

**Sterol metabolism modulates susceptibility to HIV-1 Infection**

Irma SAULLE<sup>1,2\*</sup>, Salomè Valentina IBBA<sup>1\*</sup>, Cecilia VITTORI<sup>1</sup>, Claudio FENIZIA<sup>2</sup>, Vincenzo MERCURIO<sup>1</sup>, Francesca VICHI<sup>3</sup>, Sergio LO CAPUTO<sup>4</sup>, Daria TRABATTONI<sup>1</sup>, Mario CLERICI<sup>2,5</sup>, Mara BIASIN<sup>1</sup>

<sup>1</sup>Department of Biomedical and Clinical Sciences “L. Sacco”, University of Milan, 20157 Milan, Italy; <sup>2</sup>Department of Pathophysiology and Transplantation, University of Milan, 20122 Milan, Italy; <sup>3</sup>S. Maria Annunziata Hospital, 50122 Florence, Italy; <sup>4</sup>Dipartimento di Medicina Clinica e Sperimentale, Università di Foggia, <sup>5</sup>Don C. Gnocchi Foundation ONLUS, IRCCS, 20148 Milan, Italy.

\* These authors equally contributed to this manuscript

**Acknowledgements**

HIV-1Ba-L was provided through the EU programme EVA centre for AIDS Reagents NIBSC, UK. M.C. and M.B. conceived the study; P.A.S.O. and I.S. wrote the paper; C.F. revised the paper; S.V.I., V.M., E.M.L., M.M. and D.T. performed the experiments and analysed the data; S.L.C., F.M. and F.V. selected and enrolled the subjects included in the study.

**Conflicts of interest**

The authors have no conflicts of interest.

This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

**Corresponding author:**

Irma Saulle PhD,  
Chair of Immunology, Department of Biomedical and Clinical Sciences- L. Sacco,  
University of Milan.  
Via G.B.Grassi 74, 20157 Milan, Italy.  
Phone: +39 0250319679  
e mail: irma.saulle@unimi.it

**Abstract****Background**

25-hydroxylase (CH25H) is an Interferon stimulated gene (ISG), which catalyzes the synthesis of 25-Hydroxycholesterol (25HC). 25HC intervenes in metabolic and infectious processes as controls cholesterol homeostasis and influences viral entry into host cells.

We verified whether natural resistance to HIV-1 infection in HIV-1-exposed seronegative (HESN) individuals is at least partially mediated by particularities in sterol biosynthesis.

**Methods**

Peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (MDMs) isolated from 15 sexually-exposed HESN and 15 healthy controls (HC) were *in vitro* HIV-1-infected and analyzed for: 1) percentage of IFN $\alpha$ -producing plasmacytoid Dendritic Cells (pDCs); 2) Cholesterol signaling and inflammatory response RNA expression; 3) resistance to HIV-1 infection. MDMs from 5 HC were *in vitro* HIV-1-infected in the absence/presence of exogenously added 25HC.

**Results**

IFN $\alpha$ -producing pDCs were augmented in HESN compared to HCs both in unstimulated and in *in vitro* HIV-1-infected PBMCs ( $p < 0.001$ ). An increased expression of CH25H and of a number of genes involved in cholesterol metabolism (ABCA1, ABCG1, CYP7B1, LXR $\alpha$ , OSBP, PPAR $\gamma$ , SCARB1) was observed as well; this, was associated with a reduced susceptibility to *in vitro* HIV-1-infection of PBMCs and MDMs ( $p < 0.01$ ). Notably, addition of 25HC to MDMs resulted in increased cholesterol efflux and augmented resistance to *in vitro* HIV-1-infection.

## Conclusions

Results herein show that in HESN sterol metabolism might be particularly efficient. This could be related to the activation of the IFN $\alpha$  pathway and results into a reduced susceptibility to *in vitro* HIV-1 infection. These results suggest a possible basis for therapeutic interventions to modulate HIV-1 infection.

**Keywords:** Sterol metabolism, CH25H, HIV-1, HESN,immunity

## Introduction

In the last decades several studies tried to explain the phenomenon of natural resistance to HIV-1 infection displayed by different cohorts of individuals. These individuals, referred to as HIV-1–exposed seronegative (HESN) have the extraordinary capacity to remain HIV-1 uninfected despite frequent risk behaviors, including injection drug use with needle sharing or sexual intercourse with infected partners [1,2]. Natural protection to HIV-1 infection has been associated with a wide range of genetic and immunological factors [3–5]. In particular, several publications reported a strong correlation between HIV-1 infection, resistance and the presence of an immune activation status [4,6,7]. Indeed, even if not all results are in agreement [8–11] HESNs have been convincingly shown to be characterized by an increased expression of activation markers on circulating cells and greater production of immunological effector molecules both in unstimulated condition and upon specific-stimulation [6,12–15].

Among the different immunological factors thus far correlated with the HESN phenotype, Type-I Interferons seem to play a crucial role in controlling HIV-1 infection [3,4,16–18]. Their mechanisms of action is mediated by the activation of the so called interferon stimulated genes (ISGs), a number of which can interfere with viral replication at different levels [19,20]. The ER-associated enzyme cholesterol-25-hydroxylase (CH25H) is one of these ISGs and it modulates viral replication through of its effect on cholesterol metabolism [21]. The regulation of the virus-cholesterol axis is indeed essential for the correct assembly and release of functional virions [22–24]. Both enveloped and naked viruses have to interact with the phospholipidic membrane in order to enter the cell and, once produced, new virions need to exit to spread to new targets. These steps require the virus to cross the plasma membrane of the cell twice: firstly, via fusion mediated by the envelope glycoprotein, to deliver the viral core into the cytosol; and secondly by the scission of budding virions during

release. Once inside the cell, viruses can affect lipid homeostasis and intracellular signaling in order to hijack the cell metabolism towards virus' needs [25,26].

To counteract such viral usurpation and restore a functional balance of cholesterol outflow, the infected cell can adopt different strategies. For example, the activation of liver X receptor (LXRs) and/or the inhibition of sterol regulatory element-binding protein (SREBPs) pathways result in restriction of viral replication, including HIV and HCV[25,27–30]. This makes sense considering the role played by these molecules in cholesterol metabolisms: SREBP1 provides a feedback regulatory system involved in biosynthesis and uptake of cholesterol [31] LXR, instead, favors the elimination of excess cholesterol [32] by positively regulating the expression of genes encoding lipid-transport proteins, such as ATP-binding cassette A1 (ABCA1), and ATP-binding cassette G1 (ABCG1) [33,34]. Notably, 25HC synthesized by CH25H acts exactly as a positive and negative regulator of LXR and SREPB2, respectively, thus impairing virus-cell fusion [21,35]. Actually, 25HC inhibits the growth of a wide range of enveloped viruses by inducing structural changes in the cellular membrane, thus preventing viral entry at the virus-cell fusion step[36–38]. As confirmation, recent results obtained in a biomembrane model system, showed that liposome fusion is reduced by 50% in the presence of 25HC, comparatively to cholesterol. Additional data indicated that 25HC affects the membrane fusion process through the modification of lipid membrane properties, and by direct alterations on HIV-fusion peptide structure, suggesting the possibility to employ 25HC as antiviral molecule [39,40].

