We identified a microRNA (miRNA) profile characterizing HIV lipodystrophy and explored the downstream mechanistic implications with respect to adipocyte biology and the associated clinical phenotype. miRNA profiles were extracted from small extracellular vesicles (sEV) of HIV-infected individuals with and without lipodystrophic changes and individuals without HIV, among whom we previously showed significant reductions in adipose Dicer expression related to HIV. miR-20a-3p was increased and miR-324-5p and miR-186 reduced in sEV from HIV lipodystrophic individuals. Changes in these miRNAs correlated with adipose Dicer expression and clinical markers of lipodystrophy, including fat redistribution, insulin resistance, and hypertriglyceridemia. Human preadipocytes transfected with mimic miR-20a-3p, anti-miR-324-5p or anti-miR-186 induced consistent changes in Ltbp2, Wisp2, and Neb1 expression. Knockdown of Ltbp2 (Latent-transforming growth factor beta-binding protein 2) downregulated markers of adipocyte differentiation (Fabp4, Pparg, C/ebpα, Fasn, adiponectin, Glut4, CD36), and Lamin C, and increased expression of genes involved in inflammation (IL1β, IL6, and Ccl20). Our studies suggest a unique sEV miRNA signature related to dysregulation of Dicer in adipose in HIV. Enhanced miR-20a-3p or depletion of miR-186 and miR-324-5p may downregulate Ltbp2 in HIV leading to dysregulation in adipose differentiation and inflammation, which could contribute to acquired HIV lipodystrophy and associated metabolic and inflammatory perturbations.
Altered Pattern of Circulating miRNAs in HIV Lipodystrophy Perturb Key Adipose Differentiation and Inflammation Pathways

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Abstract

We identified a microRNA (miRNA) profile characterizing HIV lipodystrophy and explored the downstream mechanistic implications with respect to adipocyte biology and the associated clinical phenotype. miRNA profiles were extracted from small extracellular vesicles (sEV) of HIV-infected individuals with and without lipodystrophic changes and individuals without HIV, among whom we previously showed significant reductions in adipose Dicer expression related to HIV. miR-20a-3p was increased and miR-324-5p and miR-186 reduced in sEV from HIV lipodystrophic individuals. Changes in these miRNAs correlated with adipose Dicer expression and clinical markers of lipodystrophy, including fat redistribution, insulin resistance, and hypertriglyceridemia. Human preadipocytes transfected with mimic miR-20a-3p, anti-miR-324-5p or anti-miR-186 induced consistent changes in Ltbp2, Wisp2, and Nebl expression. Knockdown of Ltbp2 (Latent-transforming growth factor beta-binding protein 2) downregulated markers of adipocyte differentiation (Fabp4, Pparγ, C/ebpa, Fasn, adiponectin, Glut4, CD36), and Lamin C, and increased expression of genes involved in inflammation (IL1β, IL6, and Ccl20). Our studies suggest a unique sEV miRNA signature related to dysregulation of Dicer in adipose in HIV. Enhanced miR-20a-3p or depletion of miR-186 and miR-324-5p may downregulate Ltbp2 in HIV leading to dysregulation in adipose differentiation and inflammation, which could contribute to acquired HIV lipodystrophy and associated metabolic and inflammatory perturbations.
**Introduction**

HIV lipodystrophy is the most prevalent form of acquired lipodystrophy (1). The altered fat distribution seen in HIV lipodystrophy has significant implications for metabolic risk, including cardiovascular disease (CVD) (2, 3) and non-alcoholic fatty liver disease (NAFLD) (4), which are leading contributors to morbidity and mortality in HIV. Individuals with HIV lipodystrophy can present with lipohypertrophy, with increased fat in the abdomen, viscera and dorsocervical areas, and/or lipoatrophy, with loss of fat in the face, extremities and subcutaneous depots. These changes are usually associated with insulin resistance, metabolic dysregulation and inflammation. The pathogenic mechanisms of HIV lipodystrophy remain unclear. While HIV lipodystrophy was initially attributed to antiretroviral-mediated toxic effects on adipocytes, there is an increasing appreciation that the HIV virus and its related proteins may have direct effects on key metabolic and inflammatory pathways, that may impact adipose function (5). Recent progress by our group has focused on a mechanistic hypothesis for adipose dysfunction and metabolic changes in HIV, relating to the dysregulation of Dicer (6), an integral protein in the microRNA (miRNA) processing pathway, and its subsequent effects on critical miRNAs regulating adipogenic pathways (7, 8).

MiRNAs have important regulatory functions in adipose tissue biology. Indeed, mice lacking Dicer in the adipose tissue (aDicerKO) display a marked biological dysregulation of multiple miRNAs and mRNA targets associated with lipodystrophy, and demonstrate a phenotype comprised of multiple metabolic alterations including insulin resistance, mitochondrial dysfunction, signs of oxidative stress, fatty liver, premature mortality (8, 9). These phenotypic changes in the aDicerKO mice closely resemble those seen in the lipodystrophy associated with HIV (8). Interestingly, expression of Dicer in subcutaneous adipose tissue is markedly reduced in individuals with HIV lipodystrophy, providing a mechanistic link for overlapping metabolic features between HIV lipodystrophy and the aDicerKO mice (6, 8).

Given the downregulation of adipose Dicer in individuals with HIV lipodystrophy, a human physiological model which complements the animal data from the complete genetic knockout, we sought to explore
whether these individuals with lipodystrophy demonstrated alterations in circulating miRNAs which might underlie the metabolic alterations in lipodystrophy(3, 10-12). Adipose tissue is a major contributor to the circulating miRNAs carried by small extracellular vesicles namely sEV, which have regulatory functions in distant tissues(13, 14). Building on prior work(13), we now comprehensively assess circulating miRNAs in individuals with HIV and lipodystrophy, individuals with HIV without lipodystrophy and matched uninfected individuals and perform functional studies to investigate the subsequent effects of altered miRNAs on adipose tissue. We characterize a unique pattern of differentially regulated sEV miRNAs which we show to have important gene targets in adipocytes, affecting critical adipogenic and inflammatory pathways. Thus, the current investigation identifies a novel potential mechanism for acquired HIV lipodystrophy, highlighting key downstream consequences of reduced Dicer expression, systemic dysregulation of key miRNAs, and subsequent effects of altered miRNAs on genes impacting adipose regulation.
Results

Altered Body Composition and Reduced Adipose Dicer Expression Seen in HIV Lipodystrophy

To address the role of the miRNA processing pathway in the metabolic alterations caused by HIV infection, we first studied a cohort of individuals with HIV displaying lipodystrophy, individuals with HIV and no lipodystrophy, and uninfected individuals without HIV. As shown in the Table 1, age and race were similar upon stratification into three groups (HIV/Lipo, HIV/Non-Lipo, Non-HIV).

Dorsocervical adipose tissue area was largest in the HIV/Lipo group and smallest in the non-HIV group (P<0.004). These dorsocervical adipose tissue measurements obtained by imaging confirmed our clinical assessment for lipodystrophy in which participants with lipodystrophy were recruited for presence of increased dorsocervical fullness (Table 1). In addition, we confirmed using dual-energy X-ray absorptiometry (DXA) that limb fat (6345.4±975.2 vs. 8679.7±816.9 g, P=0.04 by one way ANOVA) was lower in the HIV lipodystrophic vs. HIV non-lipodystrophic phenotypes, suggesting evidence of peripheral lipoatrophy as would be expected in lipodystrophy. As previously described(6), Dicer expression, as determined by qPCR, was most reduced among HIV/Lipo, followed by HIV/Non-Lipo and Non-HIV (2.49[0.02, 4.88] vs. 11.20[4.83, 21.45] vs. 17.69[10.72, 47.91] [median (IQR)], P=0.002) (Table 1). HIV-related characteristics among HIV/Lipo participants and HIV/Non-Lipo participants differed by duration of HIV infection (24±2 vs. 18±3 years, P=0.07), duration of antiretroviral therapy (20±2 vs. 11±2 years, P=0.007), duration of protease inhibitor use (16±3 vs. 9±2 years, P=0.08), and CD8+ count (725±115 vs. 1260±164 cells/μl, P=0.02). Overall, both HIV groups demonstrated good immunological control based upon CD4+ count and had similar viral loads. Respiratory quotient determined by indirect calorimetry was lowest among the Non-HIV participants (Table 1). Among the HIV participants, Dicer expression was significantly related to CD8 (ρ=0.51, P=0.03) count and inversely to duration of antiretroviral therapy (ART) (ρ=-0.60, P=0.009).
Altered Pattern of Circulating sEV-carried miRNAs in HIV Lipodystrophy Associated with Reduced Adipose Dicer Expression

As we have previously demonstrated, reduced adipose tissue Dicer in mice led to changes in the expression levels of circulating sEV miRNAs(13). Given the reduction in adipose Dicer expression in HIV participants with lipodystrophy, we addressed whether circulating sEV miRNAs were also changed in these individuals. To do so, we isolated serum sEV using differential centrifugation protocol(15) from HIV/Lipo, HIV/Non-Lipo and Non-HIV participants (Figure 1A). This protocol led to vesicles in the expected range for exosomes (50-200 nm), enriched in the sEV markers CD63 and TSG101 while depleted from the cellular marker CANX (Supplemental Figure 1A-C). As shown in Figure 1B, each group (Non-HIV, HIV/Non-Lipo and HIV/Lipo) displayed a distinct circulating sEV miRNA profile. Comparing the HIV/Lipo to Non-HIV participants, the levels of 7 sEV miRNAs were significantly upregulated while 12 were downregulated (Figure 2A-B). In the comparison of HIV/Non-Lipo to Non-HIV, the expression of 30 sEV miRNAs were significantly upregulated and 27 were downregulated (Figure 2A-B). The miRNA profile of HIV participants with lipodystrophy was also compared to HIV participants without lipodystrophy. In this comparison, the expression of 33 miRNAs was significantly upregulated and 35 downregulated (Figure 2A-B). Interestingly, although additional changes did not reach statistical significance, expression of many different miRNAs was generally decreased in the HIV/Lipo group as observed by the left shift in the base of the volcano plots shown in Figure 2A and the bluish tone of the upper half column for HIV/Lipo in the heatmap displayed in Figure 2B. This observation is in line with a previous observation from our group(13) and highlights that many of the differentially expressed miRNAs were downregulated. Thus, HIV infection either with or without lipodystrophy leads to significant changes in multiple circulating sEV miRNAs. Decreases in the circulating sEV miRNA population were also seen that specifically associated with lipodystrophy.

