnormal miRNA expression. In addition, other processes, such as the interaction between cytokines and their receptors, glycerophospholipids, as well as metabolism and pancreatic hormones secretion are also possible targets. Some of these alterations have been documented in the literature in physiological and pathological conditions and in different tissues (Table 3).

Lipin 1 mRNA expression

Interestingly, miR-218 was 2.5-fold more expressed in samples of HIV+ patients compared with HIV– subjects. Since genes are

expected to be down-regulated when targeted by a specific miRNA, we quantified by real-time PCR the mRNA levels of one miR-218 target, lipin 1, in subcutaneous adipose tissue. Interestingly, lipin 1 mRNA levels were 44% lower in the HIV+ group (normalized Ct: 0.620 ± 0.17 and 1.104 ± 0.19 for HIV+ patients and HIV– subjects, respectively; $P < 0.05$) compared with HIV– subjects (Figure 3), confirming our hypothesis.

Discussion

In this study, we measured the levels of 754 miRNAs in subcutaneous adipose tissue samples obtained from lipodystrophic HIV+ patients and HIV– subjects. Our results confirm the hypothesis that specific miRNAs could have a role in the development of metabolic and morphological abnormalities observed in the HIV-infected population with lipodystrophy.

Lipoatrophy and lipohypertrophy are frequently reported in LS and they are often combined. Characterization of miRNAs could give new important information about the steps of lipogenesis that could be altered by HIV and/or cART. Our hypothesis was

Table 2. Biological processes and pathways regulated by putative target genes of miRNAs

miRNA	Biological process	KEGG pathways
miR-99a	apoptotic process negative regulation of transcription from RNA polymerase II promoter cellular protein metabolic process	Wnt signalling pathway cadherin signalling pathway Wnt signalling pathway, cadherin signalling pathway
miR-874	regulation of transcription, DNA-ST multicellular organism development	endocytosis glycerophospholipid metabolism inositol phosphate metabolism neuroactive ligand-receptor interaction
miR-628-5p	signal transduction synaptic transmission ion transport, synaptic transmission	protein processing in endoplasmic reticulum amyotrophic lateral sclerosis
miR-199a-3p	apoptotic process blood coagulation negative regulation of cell proliferation	MAPK signalling pathway RNA degradation adherens junction
miR-214	signal transduction multicellular organism development positive regulation of transcription from RNA polymerase II promoter	pathways in cancer MAPK signalling pathway endocytosis
miR-125-b-1*	multicellular organism development ion transport transmembrane transport	MAPK signalling pathway Wnt signalling pathway cell adhesion molecules
miR-487-b	positive regulation of transcription from RNA polymerase II promoter blood coagulation positive regulation of transcription, DNA-ST	pathways in cancer insulin signalling pathway adipocytokine signalling pathway
miR-374a	multicellular organism development ion transport transmembrane transport	neuroactive ligand-receptor interaction RIG-I-like receptor signalling pathway
miR-532-5p	regulation of transcription, DNA-ST apoptotic process	pathways in cancer chemokine signalling pathway neurotrophin signalling pathway
miR-186	regulation of transcription, DNA-ST anterior/posterior pattern specification	chemokine signalling pathway Wnt signalling pathway melanogenesis
miR-452	positive regulation of cell proliferation negative regulation of transcription, DNA-dependent positive regulation of transcription from RNA polymerase II promoter	MAPK signalling pathway adherens junction pathways in cancer, prostate cancer
miR-30c	regulation of transcription, DNA-dependent positive regulation of transcription from RNA polymerase II promoter cell cycle	focal adhesion ubiquitin-mediated proteolysis bacterial invasion of epithelial cells
miR-196-b	negative regulation of transcription from RNA polymerase II promoter anterior/posterior pattern specification embryonic limb morphogenesis	neuroactive ligand-receptor interaction focal adhesion cell adhesion molecules
miR-let-7d	regulation of transcription, DNA-dependent cell adhesion apoptotic process	pathways in cancer MAPK signalling pathway Jak-STAT signalling pathway
miR-342-3p	regulation of transcription, DNA-ST multicellular organism development	MAPK signalling pathway axon guidance purine metabolism
miR-125-a-3p	regulation of transcription, DNA-ST transmembrane transport	pathways in cancer MAPK signalling pathway endocytosis
miR-218	apoptotic process transmembrane transport cellular lipid metabolic process	
miR-191	regulation of transcription, DNA-dependent apoptotic process negative regulation of transcription from RNA polymerase II promoter	pathogenic <i>Escherichia coli</i> infection