Furthermore, a study performed in our laboratory has shown that Tiazolides (TZD), a class of drugs with a known *in vitro* and *in vivo* activity against a wide range of pathogens, reduce HIV-1-infection (>90%) through the modulation of CH25H as well [37] These findings lead us to speculate on the possibility that the massive efflux of cholesterol from cells could deprive the virus of components necessary for the assembly of new virions and interfere with the budding process, limiting a strong virus spread [37].

As any determinant involved in the HIV-1 resistance processes could potentially be exploited in the development of novel preventative and therapeutic strategies, the analysis of immunologic correlates of the HESN phenotype is still a fascinating and prolific branch in HIV research. Based on these observations, we decided to verify whether the natural resistance to HIV-1 infection seen in HESN is at least partially dependent on a peculiar regulation of the sterol biosynthesis pathway mediated by IFN-induced CH25H expression.

## **Materials and methods**

### **Study population**

We recruited 15 HESN exposed to HIV-1 infection by unprotected heterosexual intercourse from an Italian cohort (Santa Maria Annunziata hospital, Florence, Italy) whose epidemiological and clinical characteristics as well as inclusion criteria were previously described [6]. A group of 15 HIV-1 seronegative healthy blood donors (HC) from the same hospital was used as controls. Notably, none of the subjects included in the study were receiving anticholesterol medications before or at the time of this study.

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the participating units. All subjects provided written informed consent to participate in this study.

### **PBMC isolation and monocyte derived macrophages (MDMs) differentiation.**

Peripheral blood mononuclear cells (PBMCs) from all the subjects enrolled in the study were separated as previously described [41]. Number of viable leukocytes was determined by an automatic cell counter (Digital Bio, NanoEnTek Inc, Korea).

After determining the percentage of monocytes at flow cytometer, 500,000 monocyte/well were differentiated in MDMs as previously reported [42].

### ***In vitro* HIV-1 infection of PBMCs and MDMs from HESN and HC**

$2 \times 10^6$  PBMCs isolated from 15 HESN and 15 HC were cultured in RPMI 1640 containing 20% fetal bovine serum, with or without 0.5 ng/ $1 \times 10^6$  cells HIV-1<sub>Ba-L</sub> virus with a cellular density of  $2 \times 10^6$  /ml and incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Cells were then washed and resuspended in medium containing IL-2 (15 ng/ml) (R&D Systems, Minneapolis, Minnesota, USA). Three and 7 days post *in vitro* HIV-1 infection  $1 \times 10^6$  PBMCs were used to determine IFN-alpha-producing pDC percentage and mRNA expression genes, respectively. p24 ELISA was performed on 7 days post-infection supernatants.

MDMs from 10 HESN and 10 HC subjects were cultured in RPMI 1640 containing 20% FBS and  $0.5\text{ng}/1 \times 10^6$  cells HIV-1<sub>Ba-L</sub> and incubated for 24 h at 37°C and 5% CO<sub>2</sub>. At the end of incubation, the attached macrophages were washed with PBS and cultured in complete medium containing 20% FBS for 7 days. Half of the medium was changed 3 and 5 days post infection. Seven days post infection supernatant was collected for p24 antigen ELISA quantification.

MDMs differentiated from 5 HC were overnight pretreated with 25HC (1 $\mu$ M) (Cayman Chemical, Ann Arbor, Michigan, USA). *In vitro* HIV-1-infection was performed as previously described. 25HC was administered just once at the start of our cell culture experiments. Four and 7 days post *in vitro* HIV-1 infection RNA expression and p24 antigen ELISA quantification were assessed, respectively.

### **p24 ELISA**

An HIV-1 p24 Elisa assay kit (XpressBio, Frederick, Maryland, USA) was used to measure viral p24 antigen in supernatant of PBMCs, MDMs and 25HC treated MDMs differentiated from monocytes of HESN and HC after 7 days HIV-1 infection, according to the manufacturer's protocol.

### **Flow cytometry**

Flow cytometric analyses were performed on uninfected and 3 days post infection to pDCs characterization.  $0.5 \times 10^6$  PBMCs were stained after with anti-human LIN- labeled with FITC (eBioscience, MA, USA), anti-human CD123 labeled with PE and anti-human HLADR labeled with PC7 followed by fixation, permeabilization and incubation with anti-human IFN $\alpha$  labeled with APC (Biolegend, San Diego, California, USA). At least 200 000 events were acquired in the gate of LIN-cells, using a Gallios flow cytometer (Beckman-Coulter, San Jose, California, USA). In order to calculate the percentage of monocytes in isolated PBMCs, 250,000 cells were resuspended in PBS, and stained for surface antibodies: CD36 FITC, CD4 PC5, CD14 PC7 (Beckman-coulter, Fullerton, CA).

### **RNA extraction and Retro-transcription (RT)**

RNA was extracted from  $1 \times 10^6$  PBMCs and  $5 \times 10^5$  MDMs by using the acid guanidium thiocyanate–phenol–chloroform method. RNA was dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA). One microgram of RNA was reverse transcribed into first-strand cDNA in a 20- $\mu$ l final volume containing 1  $\mu$ M random hexanucleotide primers, 1  $\mu$ M oligo dT and 200 U Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, California, USA).

### **Cholesterol signaling and inflammatory response gene expression**

Cholesterol signaling and inflammatory response gene expression by unstimulated and 7-days *in vitro* HIV-1-infected PBMCs was analyzed in a custom PCR array including a set of 48 optimized real-time PCR primer assays (SABiosciences Corporation, Frederick, MD, USA). Only targets showing >2-fold modulation were considered significant. Experiments were run on all the subjects included in the study pooled into two distinct groups (HESN and HC) and represent the mean value of the different targets analyzed in each group. Reactions were performed using a SYBR Green PCR mix (Bio-rad, CA, USA) using CFX manager 3.1 thermal cycler (Bio Rad) as previously described [41].

To validate the results obtained by array analyses, some selected significant targets were quantified by individual real-time PCR on cDNA obtained from *in vitro* HIV-1-infected MDMs differentiated from 10 HESN and 10 HC subjects. The same targets were analyzed on cDNA obtained from *in vitro* HIV-1-infected MDMs of 5 HC treated or untreated with 25HC.

### **Statistical analysis**

Data were analyzed using Student's T or ANOVA test by GRAPHPAD PRISM version 5 (Graphpad software, La Jolla, Ca, USA), and *p*-values of 0.05 or less were considered to be significant.

## Results

### Susceptibility of PBMCs and MDMs from HESN to HIV-1 infection *in vitro*

To confirm that HESN subjects are less susceptible to HIV-1 infection, PBMCs and MDMs isolated from HESN and HC were *in-vitro* infected with HIV-1 Bal. Results showed that seven days after *in vitro* HIV-1-infection p24 levels in PBMC cultures were significantly lower in HESN (mean: 57640 +/- 19590 pg/ml) compared with HC (mean value: 120234 +/- 32503 pg/ml) ( $p < 0.01$ ) (Fig. 1A). Likewise, 7 days post *in vitro* HIV-1-infection, virus-replication was reduced in MDMs from HESN (mean value: 68508 +/- 16251 pg/ml) compared to HC (mean value: 104381 +/- 15248 pg/ml) ( $p < 0.02$ ) (Fig. 1B).

### IFN $\alpha$ -producing plasmacytoid dendritic cells (pDCs) in uninfected and *in vitro* HIV-1-infected PBMCs of HESN and HC

To verify whether resistance to *in vitro* HIV-1 infection is mediated by increased production of Type-I interferons we decide to analyze the percentage of plasmacytoid dendritic cells (pDCs) in PBMCs isolated from HESN and HC. Interestingly, the percentage of IFN- $\alpha$  producing pDCs was significantly higher in unstimulated PBMCs isolated from HESN compared to HC ( $p < 0.001$ ). Such differences became even more evident analyzing 3 days post *in vitro* HIV-1 infection PBMCs isolated from HESN compared to HC ( $p < 0.0002$ ) (Figure 2). This result suggests a key role for this cellular subset in mediating antiviral immune response.

### Modulation of Cholesterol signaling and inflammatory response gene expression in uninfected and *in vitro* HIV-1-infected PBMCs and MDMs of HESN

Since Type1 Interferon expression by pDCs is increased in HESN and CH25H is an ISGs involved in cholesterol metabolism, we verified whether natural resistance to HIV-1 infection is also dependent on this pathway.

Results obtained in uninfected PBMCs from HESN and HC showed an up-regulation of genes involved in cholesterol metabolism and efflux (CH25H, CYP7B1, INSIG1, LXR $\alpha$ , LXR $\beta$ , MBTPS1, PPAR $\gamma$ , RXR $\alpha$ , RXR $\beta$ ), as well as of different membrane receptors and cholesterol transporters (ABCA1, ABCG1, CD36, LDLR, MSR1, SCARB1) in PBMCs from HESN compared to HC (Figure 3). Even the expression of different modulators involved in the inflammatory response (CD80, CD86, IFN $\alpha$ 2, IFN $\alpha$ R1, IFN $\alpha$ R2, IFN $\beta$ , IL1 $\beta$ ) and



antiviral response (IFITM3, OAS1) was increased in uninfected PBMCs from HESN compared to HC, suggesting a correlation between immune activation and the modulation of cholesterol metabolism (Figure 3).

Notably, these differences were even more pronounced upon 7-days post *in vitro* HIV-1 infection, as an up-regulation of the expression of enzymes (ACAT, CH25H, CYP7B1, INSIG1) transcription factors (LXR $\alpha$ , LXR $\beta$ , MBTPS1, PPAR $\gamma$ , RXR $\alpha$ , RXR $\beta$ ) and membrane receptors (ABCA1, ABCG1, LDLR, MSR1, SCARB1) involved in cholesterol metabolism and efflux (Figure 3) was seen in PBMCs from HESN. CH25H, CYP7B1, LXR $\alpha$  and PPAR $\gamma$  expression, in particular, was robustly (>12 fold) up-regulated in *in vitro* HIV-1-infected PBMCs from HESN. Finally, the expression of mediators of the inflammatory (CD80 CD86, IFN $\alpha$ 2, IFN $\alpha$ R1, IFN $\alpha$ R2, IFN $\beta$ 1, IL1 $\beta$ , IL6, NFKBIA), and antiviral response (IFITM1, IFITM3, OAS1) was significantly increased as well in these cells (Figure 3).

To validate the involvement of cholesterol metabolism gene expression in natural resistance to HIV-1 infection, mRNA expression of some selected targets, whose expression was increased in PBMCs, was further investigated in 7-days *in vitro* HIV-1-infected MDMs from HESN and HC by QPCR. Results confirmed the trend previously observed in PBMCs. Thus, the expression of IFN $\alpha$  was increased in *in vitro* HIV-1-infected MDMs from HESN compared to HC, though these differences did not reach statistical significance. Furthermore, the expression of ABCA1 ( $p<0.02$ ), ABCG1 ( $p<0.02$ ), CH25H ( $p<0.03$ ), CYP7B1 ( $p<0.01$ ), LXR $\alpha$  ( $p<0.009$ ), OSBP ( $p<0.02$ ), PPAR $\gamma$  ( $p<0.01$ ) and SCARB1 ( $p<0.02$ ) was significantly increased in *in vitro* HIV-1 infected MDMs from HESN compared to HC, while the expression of HMGCS1 and SREBP1 showed a trend of reduction in HESNs MDMs, although these differences were not statistically significant (Figure 4).

### **Role of 25HC in MDM resistance to *in vitro* HIV-1-infection**

To further confirm the protective role exerted by 25HC in resistance to HIV-1-infection, MDMs of 5 HC were *in vitro* HIV-1-infected the presence/absence of 25HC. Results showed that incubation of cells with 25HC reduced HIV-1-replication by nearly 60% ( $p<0.001$ ) (Figure 5A). Such control of HIV-1-replication was associated with a significant increase in the expression of IFN $\alpha$  mRNA ( $p<0.03$ ) and of nuclear transcription factor LXR $\alpha$  ( $p<0.03$ ) which, in turn, upregulated the expression of the two main factors responsible for cholesterol efflux: ABCA1 and ABCG1 ( $p<0.02$  for both) (Figure 5B).

## Discussion

The main findings of this study shed new light on a molecule whose involvement in HIV replication has been widely demonstrated: cholesterol. Indeed, it is documented that HIV entry, assembly, budding and release mainly take place in specialized cholesterol-enriched microdomains, called lipid rafts, in the plasma membrane [43]. It is also important to underline that following long-term antiretroviral therapy HIV-patients display an altered lipid profile which can result in dyslipidemia [44], and that disease progression was shown to be delayed in HIV-infected patients showing increased cholesterol metabolism and reduced cholesterol synthesis [45–47]. Further, corroborating the link between HIV-infection and cholesterol metabolism our results show that cells isolated from subjects who naturally resist to HIV-infection (HESN) manifest an alteration of several factors involved in different steps of cholesterol metabolism including synthesis, efflux and uptake, both in uninfected and in *in vitro* HIV-infected conditions. HESN subjects are, basically, more prone to release cholesterol in the extracellular milieu and to reduce its synthesis, thus favoring HIV replication, a cholesterol-dependent process.