The top differentially regulated miRNAs are shown in Figure 2B and can be clustered in three major groups: sEV miRNAs reduced in HIV lipodystrophy, others reduced in HIV without clinical evidence of
lipodystrophy, and others elevated in HIV regardless of a lipodystrophy presentation. Assessing for differences using a more stringent statistical approach (FDR < 0.05) revealed miR-20a-3p, miR-324-5p, and miR-186 as the top changed miRNAs. Notably, miR-20a-3p showed a stepwise progression and was significantly elevated in HIV/Non-Lipo compared to Non-HIV, and even further elevated in HIV/Lipo (Figure 2C). In contrast, miR-324-5p and miR-186 were specifically downregulated in the HIV/Lipo group compared to the other groups (Figure 2C). Among all participants, upregulation of miR-20a-3p correlated with downregulation of miR-324-5p (U = -0.71, P = 0.002), and miR-186 (U = -0.74, P = 0.002). In addition, downregulation of miR-324-5p correlated with downregulation of miR-186 (U = 0.54, P = 0.006) (Table 2). Interestingly, expression of these circulating miRNAs in sEV correlated significantly to adipose Dicer expression among all participants (miR-20a-3p, ρ = -0.61, P = 0.01; miR-324-5p, ρ = 0.39, P = 0.05; miR-186, ρ = 0.53, P = 0.008) (Table 3) and also strongly correlated with dorsocervical adipose tissue area (our clinical measure of lipodystrophy) (Table 3). Levels of miR-20a-3p correlated with RQ (ρ = -0.64, P = 0.008), triglycerides (ρ = 0.52, P = 0.04), insulin (ρ = 0.67, P = 0.005) and HOMA-IR (ρ = 0.50, P = 0.047), while miR-186 related to reduced CD4+ cell count (ρ = 0.55, P = 0.04) (Table 3). Taken together, these data suggest that the alteration in the levels of circulating sEV-carried miRNAs, i.e., miR-20a-3p, miR-186 and miR-324-5p, might serve as markers for the degree of lipodystrophy among individuals with HIV.

**Altered Pattern of Circulating sEV-carried miRNAs in HIV Lipodystrophy is Concordant with Changes in Adipose-specific Dicer KO mouse and Transfection with Dicer siRNA.**

sEV were isolated from the pooled serum (each sample corresponds to the serum of three mice, mixed females and males (2:1) control and aDicer KO mice (10-16 wks old) by standard ultracentrifugation protocol and subjected to RNA isolation. qPCR revealed miR-20a-3p was detectable in 2 of the 3 aDicer KO mice and none of the 5 control mice (Figure 3A). We have previously reported upon reduced miR-186 and miR-324-5p in the aDicer KO mice(13). In addition, we have now performed silencing
transfection of preadipocytes with *Dicer* siRNA. miR-20a-3p was significantly upregulated compared to control siRNA (Figure 3B). Herein, we show three different models in which miR-20a-3p is upregulated, including in the circulation of humans with HIV lipodystrophy and reduced subcutaneous adipose *Dicer* expression, in human preadipocyte cell culture models with transfection of *Dicer* siRNA in knockdown studies, and in the circulation of a murine model with adipose *Dicer* knockout.

**Gene Expression Signatures of Adipocyte Differentiation Decreased by Transfection of miR-20a-3p or the Antimers of miR-186 and miR-324-5p**

Given these previous observations, we assessed whether the increased levels of sEV-carried miR-20a-3p or decreased levels of sEV-carried miR-186 and miR-324-5p might underlie the metabolic alterations observed in HIV lipodystrophy, specifically with respect to adipocyte dysregulation in the white adipose depot, including altered patterns of adipocyte differentiation capacity. To address this question, human preadipocytes were transfected with either miR-20a-3p mimic, anti-miR-186, anti-miR-324-5p or control non-targeting miRNA and then cultured in differentiation medium for twelve additional days to give rise to mature adipocytes, after which RNA was collected and subjected to RNAseq (Figure 4A). The transfection was performed in parallel with a fluorescence-labelled miRNA or a positive control miRNA targeting aldolase-A to confirm success. Analysis of the RNAseq data revealed 125 genes significantly upregulated, and 121 genes significantly downregulated in the mimic miR-20a-3p-treated cells compared to miRNA control-treated cells (Figure 4B). Fifty-nine genes were significantly upregulated, and 203 genes were significantly downregulated when treating the cells with anti-miR-186 vs. control group, and 64 genes were significantly upregulated and 203 miRNAs significantly downregulated when treating the cells with anti-miR-324-5p (Figure 4B). Surprisingly, both anti-miRs highly overlapped in their gene expression pattern while mimic miR-20a-3p resulted in a more distinct pattern (Figure 4C and Supplemental Figures 2A-B). Among the pathways upregulated by mimic miR-20a-3p, we found several related to cellular adhesion and activity of the actin cytoskeleton (FAK-adhesion, ECM-receptor interaction, regulation of actin cytoskeleton) and others related to inflammatory processes (cytokine-
cytokine receptor activation, hematopoietic cell lineage) (Supplemental Figure 2C). Only the
proteoglycans pathway was slightly downregulated by mimic miR-20a-3p (Supplemental Figure 2C). In
contrast, both anti-miRs upregulated pathways related to catabolism, such as oxidative phosphorylation,
TCA cycle or fatty acid degradation and downregulated others related to cell adhesion and ligand
pathways such as Hippo and Wnt signaling pathways (Supplemental Figure 2D).

To identify genes that might be coordinately regulated by the upregulation of circulating sEV-carried
miR-20a-3p and the downregulation of miR-186 and miR-324-5p, we sought genes that would be
regulated by all these three the mimic miR-20a-3p, anti-miR-186 and anti-miR-324-5p in the same
direction. This analysis revealed that 7 genes were similarly upregulated and 47 genes were similarly
downregulated by these treatments (Figure 4B). The commonly regulated genes by all treatments are
shown in a heat map (Figure 4C). The gene with the most significant downregulation by all three
treatments was Latent Transforming Growth Factor Beta Binding Protein 2 (Ltbp2) as shown by volcano
plots (Figure 5A) and bar graphs (Figure 5B). In addition, other genes very significantly downregulated
by all three treatments included Nebulette (Nebl) and WNT1-Inducible-Signaling Pathway Protein 2
(Wisp2) (Figures 5A-B). Ltbp2 is thought to relate to SREBP-1b and SREBP-1c and shown to associate
with BMI-adjusted waist circumference in GWAS(16). Interestingly Nebl is a predicted target of all three
miRNAs (miR-20a-3p, miR-324-5p, and miR-186) that was identified using several miRNA-target
prediction tools (TargetScan and Diana databases, target score >85%). Wisp2 participates in the induction
of brown adipose tissue function, augmentation of insulin sensitivity, and regulation of preadipocyte
commitment and PPARγ activation(17-19). Thus, our data suggest that the increased levels of circulating
sEV-carried miR-20a-3p and decreased miR-186 and miR-324-5p associated with HIV lipodystrophy
might influence adipocyte differentiation in several key ways, by affecting pathways related to lipid
catabolism, inflammation or cellular adhesion, among others through effects on Ltbp2, Nebl and Wisp2.
In addition, utilizing the publicly available GEO database GSE28073 comparing gene expression in HIV-infected patients (20), we found a reduction of \( Ltbp2 \) in both abdominal and dorsocervical adipose tissues from HIV lipodystrophy patients compared to HIV patients without lipodystrophy (Figure 6).