Continued

Table 2. Continued

miRNA	Biological process	KEGG pathways
miR-454	cell cycle RNA splicing	pathways in cancer endocytosis
miR-149	embryo development regulation of transcription, DNA-ST multicellular organism development	focal adhesion axon guidance calcium signalling pathway dilated cardiomyopathy
miR-24	signal transduction multicellular organism development positive regulation of transcription from RNA polymerase II promoter	pathways in cancer endocytosis calcium signalling pathway

KEGG, *Kyoto Encyclopedia of Genes and Genomes*; DNA-ST, DNA-dependent signal transduction. Computational analysis of target genes of altered miRNA was performed using myMIRsite (www.itb.cnr.it/micro/index.html). Putative target genes were then analysed using the bioinformatic tool GeneCodis2.0 (<http://genecodis.dacya.ucm.es>).

Table 3. Documented or postulated effects on adipose tissue maturation of miR-24, miR-30c, miR-196, miR-99a, miR-125a, miR-125b, miR-342, miR-191 and miR-214

Cell type	miRNA	Documented or postulated effect	Reference(s)
Murine MSCs exposed to bone morphogenic protein 2	miR-24	commitment towards adipocyte cell line	13
Murine adipose tissue		up-regulation in white adipose tissue differentiation	
Human subcutaneous pre-adipocytes	miR-30c, miR-196, miR-99a, miR-125a, miR-125b	up-regulated in mature adipocytes compared with pre-adipocytes and/or differentiation process	22–25
Murine adipose tissue	miR-342	up-regulated in obesity	36
Human subcutaneous pre-adipocytes	miR-125b	down-regulated in mature adipocytes versus pre-adipocytes	8,12
Murine adipose tissue of hyperglycaemic rats	miR-125a, miR-191	up-regulated in hyperglycaemic rats	8,23
Human PBMCs	miR-214	up-regulated in hyperglycaemia and chronic renal failure	32

that miRNA abnormalities could be involved in the development of metabolic alterations observed in the HIV population on cART. It is known that miRNAs regulate gene expression and that their up- or down-regulation plays a key role in physiological processes such as cell proliferation, apoptosis and tissue differentiation.¹⁷ Reportedly, miRNAs operate in a complex functional network in which each miRNA can control several genes and, on the other hand, a single gene could be regulated by multiple miRNAs.¹⁸ Interestingly, those miRNAs whose expression was altered in HIV+ samples in our study were all up-regulated compared with controls, confirming the literature data about overexpression of miRNAs in HIV-infected patients on therapy with viral suppression,¹⁹ in contrast to miRNA down-regulation in patients with uncontrolled HIV viral load.^{20,21} To gain further insight into the underlying molecular mechanisms behind this dysregulation, we attempted to infer the functional consequences of the up-regulation of those miRNAs that we have found to be increased in HIV+ samples.

Lipodystrophy could be a consequence of a reduction in the differentiation of adipose cells. And yet, in our study, we discovered an up-regulation of miR-24, which prompts commitment towards adipose differentiation in murine MSCs exposed to bone

morphogenic protein 2.¹³ These data and the reported localization of MSCs in adipose tissue indicate that MSCs of adipose tissues of HIV-infected subjects would preferentially differentiate into adipocytes rather than osteoblasts, myocytes and chondrocytes. These results also suggest that the factors responsible for stimulating differentiation towards adipose cells are conserved in the subcutaneous adipose tissue of HIV+ patients on cART. Moreover, we found an up-regulation of some miRNAs that are typically overexpressed during adipocyte maturation phases, such as miR-30c, miR-125a, miR-125b and miR-99a, suggesting that the normal conversion from pre-adipocytes into adipocytes is not affected.^{22–25} These results point towards an overproduction or an increased activation of maturation processes of adipocytes.