The triggering factors responsible for cholesterol pathway alteration in HESN seem to be type I interferons (IFNs), mainly produced by plasmacytoid DCs (pDCs), whose percentage was significantly augmented in uninfected as well as *in vitro* HIV-1-infected PBMCs from HESN. IFNs are considered one of the most potent anti-viral signaling molecules; their effect being mediated by the upregulation of several ISGs [3,4,21] among which, CH25H is suggested to play a pivotal role. The expression of this enzyme, which catalyzes the formation of 25HC from cholesterol [48] was, indeed, significantly augmented in both uninfected and *in vitro* HIV-1-infected PBMCs and MDMs from HESN and is likely to be at least in part responsible for the 25HC-dependent upregulation of LXR $\alpha$  and downregulation of SREBP1 [21,35], as we observed in our experimental setting. LXR $\alpha$ , in particular, behaves as an oxysterol sensor of intracellular cholesterol homeostasis [21] and, when being produced in great abundance, blocks new cholesterol biosynthesis while promoting its efflux [21]. The activation of such strategy is corroborated by the observation that, among others, the expression of ABCA1 and ABCG1, two transmembrane proteins responsible for cholesterol efflux from cells, is significantly augmented, while the expression of HMGCS1, a key enzyme of cholesterol synthesis, is reduced in cells from HESN compared to HC. Other genes whose expression was raised in HESN MDMs include: oxysterol-binding protein

(OSBP), which endorses the mobilization of lipids from endoplasmic reticulum to Golgi, together with the downregulation of newly synthesized cholesterol [49] and peroxisome proliferated factor (PPAR)  $\gamma$ , that manages the removal of intracellular cholesterol [50]. Notably, the peculiar expression of these genes seen in HESN cells was greatly enhanced following *in vitro* HIV-1-infection, suggesting that cells of these individuals are programmed to set up an antiviral defensive strategy mediated by the modulation of the cholesterol metabolism.

To further validate these findings MDMs from 5 HCs were *in vitro* HIV-1-infected after being pre-incubated with 25HC. Of note, HIV-1 replication in 25HC-treated MDMs was significantly reduced and was associated with a significant increase in mRNA expression of type I IFNs, LXR $\alpha$  as well as of factors favoring cholesterol release such as ABCG1 and ABCA1. The ability of exogenous 25HC to control virus replication has already been demonstrated for different virus and cellular types [38,51–54], including HIV [21], but, to our knowledge, this is the first time that such protective effect has been documented in human MDMs.

Another aspect that should be considered concerns the state of immune activation observed in HESN PBMCs both in uninfected and in *in vitro* HIV-1-infected condition. For example, it was recently reported that cholesterol efflux activation results in the suppression of the inflammasome assembly [55], and that inhibition of the NLRP3 inflammasome decreases foam cell formation of THP-1 macrophages by stimulating cholesterol efflux [56]. Nevertheless, 25HC has been also described as an amplifier of inflammatory signaling [57] and, even more important, persistent activation of the immune system was reported in patients with hypercholesterolemia, even when cholesterolemia is lowered by statins [58]. This condition was ascribed to the ability of monocytes to retain a long-term pro-inflammatory phenotype after a brief exposure to a triggering factor, a phenomenon defined 'trained immunity'. It's, therefore, tempting to speculate that regardless of cholesterol reduction, monocytes from HESN are more prone to develop a trained immunity phenotype which can shield them from HIV-exposure through the release of pro-inflammatory cytokines. Further analyses are required to shed light on the role of this intriguing mechanism in natural resistance to HIV-infection.

The analyses performed in this study do not allow to exclude that the modulation of cholesterol content seen in HESN cells is, as has been previously suggested, at least in part

the result of genetic or epigenetic regulation of one or more factors [59,60]. Thus, ABCA1 mRNA expression was shown to be increased in HIV-infected non progressors (NPs) as an inherited trait [45]. Additionally, two GWASs for HIV-1 infection susceptibility suggested a role of variants within or in proximity of the *CYP7B1* gene in this phenomenon [61,62]. The *CYP7B1* gene encodes an enzyme that is involved in cholesterol catabolism by inactivation of oxysterols [63] but it also controls numerous immune functions, via its catabolite 25HC [64]. Notably, one of such effect is IgA synthesis [65], a class of antibodies previously associated with resistance to HIV-1-infection [66,67]. However, comparisons of allelic and genotypic frequencies among three different HESN cohorts indicate that the two GWAS-defined variants in the *CYP7B1* region do not strongly influence HIV-1 infection susceptibility [68]. We can, therefore, presume that the increased expression of *CYP7B1* we observed in cells of our HESN upon *in vitro* HIV-1 infection is not genetically determined. Further studies will nevertheless be necessary to verify whether the peculiarities of cholesterol metabolism seen in HESN are genetically driven or secondary to environmental setting.

This is not the first report trying to establish a correlation between natural resistance to HIV-infection and cholesterol metabolism. In 2013, a study investigating nutritional status and metabolic disorders in a cohort of prepubertal HESN children showed a higher prevalence of dyslipidemia and hypercholesterolemia [69]. The authors concluded that intrauterine HIV-exposure could be responsible for these differences, suggesting that even in the absence of productive infection, HIV is able to disrupt this pathway. More recently, Tort and colleagues explored cholesterol efflux in HIV-patients as well as in sexually-exposed HESN [70]. Results showed that low cholesterol efflux in HIV-patients was associated with reduced CD4+T-cells as well as with higher viral loads but, contrarily to our findings, a lower cholesterol efflux was seen in HESN compared to HC. Differences in the methods used to investigate the same mechanism could justify this discrepancy. Thus, whereas an assay on THP-1 cellular line using ApoB-depleted plasma from HESN as cholesterol acceptor was used by Tort, we analyzed the expression of determinants involved in cholesterol homeostasis on primary cells.

Results herein are only the first step in understanding the mechanisms that operate in the ability of some individuals to spontaneously control HIV-1 infection. It is likely that several mechanisms that involve numerous molecular players are responsible for this phenomenon, and innovative approaches are needed to identify the contribution of cholesterol metabolism

to each mechanism. Although preliminary, though, these results suggest the possibility that modulation of cholesterol metabolism could have a role in preventing or controlling HIV-1 infection.