Assessment of Adipocyte Differentiation and Inflammatory Phenotype in Knockdown Studies of Transfected Human Adipocytes

In order to determine the effects of the downregulation of \( Ltbp2, \) \( Nebl \) and \( Wisp2 \) and their potential synergy in adipocyte differentiation, we performed silencing transfection of preadipocytes with either \( Ltbp2 \) siRNA, \( Nebl \) siRNA, \( Wisp2 \) siRNA individually, or a cocktail of all three siRNA (containing 1/3 of each siRNA dose) one day prior to induction of differentiation. As a control, a non-targeting siRNA was used in parallel. RNA was collected at the end of the differentiation and subjected to RNAseq (Figure 7A). As shown in Supplemental Figure 3, single siRNA treatment efficiently downregulated (>80% reduction) the target genes. Triple siRNA combination efficiently downregulated \( Ltbp2 \) (~80% reduction) but had a weaker but still significant reduction on \( Nebl \) (~50% reduction) and had a very modest non-significant effect on \( Wisp2 \) (~20% reduction) (Supplemental Figure 3). As the triple siRNA combination had 1/3 of the dose of each siRNA, this differential targeting capacity for each target is likely due to lower efficiency at slightly lower doses.

We next analyzed how the downregulation of \( Ltbp2, Nebl, Wisp2 \) might affect processes involved in lipodystrophy such as adipocyte differentiation. We found that knockdown of \( Ltbp2 \) significantly downregulated multiple genes markers of adipocyte differentiation such as \( Fabp4, \) \( Ppar\gamma, \) \( C/epsilon, \) \( Fasn, \) adiponectin, \( Glut4, \) and \( CD36 \) (Figure 7B). Knocking down \( Wisp2 \) also led to a general decrease of various of these adipocyte differentiation markers, with effects on \( Fabp4, \) \( Ppar\gamma, \) \( Fasn, \) adiponectin, \( Glut4 \) and \( Srebp1c \) reaching statistical significance (Figure 7B). In contrast, \( Nebl \) siRNA did not lead to any significant changes. Combined knockdown of these targets using a combination of \( Ltbp2 \) siRNA, \( Nebl \)
siRNA, and Wisp2 siRNA was additionally assessed in a supportive analysis, and resulted in a significant
downregulation of Fabp4, Pparγ, C/epba, Fasn, adiponectin, Glut4, and CD36 (Supplemental Figure
4B). Downregulation of genes related to adipocyte differentiation were generally lower with Ltbp2 siRNA
in contrast to the triple combined siRNA (but at one-third of the concentration), suggesting the effect was
driven predominantly by Ltbp2 siRNA. Mutations in lamins A and C have been associated with
lipodystrophy and metabolic complications(21-23). In our studies, compared to the control siRNA, there
was a significant downregulation of Lamin C expression in both Ltbp2 siRNA and Wisp2 siRNA groups,
while Lamin A expression did not appear to differ among all groups (Figure 7C and Supplemental
Figure 4C). We also demonstrate decreased adipocyte differentiation in histological studies utilizing oil-
red-o staining. Histologic images and quantitative data show reduced oil-red-o staining after adipocytes
were transfected with siRNA for Ltbp2 and Wisp2 when compared to the control (Figure 8 and
Supplemental Figure 5).

Another process intimately related to lipodystrophy is the activation of inflammatory pathways. Again,
Ltbp2 knockdown had the greatest effects, leading to upregulation of IL1b, IL6 and Ccl20 while the other
treatments had no or very minor effects (Figure 7D and Supplemental Figure 4D). We saw consistent
but nonsignificant trends toward increased expression of IFNβ in response to knockdown of Ltbp2, Nebl
and Wisp2. Thus, our data suggest that Ltbp2 is important for a proper adipocyte differentiation and
control of inflammation. The downregulation of Ltbp2 induced by elevated sEV-carried miR-20a-3p or
decreased sEV miR-186 and miR-324-5p in HIV lipodystrophy patients may underlie the impairment of
adipose tissue development/differentiation and the increased activation of inflammatory processes seen in
such patients and could be a potential target for future clinical studies (Figure 9).

No effects of knockdown of Ltbp2, Nebl or Wisp2 were seen on markers of mitochondrial function,
Tomm20 and mtND2. When assessing effects on markers of oxidative stress, no effects were seen on
Catalase, Sod1, Sod2 or NADPHox-p40phos, but Hmox1 was decreased significantly in response to Ltbp2 (Supplemental Figure 6).

In exploratory analyses of effects on brown fat gene expression, we applied a beiging differentiation protocol to the preadipocytes. We observed non-significant decreases in brown adipose maker Ucp1 with Ltbp2, Nebl and Wisp2 siRNA compared to the control. In addition, Prdm16, a key beige adipose marker, demonstrated a relative decrease following transfection of Ltbp2, Nebl, and Wisp2 siRNA when compared to the control (Supplemental Figure 7).
The HIV population frequently presents with a redistribution of fat and metabolic abnormalities, including dorsocervical adipose lipohypertrophy, ectopic fat accumulation, subcutaneous adipose dysfunction, insulin resistance and dyslipidemia. These features closely resemble the phenotype of adipose-specific DicerKO mice, surmising a role for adipose-derived miRNAs in the pathogenesis of this syndrome. Indeed, downregulation of Dicer expression in fat and subsequent dysregulation of adipose-derived miRNAs, many of which may be secreted in sEV and function as adipokines, could provide a novel mechanism for altered adipose function and related metabolic abnormalities in HIV lipodystrophy. Interestingly, HIV-related accessory proteins, for example VPR and TAT, have been reported to suppress Dicer expression. While suppression of Dicer may have evolved as a mechanism to enhance infectivity of the HIV virus, there may be unintended metabolic consequences leading to a lipodystrophic phenotype.

To begin to investigate the role of Dicer in adipose and metabolic regulation in HIV, we previously assessed subcutaneous adipose expression of Dicer in a group with well-defined lipodystrophy and demonstrated reductions in adipose Dicer expression in association with key brown fat genes and clinical parameters. In the current study, we have performed sEV miRNA profiling in this well-phenotyped cohort of individuals with and without HIV in whom we have previously characterized adipose expression of Dicer. We hypothesized a unique miRNA signature among those with HIV lipodystrophy in relationship to dysregulation of Dicer. In these analyses evaluating sEV miRNA profiles, we identified three specific miRNAs (miR-20a-3p, 186, and 324-3p) as the top differentially regulated miRNAs using a stringent statistical approach. Notably these miRNAs demonstrated a unique signature strongly related to Dicer expression and well-defined clinical lipodystrophy characteristics, including increased dorsocervical adipose tissue, as well as key metabolic and immune indices. In support of our findings, we were able to demonstrate upregulation of miR-20a-3p across three adipose-specific models of reduced Dicer expression: 1) in the circulation among patients with HIV lipodystrophy and reduced Dicer...
expression in the abdominal subcutaneous depot, 2) in the circulation of adipose-specific Dicer KO mouse, and 3) and in a preadipocyte culture model transfected with Dicer siRNA. Follow up experiments to manipulate these identified miRNA pathways in human cultured preadipocytes led to identification of novel corresponding adipose-specific gene targets of these miRNAs. Subsequent knockdown experiments of these genes through silencing transfection studies demonstrated key metabolic roles relevant to glucose and lipid metabolism.

We studied an HIV population who were well-treated on ART, a group which is representative of the large population of aging individuals who are living with chronic HIV infection and demonstrate good immunologic control, but nonetheless have an increased risk for mortality driven in part by metabolic complications. The HIV lipodystrophy cohort was identified based on the presence of dorsocervical lipohypertrophy on physical exam and confirmed based on quantitative measurements on MRI imaging. Moreover, the cohort demonstrated reduced extremity fat via DXA, suggesting the common phenotype of loss of subcutaneous fat. Dicer expression was lowest in those with imaging confirmed lipodystrophy, followed by HIV without lipodystrophy and then non-HIV controls(6).

We have previously shown the dorsocervical adipose tissue is a beige-fat like adipose depot that may develop as a compensatory mechanism to lipodystrophy(26). In a prior study we showed that reduced Dicer expression in subcutaneous adipose tissue was related inversely to dorsocervical adipose tissue accumulation. We now demonstrate that increased dorsocervical adipose tissue is strongly correlated to the unique miRNA signature we have identified, such that greater dorsocervical adipose tissue is associated with upregulation of miR-20a-3p and downregulation of miR-186 and miR-324-5p. Moreover, in exploratory analyses, we observed potential effects of key dysregulated genes in this study, Ltbp2, Nebl and Wisp2 on two genes involved in browning of white adipocytes, Ucp1 and Prdm16. Further studies should assess the effects of dicer related downstream effect on brown and beige fat pathways in specific adipose depots.
In our study, pathway analyses revealed that highly relevant clusters important to metabolism and cytokine signaling were significantly affected by miR-20a-3p mimic as well as anti-miR-186 and anti-miR-324. These additional analyses provide supporting evidence that the three top differentially regulated miRNAs identified in this study are linked to adipocyte biology and physiologic systems applicable to HIV lipodystrophy. In addition, there may be clinical utility to evaluating whether these 3 sEV miRNAs could serve as a diagnostic tool using a simple, non-invasive technique to identify a clinical presentation of HIV lipodystrophy, for which objective measures may be lacking. Moreover, we saw that upregulation of miR-20a-3p related to greater triglycerides and insulin resistance, further suggesting a link to the clinical lipodystrophy phenotype. Further studies may identify other miRNAs potentially important in mediating metabolic effects in relationship to reduced Dicer in HIV.