However, the decreased amount of subcutaneous adipose tissue in lipodystrophic HIV+ patients clearly indicates that other regulatory mechanisms are operating as well, but in the opposite way. Reportedly, lipodystrophic subjects have a reduced number of adipose cells and an increased number of apoptotic cells, suggesting an increased turnover of adipose cells.^{26–28}

It has been suggested that lipodystrophy might be the result of an alteration in the steady-state of adipose tissue related to an immune dysregulation and that it is promoted by cART.²⁹ In

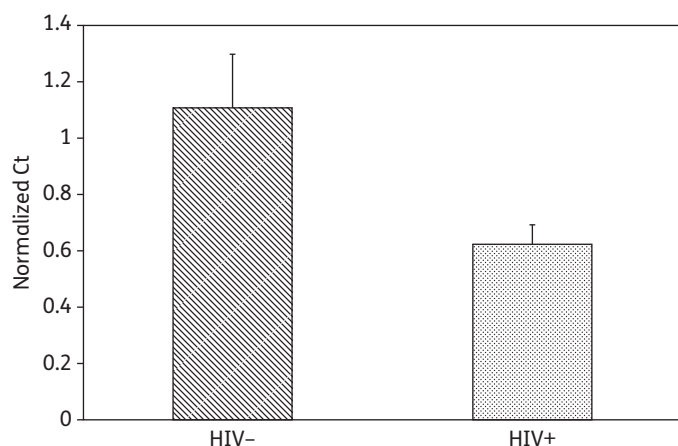


Figure 3. Analysis of lipin 1 mRNA expression. The lipin 1 gene was identified as a putative target gene of miR-218. Levels of lipin 1 mRNA were determined by real-time PCR in subcutaneous adipose tissue of HIV- subjects (controls) and HIV+ patients. Ct lipin 1 values were normalized with respect to Ct 18S values, obtained by RQ Manager 1.2 (Applied Biosystems) and expressed as mean \pm SEM ($n=8$). Statistical analysis between HIV- and HIV+ groups was performed applying a paired Student's *t*-test. * $P<0.05$ for HIV+ versus HIV-.

particular, an increased release of TNF- α might be responsible for the inhibition of adipose cell differentiation and triglyceride esterification, reduced deposition of fat within adipose cells and promotion of lipolysis.

The dysregulation of miRNA expression might be directly associated with the origin of metabolic and morphological alterations or be the result of an inflammatory pathway.

Subcutaneous adipose tissue of HIV+ patients was previously defined as a site of inflammation, with production of several cytokines.^{30,31} This could be one of the reasons for the increased turnover of adipocytes. This hypothesis was confirmed by the up-regulation of miR-214, which reportedly is associated with monocyte inflammatory responses in other clinical conditions in which fat redistribution has also been reported, such as hyperglycaemia and chronic renal disease.³² Abnormal adipose cell differentiation and morphology have been described in HIV+ patients and correlated with altered expression of sterol regulator element-binding protein 1, which in turn could induce increased insulin resistance.³³ miR-125a and miR-191 are frequently found to be up-regulated in hyperglycaemic rats,³⁴ a result consistent with the association between miRNA dysregulation and metabolic disturbances which we observed in the adipose tissue of our patients.

The complex perturbation of metabolism and maturation is also confirmed by significantly reduced levels of lipin 1 in the adipose tissue of HIV-infected patients with lipodystrophy.³⁵ Lipin 1 is expressed both in pre-adipocytes and adipocytes and promotes the expression of adipogenic transcription factors including CAAT-enhancer-binding protein α and peroxisome proliferator-activated receptor γ , as well as that of genes involved in lipogenesis and lipid storage in mature adipocytes. Reduction of lipin 1 synthesis could lead to changes in the maturation processes of adipocytes and pre-adipocytes. We demonstrated an inverse correlation between lipin 1 mRNA levels and those of miR-218,

supporting a role for miRNA dysregulation in determining severe metabolic and morphological consequences.

Our study has some limitations. Lipodystrophy diagnosis was based only on clinical signs and so it could be inaccurate. Moreover, the interpretation of miRNA level abnormalities is difficult due to multiple functions attributed to each single miRNA.

The results of the study provide a detailed analysis of miRNA expression in subcutaneous adipose tissue, adding new insights into the pathogenesis of LS in HIV+ patients.

Lipin 1 was confirmed to be one of the most important factors linked to LS pathogenesis, its down-regulation being mediated by the up-regulation of miR-218.

Further experiments involving *in vitro* miRNA silencing are needed to confirm the role of miRNA dysregulation in adipocyte differentiation and maturation.

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Transparency declarations

None to declare.

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