## References

- 1 Imagawa DT, Lee MH, Wolinsky SM, Sano K, Morales F, Kwok S, *et al.* **Human Immunodeficiency Virus Type 1 Infection in Homosexual Men Who Remain Seronegative for Prolonged Periods.** *N Engl J Med* 1989; **320**:1458–1462.
- 2 Miyazawa M, Lopalco L, Mazzotta F, Caputo S, Veas F, Clerici M. **The ‘immunologic advantage’ of HIV-exposed seronegative individuals.** *Aids* 2009; **23**:161–175.
- 3 Fenizia C, Saulle I, Clerici M, Biasin M. **Genetic and epigenetic regulation of natural resistance to HIV-1 infection: new approaches to unveil the HESN secret.** *Expert Rev Clin Immunol* 2020; **0**:null.
- 4 Fenizia C, Rossignol J-F, Clerici M, Biasin M. **Genetic and immune determinants of immune activation in HIV-exposed seronegative individuals and their role in protection against HIV infection.** *Infect Genet Evol* 2018; **66**:325–334.
- 5 Biasin M, Sironi M, Saulle I, de Luca M, la Rosa F, Cagliani R, *et al.* **Endoplasmic reticulum aminopeptidase 2 haplotypes play a role in modulating susceptibility to HIV infection.** *AIDS Lond Engl* 2013; **27**:1697–1706.
- 6 Saulle I, Biasin M, Gnudi F, Rainone V, Ibba SV, Caputo SL, *et al.* **Short Communication: Immune Activation Is Present in HIV-1-Exposed Seronegative Individuals and Is Independent of Microbial Translocation.** *AIDS Res Hum Retroviruses* 2015; **32**:129–133.
- 7 Sironi M, Biasin M, Cagliani R, Forni D, De Luca M, Saulle I, *et al.* **A common polymorphism in TLR3 confers natural resistance to HIV-1 infection.** *J Immunol Baltim Md 1950* 2012; **188**:818–823.
- 8 Card CM, Ball TB, Fowke KR. **Immune quiescence: a model of protection against HIV infection.** *Retrovirology* 2013; **10**:141.
- 9 Lajoie J, Kimani M, Plummer FA, Nyamiobo F, Kaul R, Kimani J, *et al.* **Association of sex work with reduced activation of the mucosal immune system.** *J Infect Dis* 2014; **210**:319–329.
- 10 Taborda NA, Hernández JC, Lajoie J, Juno JA, Kimani J, Rugeles MT, *et al.* **Short Communication: Low Expression of Activation and Inhibitory Molecules on NK Cells and CD4(+) T Cells Is Associated with Viral Control.** *AIDS Res Hum Retroviruses* 2015; **31**:636–640.
- 11 Lajoie J, Birse K, Mwangi L, Chen Y, Cheruiyot J, Akolo M, *et al.* **Using safe, affordable and accessible non-steroidal anti-inflammatory drugs to reduce the**

- number of HIV target cells in the blood and at the female genital tract. *J Int AIDS Soc* 2018; **21**:e25150.
- 12 Biasin M, Caputo SL, Speciale L, Colombo F, Racioppi L, Zagliani A, *et al.* **Mucosal and systemic immune activation is present in human immunodeficiency virus-exposed seronegative women.** *J Infect Dis* 2000; **182**:1365–1374.
  - 13 Tomescu C, Seaton KE, Smith P, Taylor M, Tomaras GD, Metzger DS, *et al.* **Innate activation of MDC and NK cells in high-risk HIV-1-exposed seronegative IV-drug users who share needles when compared with low-risk nonsharing IV-drug user controls.** *J Acquir Immune Defic Syndr* 1999 2015; **68**:264–273.
  - 14 Tomescu C, Duh F-M, Lanier MA, Kapalko A, Mounzer KC, Martin MP, *et al.* **Increased plasmacytoid dendritic cell maturation and natural killer cell activation in HIV-1 exposed, uninfected intravenous drug users.** *AIDS Lond Engl* 2010; **24**:2151–2160.
  - 15 Tomescu C, Abdulhaqq S, Montaner LJ. **Evidence for the innate immune response as a correlate of protection in human immunodeficiency virus (HIV)-1 highly exposed seronegative subjects (HESN).** *Clin Exp Immunol* 2011; **164**:158–169.
  - 16 Sandler NG, Bosinger SE, Estes JD, Zhu RTR, Tharp GK, Boritz E, *et al.* **Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression.** *Nature* 2014; **511**:601–605.
  - 17 Veazey RS, Pilch Cooper HA, Hope TJ, Alter G, Carias AM, Sips M, *et al.* **Prevention of SHIV transmission by topical IFN- $\beta$  treatment.** *Mucosal Immunol* 2016; **9**:1528–1536.
  - 18 Cervantes CAC, Oliveira LMS, Manfrere KCG, Lima JF, Pereira NZ, Duarte AJS, *et al.* **Antiviral factors and type I/III interferon expression associated with regulatory factors in the oral epithelial cells from HIV-1-serodiscordant couples.** *Sci Rep* 2016; **6**:25875.
  - 19 Sironi M, Biasin M, Cagliani R, Gnudi F, Saulle I, Ibba S, *et al.* **Evolutionary analysis identifies an MX2 haplotype associated with natural resistance to HIV-1 infection.** *Mol Biol Evol* 2014; **31**:2402–2414.
  - 20 Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, *et al.* **Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals.** *J Infect Dis* 2007; **195**:960–964.
  - 21 Liu S-Y, Aliyari R, Chikere K, Li G, Marsden MD, Smith JK, *et al.* **Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol.** *Immunity* 2013; **38**:92–105.
  - 22 Lorizate M, Kräusslich H-G. **Role of lipids in virus replication.** *Cold Spring Harb Perspect Biol* 2011; **3**:a004820.

- 23 Martín-Acebes MA, Blázquez A-B, de Oya NJ, Escribano-Romero E, Shi P-Y, Saiz J-C. **A single amino acid substitution in the core protein of West Nile virus increases resistance to acidotropic compounds.** *PLoS One* 2013; **8**:e69479.
- 24 Mazzon M, Mercer J. **Lipid interactions during virus entry and infection.** *Cell Microbiol* 2014; **16**:1493–1502.
- 25 Chukkapalli V, Heaton NS, Randall G. **Lipids at the interface of virus-host interactions.** *Curr Opin Microbiol* 2012; **15**:512–518.
- 26 Boasso A, Royle CM, Doumazos S, Aquino VN, Biasin M, Piacentini L, *et al.* **Overactivation of plasmacytoid dendritic cells inhibits antiviral T-cell responses: a model for HIV immunopathogenesis.** *Blood* 2011; **118**:5152–5162.
- 27 Moog C, Aubertin AM, Kirn A, Luu B. **Oxysterols, but not cholesterol, inhibit human immunodeficiency virus replication in vitro.** *Antivir Chem Chemother* 1998; **9**:491–496.
- 28 Pezacki JP, Sagan SM, Tonary AM, Rouleau Y, Bélanger S, Supekova L, *et al.* **Transcriptional profiling of the effects of 25-hydroxycholesterol on human hepatocyte metabolism and the antiviral state it conveys against the hepatitis C virus.** *BMC Chem Biol* 2009; **9**:2.
- 29 Dubrovsky L, Van Duyne R, Senina S, Guendel I, Pushkarsky T, Sviridov D, *et al.* **Liver X receptor agonist inhibits HIV-1 replication and prevents HIV-induced reduction of plasma HDL in humanized mouse model of HIV infection.** *Biochem Biophys Res Commun* 2012; **419**:95–98.
- 30 Morrow MP, Grant A, Mujawar Z, Dubrovsky L, Pushkarsky T, Kiselyeva Y, *et al.* **Stimulation of the liver X receptor pathway inhibits HIV-1 replication via induction of ATP-binding cassette transporter A1.** *Mol Pharmacol* 2010; **78**:215–225.
- 31 Brown MS, Goldstein JL. **Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL.** *J Lipid Res* 2009; **50**:S15–S27.
- 32 Tontonoz P, Mangelsdorf DJ. **Liver X receptor signaling pathways in cardiovascular disease.** *Mol Endocrinol* Published Online First: 2003. doi:10.1210/me.2003-0061
- 33 Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, *et al.* **Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR $\alpha$ .** *Proc Natl Acad Sci U S A* 2000; **97**:12097–12102.
- 34 Kennedy MA, Venkateswaran A, Tarr PT, Xenarios I, Kudoh J, Shimizu N, *et al.* **Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein.** *J Biol Chem* 2001; **276**:39438–39447.
- 35 Blanc M, Hsieh WY, Robertson KA, Kropp KA, Forster T, Shui G, *et al.* **The transcription factor STAT-1 couples macrophage synthesis of 25-hydroxycholesterol to the interferon antiviral response.** *Immunity* 2013; **38**:106–118.

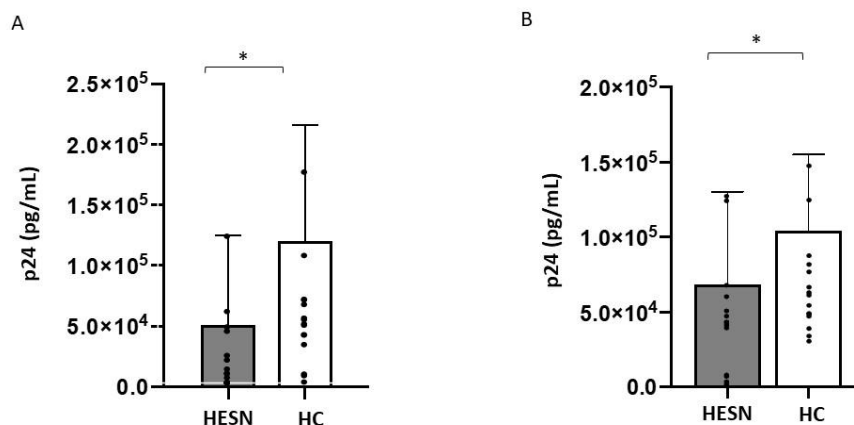
- 36 Cagno V, Civra A, Rossin D, Calfapietra S, Caccia C, Leoni V, *et al.* **Inhibition of herpes simplex-1 virus replication by 25-hydroxycholesterol and 27-hydroxycholesterol.** *Redox Biol* 2017; **12**:522–527.
- 37 Civra A, Cagno V, Donalisio M, Biasi F, Leonarduzzi G, Poli G, *et al.* **Inhibition of pathogenic non-enveloped viruses by 25-hydroxycholesterol and 27-hydroxycholesterol.** *Sci Rep* 2014; **4**:7487.
- 38 Shrivastava-Ranjan P, Bergeron É, Chakrabarti AK, Albariño CG, Flint M, Nichol ST, *et al.* **25-Hydroxycholesterol Inhibition of Lassa Virus Infection through Aberrant GP1 Glycosylation.** *mBio* 2016; **7**. doi:10.1128/mBio.01808-16
- 39 Gomes B, Gonçalves S, Disalvo A, Hollmann A, Santos NC. **Effect of 25-hydroxycholesterol in viral membrane fusion: Insights on HIV inhibition.** *Biochim Biophys Acta Biomembr* 2018; **1860**:1171–1178.
- 40 Gomes B, Sanna G, Madeddu S, Hollmann A, Santos NC. **Combining 25-Hydroxycholesterol with an HIV Fusion Inhibitor Peptide: Interaction with Biomembrane Model Systems and Human Blood Cells.** *ACS Infect Dis* 2019; **5**:582–591.
- 41 Saulle I, Ibba SV, Torretta E, Vittori C, Fenizia C, Piancone F, *et al.* **Endoplasmic Reticulum Associated Aminopeptidase 2 (ERAP2) Is Released in the Secretome of Activated MDMs and Reduces in vitro HIV-1 Infection.** *Front Immunol* 2019; **10**. doi:10.3389/fimmu.2019.01648
- 42 Merlini E, Tincati C, Biasin M, Saulle I, Cazzaniga FA, d'Arminio Monforte A, *et al.* **Stimulation of PBMC and Monocyte-Derived Macrophages via Toll-Like Receptor Activates Innate Immune Pathways in HIV-Infected Patients on Virally Suppressive Combination Antiretroviral Therapy.** *Front Immunol* 2016; **7**:614.
- 43 Waheed AA, Freed EO. **Lipids and membrane microdomains in HIV-1 replication.** *Virus Res* 2009; **143**:162–176.
- 44 Bowman ER, Kulkarni M, Gabriel J, Cichon MJ, Riedl K, Belury MA, *et al.* **Altered Lipidome Composition Is Related to Markers of Monocyte and Immune Activation in Antiretroviral Therapy Treated Human Immunodeficiency Virus (HIV) Infection and in Uninfected Persons.** *Front Immunol* 2019; **10**. doi:10.3389/fimmu.2019.00785
- 45 Rappocciolo G, Jais M, Piazza P, Reinhart TA, Berendam SJ, Garcia-Exposito L, *et al.* **Alterations in Cholesterol Metabolism Restrict HIV-1 Trans Infection in Nonprogressors.** *mBio* 2014; **5**. doi:10.1128/mBio.01031-13
- 46 Prasad VR, Bukrinsky MI. **New Clues to Understanding HIV Nonprogressors: Low Cholesterol Blocks HIV Trans Infection.** *mBio* 2014; **5**. doi:10.1128/mBio.01396-14
- 47 DeLucia DC, Rinaldo CR, Rappocciolo G. **Inefficient HIV-1 trans infection of CD4+ T cells by macrophages from HIV-1 nonprogressors is associated with altered membrane cholesterol and DC-SIGN.** *J Virol* Published Online First: 11 April 2018. doi:10.1128/JVI.00092-18



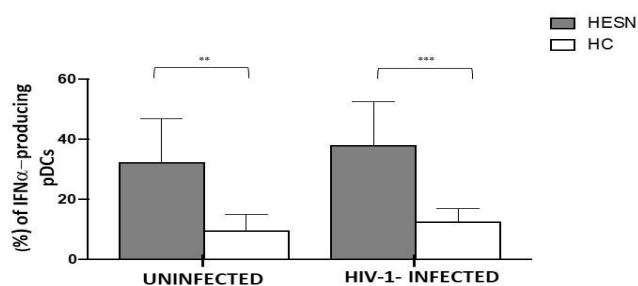
- 48 Lund EG, Kerr TA, Sakai J, Li W-P, Russell DW. **cDNA Cloning of Mouse and Human Cholesterol 25-Hydroxylases, Polytopic Membrane Proteins That Synthesize a Potent Oxysterol Regulator of Lipid Metabolism.** *J Biol Chem* 1998; **273**:34316–34327.
- 49 Amako Y, Sarkeshik A, Hotta H, Yates J, Siddiqui A. **Role of oxysterol binding protein in hepatitis C virus infection.** *J Virol* 2009; **83**:9237–9246.
- 50 Monsalve FA, Pyarasani RD, Delgado-Lopez F, Moore-Carrasco R. **Peroxisome Proliferator-Activated Receptor Targets for the Treatment of Metabolic Diseases.** *Mediators Inflamm* 2013; **2013**. doi:10.1155/2013/549627
- 51 Zhang Y, Wang L, Huang X, Wang S, Huang Y, Qin Q. **Fish Cholesterol 25-Hydroxylase Inhibits Virus Replication via Regulating Interferon Immune Response or Affecting Virus Entry.** *Front Immunol* 2019; **10**. doi:10.3389/fimmu.2019.00322
- 52 Wang J, Zeng L, Zhang L, Guo Z-Z, Lu S-F, Ming S-L, *et al.* **Cholesterol 25-hydroxylase acts as a host restriction factor on pseudorabies virus replication.** *J Gen Virol* 2017; **98**:1467–1476.
- 53 Dong H, Zhou L, Ge X, Guo X, Han J, Yang H. **Antiviral effect of 25-hydroxycholesterol against porcine reproductive and respiratory syndrome virus in vitro.** *Antivir Ther* 2018; **23**:395–404.
- 54 Hamirally S, Kamil JP, Ndassa-Colday YM, Lin AJ, Jahng WJ, Baek M-C, *et al.* **Viral Mimicry of Cdc2/Cyclin-Dependent Kinase 1 Mediates Disruption of Nuclear Lamina during Human Cytomegalovirus Nuclear Egress.** *PLoS Pathog* 2009; **5**. doi:10.1371/journal.ppat.1000275
- 55 Tall AR, Westerterp M. **Inflammasomes, neutrophil extracellular traps, and cholesterol.** *J Lipid Res* 2019; **60**:721–727.
- 56 Chen L, Yao Q, Xu S, Wang H, Qu P. **Inhibition of the NLRP3 inflammasome attenuates foam cell formation of THP-1 macrophages by suppressing ox-LDL uptake and promoting cholesterol efflux.** *Biochem Biophys Res Commun* 2018; **495**:382–387.
- 57 Gold ES, Diercks AH, Podolsky I, Podyminogin RL, Askovich PS, Treuting PM, *et al.* **25-Hydroxycholesterol acts as an amplifier of inflammatory signaling.** *Proc Natl Acad Sci* Published Online First: 3 July 2014. doi:10.1073/pnas.1404271111
- 58 Bekkering S, Stiekema LCA, Bernelot Moens S, Verweij SL, Novakovic B, Prange K, *et al.* **Treatment with Statins Does Not Revert Trained Immunity in Patients with Familial Hypercholesterolemia.** *Cell Metab* 2019; **30**:1–2.
- 59 de Almeida ERD, Reiche EMV, Kallaur AP, Flauzino T, Watanabe MAE. **The roles of genetic polymorphisms and human immunodeficiency virus infection in lipid metabolism.** *BioMed Res Int* 2013; **2013**:836790.
- 60 Echeverria P, Guardiola M, González M, Vallvé JC, Bonjoch A, Puig J, *et al.* **Association between polymorphisms in genes involved in lipid metabolism and**

- immunological status in chronically HIV-infected patients.** *Antiviral Res* 2015; **114**:48–52.
- 61 Limou S, Delaneau O, van Manen D, An P, Sezgin E, Le Clerc S, *et al.* **Multicohort genomewide association study reveals a new signal of protection against HIV-1 acquisition.** *J Infect Dis* 2012; **205**:1155–1162.
- 62 Lingappa JR, Petrovski S, Kahle E, Fellay J, Shianna K, McElrath MJ, *et al.* **Genomewide association study for determinants of HIV-1 acquisition and viral set point in HIV-1 serodiscordant couples with quantified virus exposure.** *PLoS One* 2011; **6**:e28632.
- 63 Yantsevich AV, Dichenko YV, Mackenzie F, Mukha DV, Baranovsky AV, Gilep AA, *et al.* **Human steroid and oxysterol 7 $\alpha$ -hydroxylase CYP7B1: substrate specificity,azole binding and misfolding of clinically relevant mutants.** *FEBS J* 2014; **281**:1700–1713.
- 64 Martin C, Bean R, Rose K, Habib F, Seckl J. **cyp7b1 catalyses the 7 $\alpha$ -hydroxylation of dehydroepiandrosterone and 25-hydroxycholesterol in rat prostate.** *Biochem J* 2001; **355**:509–515.
- 65 Bauman DR, Bitmansour AD, McDonald JG, Thompson BM, Liang G, Russell DW. **25-Hydroxycholesterol secreted by macrophages in response to Toll-like receptor activation suppresses immunoglobulin A production.** *Proc Natl Acad Sci U S A* 2009; **106**:16764–16769.
- 66 Mazzoli S, Trabaironi D, Caputo SL, Piconi S, Blé C, Meacci F, *et al.* **HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals.** *Nat Med* 1997; **3**:1250–1257.
- 67 Mazzoli S, Lopalco L, Salvi A, Trabattoni D, Caputo SL, Semplici F, *et al.* **Human Immunodeficiency Virus (HIV)-Specific IgA and HIV Neutralizing Activity in the Serum of Exposed Seronegative Partners of HIV-Seropositive Persons.** *J Infect Dis* 1999; **180**:871–875.
- 68 Sironi M, Biasin M, Pontremoli C, Cagliani R, Saulle I, Trabattoni D, *et al.* **Variants in the CYP7B1 gene region do not affect natural resistance to HIV-1 infection.** *Retrovirology* 2015; **12**:80.
- 69 Claudio CC, Patin RV, Palchetti CZ, Machado DM, Succi RC de M, Oliveira FLC. **Nutritional status and metabolic disorders in HIV-exposed uninfected prepubertal children.** *Nutr Burbank Los Angel Cty Calif* 2013; **29**:1020–1023.
- 70 Tort O, Escribà T, Egaña-Gorroño L, de Lazzari E, Cofan M, Fernandez E, *et al.* **Cholesterol efflux responds to viral load and CD4 counts in HIV+ patients and is dampened in HIV exposed.** *J Lipid Res* 2018; **59**:2108–2115.

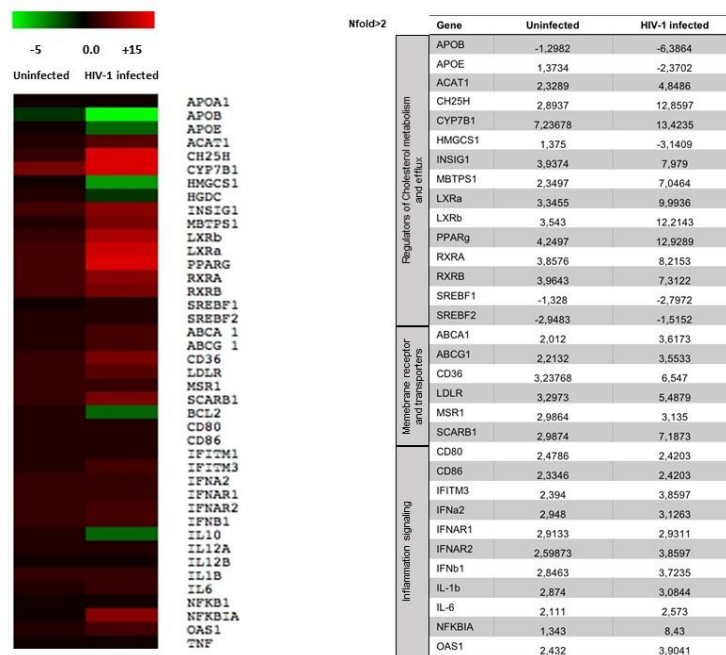
**Figure 1. Susceptibility to in vitro HIV-1 infection was reduced in peripheral blood mononuclear cells (PBMCs) and human monocyte derived macrophages (MDMs).** (A) PBMCs from 15 HESN (gray bar) and 15 HC (white bars) were in vitro HIV-1-infected with a R5 HIV1Ba-L. (B) MDMs from from 10 HESN (gray bar) and 10 HC (white bars) were in vitro infected with a R5 HIV1Ba-L. P24 concentration was measured by ELISA in 7 days post in vitro HIV-1 infection supernatants in all the experiments. Mean values +/- SEM are shown. \* $p < 0.05$ .



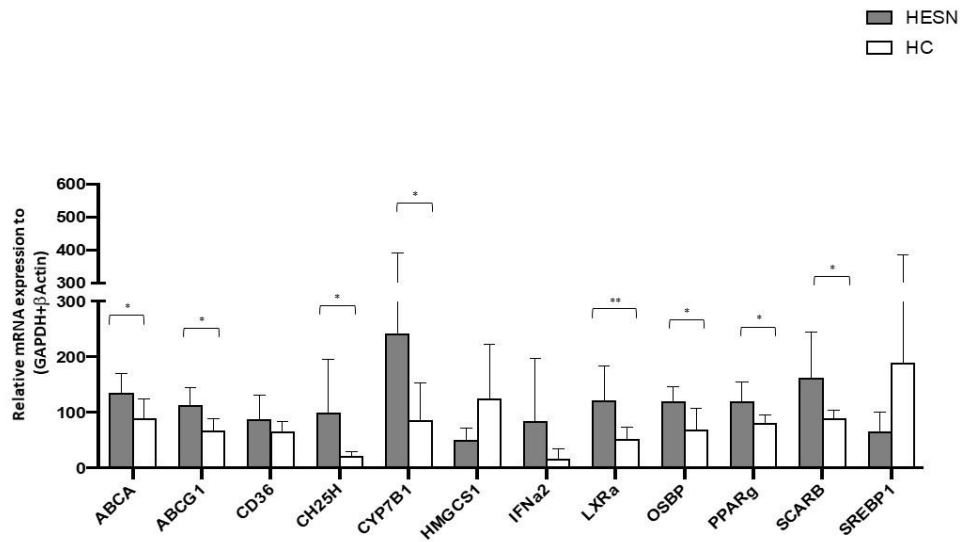
**Figure 2. The percentage of IFN $\alpha$  producing plasmacytoid dendritic cells (pDCs) was increased in HESN compared to HC in both uninfected and HIV-1-infected PBMCs.** (A) Percentage of IFN $\alpha$  producing pDC from 15 HESN (gray bars) and 15 HC (white bars) in uninfected and HIV-1 infected condition. Mean values +/- SEM are shown. \*\*  $p < 0.001$ , \*\*\*  $p < 0.001$



**Figure 3. mRNA expression of 43 genes that are part of cholesterol signaling and inflammatory response was significantly altered in uninfected and HIV-infected PBMCs from HESN.** Real-time PCR Array was performed on uninfected and in vitro HIV-1-infected peripheral blood mononuclear cells (PBMCs) from 15 HESN and 15 HC. Samples were pooled into two distinct groups (HESN and HC). Gene expression (*n*fold) is shown as a color scale from green to red (MEV multiple experiment viewer software). The *N*fold of HESN vs HC untreated and HIV-infected are shown. Only targets showing at least >2-fold modulation are shown in table.



**Figure 4. mRNA expression of genes involved in cholesterol metabolism and efflux was significantly altered in HIV-1-infected MDMs from HESN.** mRNA expression of genes involved in cholesterol metabolism and efflux was evaluated by Real time PCR in 7-days *in vitro* HIV-1-infected MDMs from 10 HESN (grey bars) and 10 HC (white bars). Mean values +/- SE are shown. \*  $p < 0.05$ . \*\*  $p < 0.01$ .



ACCF

**Figure 5. p24 viral antigen concentration and mRNA expression of genes involved in cholesterol metabolism and efflux were significantly different in in vitro HIV-1-infected MDMs treated with 25hydroxy cholesterol (25HC)** (A) MDMs from 5 HC were in vitro HIV-1-infected with a R5 HIV1Ba-L with (dark gray bars) or without 25HC (light gray bars) and p24 viral antigen was measured by ELISA 7days post infection. (B) mRNA expression analyses of genes involved in cholesterol metabolism and efflux was higher in MDMs from 25HC-treated (dark gray bars ) compared to untreated (light gray bars) MDMs 7-days post in vitro HIV-1-infection. Mean values +/- SEM are shown. \*p < 0.05.