There are limited adipose and metabolic-related data about the roles of the three top differentially regulated miRNAs identified in this study in other cellular and disease models. A recent study of mycobacterium tuberculous suggested that overexpression of miR-20a-3p contributed to mycobacterial survival in a macrophage cell line and may aid in the host immune response(27). In this way, changes in miR-20a-3p and other miRNAs could help to enhance survival of HIV in the host and have a secondary consequence of metabolic complications. In addition, miR-20a-3p has also been implicated in MAPK signaling pathway and phosphatidylinositol signaling system(28). Consistent with the current study, miRNA-20a-3p has been shown to be elevated among type 2 diabetes mellitus patients compared to healthy individuals when isolated from circulating plasma ectosomes(29). A recent study showed that miR-186 is upregulated in a T-lymphoblast cell line following acute HIV infection, and this is associated with reduced Dicer expression in these cells(30). In our study, we have shown, in contrast, reduced miR-186 in circulating sEV from chronically treated HIV-infected patients on ART demonstrating reduced Dicer expression in the adipose depot. With regards to miR-324-5p, which we show to be downregulated in HIV lipodystrophy, this miRNA has also been shown to be downregulated in circulating sEV of...
patients with type 2 diabetes mellitus while demonstrating an inhibitory effect on diabetes-related inflammation (31). miR-324 overexpression has been linked to enhanced glucose uptake in oxygen glucose deprived neurons (32) and also has been reported to promote Wnt signaling (33). Consistent with this, we found downregulated Wnt signaling in adipocytes following treatment with anti-miR-324-5p.

In the context of HIV lipodystrophy, few other miRNAs have been reported to have a potential role. One study demonstrated that miR-218 was upregulated in the subcutaneous adipose of patients with HIV lipodystrophy, and this was associated with reduced lipin1 levels, a putative target of this miRNA. Furthermore, co-treatment of 3T3-L1 cells with a miR-218 mimic and lopinavir/ritonavir decreased Glut4 levels (34, 35). This prior study also reported that 21 miRNAs out of 754 were overexpressed by 2-fold in the subcutaneous adipose among 8 individuals with HIV lipodystrophy compared to 8 uninfected individuals. Interestingly, in contrast to our findings in sEV miRNAs, the authors found that miR-186 was upregulated in the adipose depot, but did not report any data on miR-20a-3p or miR-324-5p in the adipose tissue or on altered pattern of circulating miRNA.

We have previously reported significant miRNAs affected in the aDicer KO model (8), and now show general concordance between findings in these mouse and human studies, for example in the relative increase in miR-20a-3p and reductions in reduced miR-186 and miR-324-5p. Some differences between studies could be due to selective sorting of certain miRNAs into sEV (36, 37), differing biology in the mouse vs. human, or differences in the mechanism of Dicer dysregulation in these models, e.g., adipose-specific total genetic knockout of Dicer in the mouse study (8) vs. physiological regulation in multiple tissues by HIV-related factors in the current study. Taken together, these data from multiple experiments, including the observation of increased miR-20a-3p expression in preadipocytes in response to Dicer siRNA, suggest an effect of reduced dicer to increase miR-20a-3p and affect other miRNAs.
One interesting aspect of these studies was the finding that $Ltbp2$ transcription was significantly downregulated in human preadipocyte cultures by all three miRNAs observed to be altered in HIV lipodystrophy. Similarly, expression of $Nebl$ and $Wisp2$ were also decreased by all 3 miRNAs. Furthermore, we showed that $Ltbp2$ was reduced in both abdominal and dorsocervical adipose tissue biopsies from another cohort of HIV patients who developed lipodystrophy when compared to adipose tissue from HIV patients that did not develop lipodystrophy(20), further indicating a critical role of $Ltbp2$ in HIV lipodystrophy. Moreover, in the present study, we demonstrated that downregulation of $Ltbp2$ in adipocytes by siRNA affected key regulators of adipocyte differentiation, metabolic regulation, cellular structure and inflammation. Indeed, knockdown of $Ltbp2$ in human cell culture of preadipocytes consistently resulted in downregulation of several key genes associated maturation of adipocytes, including $C/ebp\alpha$ and $CD36$, highlighting the role of $Ltbp2$ as a potential mechanistic factor contributing to subcutaneous adipose dysfunction and related metabolic abnormalities in HIV lipodystrophy. To complement these changes in gene expression, we also found reduced oil-red-o expression in adipocytes transfected with $Ltbp2$. Of note, prior work in HIV has shown adipose dysfunction among individuals with HIV, with lower mRNA concentrations of key adipogenic differentiation factors, including $C/ebp\alpha$ and $\beta$, $PPAR\gamma$, and $Srebp1c$(38). This prior work also demonstrated $Srebp1c$ mRNA concentrations correlated negatively with inflammatory cytokine levels(38).

Several genes downregulated by knockdown of $Ltbp2$ are also linked to fatty acid oxidation and lipid metabolism pathways, such as $FASN$, $Fabp4$ and adiponectin. Impaired fatty oxidation is a characteristic of HIV lipodystrophy(39, 40). Adiponectin, $GLUT4$ and $PPAR\gamma$, integral genes to insulin action and sensitivity, were also dampened by $Ltbp2$ downregulation. Insulin resistance is a common clinical finding in HIV lipodystrophy often presenting in association with other metabolic sequelae, such as ectopic fat accumulation and inflammation(10, 12, 41). While existing miRNA databases do not report $Ltbp2$ as a target of our identified miRNAs, our data reveal a clear link between these miRNAs and $Ltbp2$. Knockdown of $Wisp2$ also resulted in downregulation of markers of adipocyte differentiation though to a
somewhat lesser degree than Ltbp2. In that regard, combined loss of Ltbp2, Nebl, and Wisp2 did not have an additive effect across critical metabolic genes above that of Ltbp2, suggesting that these genes act in the same pathway.

Mutations in Lmna and Pparγ are well-recognized in familial partial lipodystrophies and therefore could play a role in this acquired form of lipodystrophy. In this study, we determined that knockdown of Ltbp2 and Wisp2 resulted in significant downregulation of Lamin C. The Lmna gene encodes both lamins A and C—isoforms generated by alternative splicing of Lmna. Lmna gene mutations generally disrupt both lamin A and lamin C in partial lipodystrophy(42). Missense mutations on the Lmna gene, predominantly identified on exon 8, have been reported to cause a lipodystrophic phenotype(43, 44). There are reports of lamin A specific mutations in partial lipodystrophy(43) and lamin C specific mutations in generalized lipodystrophy(42). Interestingly, protease inhibitors have been linked to altered lamin C(45). We demonstrate reduced Lamin C in response to knockdown of key genes dysregulated by miRNAs in HIV lipodystrophy in cultured human preadipocytes. This occurs in vitro, independent of any ART exposure, but in vivo, it is possible that ART use may further compound this effect on Lamin C. The altered expression of these genes regulated by Ltbp2 expression may contribute to fat dysregulation and subsequent metabolic complications in HIV lipodystrophy.

Finally, three inflammatory markers, IL1β, IL6 and Ccl20, were significantly increased with knockdown of Ltbp2. HIV lipodystrophy is associated with an inflammatory phenotype, i.e., subcutaneous adipose tissue shows an increased infiltration with inflammatory cells. Importantly, IL-6, and to a lesser extent IL-1β, have been linked to HIV lipodystrophy(46-48). Of note, knockdown of Ltbp2 was also associated with reduced Hmox1. Knockdown of Hmox1 could have an inflammatory effect as HMOX1 catalyzation can result in anti-inflammatory responses by up-regulation of interleukin 10 (IL-10) and interleukin 1 receptor antagonist (IL-1RA) expression(49, 50) Our data suggest altered miRNAs regulate Ltbp2, which
in turn may increase inflammatory gene expression in adipose tissue, thus providing an additional mechanism for this effect. Other inflammatory markers, including IFNβ, were generally increased but did not reach significance with knockdown of Ltbp2. Dicer has recently been identified to repress interferons and the antiviral response in other cell types(51), thus our data showing a trend towards increased interferon with reduced Dicer show a potential consistent effect in adipocytes.

This study has a number of strengths, and some limitations. A strength of our study is the translational exploration of a clinical phenotype. Leveraging our preclinical model of the aDicerKO mouse, we evaluated the implications of reduced adipose Dicer expression in HIV lipodystrophy on sEV-carried miRNAs and further tested manipulation of these miRNAs leveraging RNAseq to arrive at key genes contributing to critical adipogenic and inflammatory pathways. We were unable to collect additional subcutaneous adipose tissue samples in our human subjects to complement our RNA seq studies conducted using cultured human preadipocytes, but we validated the potential role of Ltbp2 in a relevant HIV database of adipose gene expression(20). Our data profiling circulating sEV patterns do not allow us to discern whether reduced Dicer in subcutaneous adipose is directly affecting local miRNAs, and whether such altered miRNAs affect local adipogenic pathways in a paracrine fashion, distant adipose tissues or other organs. However, our adipocyte transfection study using Dicer siRNA suggest the adipocyte may be directly involved in a paracrine like effect. Future studies should now investigate this newly discovered miRNA signature and related target genes to determine effects on key adipogenic pathways in critical adipose depots including browning of white adipose tissue and oxidative stress, as well as metabolic pathways in other critical organs, such as the liver, to determine how this mechanism contributes to overall metabolic dysregulation in HIV. Future studies should also assess if these pathways are important in other conditions of reduced Dicer, such as aging and overfeeding(9, 52), which are characterized by metabolic dysregulation.
In the current investigation, we were unable to disentangle any specific contribution of ART to reduce Dicer and dysregulate circulating miRNAs. Nonetheless, our experiments in adipocyte cultures were performed in the absence of any ART, suggesting that the downstream consequences of reduced Dicer and altered miRNAs on adipose function occur independently of ART. Further studies should investigate this question.

