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## Lipid Metabolism in Patients Infected with Nef-deficient HIV-1 Strain

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### Abstract

**Background**—HIV protein Nef plays a key role in impairing cholesterol metabolism in both HIV infected and bystander cells. The existence of a small cohort of patients infected with Nef-deficient strain of HIV presented a unique opportunity to test the effect of Nef on lipid metabolism in a clinical setting.

**Methods**—Here we report the results of a study comparing six patients infected with Nef-deficient strain of HIV ( NefHIV) with six treatment-naïve patients infected with wild-type HIV (WT HIV). Lipoprotein profile, size and functionality of high density lipoprotein (HDL) particles as well as lipidomic and microRNA profiles of patient plasma were analyzed.

**Results**—We found that patients infected with NefHIV had lower proportion of subjects with plasma HDL-C levels <1 mmol/l compared to patients infected with WT HIV. Furthermore, compared to a reference group of HIV-negative subjects, there was higher abundance of smaller under-lipidated HDL particles in plasma of patients infected with WT HIV, but not in those infected with NefHIV. Lipidomic analysis of plasma revealed differences in abundance of

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#### Conflict of interests

The authors have no conflict of interest to disclose.

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phosphatidylserine and sphingolipids between patients infected with NefHIV and WT HIV. MicroRNA profiling revealed that plasma abundance of 24 miRNAs, many of those involved in regulation of lipid metabolism, was differentially regulated by WT HIV and NefHIV.

**Conclusion**—Our findings are consistent with HIV protein Nef playing a significant role in pathogenesis of lipid-related metabolic complications of HIV disease.

## Keywords

HIV; Lipoproteins; Lipids; HDL Metabolism; Dyslipidemias; MicroRNA; Nef

## 1. Introduction

HIV disease is characterized by severe metabolic complications including dyslipidemia and atherosclerosis [1, 2]. Adverse side-effects of antiretroviral regimens were originally blamed for these complications, however, as development of better treatment regimens with reduced effect on lipid metabolism did not eliminate dyslipidemia [3] and high risk of atherosclerosis [4], it is becoming increasingly clear that HIV disease itself makes a substantial contribution to the pathogenesis of these complications. We have demonstrated that HIV protein Nef inhibits cholesterol efflux causing cholesterol accumulation in HIV-infected macrophages [5]. The same effect was observed in uninfected cells treated with recombinant Nef or with plasma containing soluble Nef released from infected cells [6]. Furthermore, recombinant Nef injected in mice in vivo caused atherosclerosis and dyslipidemia supporting a key role of Nef in pathogenesis of HIV-associated metabolic abnormalities [7]. However, no clinical evidence supporting the role of Nef in lipid dysregulation is available.

In this study we analyzed plasma samples of six patients infected with Nef-deficient strain of HIV-1 (NefHIV), all members of the Sydney Blood Bank Cohort (SBBC). Pathogenicity and immunogenicity of this strain have been described in previous publications [8–11] and summarized in a recent review [12]. In brief, all patients were infected with the same strain of HIV-1 through blood transfusion; they were slow-progressors or non-progressors and remained asymptomatic for an extended period of time [12]. Lipid metabolism in these patients was never investigated and, considering a potentially key role of Nef in the pathogenesis of HIV related impairment of lipid metabolism, these patients provided a unique opportunity to elucidate the role of Nef in HIV-associated metabolic disorders in a clinical setting.

## 2. Methods

### 2.1. Patients

Patients infected with Nef-deficient strain of HIV-1 (NefHIV, n=6) were all members of the SBBC cohort. Clinical and immunological parameters of these patients were originally described in several publications [8–11]; age, sex, CD4+ cell count and viral load values for these patients are shown in Table 1 in comparison to the same parameters in WT HIV subjects. All patients, except patient D36, were not receiving antiretroviral therapy. Two subjects (C49, C64) were postmenopausal females, all other patients were males.

The NefHIV-infected patients were matched with patients infected with Nef-positive (WT) HIV-1 strain. Patients infected with Nef-positive strain of the virus (n=6) were selected from a cohort of treatment-naïve HIV patients described in our previous study [13]. All subjects were males. Reference values for HIV-negative subjects were obtained by analyzing plasma samples of six HIV negative subjects (all males) selected from a group of healthy volunteers from the blood bank of the Baker IDI Heart and Diabetes Institute.

All subjects were not undergoing any lipid-lowering therapy and did not have a history of cardiovascular disease. All samples were stored at  $-80^{\circ}\text{C}$  and were analyzed retrospectively.