In summary, we conclude miR-20a-3p functions as a key marker among individuals with HIV lipodystrophy with altered adipose tissue Dicer expression. Overexpression of miR-20a-3p was shown to promote metabolic perturbations, potentially through the downregulation of Ltbp2 in the adipocyte, leading to dysregulation in critical markers of adipose differentiation and inflammation, and a relevant lipodystrophy gene, Lamin C. As such, these studies provide a plausible mechanistic schema, whereby miR-20a-3p downregulates Ltbp2 leading to adipocyte dysfunction in HIV lipodystrophy. This study demonstrates a novel pathway that can be potentially targeted to improve adipose dysfunction in this population.
Methods

Study participants

Eighteen participants with HIV, 9 with and 9 without lipodystrophy, and 9 uninfected participants without HIV were previously recruited in an observational study to assess Dicer expression in the abdominal subcutaneous adipose tissue(6). All participants were male between the ages of 18-60 years with a body mass index 18-35.0 kg/m². Among participants with HIV, ART use had been stable for >12 months. A single investigator assessed for HIV-related lipodystrophic changes in fat. Lipodystrophy was characterized by the presence of excess dorsocervical fat accumulation. Magnetic resonance imaging (MRI) also confirmed the examination (see MRI methods below). Major exclusion criteria included hemoglobin <10.0 g/dL; known history of diabetes and use of anti-diabetic medications; abnormal thyroid function; use of glucocorticoids, growth hormone, growth hormone releasing hormone or other anabolic therapies within 3 months of enrollment, current substance abuse, or active or serious chronic medical conditions other than HIV.

Assessment of fat redistribution

MRI of the neck was performed on a Siemens 3T Trio magnetic resonance system using phased array neck and body matrix coils. A volumetric 3D Dixon gradient-echo multiecho pulse sequence with 6 echo-times (repetition time = 20ms, echo-time = 2.46, 6.15, 9.84, 12.3, 14.76, 17.22 ms, flip angle = 5, slice thickness = 3mm, field of view = 42cm, matrix = 256 x 256) was employed. Sagittal images were reconstructed to identify the level of C7 vertebral body. Axial images at the level of C7 were reconstructed and used for measurement of dorsocervical adipose tissue area. Vertical reference lines were placed along the lateral border of the vertebral body of C7 and projected over dorsocervical adipose tissue, providing standardized lateral boundaries for the fat depot. Dorsocervical adipose tissue was demarcated anteriorly by the paraspinal muscles and posteriorly by the dorsocervical skin. Area measurements within these boundaries were expressed in cm². In addition, MRI were acquired using an axial T1-weighted fat suppressed pulse sequence obtained at the level of L4 vertebral body for
determination of visceral and subcutaneous fat areas utilizing commercial software (Vitrak, Merge eFilm, Milwaukee, WI).

Biochemical and metabolic assessment

Participants presented after an overnight fast for blood sampling. CD4+ and CD8+ T cell counts were assessed by flow cytometry. HIV viral load was determined by ultrasensitive RT PCR (Roche COBAS amplicor, Indianapolis, IN). As previously described, Dicer expression was determined from subcutaneous adipose tissue biopsies (6).

Abdominal fat biopsy

Surgical sampling of subcutaneous abdominal fat was performed under local anesthesia with 1% lidocaine, using a 4-mm diameter punch biopsy. Specimens were snap frozen in a dry ice/ethanol bath and immediately transferred to -80°C.

Animals

All animal studies were conducted in compliance with the regulations and ethics guidelines of the NIH and were approved by the IACUC of the Joslin Diabetes Center. Generation of adipocyte-specific Dicer knockout mice (ADicerKO; Adiponectin-Cre+ Dicerfl/fl) and control mice (Adiponectin-Cre-Dicerfl/fl) were described elsewhere (8). Female and male mice aged between 10-18 weeks were used for the experiments. Mice were anesthetized with avertin, and blood was collected by cardiac puncture.

Human preadipocytes and white adipogenesis differentiation

Human white preadipocytes were provided by the adipocyte core of the Boston Nutrition Obesity Research Center (BNORC). Abdominal subcutaneous fat tissue was obtained from subjects undergoing plastic surgery. Donors were 30-60 years old, females, body mass index range 21.7-45.7 kg/m² and non-diabetic. Adipose stromal cells were isolated as previously described14. Briefly, minced tissue was treated
with collagenase solution (1mg/ml HBSS) (Type 1, Worthington Biochemical, Lakewood NJ) at 37°C
100 rpm for 2 hours. The digested tissue was filtered through a 250 μm mesh (Component Supply, Inc. Smithville Hwy, Sparta, TN). Cells were centrifuged at 500 g for 10 min at room temperature. The red blood cells in the cell pellets were lysed (0.154mM NH4Cl, 10 mM K2HPO4 and 0.1mM EDTA, pH 7.3). The washed cells were plated using alpha MEM Media (Gibco Thermo Fisher, Scientific Waltham, MA) with 10% FBS (Gemini Bio Products, West Sacramento, CA) 100 units/ml penicillin, 10μg/ml streptomycin (Pen/Strep) (Corning, Corning NY). For the experiments, cells were grown in DMEM high glucose with 20% FBS and 1 % Pen/Strep until confluency. At day 0 of the differentiation protocol, cells were pre-induced using growth media supplemented with 500 nM insulin, 2 nM 3,3’,5’-triiodo-L-thyronine (T3) and 1 μM rosiglitazone. This medium was renewed every 2-3 days. At day 6, cell differentiation was induced using growth medium supplemented with 500 nM insulin, 2 nM T3, 1 μM rosiglitazone, 0.54 mM 3-isobutyl-1-methylxanthine (IBMX), 33 uM biotin, 17 uM pantothenate, 0.1 μM dexamethasone and 30 μM indomethacin. This medium was renewed every 2-3 days for 12 additional days, after which cells can be considered fully differentiated (day 18 of differentiation protocol). All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. For the mimic and anti-miRs transfection, 83 nM mimic miR-20a-3p (Horizon Discovery-Dharmacon, Lafayette, CO) and anti-miR-186 and anti-miR-324-5p (miRVana, Thermofisher) mixed with lipofectamine RNAiMAX (Thermofisher) was done at day 5 after starting pre-induction phase. As controls, a positive control miRIDIAN microRNA Mimic targeting aldolase-A and a miRIDIAN Dy-547-labelled miRNA Transfection Control (both from Horizon Discovery-Dharmacon) were transfected in parallel. For the transfection with siRNAs against Ltbp2, Nebl, Wisp2 or non-targeting control siRNA, we used 16.67 nM each single siRNA in combination with lipofectamine RNAiMAX. For the triple siRNA combination, 1/3 of each siRNA of the above siRNAs was used (5.55 nM each). Transfection with Dicer siRNA (Horizon Discovery Dharmacon) was also performed at day 5 after starting pre-induction phase.
One day later differentiation was induced as described above and samples were collected for RNA isolation 6 days after differentiation induction.

**Human preadipocytes and beige adipogenesis differentiation**

Human white preadipocytes were obtained and grown until confluence as described in the previous section. After reaching confluence, cells were transfected with siRNAs against *Ltbp2, Nebl, Wisp2* or the triple combination as done for white adipogenesis above. One day after transfection with siRNAs, beige adipogenesis was induced based on the protocol described by Singh et al. (53), with slight modifications. Briefly, confluent cells were induced to beige differentiation by adding the following reagents resuspended in DMEM/F12 (Thermofisher, Waltham, MA, 11965118) with 10% FBS and 1% Pen/Strep: 200 ng/mL recombinant hIGF-I (Peprotech, Rocky Hill, NJ 100-11), 8 ng/mL human basic FGF (Miltenyi Biotech, San Jose, CA, 130-093-838), 100 ng/mL human BMP7 (R&D systems, Minneapolis, MN, 354-BP), 10 μM Y27632 (Cayman Chem, Ann Arbor, MI, 10005583), 2 μM rosiglitazone (Sigma, R2408), 1 nM T3 (Sigma, T6397), 1 μM dexamethasone (Sigma, D4902) and 500 μM IBMX (Sigma, I5879). Medium was replaced every three days. Lipid droplet accumulation was readily observable a few days after induction and increased with time until reaching approximately 80-90% of the plate at day 21 after induction. At this day, cells were washed with PBS and added TRIzol for RNA isolation as described below.

**Small extracellular vesicle isolation**

sEV isolation was performed using standard differential ultracentrifugation protocol (15) on 2-3 mL of serum from each of the 27 participants [9 HIV lipodystrophy (HIV/lipo), 9 HIV without lipodystrophy (HIV/non-lipo), 9 non-HIV]. Serum was collected in a tube without anticoagulant and underwent centrifugation at 4°C at setting 2800rpm for 10 min. Briefly, serum was diluted with PBS and successively centrifuged at 2,000 g for 10 min and 10,000 g for 30 min. The supernatant was then
ultracentrifuged at 100,000 g for 70 min using a SW-28 rotor to pellet the sEV. Pellets were washed with PBS, centrifuged again at 100,000 g for additional 70 min and either resuspended in PBS for nanoparticle tracking analysis, immunoblotting, nanoparticle tracking analysis and electron microscopy or in TRIZol reagent (Thermofisher) for RNA isolation and subsequent miRNA profiling. For the sEV isolation of mouse serum, blood obtained by cardiac puncture sat for 30 min at room temperature to allow coagulation prior centrifuging twice at 500 g for 5 min and 2,000 g for 10 min to remove cells and cellular debris. Supernatant was mixed 1:4 with double filtered PBS and centrifuged at 10,000 g for 30 min to remove large vesicles. Supernatants containing serum sEV were mixed in pools, each consisting of 10,000 g-derived supernatants from two females and one male of the same genotype, having the same age distribution in all control and ADicerKO samples. These pools were ultracentrifuged at 100,000 g for 70 min using a SW-28 rotor (Beckman Coulter, Indianapolis, IN). The pellets containing the sEV were washed with double-filtered PBS and centrifuged again at 100,000 g for 70 min. After removing all supernatant, pellets were resuspended in TRIZol and proceeded to RNA isolation, miRNA retrotranscription and PCR assay as described below.

Nanoparticle tracking analysis (NTA)

Vesicle concentration and size distribution was determined by the dynamic light scattering technology using a Nanosight LM10 (Malvern Panalytical, Malvern, United Kingdom) at the Nanosight Nanoparticle Sizing & Quantification Facility at Massachusetts General Hospital (Charlestown, MA). The script was programmed to take 5 videos for 30 sec each for each sample. Samples were diluted 1:50 prior running Nanosight system. For the calculation of the sEV concentration, this dilution and the volume of original serum where they were isolated from were taken into account.

Immunoblotting

Serum sEV and human 293T cells were resuspended in RIPA lysis buffer (Millipore Sigma, Burlington, MA) containing SDS 0.1% and protease and phosphatase inhibitors (Biotool, York, United Kingdom) and
incubated on ice for 30 min prior to centrifugation at 12,000 g for 10 min. Supernatants were used for western blotting in SDS-PAGE electrophoresis. Protein concentration was determined by a BCA kit (Thermofisher). The following antibodies were used: TSG101 (sc-7964, Santa Cruz, Dallas, TX), CD63 (ab68418, Abcam, Cambridge, United Kingdom) and calnexin (CANX) (ab22595, Abcam, Cambridge, United Kingdom).

Oil-Red-O (ORO) staining

Human adipocytes were grown, differentiated and transfected against Ltbp2, Nebl, Wisp2 and the triple combination as described above. Cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min. Cells were washed with PBS again and incubated for 1 hour with ORO working solution. To make ORO working solution, ORO stock solution (0.5% ORO in isopropanol) was mixed 3:2 with water and filtered. After the one-hour incubation, cells were washed with water and representative photographs were taken immediately. Quantification of ORO signal was performed by ImageJ (NIH). Briefly, RGB (red/green/blue) channels were separated in three pictures. Signal from red-containing pictures was quantified by selecting a 0-50 threshold and later calculated the percentage of positive area.

Electron microscopy

CD63 immunogold staining of sEV preparations was performed at Electron Microscopy Facility at Harvard Medical School (Boston, MA). sEV were isolated by ultracentrifugation as described above and adsorbed to a hydrophilic carbon coated grid. After blocking with BSA, grids were incubated with antibody against the sEV marker CD63 (BD Pharmingen 556019, Franklin Lakes, NJ) and later an IgG secondary antibody (Abcam ab6709). The antibody complex was detected using Protein A-gold (10 nm). The grids were examined in a JEOL 1200EX transmission electron microscope, and images were recorded with an AMT 2k CCD camera.

RNA isolation and miRNA profiling
Upon addition of chloroform, samples were centrifuged at 12,000 g for 15 min. The upper phase was collected and mixed with isopropanol, ammonium acetate (250 mM) and RNA-grade glycogen (1 μg/mL, Thermofisher) and incubated overnight at -20°C. Samples were then centrifuged at 12,000 g for 30 min, washed twice with 75% ethanol and resuspended in nuclease-free water. The RNA concentration was assessed by Nanodrop, and equal amounts of RNA for each sample were used for miRNA profile analysis. This was accomplished using a quantitative real-time PCR (qPCR)-based kit (RA660A-1, System Biosciences, Palo Alto, CA) following manufacturer’s instructions. For single detection of miRNAs, RNA was retrotranscribed using miRCURY LNA Starter kit (Qiagen, Hilden, Germany) and assessed by PCR using highly-specific miRCURY LNA primers (Qiagen).

RNA extraction from human adipocytes was performed as described for sEV RNA isolation. Reverse transcription was done using High Capacity Reverse transcription kit (Applied Biosystems, Waltham, MA) following manufacturer’s instructions.

**RNA sequencing**

The Total-RNA samples from human adipocytes were quantified using an Agilent 4200 TapeStation instrument, with a corresponding Agilent TapeStation RNA assay. The resulting RIN (RNA Integrity Number) scores and concentrations were taken into account for qualifying samples to proceed.

The samples were normalized to 200 ng of input in 50μL (4 ng/μL), and the mRNA was captured using oligo-dT beads as part of the KAPA mRNA HyperPrep workflow. cDNA synthesis, adapter ligation, and amplification were conducted subsequently as part of the same workflow. Following amplification, residual primers were eluted away using KAPA Pure Beads in a 0.63x SPRI-based cleanup.
The resulting purified libraries were run on an Agilent 4200 Tapestation instrument, with a corresponding Agilent High Sensitivity D1000 ScreenTape assay to visualize the libraries and check that the size and concentrations of the libraries matched the expected product. qPCR with the KAPA Library Quantification kit, which uses primers complementary to the sequencing flow-cell oligos, was run to confirm the functional concentration. Molarity values obtained from this assay were used to normalize all samples in equimolar ratio for one final pool. The pool was denatured and loaded onto an Illumina NextSeq 500 instrument, with a High-Output 75-cycle kit to obtain Single-End 75bp reads. The pool was loaded at 1.6 pM, with 5% PhiX spiked in as a sequencing control. The base call files were demultiplexed through the Harvard BPF Genomics Core's pipeline, and the resulting fastq files were used in subsequent bioinformatic analysis.

**Statistical analysis**

Normality of distribution was determined using the Shapiro-Wilk test. Data are presented as mean ± standard error of mean or median [interquartile range (IQR)], depending on normality of the distribution. Categorical variables are reported as proportions. Primary demographic comparisons were made between all three groups, non-HIV, HIV non-lipodystrophic and HIV lipodystrophic by overall comparison among the group by the appropriate test: ANOVA or Kruskal-Wallis test. Univariate regression was performed using Spearman’s correlation coefficient.

For miRNA profiling analysis, the data from two participants (one HIV/Lipo and another HIV/Non-Lipo) were removed due to high degree of hemolysis that significantly affected the miRNA profile results (Supplemental Table 1). As an initial step, although 1113 different miRNAs were measured, only those with Ct>35 in at least 5 samples per group (997 miRNAs) were considered expressed and therefore remained for further analysis. Expression data for miRNA expression was normalized to the mean Ct of all miRNAs for each sample. All samples displayed a similar Ct distribution. miRNAs nomenclature was based on miR-Base database version 22 (www.mirbase.org). To discover differential
miRNA expression among groups, we use Limma, an R package for linear modeling that powers
differential expression analyses (54). The same statistical method was used for analysis of RNAseq data.
For RNASeq, genes meeting criteria for an absolute fold change 1.5 and P<0.05 were initially considered
significant (Supplemental Tables 2-7). For multiple comparisons related to miRNA and RNAseq, a false
discovery rate was then applied as indicated in each experiment. Statistical significance was defined as
FDR<0.05 for sEV miRNA profiling. Data for Ltbp2 expression in HIV non-lipodystrophic and
lipodystrophic abdominal subcutaneous and dorsocervical adipose tissues was obtained from dataset
repository GSE28073 (20). For gene expression analysis, statistical significance was performed by
ANOVA test followed by Dunnett’s Test comparing each group to the control group, controlling for
multiple comparisons. Gene target prediction of miRNAs was performed using Targetscan and Diana-
Tools software (55, 56), selecting a score >85. For hierarchical cluster analysis, moderated F-test was used
to detect genes that are differentially expressed between any two groups (namely ctrl, miR-20a-3p, anti-
miR-186, and anti-miR-324-5p). 1031 genes were selected that had FDR < 0.05 in the F-tests.
Hierarchical cluster analysis was conducted based on the Euclidean distance of these selected genes. We
defined 8 clusters according to the hierarchical tree. All statistical analyses were performed using SAS
JMP (version 15), SPSS (version 20, IBM) and R.

Study Approval

The study was approved by the Mass General Brigham Research Committee (Massachusetts General
Hospital, Boston, MA, USA). All individuals provided written informed consent prior to inclusion in the
study.

Author Contributions:

SS: Study design, patient recruitment, data analysis, drafting and critical review of manuscript. Assigned
primary co-first author due to patient recruitment and conceptualization of the project.