Plasma samples of both groups of HIV-infected patients used in this study were from the previous studies [9, 13]; original human ethics approvals permitted for the extension of analysis of the collected samples.

**Lipoprotein profile**—Total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were analyzed using Cobas blood analyser. Apolipoprotein A-I and apolipoprotein B concentrations were analyzed using ELISA kits (Mabtech, Sweden).

## 2.2. HDL size

Distribution of apoA-I among HDL subfractions was analyzed by non-denaturing PAGE followed by immunoblotting using antibody against human apoA-I as described previously [14]. The following definitions of HDL subfractions were used: HDL<sub>3c</sub>, 7.2–7.8 nm; HDL<sub>3b</sub>, 7.8–8.2 nm; HDL<sub>3a</sub>, 8.2–8.8 nm; HDL<sub>2a</sub>, 8.8–9.7 nm; HDL<sub>2b</sub>, 9.7–12 nm.

## 2.3. Cholesterol efflux

Cholesterol efflux assay was performed using THP-1 human macrophages activated with LXR agonist as described previously [15]. 1% plasma or 1.1% apoB-depleted plasma (obtained as described previously [15]) were used as an acceptor.

## 2.4. Lipidomic analysis

Lipidomic analysis was performed as described previously [16]. In brief, lipids from 10  $\mu\text{l}$  of plasma were extracted using a modified, single phase Folch method; the analysis was done in triplicates. The analysis was performed by liquid chromatography electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) using a Agilent 1200 liquid chromatography system, and Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ion spray source ( $350^{\circ}\text{C}$ ) and Analyst 1.5 and MultiQuant data systems using a Zorbax C18, 1.8  $\mu\text{m}$ ,  $50 \times 2.1\text{-mm}$  column (Agilent Technologies). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding internal standard.

## 2.5. Small RNA deep sequencing and bioinformatics analysis

RNA from plasma was extracted using the miRNeasy Mini Kit (Qiagen, Australia). The manufacturers' protocol was followed with a slight modification involving the use of Trizol LS (Life Technologies, Australia). The small RNA yield, composition and quality was analyzed using the Agilent 2100 Bioanalyser with the Small RNA kit (Agilent

Technologies). Sequencing adapters were ligated onto the small RNA sample followed by conversion into cDNA libraries using the Ion Total RNA-Seq Kit V2 (Life Technologies, Australia) and prepared for deep sequencing on the Ion Torrent Personal Genome Machine (PGM™). Pooled libraries with unique barcodes were loaded on 318™ sequencing chips and run on the Ion Torrent PGM (Life Technologies, Australia) using the Ion PGM™ 200 Sequencing Kit (Life Technologies). The Torrent Suite 4.2.1 was used to manage the Ion Torrent PGM™ to process raw signals and perform base calling. The sequences were then assessed for quality, and primer-adaptor sequences were trimmed by the Torrent Suite software, followed by alignment to the human reference genome (HG19) using bowtie 2 followed by a second pass through TMAP using Partek Flow. The trimmed and aligned data was mapped to known miRNA using miRBase V.20. The number of reads for each miRNA was adjusted to reads per million (RPM) and normalized using the lognormal linear method across all samples. Samples containing less than 5 reads per million were removed. The data was then transferred to Partek Genomics Suite for statistical analysis, hierarchical clustering and to identify unique miRNA in each sample type. TargetScan, mirDB and TarBase were interrogated to predict targets for the selected miRNAs. Raw sequencing files in .BAM format for each sample sequenced can be downloaded from <http://www.ebi.ac.uk/ena/data/view/PRJEB7984>

### 3. Results

#### 3.1. Plasma lipoproteins

Patient plasma lipoprotein and apolipoprotein levels are shown in Table 1. Total cholesterol, triglyceride and apolipoprotein B levels were similar between the groups, while LDL-C levels trended higher in WT HIV group. In comparison to a reference group of HIV-negative subjects level of HDL-C was reduced by 25% in WT HIV infected patients, but only by 8% in NefHIV infected subjects, however, due to limited power of the study these substantial differences did not reach statistical significance. However, when we defined hypoalphalipoproteinemia (HALP) as HDL-C below 1 mmol/l, the proportion of subjects with HALP was significantly lower among patients infected with NefHIV compared to those in WT HIV group (chi-square,  $P=0.05$ ). Levels of apoA-I were similar in both HIV-infected groups, consequently, the HDL-C/apoA-I ratio, which may be considered a surrogate marker of HDL lipidation, was 20% higher in NefHIV patients, but again, the difference was not statistically significant.

#### 3.2. HDL structure and functionality

We previously demonstrated that Nef reduces abundance and functionality of ABCA1 [5, 6, 17]; the most likely consequence of this effect at systemic level would be changes in lipidation of HDL, which, in turn, may affect HDL functionality. An indication of this was a higher ratio of HDL-C/apoA-I in NefHIV patients compared to HIV patients (Table 1). We therefore analyzed the distribution of HDL among particles of different sizes. In patients infected with WT HIV the proportion of large HDL<sub>2b</sub> particles (9.7–12 nm) was reduced relative to that in the reference group of uninfected subjects, whereas this effect was partially eliminated in patients infected with NefHIV (Fig. 1A). The proportion of smaller HDL<sub>3a</sub> particles (8.2–8.8 nm) was increased in plasma of WT HIV infected patients, but not

in patients infected with NefHIV. Proportion of HDL<sub>3b</sub> particles (7.8–8.2 nm) was increased in plasma of patients from both WT HIV and NefHIV groups, while the proportion of smallest HDL<sub>3c</sub> particles (7.2–7.8 nm) was not affected by HIV status. Thus, HIV infection reduced the proportion of large fully lipidated HDL particles and increased the proportion of smaller under-lipidated HDL, this effect was partially eliminated in patients infected with NefHIV.

To investigate the effect of these changes in HDL structure on HDL functionality, the ability of whole plasma and of HDL to support cholesterol efflux from human macrophages was tested. Macrophages were activated with LXR agonist to ensure that most of the efflux represents specific ABCA1/G1-dependent cholesterol efflux. When the efflux to whole plasma was tested, we found no statistically significant difference between the groups in the capacity of plasma to support cholesterol efflux (Fig. 1B). Next, we measured the efflux to plasma depleted of apoB-containing lipoproteins (*i.e.* plasma where HDL is the only available lipoprotein). We found that while there was no difference between the efflux to apoB-depleted plasma from HIV-negative subjects and subjects infected with NefHIV, cholesterol efflux to plasma from WT HIV group was higher (Fig. 1C). We hypothesized that increased cholesterol efflux to HDL from WT HIV-infected subjects was due to increased proportion of small HDL<sub>3a</sub> particles, and indeed when data for all three groups were combined, there was a correlation between the capacity of HDL to support cholesterol efflux and proportion of HDL<sub>3a</sub> particles ( $r=0.50$ ;  $p<0.05$ ).

### 3.3. Lipidomic profiling

We have previously reported the effects of HIV infection on plasma lipidomic profile [16]. Using a similar approach, we compared lipidomic profile of patients infected with WT or Nef HIV. The full lipidomic profile (330 lipid species) is provided in the accompanying Data in Brief article [18], and lipid species significantly different between the groups are shown in Table 2. Compared to the reference group of uninfected subjects, three species of phosphatidylserine (PS) and PS as a group were reduced in WT HIV group, but not in NefHIV group. Lipid species affected in NefHIV group but not affected in WT HIV group, include individual species of dihydroceramide (dhCer), ceramide, alkylphosphatidylethanolamine, phosphatidylethanolamine (PE) and two species of sphingomyelin. Individual species of phosphatidylinositol and dhCer were increased in both HIV infected groups. One species each of dihexosylceramide, PE and PS were only increased in NefHIV group.

When data for all three groups were combined, there was positive correlation between the proportion of HDL<sub>2a</sub> particles (which was similar in the three groups) and plasma abundance of phosphatidylcholine ( $r=0.69$ ,  $p<0.002$ ), cholesterol ( $r=0.54$ ,  $p<0.05$ ), alkenylphosphatidylcholine ( $r=0.47$ ,  $p<0.05$ ) and phosphatidylethanolamine ( $r=0.50$ ,  $p<0.05$ ); all these lipid species are major constituents of large lipidated HDL particles. There was also negative correlation between cholesterol efflux to whole plasma and the abundance of cholesteryl esters ( $r=-0.50$ ,  $p<0.05$ ) and ceramide ( $r=-0.45$ ,  $p<0.05$ ).

### 3.4. miRNA profiling

Abundance of small RNAs extracted from plasma samples was analyzed by deep sequencing. RNA from two patients infected with NefHIV did not pass quality control testing and were not sequenced. Unsupervised hierarchical clustering was performed on significantly differentially expressed miRNA using Euclidean average linkage by miRNA. The full dataset is presented in Supplemental Dataset 1, and differentially expressed miRNAs are shown in Fig. 2. Twenty-four miRNAs were found to be significantly deregulated in patients infected with WT HIV compared to the NefHIV groups. These 24 miRNAs were observed to show similar or recovered expression levels within NefHIV and uninfected subject groups but were significantly deregulated in HIV patients. There are two major nodes of the dendrogram. Node 1 contains 4 miRNAs which were found to be up-regulated in WT HIV group. Node 2 contains 20 miRNA which were found to be down-regulated in WT HIV group. Predicted and validated targets for these miRNAs that are related to lipid metabolism and/or HIV infection are listed in Table 3.

## 4. Discussion

Metabolic complications are an important part of HIV disease and include impairment of cellular and systemic lipid metabolism and enhanced development of atherosclerosis. We have demonstrated the key role of HIV protein Nef in disturbances of cellular cholesterol metabolism caused by HIV infection [5]. Nef is an HIV accessory protein expressed early in infection; it is located on the plasma membrane of infected cells (for review see [19]) and is also released from infected cells. The documented effects of released Nef on uninfected cells include apoptosis [20], endothelial dysfunction [21], impairment of immune response [22], and impairment of cholesterol metabolism. The effects of extracellular Nef on lipid metabolism in bystander cells include down regulation of ABCA1 and inhibition of cholesterol efflux in vitro and hypoalphalipoproteinemia and hypertriglyceridemia in vivo [6, 7]. Furthermore, Nef affects the spectrum of miRNAs released from infected cells in exosomes, including several miRNAs with established role in cholesterol metabolism, such as miR-33a\*, miR-16b, miR-145, miR-144\* [23]. Collectively, these findings suggest that Nef released from infected cells may be an important factor in pathogenesis of metabolic complications of HIV disease, but this concept was never tested in a clinical setting. Patients infected with NefHIV provided a unique opportunity to close this gap.

The main finding of this study is a lower prevalence of hypoalphalipoproteinemia in patients infected with NefHIV compared to patients infected with WT HIV. Furthermore, the proportion of smaller, presumably immature, HDL was increased in WT HIV infected patients similar to what was documented for SIV infection [6]. This effect was not observed in patients infected with NefHIV. The HDL-C/apoA-I ratio trended to be higher in NefHIV infected patients, also suggesting that HDL particles in these patients are more lipidated. These findings are consistent with our previous observations implicating Nef in reducing liver ABCA1 and impairing generation and/or maturation of HDL particles [6, 7]. There was no effect of HIV, with or without Nef, on the ability of whole plasma to support cholesterol efflux, which is consistent with our previous findings [3, 13]. However, when the efflux to HDL was measured, HDL from WT HIV group, but not from NefHIV group, had

higher functionality in cholesterol efflux assay. The most likely explanation of this finding is higher proportion of small, presumably under-lipidated HDL particles in plasma of WT HIV infected patients compared to two other groups; small HDL particles are better acceptors of cholesterol compared to larger fully lipidated HDL particles [24]. This finding is inconsistent with findings of another recent study where the efflux to apoB-depleted plasma from WT HIV infected patients was reduced [25]; however, patient demographics in this study was different. Our hypothesis, however, is that the primary effect of extracellular Nef is in reducing abundance of ABCA1 in both hepatic and extra-hepatic cells. In hepatocytes, reduction of ABCA1 would reduce plasma levels of HDL. In extra-hepatic cells, reduction of ABCA1 would reduce capacity of the cells for cholesterol efflux; contribution of the inhibition of ABCA1 in macrophages used in this study was not tested as cells were neither infected with HIV nor treated with Nef.

Lipidomic analysis of patient plasma showed a significant difference between WT HIV and uninfected subjects as well as between WT HIV and NefHIV groups in several species of phosphatidylserine. The lower level of phosphatidylserine in plasma from WT HIV group relative to NefHIV groups may reflect different circulating microparticle levels in these groups. Phosphatidylserine is a major lipid in the platelet plasma membrane and in platelet derived microparticles, thus the lower level observed in plasma of WT HIV group may reflect a role of Nef in suppressing platelet activation and/or microparticle production. Also of interest is the lipid species that show a significant difference between the uninfected group and the NefHIV group, but no difference between uninfected and WT HIV groups. These lipids include primarily sphingolipids (dihexosylceramide, ceramide and sphingomyelin) which were elevated in the NefHIV infected patients. We have previously reported that ceramides, while not associated with HIV itself, are positively associated with risk of future cardiovascular events in HIV infected individuals, and that sphingomyelin, which was negatively associated with WT HIV, also showed a positive association with future cardiovascular events [16].

Profiling of plasma miRNA showed that the abundance of 24 miRNAs was likely dependent on the presence of Nef. Predicted targets of these miRNAs included ABCA1 itself (hsa-miR-199b-3p) and two factors directly involved in regulation of ABCA1 abundance, ABCA12 (hsa-miR-409-3p) [26] and calnexin (hsa-miR-320a) [27]. MicroRNAs apparently regulated by Nef were also predicted to target a number of other ABC transporters (ABCA10, ABCA13, ABCB9), lipoprotein receptors (LDL receptor, VLDL receptor, several members of LDL receptor related protein family) and ligands (apolipoprotein A-II) and enzymes involved in lipid biosynthesis (members of long-chain acyl-CoA synthetase family, neutral sphingomyelinase). Fifteen of these miRNAs, or their close homologs, were also found to be regulated by Nef in exosomes released from Nef-transfected cells [23], although the direction of change was not always the same. Despite relatively large changes in the abundance of these miRNAs, this did not cause profound changes in plasma lipoprotein profile. However, the target cells of these miRNA are not necessarily the cells involved in regulation of plasma lipoprotein metabolism, but may be cells where changes in intracellular cholesterol metabolism play a role in pathogenesis of common complications of HIV, such as cells of vessel wall involved in atherosclerosis or  $\beta$ -cells involved in diabetes. Thus, within a limitation of analysis restricted to circulating miRNAs and the fact that not

all targets were validated, our findings are consistent with a hypothesis that alteration of expression and/or release of miRNAs may be an important mechanism by which HIV Nef dysregulates lipid metabolism in HIV disease.

## 5. Limitations

This study has several important limitations. First, the group size of NefHIV infected patients was small. Unfortunately, the cohort of these patients is unique and no more patients infected with NefHIV are known to us, therefore, this cohort could not be expanded. A change in the treatment guidelines shifting initiation of treatment to primary care also prevented expansion of the matching treatment-naïve WT HIV group. The NefHIV group included two female patients, but both were of postmenopausal age, therefore, this should not have greatly affected the results. Plasma samples from patients of NefHIV group were collected at a different time and by different operators and were stored for longer time compared to samples in WT HIV group, potentially contributing to the observed variability. Finally, infection with Nef-deficient HIV resulted in much lower viral loads and in a milder presentation of several elements of immunologic dysfunction compared to patients infected with WT HIV. This makes it difficult to distinguish between the direct effects of the deficiency of Nef and indirect effects of milder impairment of immunologic status and lower viral load. This compromise is difficult to avoid as viral loads similar to those in patients infected with NefHIV are only achievable after effective treatment with HAART, which would represent an even bigger confounding factor. Despite these limitations, the findings of this study support the hypothesis that Nef secreted from HIV infected cells plays, directly or indirectly, a major role in pathogenesis of metabolic complications of HIV disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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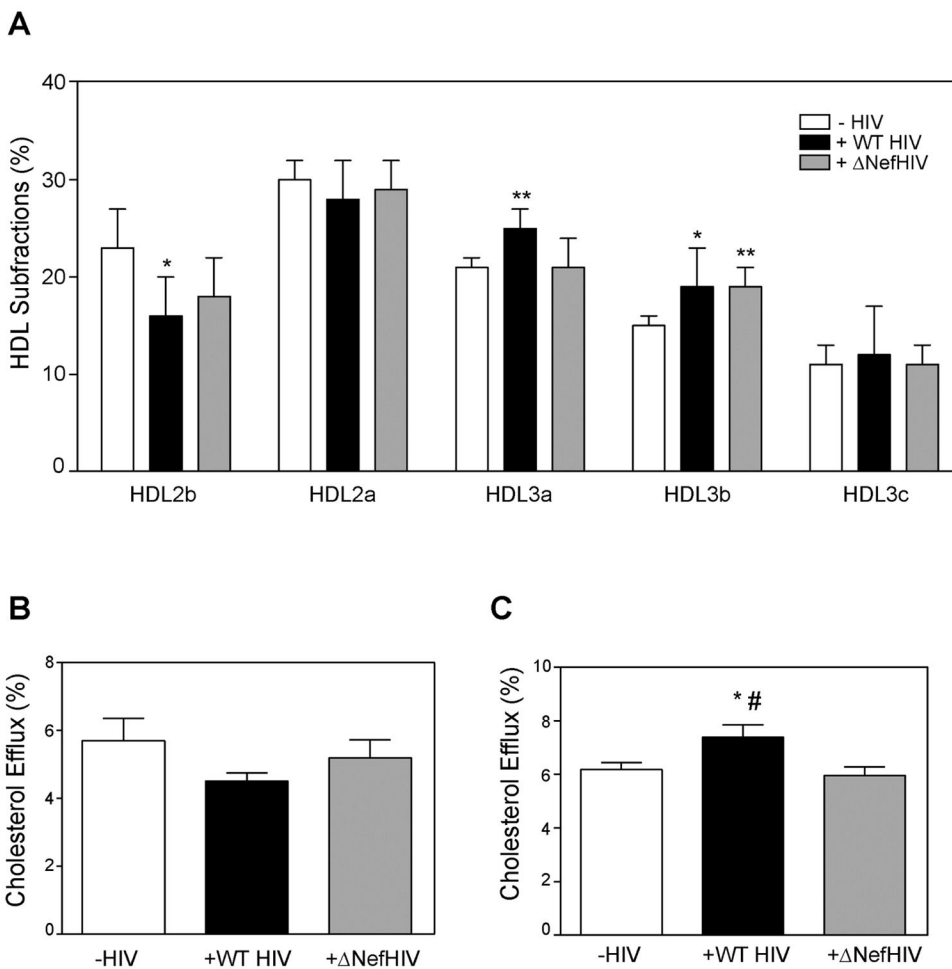


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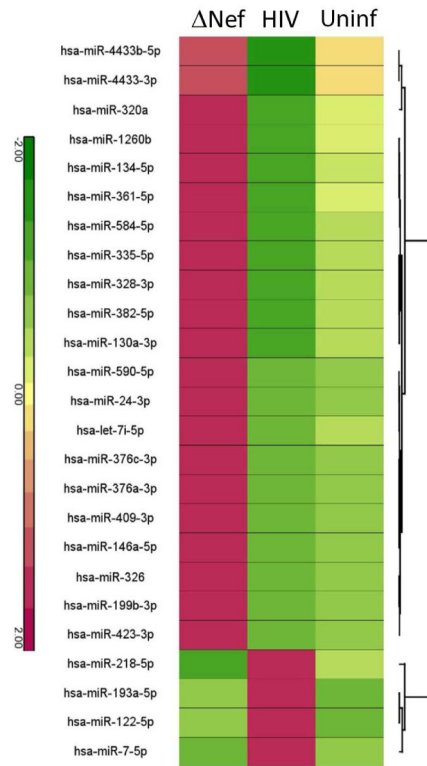
### Highlights

- Patients infected with Nef-deficient strain of HIV( NefHIV) have reduced prevalence of hypoalphalipoproteinemia
- Several lipid classes in plasma lipidomic profile are different in patients infected with WT HIV and NefHIV
- HIV induced changes in profile of lipid metabolism related microRNA are attenuated in NefHIV patients
- Findings are consistent with Nef playing a significant role in lipid abnormalities in HIV patients



**Figure 1. The effect of HIV and NefHIV infection on the structure (A) and function (B, C) of plasma high density lipoprotein**

**A** – Analysis of distribution of HDL among different subfractions in plasma of HIV-negative subjects and patients infected with WT HIV or NefHIV. \* $p < 0.05$ ; \*\* $p < 0.01$  versus HIV negative subjects. **B**- Cholesterol efflux from human THP-1 monocyte-macrophages to 1% plasma from HIV-negative subjects and patients infected with WT HIV or NefHIV. **C**- Cholesterol efflux from human THP-1 monocyte-macrophages to 1.1% apoB-depleted plasma from HIV-negative subjects and patients infected with WT HIV or NefHIV. \* $p < 0.05$  versus HIV-negative; # $p < 0.05$  versus NefHIV.



**Figure 2. Hierarchical clustering of differentially regulated miRNA in WT HIV and NefHIV patients**

Abundance of miRNAs that show similar or recovered expression levels within NefHIV patients and uninfected patients however significantly deregulated in HIV patients. Lines connect pairs with  $p < 0.01$ .

**Table 1**

Parameters of HIV Disease and Plasma Lipoprotein Profile

Subject	CD4+ (cells/ $\mu$ l)	Viral load ( $10^4$ /ml)	Age	Sex	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	TG (mmol/l)	apoA-I (g/l)	HDL-C/apoA-I	apoB (g/L)
<b>HIV Negative</b>											
C1	ND	-	40	M	4.7	2.9	1.4	0.8	2.19	0.64	0.7
C2	ND	-	37	M	4.9	2.7	1.3	2.1	1.75	0.74	0.9
C3	ND	-	39	M	4.0	2.3	1.4	0.7	1.86	0.75	0.8
C4	ND	-	44	M	4.2	1.1	1.5	3.6	2.24	0.67	0.4
C5	ND	-	42	M	3.9	2.3	1	1.2	1.76	0.57	0.8
C6	ND	-	42	M	4.3	2.8	1.2	0.8	1.86	0.65	0.9
<b>Mean<math>\pm</math>SD</b>	<b>ND</b>	<b>-</b>	<b>41<math>\pm</math>3</b>		<b>4.3<math>\pm</math>0.4</b>	<b>2.4<math>\pm</math>0.7</b>	<b>1.3<math>\pm</math>0.2</b>	<b>1.5<math>\pm</math>1.1</b>	<b>1.9<math>\pm</math>0.2</b>	<b>0.67<math>\pm</math>0.07</b>	<b>0.8<math>\pm</math>0.2</b>
<b>WT HIV</b>											
H1	588	0.07	50	M	4.2	2.5	0.8	1.8	1.2	0.67	0.7
H2	774	0.005	58	M	6.0	3.6	1.8	1.3	2.0	0.90	0.8
H3	400	8.4	51	M	4.5	2.6	1.5	0.7	1.8	0.83	0.6
H4	405	2.5	55	M	4.0	2.6	0.7	1.6	1.2	0.58	0.7
H5	190	10.0	52	M	4.7	3.5	0.6	1.1	1.1	0.55	0.8
H6	414	2.2	50	M	4.0	2.3	0.7	2.0	1.3	0.54	0.7
<b>Mean<math>\pm</math>SD</b>	<b>461<math>\pm</math>198</b>	<b>3.9<math>\pm</math>4.3</b>	<b>53<math>\pm</math>3</b>		<b>4.5<math>\pm</math>0.8</b>	<b>2.9<math>\pm</math>0.6</b>	<b>1.0<math>\pm</math>0.5</b>	<b>1.4<math>\pm</math>0.5</b>	<b>1.4<math>\pm</math>0.4*</b>	<b>0.68<math>\pm</math>0.15</b>	<b>0.7<math>\pm</math>0.1</b>
<b>NefHIV</b>											
C49	1006	0	44	F	3.4	2.1	0.7	1.8	1.2	0.58	0.8
C54V	1054	0.04	71	M	3.2	1.4	1.5	0.6	1.4	1.07	0.4
C64	850	0	73	F	5.7	3.1	2.0	1.3	1.9	1.05	0.9
C98	435	0.02	61	M	5.0	3.1	1.2	1.4	1.5	0.80	1.1
C135	480	0	53	M	4.2	2.6	1.1	0.8	1.2	0.92	0.8
D36	ND	ND	40	M	4.0	2.4	0.8	1.8	1.3	0.62	0.8
<b>Mean<math>\pm</math>SD</b>	<b>765<math>\pm</math>291<sup>#</sup></b>	<b>0.01<math>\pm</math>0.02<sup>#</sup></b>	<b>57<math>\pm</math>14</b>		<b>4.2<math>\pm</math>1.0</b>	<b>2.5<math>\pm</math>0.6</b>	<b>1.2<math>\pm</math>0.5</b>	<b>1.3<math>\pm</math>0.5</b>	<b>1.4<math>\pm</math>0.3*</b>	<b>0.84<math>\pm</math>0.21</b>	<b>0.8<math>\pm</math>0.2</b>

Means  $\pm$  SD are shown.

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\* p<0.02 vs HIV Negative (t-test);

# p<0.05 vs WT HIV (Rank Sum Test); ND – not determined

† Data for NeHIV group are taken from [9]; data for WT HIV group are taken from [13].

**Table 2**

## Lipidomic analysis

Lipid Species	HIV Negative	WT HIV	Nef-HIV
PS 36:1	1418.5 ± 935.8	259.5 ± 259.9**	7733.5 ± 9425.3 <sup>#</sup>
PS 38:3	299.8 ± 158.4	53.8 ± 91.9*	1559.4 ± 1791.7 <sup>#</sup>
PS 40:5	123.0 ± 65.5	13.6 ± 33.4**	590.9 ± 659.4 <sup>#</sup>
PS (total)	2161.5 ± 1317.7	334.2 ± 121.4*	10,772.2 ± 12,619.5 <sup>#</sup>
dhCer 20:0	20.8 ± 5.6	23.4 ± 9.1	115.7 ± 125.8*
Cer 18:0	80.4 ± 16	102.9 ± 26.6	140.7 ± 76.9*
SM 32:0	423.0 ± 84.8	512.0 ± 136.4	666.2 ± 176.0*
SM 34:0	4,369 ± 686.2	5,402.1 ± 1,442.6	6,326 ± 803.1**
PE(O-36:6)	34.3 ± 10.4	71.7 ± 44.9	95.4 ± 32.5**
dhCer (total)	519.0 ± 77.7	571.9 ± 254.3	1439.2 ± 1033.8*
PI 34:0	153.5 ± 58.0	362.2 ± 298.8*	342.8 ± 287.7
dhCer 16:0	46.3 ± 6.7	71.5 ± 15.7*	120.7 ± 70.5**
dhCer 18:0	46.1 ± 11.4	79.6 ± 22.0*	194.1 ± 184.3*
DHC 20:0	111.3 ± 38.4	85.0 ± 38.6	256.2 ± 249.5 <sup>#</sup>
PE(22:6/0:0)	1378.0 ± 402.6	1723.5 ± 836.7	1042.8 ± 168.4 <sup>#</sup>
PS 40:6	128.4 ± 53.9	36.6 ± 49.7	572.1 ± 619.6 <sup>#</sup>

Abbreviations: dhCer, dihydroceramide; Cer, ceramide; DHC, dihexosylceramide; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin;

\* p < 0.05 vs HIV Negative;

\*\* p < 0.01 vs HIV Negative;

<sup>#</sup> p < 0.05 vs WT HIV (ANOVA)



Table 3

Selected predicted targets of miRNAs depended on presence of Nef

microRNA	Predicted targets	Validated targets	Gene name	Reference to HIV
hsa-miR-199b-3p	ABCA1		ATP-binding cassette transporter A1	Many
	ABCA13		ATP binding cassette, transporter A13	
	LRP2		LDL receptor-related protein 2	
hsa-miR-409-3p	ABCA12		ATP-binding cassette, transporter A12	Many
	ABCB10		ATP-binding cassette, transporter B10	
	LRP		LDL receptor-related protein	
hsa-miR-320a	CANX		Calnexin	PMID:22412921
	XPO1/CRM1		Exportin-1	PMID: 25170080
				PMID: 22318151
hsa-miR-24-3p	ABCB9	ABCB9	ATP-binding cassette transporter B9	
hsa-miR-382-5p	LRP12		LDL receptor-related protein 12	
hsa-miR-130a-3p	LRP8		LDL receptor-related protein 8	
	LDLR	LDLR	LDL Receptor	PMID:24191141
	ACSL4		acyl-CoA synthetase long-chain family member 4	
hsa-miR-4433b-5p	PLCB1		phospholipase C, beta 1	PMID:23555755
	CD69		CD69	PMID:25340508
	VLDLR		VLDL Receptor	PMID:25394062
hsa-miR-7-5p	SMPD3		Neutral sphingomyelinase II	
	APOA2		Apolipoprotein A2	PMID:21978398
	IRS2	IRS2	Insulin receptor substrate 2	PMID:15590971
hsa-miR-218-5p	ACSL4	ACSL4	acyl-CoA synthetase long-chain family member 4	
	IDE	IDE	insulin-degrading enzyme	PMID:17026490
	ACSL1		acyl-CoA synthetase long-chain family member 1	
hsa-miR-376c-3p	VLDLR		VLDL receptor	PMID:25394062
	ACSL1		acyl-CoA synthetase long-chain family member 1	
	ALCAM		activated leukocyte cell adhesion molecule	PMID25420915
hsa-miR-590-5p	IL12A		Interleukin 12A	PMID: 12444143
hsa-miR-326	SLC2A1/GLUT1		MFS transporter, solute carrier family 2	PMID: 24335483
hsa-miR-193a-5p	SRF		Serum response factor	PMID: 12120892

microRNA	Predicted targets	Validated targets	Gene name	Reference to HIV
hsa-miR-146a-5p	CCL8/MCP-2	CCL8/MCP-2	Chemokine (C-C motif) ligand 8	PMID: 20181935
hsa-let-7f-5p	TBP	TBP	TATA Box motif	PMID: 25336585
hsa-miR-122-5p	HMOX1 BACH1	HMOX1 BACH1	Heme oxygenase (decycling) 1 BTB and CNC homology 1	PMID: 24752012 PMID: 24752012
hsa-miR-134-5p	SHP	SHP	Src homology-2 domain-containing protein tyrosine phosphatase	PMID: 18776219 PMID: 23029125