RGM: Study design, data analysis, drafting and critical review of manuscript.
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**Figure 1:** Isolation of circulating sEV miRNAs correlating to HIV infection and lipodystrophy.

A) Diagram representing the sEV isolation method. Exosomes were isolated from HIV/Lipodystrophy, HIV/Non-Lipodystrophy, and Non-HIV participants (n=9 per group) by differential centrifugation protocol and subjected to qPCR-based miRNA profiling.

B) Heatmap showing the group average expression for each miRNA measured in serum. Red color indicates high expression, blue indicates low expression. Left column (Non-HIV, indicated by green box at top); Middle column (HIV Non-Lipo, indicated by blue box at top), HIV Non-Lipo; Right column, (HIV Lipo, indicated by pink box at top).

n=8-9 per group applies to all the panels in this figure.
Figure 2

(A) HIV/Non-Lipo vs. Non-HIV
- 30 miRNAs↑
- 27 miRNAs↓

(B) HIV/Lipo vs. HIV/Non-Lipo
- 33 miRNAs↑
- 35 miRNAs↓

(C) HIV/Lipo vs. Non-HIV
- 7 miRNAs↑
- 12 miRNAs↓

Reduction with HIV Lipodystrophy
- hsa-miR-20a-3p
- hsa-miR-412-3p

Reduction with HIV Non-Lipodystrophy
- hsa-miR-324-5p
- hsa-miR-512-3p

Elevation with HIV +/− Lipodystrophy
- hsa-miR-186-5p
- hsa-miR-542-5p

Non-HIV
HIV/Non-Lipo
HIV/Lipo

hsa-miR-20a-3p
hsa-miR-186-5p
hsa-miR-324-5p
hsa-miR-542-5p

Normalized miRNA expression

Non-HIV
HIV/Non-Lipo
HIV/Lipo

hsa-miR-20a-3p
hsa-miR-186
hsa-miR-324-5p

Three asterisks (***), one asterisk (*), and two asterisks (**) indicate statistical significance.
Figure 2: Circulating sEV miRNAs correlating to HIV infection and lipodystrophy.

A) Volcano plots representing the fold change and statistical significance for circulating sEV miRNAs for each of the group comparisons. The top regulated miRNAs are highlighted. The numbers in the rectangles indicate the number of upregulated and downregulated exosomal miRNAs (P<0.05). The three miRNAs further studied (miR-20a-3p, miR-186 and miR-324-5p) are highlighted in a red circle.

B) Heatmap showing the abundance of the top regulated circulating sEV miRNAs shown in B in the conditions stated on the right side. High expression as indicated by normalized Ct is shown in red and low expression in blue. Each column represents an individual sample.

C) Normalized sEV miRNA expression obtained from miRNA profiling in each group for miR-20a-3p, miR-186 and miR-324-5p.

Data are expressed as mean ±SEM. *P<0.05, **P<0.01, ***P<0.001. n=5-9 per group applies to all the panels in this figure, statistical comparison in A-C is assessed by Limma analysis.
**Figure 3:** Expression of miR-20a-3p in alternative models of reduced *Dicer* expression

A) Normalized sEV miRNA expression for miR-20a-3p in adipose specific *Dicer* KO (a*Dicer* KO) mice vs. control.

B) Normalized sEV miRNA expression for miR-20a-3p in adipocytes transfected with *Dicer* siRNA vs. control.

Data are expressed as mean ± SEM. *P<0.05. n=5 sera pools for control mice group (each pool derives from sera of 3 mice), n=3 sera pools for a *Dicer* KO mice group (each pool derives from sera of 3 mice), n=3 per group applies for panel B. N.D., not detected.
Figure 4

A

Transfection with either:
1 day before induction (day +5)

- non-targeting control miRNA
- mimic miR-20a-3p
- anti-miR-186
- anti-miR-324-5p

Human preadipocytes
(abdominal sc)

Start preinduction
(day 0)

Human adipocytes

Start induction
(day 6)

RNA isolation
(day 18)

B

control vs
mimic miR-20a-3p
control vs
anti-miR-186

control vs
anti-miR-324-5p

C

control
mimic miR-20a-3p
anti-miR-186
anti-miR-324-5p

Gene expression changes:

- Upregulated genes
- Downregulated genes

Gene expression matrix with fold changes.
**Figure 4:** Overexpression of miR-20a-3p and inhibition of miR-186 and miR-324-5p altered transcriptome of differentiating adipocytes.

A) Diagram representing the experimental setup.

B) Venn-diagram showing the number of genes upregulated (red) and downregulated (blue) for each comparison and the overlap among them.

C) Heatmap showing the commonly regulated genes by all treatments from the center of the Venn diagram in B. Red indicates high expression, blue indicates low expression.

n=4 per group in all the panels, statistical comparisons in B-C assessed by Limma analysis.
Figure 5

A

mimic miR-20a-3p vs control

anti-miR-186 vs control

anti-miR-324-5p vs control

Z-score adjusted log$_2$ CPM

B

gene expression (over control)

Ltpbp2

Neb1

Wisp2

* * *
**Figure 5:** Overexpression of miR-20a-3p and inhibition of miR-186 and miR-324 of differentiating adipocytes demonstrates downregulation of *Ltbp2*, *Nebl*, and *Wisp2*.

A) Volcano plots illustrating the fold change and statistical significance for the profile of each comparison. The dashed line in each plot indicates P-value 0.05. The genes that are subject of further study (*Ltbp2*, *Nebl* and *Wisp2*) are highlighted in blue.

B) Bar graphs showing the degree of downregulation of *Ltbp2* (left), *Nebl* (middle) and *Wisp2* (right graph) induced by each of the treatments.

Data are expressed as mean ± SEM. *P<0.05. n=4 per group in all the panels, statistical comparisons in A-B assessed by Limma analysis.
Figure 6: *Ltbp2* expression is downregulated in another cohort of HIV-infected patients with lipodystrophy

*Ltbp2* gene expression in the abdominal subcutaneous and dorsocervical adipose tissue depots from a cohort of HIV-infected patients without or with lipodystrophy.

n=11 for non-lipodystrophic patients, n=17 for lipodystrophic patients. Data are expressed as mean ± SEM. *P<0.05, **<0.01, statistical comparisons was assessed by Limma analysis
Figure 7

A. Transfection with either:
- non-targeting control siRNA
- Ltbp2 siRNA
- Nebl siRNA
- Wisp2 siRNA

1 day before induction (day +5)

Human preadipocytes
(abdominal sc)

Start preinduction
(day 0)

Start induction
(day 6)

RNA isolation
(day 18)

Human adipocytes

B. Differentiation markers

<table>
<thead>
<tr>
<th>Gene</th>
<th>control</th>
<th>Ltbp2 siRNA</th>
<th>Nebl siRNA</th>
<th>Wisp2 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabp4</td>
<td></td>
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<tr>
<td>PPARγ</td>
<td></td>
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<tr>
<td>C/EBPα</td>
<td></td>
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<tr>
<td>Fasn</td>
<td></td>
<td></td>
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<td>Adiponectin</td>
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<td>Glut4</td>
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<tr>
<td>CD36</td>
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<tr>
<td>Srebp1c</td>
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C. Lamins

<table>
<thead>
<tr>
<th>Lamin A</th>
<th>Lamin C</th>
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D. Inflammatory markers

<table>
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<tr>
<th>Gene</th>
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<th>Ltbp2 siRNA</th>
<th>Nebl siRNA</th>
<th>Wisp2 siRNA</th>
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<tbody>
<tr>
<td>IL1β</td>
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<td>TNF</td>
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<td>IL6</td>
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<tr>
<td>CCL20</td>
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<td></td>
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</tr>
<tr>
<td>CXCL5</td>
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<tr>
<td>CXCL1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 7: *Ltbp2* regulates adipocyte differentiation, *Lamin C* expression and inflammation

A) Diagram representing the experimental setup.

B) Effect of the treatment of preadipocytes with negative control-, *Ltbp2-, Nebl-,* and *Wisp2*- siRNA on the indicated differentiation markers.

C) Effects of the indicated treatments on the expression of *Lamin A* and *Lamin C*.

D) Effects of the indicated treatments on the expression of the inflammatory markers shown below.

Data are expressed as mean ±SEM. *P<0.05, **<0.01. n=8 in all panels, statistical comparisons in B-D was performed by ANOVA followed by Dunnett’s Test comparing individual groups to control.
A

Control siRNA

Control siRNA (zoomed in)

Ltbp2 siRNA

Nebl siRNA

Wisp2 siRNA

B

ORO+ area (as percentage of total area)

control
Ltbp2 siRNA
Nebl siRNA
Wisp2 siRNA

Figure 8
Figure 8: Knockdown of *Ltbp2* demonstrates reduced adipocyte differentiation

A) Histologic images of adipocytes treated with *Ltbp2*, *Neb1*, and *Wisp2* siRNA stained with oil-red-O

B) Quantitative measure of oil-red-O staining as a percentage of total area

Scale bar: 100 μm.

n=4, data are expressed as mean ± SEM. *P<0.05, **P<0.001 statistical comparisons was performed by ANOVA followed by Dunnett’s Test comparing individual groups to control.
Accessory proteins of the Human Immunodeficiency Virus (HIV) may suppress Dicer expression in the adipose tissue. Lack of Dicer in adipose tissue leads to lipodystrophic changes in fat redistribution and significant metabolic dysregulation and alteration of the levels of circulating exosomal miRNAs, i.e. sEV-carried miR-20a-3p is upregulated and miR-324-5p and miR-186 are down regulated in serum taken from lipodystrophic patients with HIV. These sEV would target preadipocytes inducing downregulation of Ltbp2, Nebl, Wisp2, which would subsequently impair adipocyte differentiation, inflammation and Lamin C expression. Thus, reduced dicer expression in the adipose among HIV-infected patients may contribute to an altered miRNA signature and adipocyte differentiation and inflammation, which could have potential clinical implications relevant to HIV lipodystrophy. Other tissues affected in HIV lipodystrophy, such as the heart, liver, and muscle, could also be relevant targets of this pathway.
Table 1. Baseline demographic and clinical characteristics among HIV/lipodystrophy, HIV/Non-lipodystrophy, and non-HIV participants

<table>
<thead>
<tr>
<th></th>
<th>Non-HIV (n=9)</th>
<th>HIV Non-Lipodystrophic (n=9)</th>
<th>HIV Lipodystrophic (n=9)</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>55±2</td>
<td>52±3</td>
<td>56±2</td>
<td>0.43</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
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<tr>
<td>Caucasian</td>
<td>78</td>
<td>67</td>
<td>78</td>
<td>0.68</td>
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<tr>
<td>African American</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td></td>
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<tr>
<td>Other</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td></td>
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<tr>
<td><strong>HIV Parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration HIV (years)</td>
<td>N/A</td>
<td>18±3</td>
<td>24±2</td>
<td>0.07</td>
</tr>
<tr>
<td>Duration ART use (years)</td>
<td>N/A</td>
<td>11±2</td>
<td>20±2</td>
<td>0.007</td>
</tr>
<tr>
<td>Duration PI use (years)</td>
<td>N/A</td>
<td>9±2</td>
<td>16±3</td>
<td>0.08</td>
</tr>
<tr>
<td>Duration NRTI use (years)</td>
<td>N/A</td>
<td>11±2</td>
<td>16±1</td>
<td>0.08</td>
</tr>
<tr>
<td>Duration NNRTI (years)</td>
<td>N/A</td>
<td>9±4</td>
<td>10±1</td>
<td>0.95</td>
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<tr>
<td>CD4 cell count (#/mm3)</td>
<td>N/A</td>
<td>681±90</td>
<td>482±90</td>
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<tr>
<td>CD8 cell count (#/mm3)</td>
<td>N/A</td>
<td>1260±164</td>
<td>725±115</td>
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<tr>
<td>Undetectable viral load (%)</td>
<td>N/A</td>
<td>78</td>
<td>67</td>
<td>0.60</td>
</tr>
<tr>
<td>HIV RNA viral load (copies/mL, log10)</td>
<td>N/A</td>
<td>1.28 (1.28, 1.30)</td>
<td>1.28 (1.28, 1.41)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Body Composition Parameters</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.8±1.3</td>
<td>29.5±1.5</td>
<td>29.9±1.3</td>
<td>0.98</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>103.4±3.9</td>
<td>108.0±4.4</td>
<td>108.1±3.7</td>
<td>0.65</td>
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<tr>
<td>Abdominal VAT area (cm²)</td>
<td>216.7±35.2</td>
<td>229.2±33.2</td>
<td>260.1±34.3</td>
<td>0.67</td>
</tr>
<tr>
<td>Abdominal SAT area (cm²)</td>
<td>281.0±35.7</td>
<td>308.1±30.8</td>
<td>300.7±46.4</td>
<td>0.88</td>
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<tr>
<td>DCAT area (cm²)</td>
<td>6.6±0.8</td>
<td>7.1±0.7</td>
<td>12.5±1.3</td>
<td>0.004</td>
</tr>
<tr>
<td>-----------------</td>
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</table>

**Metabolic Parameters**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory Quotient</td>
<td>0.72±0.03</td>
<td>0.82±0.03</td>
<td>0.81±0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>122±24</td>
<td>141±18</td>
<td>149±24</td>
<td>0.69</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>51 (41, 71)</td>
<td>42 (34, 46)</td>
<td>49 (35, 66)</td>
<td>0.19</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>107 (89, 153)</td>
<td>114 (88, 146)</td>
<td>79 (57, 136)</td>
<td>0.36</td>
</tr>
<tr>
<td>Serum Insulin (IU/mL)</td>
<td>8.0 (4.0, 9.5)</td>
<td>7.7 (5.8, 10.0)</td>
<td>9.9 (4.0, 11.3)</td>
<td>0.56</td>
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<tr>
<td>HOMA-IR</td>
<td>1.61 (0.81, 2.29)</td>
<td>1.67 (1.35, 2.06)</td>
<td>1.98 (0.78, 2.18)</td>
<td>0.97</td>
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</tbody>
</table>

**Adipose Tissue Gene Expression**

<table>
<thead>
<tr>
<th>Dicer^A</th>
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<tbody>
<tr>
<td></td>
<td>17.69 (10.72, 47.91)</td>
<td>11.20 (4.83, 21.45)</td>
<td>2.49 (0.02, 4.88)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Normally distributed data reported as mean ± standard error of the mean; Non-normally distributed data reported as median (interquartile range). Overall P value obtained by ANOVA or Kruskal-Wallis Test.

^Gene expression values are normalized to TBP, results are expressed as ratios in arbitrary units.

Abbreviations: ART, antiretroviral therapy; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; N/A, non-applicable; BMI, body mass index; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; DCAT, dorsocervical adipose tissue; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance
### Table 2. Correlations between small extracellular vesicles miRNAs among all participants

<table>
<thead>
<tr>
<th></th>
<th>miR-20a-3p</th>
<th></th>
<th>miR-324-5p</th>
<th></th>
<th>miR-186</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P-Value</td>
<td>ρ</td>
<td>P-Value</td>
<td>ρ</td>
<td>P-Value</td>
</tr>
<tr>
<td>miR-20a-3p</td>
<td>-0.71</td>
<td>0.002</td>
<td>-0.74</td>
<td>0.002</td>
<td></td>
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<tr>
<td>miR-324-5p</td>
<td></td>
<td></td>
<td>0.54</td>
<td>0.006</td>
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<tr>
<td>miR-186</td>
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</tbody>
</table>

Relationships determined by Spearman's Correlation Coefficient.
Table 3. Correlations between small extracellular vesicles miRNA and metabolic parameter among all participants

<table>
<thead>
<tr>
<th></th>
<th>miR-20a-3p</th>
<th></th>
<th>miR-324-5p</th>
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<th>miR-186</th>
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</thead>
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<tr>
<td></td>
<td>ρ</td>
<td>P-Value</td>
<td>ρ</td>
<td>P-Value</td>
<td>ρ</td>
<td>P-Value</td>
</tr>
<tr>
<td>Adipose Parameters</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Abdominal SAT (cm²)</td>
<td>-0.09</td>
<td>0.73</td>
<td>-0.25</td>
<td>0.25</td>
<td>-0.14</td>
<td>0.95</td>
</tr>
<tr>
<td>Abdominal VAT (cm²)</td>
<td>0.40</td>
<td>0.12</td>
<td>-0.18</td>
<td>0.41</td>
<td>-0.08</td>
<td>0.71</td>
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<tr>
<td>DCAT (cm²)</td>
<td>0.64</td>
<td>0.007</td>
<td>-0.71</td>
<td>0.0001</td>
<td>-0.64</td>
<td>0.001</td>
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<tr>
<td>Adipose Dicer Expression</td>
<td>-0.61</td>
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<td>0.39</td>
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<td>0.53</td>
<td>0.008</td>
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<td>Metabolic Parameters</td>
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<tr>
<td>Respiratory Quotient</td>
<td>0.64</td>
<td>0.008</td>
<td>-0.33</td>
<td>0.10</td>
<td>-0.25</td>
<td>0.24</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>0.52</td>
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<td>0.11</td>
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<td>HDL (mg/dL)</td>
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<td>-0.05</td>
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<td>0.23</td>
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<tr>
<td>LDL (mg/dL)</td>
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<td>Serum Insulin (IU/mL)</td>
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<td>0.005</td>
<td>-0.31</td>
<td>0.13</td>
<td>-0.27</td>
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<tr>
<td>HOMA-IR</td>
<td>0.50</td>
<td>0.047</td>
<td>-0.16</td>
<td>0.45</td>
<td>-0.16</td>
<td>0.46</td>
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<tr>
<td>HIV-Related Parameters</td>
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</tr>
<tr>
<td>Log₁₀ HIV Viral Load</td>
<td>0.51</td>
<td>0.11</td>
<td>-0.18</td>
<td>0.51</td>
<td>0.34</td>
<td>0.22</td>
</tr>
<tr>
<td>CD4+ Cell Count (#/mm³)</td>
<td>-0.02</td>
<td>0.96</td>
<td>0.05</td>
<td>0.86</td>
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<td>0.04</td>
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<tr>
<td>CD8+ Cell Count (#/mm³)</td>
<td>-0.10</td>
<td>0.79</td>
<td>0.29</td>
<td>0.28</td>
<td>0.48</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Relationships determined by Spearman's Correlation Coefficient

Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; DCAT, dorsocervical adipose tissue; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance