LIPIDOMIC PROFILE OF IMMUNOLOGICAL NON-RESPONDERS HIV-POSITIVE PATIENTS

Final Degree Thesis

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Summary

The human immunodeficiency virus infection (HIV) is widespread around the world with approximately 38 million people infected. The biggest problem of this virus is the inability to eradicate it due to its persistence mechanisms in human cells. The combined antiretroviral treatment (ART) reduces the HIV viremia and improves the $CD4^+$ T-cells count and CD4/CD8 ratio. Recovery of $CD4^+$ T-cell proliferation and decreased $CD4^+$ lymphocyte destruction does not occur in some patients after ART initiation. These patients are called "immune non-responders" or "poor immune responders" (INR). The prognosis of patients with poor immune recovery is much worse as they have a greater number of diseases related or non-related to acquired immunodeficiency syndrome (AIDS).

The early identification of INRs could improve their prognosis by trying to reduce its possible effects. Hence, the study of molecules for early identification of these patients is innovative and of great importance. The omics approach allows the simultaneously study of a great quantity of molecules at different stages of the HIV infection.

In this pilot study, the lipidomics of HIV-positive patients before and after initiation of antiretroviral treatment were analysed in $CD4^+$ T- and $CD8^+$ T-cells. Using lipidomics, we mostly identified important difference in the lipid profiles in the $CD4^+$ T-cell between the immunological responders (IRs) and immunological non-responders. The most statistically significant differences among groups were achieved in concentration of PC 30:0 and TG 60:4.

Lipids are involved in many cellular processes including cell membrane formation, transport of molecules across the membrane, cell signalling and inflammatory processes, among others. Altered concentrations of these lipids in INR patients may be related to their poor immune system recovery. Thus, the obtained lipid species could be further analysed in future research as possible biomarkers of immunological non-responders.

Introduction

Human immunodeficiency virus

The Human Immunodeficiency Virus (HIV) is a retrovirus first isolated in 1983 when a big epidemic was confronting the world, the Acquired Immunodeficiency Syndrome (AIDS)[1] .The HIV is a retrovirus originated from a simian lentivirus (simian immunodeficiency virus, SIVs) into chimpanzee reservoirs[2]. The chimpanzees show similar symptoms as AIDS patients. The principal theory is that there was a zoonotic transmission and recombination between primates and humans that led to the emergence of HIV. The cross-species transmission is more likely to succeed among species which share a large part of their genetic code. The human and primates have a close genetic relationship sharing almost the 99% of their genome[3].

Two different types of HIV were identified, HIV-1 and HIV-2, which are genetically and serologically different. The HIV-1 was first isolated in 1983, while the HIV-2 was isolated later when some serological differences where observed in the HIV coming from several patients [4]. Worldwide, the HIV-1 is the most aggressive and spread in Europe. By contrast, the HIV-2 is less pathogenic and normally located in Africa. The research studies showed that the $CD4^+$-T cell decline is slower in the HIV-2 infected patients; and that the transmission and viral load is lower compared to HIV-1 infected patients. Thus, these studies confirmed that HIV-1 is more aggressive than HIV-2 [5].

According to latest data of the Joint United Nations Programme on HIV/AIDS (UNAIDS), 38 million people live with HIV all over the world, but only 26 million have access to the antiretroviral therapy (ART). From the start of the pandemic in the early 80s, approximately 76 million people have been infected with HIV and 33 million have died. Thus, the need to research and study the possible HIV treatments accessibly to the entire world's population is mandatory. And the importance of studying all the mechanisms that the virus uses to infect and remain in the host, which makes impossible to eradicate the virus from the organism, is determinant to find a functional cure [6].

Structure and Genome

The HIV-1 RNA genome is made up of 9181 base pairs with 10 genes which encodes for 10 proteins. The genome is flanked by 2 long terminal repeat sequences (LTR). The proteins encoded by the genome are: Gag, Pol, Vif, Vpr, Vpu, Env, Antisense protein (ASP), Nef, Tat and Rev **(Figure 1)** [7]. These genes have structural and regulatory functions as well as being involved in the virus replication and infection of the host cell. The gag and env genes encode structural proteins which form the capsid and the envelope, respectively [8]. On the other hand, the pol gene encodes for enzymatic proteins related to DNA synthesis and integration [9]. The rest of the genes have regulatory functions (tat and rev) or act as accessory genes (nef, vif and vpr) [8].

The HIV-1 genome has large mutation and recombination rates which happened to be one of the reasons that nowadays there is not a HIV cure [10].

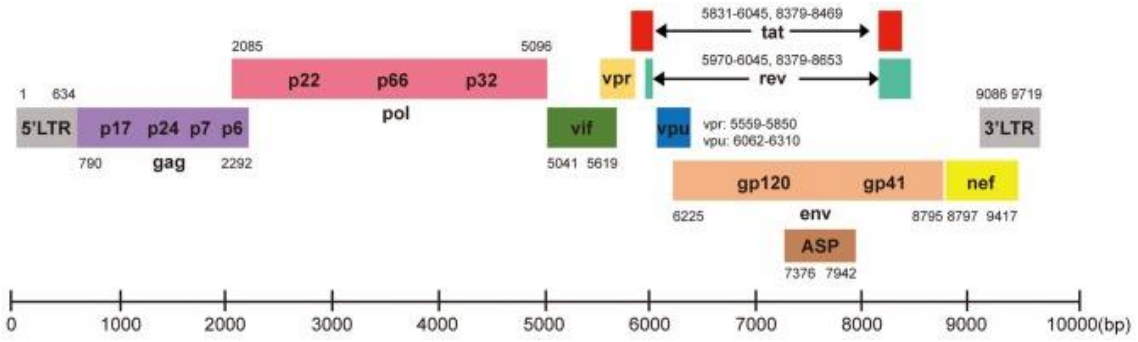

Figure 1. HIV virion RNA genome. The genome encodes for 10 proteins: Gag, Pol, Vif, Vpr, Vpu, Env, ASP, Nef, Tat and Rev [7].

The HIV virion has to maturate to fully accomplish the structure and infective capacity. Therefore, there are several structural differences between the immature and mature virion **(Figure 2)** which are the two morphological forms of the virion.

The HIV virion has a spherical shape with three principal parts: core, capsid and envelope. The core contains two RNA strains and the reverse transcriptase which is a RNA-dependent DNA polymerase. The two RNA molecules form a ribonucleoprotein complex with the Nucleocapsid proteins (NC) to maintain a stable structure. The envelope consists of a lipid bilayer where we can found the viral envelope protein (Env).

The principal difference between the immature and mature virion is the capsid organization. The mature virion capsid is composed by the Gag protein products of maturation obtained by proteolysis. The capsid has a cone shape formed by pentamers and hexamers of the Capsid Protein (CA) **(Figure 2)** [11]**.**

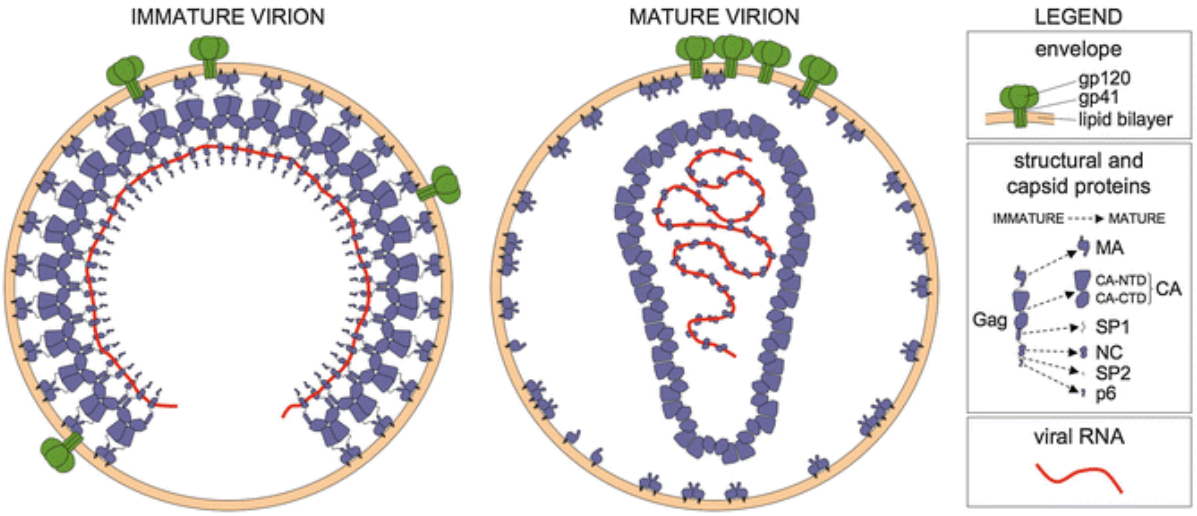

Figure 2. Scheme of immature and mature virion structure [11].

Viral replication cycle

The cycle life of the HIV-1 virus is related with the mechanism of the human host cell and described in 5 different stages. Being the first one the viral attachments and fusion, and the last one the virus maturation. Firstly, the virion binds to the receptor, CD4 molecule (identified in 1984), and a co-receptor, the CXC-chemokine receptor 4 or CXC-chemokine receptor 5 (identified in the 1996) [1]. Then, the viral envelope and the cell membrane fuse, hence the HIV enters into the host cell. The reverse transcription is the next stop to obtain the viral cDNA which enters into the cell nucleus to integrate into the DNA of the host. When the viral DNA is integrated into the DNA, transcription and translation allow the production of the viral proteins and RNA. When all the viral products are ready, assembly takes place. Finally, the virion exits (budding) and matures outside the cell **(Figure 3)** [12].

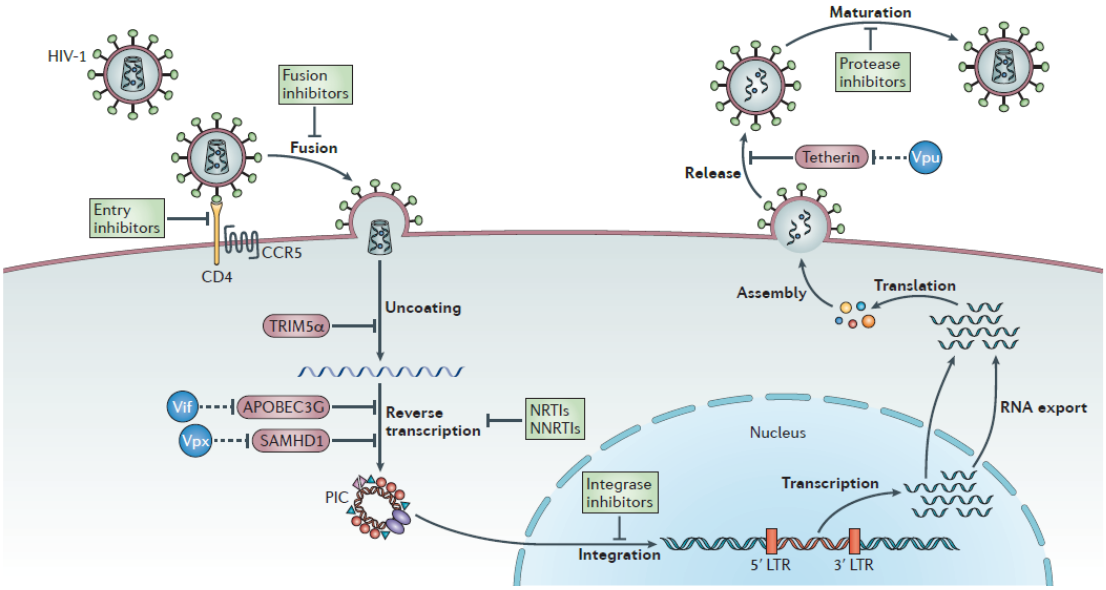

Figure 3. Stages of the viral replication cycle. Starting from the infection of the cell to maturation of the virion [1].

HIV development and progression

The progression of the HIV in the host could be measured by using circulating biomarkers as the $CD4^+$ T-cell count (cells/mm^3), the viral load (RNA copies/mL) **(Figure 4)** [13], the CD4/CD8 ratio, the p24 antigen (HIV viral protein) or HIV antibodies. The $CD4^+$ T-cell count biomarker, along with viral load, is the most widely used to monitor the progression of infection in HIV-positive patients[14].

HIV infection is characterised by a decrease in $CD4^+$ T-cells and an increase in $CD8^+$ T-cells. Therefore, the CD4/CD8 ratio decreased in HIV-positive patients. Thus, like viral load and $CD4^+$ T-cell count, the CD4/CD8 ratio may act as a biomarker of immune response activation and immune dysregulation [15,16].

During the initial infection, the patient enters into the acute phase where the viral RNA levels increase. The $CD4^+$ T-cell count suffers a fast decrease and the amount of p24 start to increase drastically[13]. The CD4/CD8 ratio decreases during the acute phase. Furthermore, in most patients these CD4/CD8 ratio values are maintained throughout the progression of the infection[15]. During this stage, the replication and multiplication of the virus is fast and the host has a great capacity to transmit the virus. Thus, there is very important to detect the HIV infection early to could initiate the antiretroviral therapy. This phase takes approximately 2-4 weeks minimum and the main symptoms are flu-like symptoms such as headaches, fever, among others... At the end of the acute phase, there is a short recovery period. During this period, the $CD4^+$ T-cell count increases, while the viral load decreases.

At the beginning of the chronic phase, the $CD4^+$ T-cell count decreases steadily, the viral load and the levels of antibodies start to increase at the same time that the levels of p24 antigen decreases due to the formation of the antibodies-p24 antigen complex. During this phase, the patient is in a latent phase. The replication of the virus slows down, so the virus does not multiply at the same rate as in the previous phase. Finally, the flu-like symptoms have disappeared so it is an asymptomatic phase.

The chronic stage can last between 5-10 years depending on the individual. At this moment, the progression of the disease could vary according to the patient and the ART treatment. This period time is accomplished if the patient hasn't had any antiretroviral treatment because the disease progresses and the patient can lead to AIDS. In fact, AIDS is the late stage of HIV infection that occurs when the body's immune system is badly damaged because of the virus. The AIDS phase is characterized by the reduction of the $CD4^+$ T-cells count and the viral load increase **(Figure 4)** [13,14]. The CD4/CD8 ratio is maintained during all the HIV-infection phases. Non-recovery of CD4/CD8 ratio pre-infection values during disease progression and treatment of HIV patients is due to $CD8^+$ T-cell proliferation. The $CD8^+$ T-cells are involved in the immune response against the HIV virus that remains in the host event the successfully response to the antiretroviral treatment [16]. During this phase different comorbidities can appear due to the weak immune system that AIDS provokes.

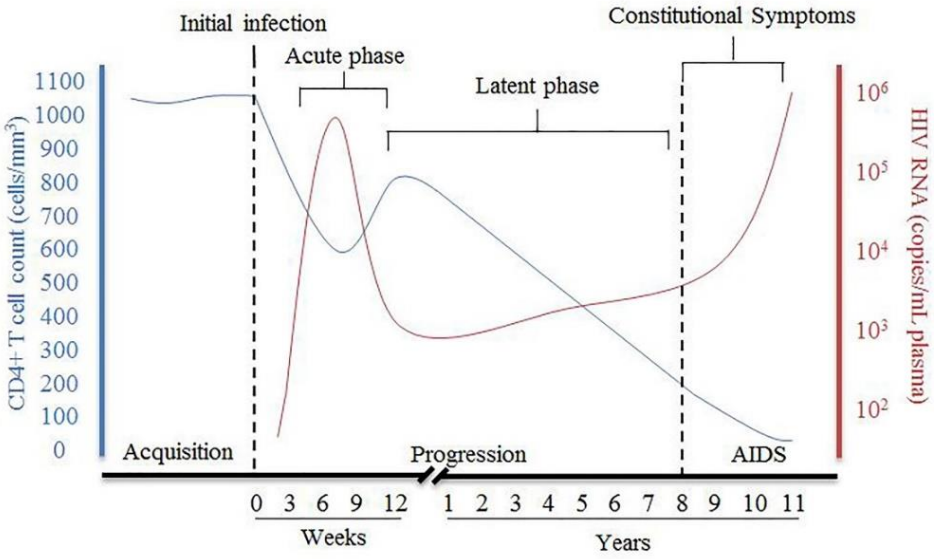

Figure 4. Phases of the HIV progression. The infection progression was marked by the $CD4^+$ T-cell count and the viral load [13].

HIV treatment

The principal HIV treatment is the ART therapy. It is based in the combination of different antiretroviral drugs. This therapy has reduced the mortality and morbidity of the AIDS and it was approved in 1987. It has also reduced the expansion of the infection. The ART cannot eliminate completely the HIV of the organism due to the mechanisms of persistence: persistent replication, latent cellular reservoir, the immune system incapacity to recognise and eliminate the infected cells, and virus accommodation in anatomical reservoirs as the Gut-associated lymphoid tissue (GALT) [17].

ART turns AIDS into a chronic disease. Therefore, the medication must be administered for life. Although ART treatments have been substantially improved and reduced their toxicity, HIV-positive people still developing much clinical outcomes associated to ART toxicity as well as non-AIDS associated co-morbidities. The co-morbidities could decrease the life quality of the individual or even provoke death. The co-morbidities could be cancer, co-infection with other virus such as hepatitis C virus (HCV), cardiovascular disease (CVD), renal insufficiency, pneumonia or metabolic syndrome, among others [18,19].

The principal aim of the ART is preventing the replication of the HIV. To accomplish this goal different types of drugs are used. Each one of the ART drugs inhibit different stages of the HIV replication cycle [20]. The drug classes used includes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 antagonists, integrase strand transfer inhibitor (INSTIs), attachment inhibitors, post-attachment inhibitors or pharmacokinetic enhancers (boosters). The drugs can be combined in several ways depending of the patient [12].

In the last years, other techniques have been study as treatment to the HIV infection. Some of the techniques are based in the elimination of the latent cellular reservoir through suppression of HIV transcription. Other research studies based the treatment in early initiation of ART treatment and pharmacological reactivation of latency using shock and kill. Other techniques use immunological methods[17] or are based in the vaccine finding and the use of neutralising monoclonal antibodies [21]. Other clinical studies are using gene therapy in which the individual own cells can be modified or RNA therapies [22]. The problem is that all of these techniques are under development and currently under study. Therefore, the best treatment at the moment is the ART therapy.

Immunological non-responders and responders

The HIV infection morbidity and mortality successfully decreases during ART suppression treatment. The ART aim is to increase the $CD4^+$ T-cell proliferation and decrease the destruction. The antiretroviral therapy also regulates the HIV viral load by inhibition of the virus replication. Nevertheless, part of HIV-infected patients does not recover the $CD4^+$ T-cell count after initiation of the viral suppression treatment. These patients control the HIV viremia but they are called patients with discordant response due to the lower $CD4^+$ T-cell count threshold [23,24].

The main biomarker used to mark the disease progression during the infection and the treatment outcome is the $CD4^+$ T-cells counts. Other biomarkers can be used as the viral load, the CD4/CD8 ratio and the $CD4^+$ T-cells count increase [13,15]. But nothing could predict the evolution of HIV patients before initiation of ART therapy.

The immunological responders (IRs) were defined as the HIV-positive patients who are under treatment during 2 years or more and react positively to the ART therapy. These patients, after starting medication treatment, increases successfully their $CD4^+$ T-cells counts (more than >250 cells/µl) and decreases the viral load to undetectable levels (<50 copies/ml). Another biomarker to identify the IRs is the absolute increase of more than 100 $CD4^+$-T cells /µl from the baseline after 1 year of the initiation of antiretroviral treatment [23].

The immunological non-responders (INRs) were defined as the HIV-positive patients who successfully response to ART (viral load is under 50 copies/ml) but whose $CD4^+$ T-cells count is under 250 cells/µl after 2 years or more of ART. These patients have a prevalence of 10-40% depending of the study population and the INRs description. The INRs have a higher rate of morbidity and mortality due to HIV related and non-related diseases [23,24].The infections and illnesses could be related with the non-immunity recovery [25]. Hence, the incomplete immune reconstitution puts INRs in more danger than the IRs [26]. Some cohort research studies have differentiated the inflammatory pattern of INRs from that of IRs. This fact shows that persistent inflammation may differ between these two types of patients [27]. The study of the differences between the INRs and IRs is important, so the INRs can be identified in early stages and treated to achieve the immune recovery.

Mechanisms related with the immunological non-responders individuals

The incomplete immune reconstitution is related with several factors. The low immune recovery could be due to problems with the $CD4^+$ T-cell production or destruction, as well as other factors such as age or genetics, among others.

The mechanisms related with the $CD4^+$ T-cell production are involved with the production in the bone marrow and thymus. The hematopoietic progenitor cells (HPCs) and the hematopoietic cells can be infected by HIV due to the expression of the receptors and co-receptors. Therefore, the production of these cells could be altered by the HIV infection. Recent studies have elucidated INRs differences in the production of several lymphocytes lineages. Moreover, some research studies have found that cytokines influence the reconstruction of the immune system. And some cytokines are essential to proliferation and production of T-cells as the Interleukine-7 (IL-7), its receptor (IL-7R) and Interleukine-6 (IL-6) **(Figure 5)** [23,27].

The mechanisms related with the $CD4^+$-T cells destruction involved several factors. Some factors such as the T-cell exhaustion occasioned by the immune checkpoint receptors (ICRs). Also, the increase concentration or the hyperproliferation of the $CD4^+$ T-cells, due to the immune activation and inflammation which provokes cell apoptosis. The immune activation could also be as a consequence of the dysbiosis of the gut microbiota, the microbial translocation and coinfections **(Figure 5)** [23]**.**

There are other mechanisms and factors affecting the immune reconstitution as male sex, older age, genetic factors, metabolic characteristics or ART treatment [23,25].

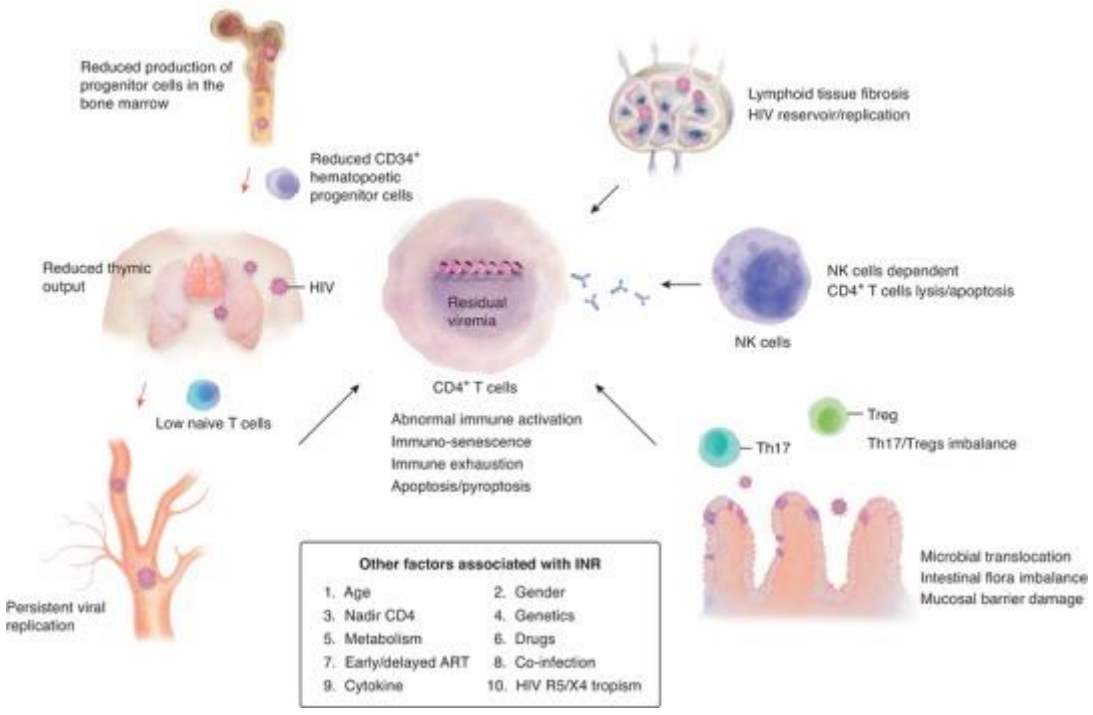

Figure 5. Factors associated to poor immune recovery of HIV-positive patients. The principal factor associated with poor immune recovery is the residual viremia in the $CD4^+$ T-cells. The other factors contributed to exacerbate the residual viremia such as persistent viral replication, reduced thymic output, microbial translocation, cell apoptosis, HIV reservoirs[23]…

Omics studies in HIV studies

The omic studies are a set of biological science based on the study of several molecules to understand their paper in the cellular pathways that become more popular and useful in the latest years. The omics studies are used to understand the pathways involved in diseases and find possible treatment in biomedicine and biosciences.

The omics sciences could be represented as a cascade. The cascade is based on the study of cellular molecules from gens to metabolites. The cascade is formed by genomics, epigenomics, transcriptomics, proteomics and metabolomics **(Figure 6)**. The omics sciences enable the study of cellular processes as a whole. They provide an overall picture of the interactions occurring in the cell or organism at a specific point in time. These types of techniques are used in early research to screen a large number of compounds simultaneously. A selection of a smaller group of compounds is carried out. The omic sciences have been used in the latest years in biomedical sciences to identify biomarkers, test possible treatments and analyse biological and chemical compounds unbalance [28].

Concretely, metabolomics is an emerging field and is broadly defined by the large-scale study and identification of the pathways and chemical processes of the metabolome in a given cell or organism [29]. The metabolomics includes two different substudies, lipidomics and glycomics. Lipidomics is the large-scale study of the structure, function and pathways of the lipidome produced in a cell or organism just like their interactions with other compounds in the cell [30].

The lipidome plays an essential role in the cellular metabolism. The lipids are involved in the maintenance of membrane structure, cell signalling, energy production processes, transport across the membrane, among others. The last years, lipidomics science has given the opportunity to study the implication of cellular lipid species in some diseases such as cancer, metabolic syndrome, personalised medicine... Multi-omics studies have been carried out to study the interaction of all the different compounds and their implication in a disease [31]. Recent untargeted metabolomics profiling studies have shown the dyslipidemia and lipid abnormalities shown by HIV infected patients. Moreover, the lipid abnormalities were linked with inflammation biomarkers (IL-6 and IFN-α) and microbial translocation [32].

Recent studies have identified some metabolites and lipids that differ between the INRs and IRs after initiating ART therapy, indicating that specific metabolites and lipid species can be used as biomarkers. Some of the metabolites identified were the L-tyrosine and L-glutamate, both involved in proinflammatory cytokines production and immune activation [24]. Regarding the lipid profile, most of the INRs showed differences in the general concentration of HDL and VLDL before ART therapy. The HDL molecules, as apoliprotein A-I, are involved in inflammatory processes and the glucose metabolism [33].

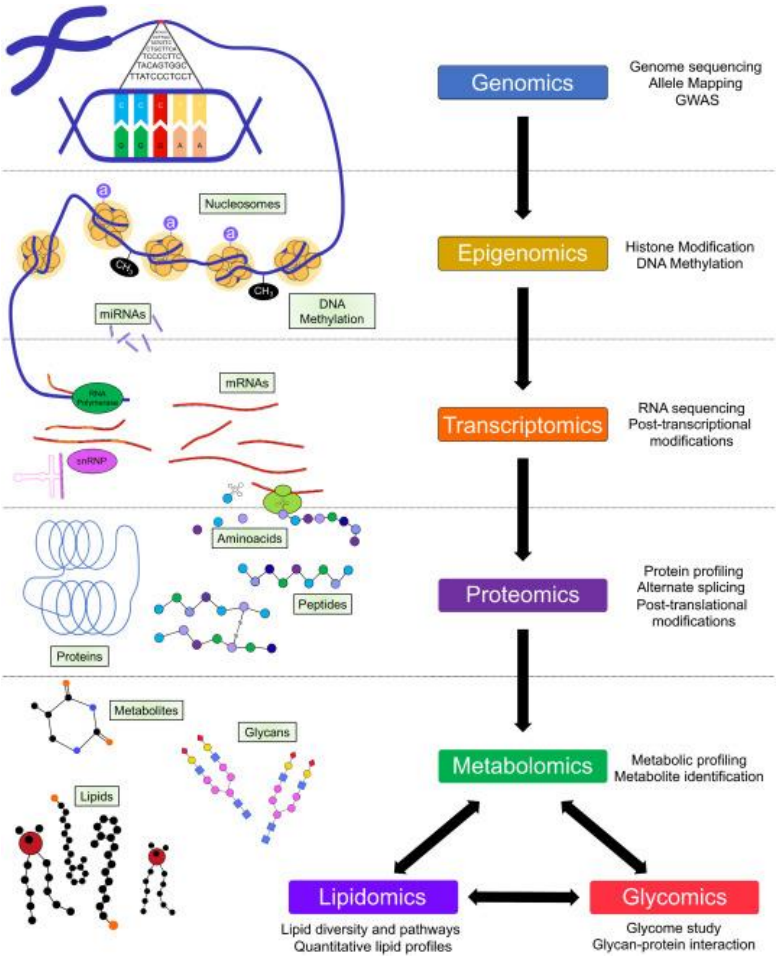

Figure 6. From the study of the genome, throughout the epigenetic changes, transcription to mRNAs, to translation into proteins and formation of metabolites [44]

Hypothesis and Objectives

The main hypothesis of this thesis is the capacity of the lipid profile to become a prognosis biomarker in $CD4^+$ and $CD8^+$ lymphocytes in HIV-positive patients who have a low $CD4^+$ T-cell recovery. Hence, some lipid species could work as marker of poor immune recovery.

The main objective is to find lipidomic biomarkers in $CD4^+$ T- and $CD8^+$ T-cells, to identify and differentiate the patients who will be responders to the antiretroviral treatment and the patients who will be non-responders to the antiretroviral treatment.

As been said, the identification of immunological non-responders HIV-positive patients could improve their prognosis and avoid the development of some co-morbidities due to the anticipation in the diagnosis.

The secondary objectives are:

- Identify de main lipids in the principal metabolic pathways that are different between the immunologic responders and non-responders.
- Identify the main lipids in the principal metabolic pathways that are different between patients with low $CD4^+$ T-cell count (cases) compared to those patients with high $CD4^+$ T-cell count (control) at the beginning of the antiretroviral treatment.

Materials and methods

Participants selection and characteristics

The study cohort included 100 HIV-patients from five different hospitals from Catalonia. The hospitals are: Hospital Universitari Joan XXIII, Hospital Can Ruti/IrsiCaixa, Hospital Vall d'Hebrón, Hospital Clínic de Barcelona and Hospital Sant Pau. The cohort consisted of HIV-positive patients was divided into two groups, controls (n=36) and cases (n=64).

From the initial cohort, only the 31 patients corresponding to Hospital Universitari Joan XXIII in Tarragona were analysed for the present work **(Figure 7)**. The selection of only 31 patients was due to the inability to obtain lipidomics results for the rest of the patients in time. The patients were divided in controls (n=19) and cases (n=12), categorization of these groups was based on the $CD4^+$ T-cell counts before taking ART treatment. The control patients had > 350 cells/µl, meanwhile the cases had < 200 cells/µl before starting ART therapy. Moreover, the cases were also divided in IRs (n=7) and INRs (n=5) depending on the $CD4^+$ T-cell counts after 48 weeks of initiation of the ART therapy. After the treatment, the IR patients were described by a $CD4^+$ T-cell count >250 cells/µl and the INR patients were defined by ≤250 cells/µl **(Figure 7)**.

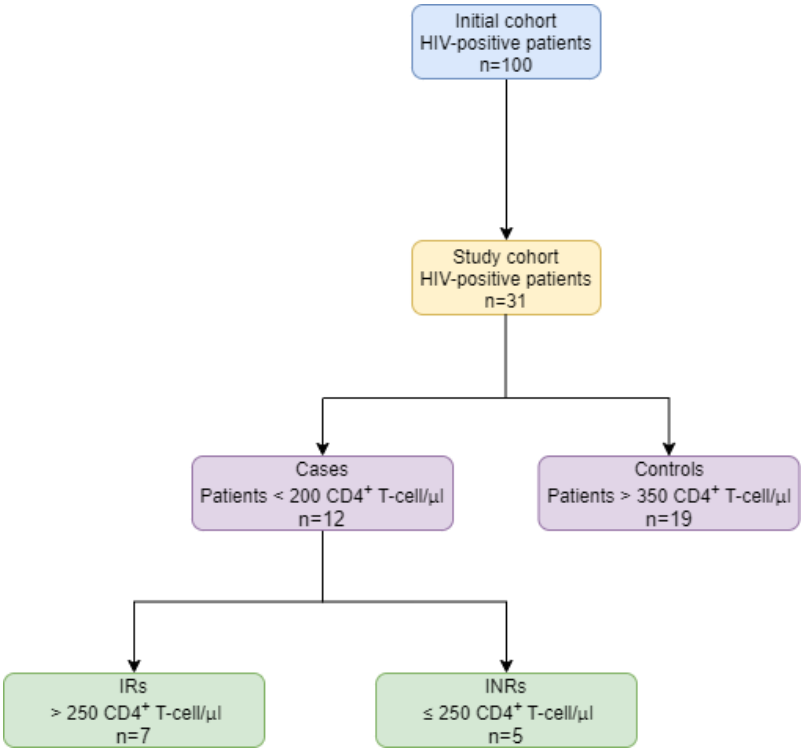

Figure 7. Flowchart of the groups studied in the investigation. The 69 HIV-positive patients excluded from the final cohort (n=13) did not belong to the Joan XXIII Hospital. The exclusion of 69 patients from the study cohort was due to the delay in obtaining lipidomics results.

Cells isolation

The initial sample was total blood, approximately 6 tubes Leucosep (9 ml) of each patient for the extraction of the PBMCs with Ficoll. Once the PBMCs were obtained, the $CD4^+$ T- and $CD8^+$ T-cells were isolated in an Automacs using MicroBeads. The cells were stored into 3 tubs/per subcellular type at -80ºC. Each tube of cells was subjected to lipidomics analysis.

Lipidomics

Hydrophobic lipids were obtained from the cells by liquid-liquid extraction with chloroform:methanol (2:1) and subsequent sonication to rupture the cell membrane. The NaCl was added to extract more hydrophobic lipids through the formation of a layer containing hydrophilic lipids. The separation of the hydrophobic lipids was performed by UHPLC-qTOF (model 6550 of Agilent, USA) using a C18 column (Kinetex EVO C18 Column, 2.6 µm, 2.1 mm X 100 mm). The match of the accurate mass and tandem mass spectrum was used to identify the lipid species.

Statistical analysis

The statistical analyses performed were based on the groups previously defined: control were defined as HIV-positive patients with higher $CD4^+$ T-cell count, and cases were defined as HIV-positive patients with lower $CD4^+$ T-cell count before initiating ART treatment. The cases were subdivided as IRs and INRs. From the same patient, the information obtained by the analysis was divided depending on $CD4^+$ T- and $CD8^+$ T-cells data.

First, the crude data obtained from lipidomics analyses should be normalized using the cell number from each sample. The identification of the outliers was done by box plot graphical analysis. Hence, the patients with out-of-range lipid profiles were eliminated for the following analyses. The U Mann Whitney analysis allowed us to compare two groups with independent and non-parametric data. While the Wilcoxon analysis allowed us to compare the two cell types which are dependent data. The IBM SPSS 22 was the statistical software used to perform a large number of analyses including Wilcoxon and Mann Whitney tests. Metaboanalyst is an online open statistical programme specifically designed for the analysis of metabolites, in our case was used to analyse the lipidomics data. The graphics representation was performed by GraphPad Prism 8.0.2 which is a graphical software and it enabled to analyse the data collected.

Results

The baseline clinical characteristics of the 31 patients included in this study were represented in the **Table 1**. The characteristics were obtained from the database of the Hospital Universitari Joan XXIII. The patients median age was different in the controls, immunological responders and immunological non-responders (39, 43 and 34,5 years), with more than 50% of males per group. The homosexuality risk factor (57,1 %) and HCV co-infection (28,6 %) was similar between controls and IRs in contrast with INRs (25%), on the other hand the heterosexually risk factor (75%) and Hepatitis C virus co-infection (50 %) was higher in INRs. Based on the classification criteria of the groups, before initiation of ART treatment the controls had 370 [290-420] $CD4^+$ T-cells/µL and 0.51 [0.42-0.64] CD4/CD8 ratio while cases (IRs and INRs) had lower $CD4^+$ T-cell counts (150 [130-170];125 [30-168]) and CD4/CD8 ratio (0.19 [0.14-0.26]; 0.094 [0.03-0.16])

Table 1. Patients baseline clinical characteristics. Qualitative variables were expressed by number of patients (percentage). Quantitative variables were expressed by median [interquartile range]. The cells count and ratio median expressed were baseline values. IR, immunological responders; INR, immunological non-responders; HCV, hepatitis C virus. The patient groups were different qualitatively and quantitatively in age, sex, risk factor, HCV co-infection, CD4+ T- and CD8+ T-cell count and CD4/CD8 ratio.

Clinical characteristics	Controls	IRs	INRs
Age (years)	39 [30-48]	43 [34-51]	34.5 [28-42]
Male	6 (85.7)	4 (57.1)	3 (75.0)
Risk factor			
Homosexual/bisexual	4 (57.1)	4 (57.1)	1 (25.0)
Heterosexual	2 (28.6)	3 (42.9)	3 (75.0)
Unknown	1 (14.3)		
HCV co-infection (positive)	2 (28.6)	2 (28.6)	2 (50.0)
Baseline			
$CD4^+$ T-cell count (cells/µl)	370 [290-420]	150 [130-170]	125 [30-168]
$CD8^+$ T-cell count (cells/µl)	640 [390-1010]	670 [580-1290]	1020 [710-1443]
CD4/CD8 ratio	0.51 [0.42-0.64]	0.19 [0.14-0.26]	0.094 [0.03-0.16]

The definition of controls and cases had already marked a difference between these two study groups. The groups were divided by cell type ($CD4^+$ T- and $CD8^+$ T-cell) to compare them.

Lipidomic profile differences between controls and cases

First, in order to identify the significant differences between HIV-positive patient with high $CD4^+$ T-cell count (control) and those patients with low $CD4^+$ T-cell count (cases) at the beginning of the antiretroviral treatment the statistical test were applied in the two different cellular types.

Regarding the $CD4^+$ T-cell type, the significant differences between controls and cases ($p<0.05$) showed by the statistical analysis cases were mainly seen in concentration of phosphatidylcholine (PC) and sphingomyelin (SM), but also in concentration of some lysophospatidylcholines (LPC), which are from same lipid family of PC **(Table 3; Annex 1)**. Then, to confirm the lipid profile contrast between these two groups, we selected the statistically significant lipids ($p<0.05$) to represent the data in a principal component analysis (PCA) and

Random Forest (RF) analysis. As shown in the **Figure 8**, the PCA verified the distinction between the two groups, controls and cases. There were three patients who have a similar profile to the controls.

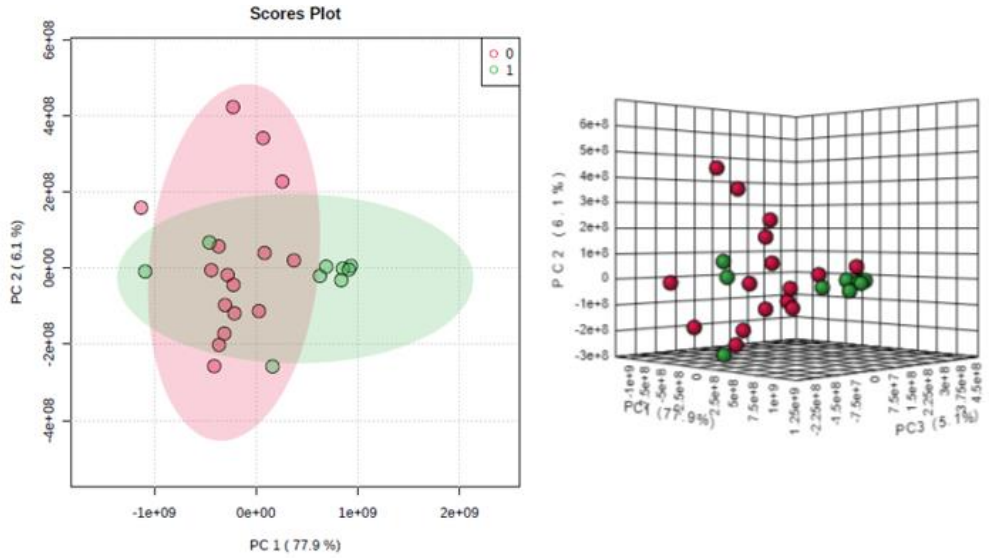

Figure 8. 2D and 3D Principal Component Analysis (PCA) score plot of statistically significant (p<0.05) lipid compounds in the CD4⁺ T-cells. The graphic showed the difference between control (red dots) and cases (green dots) patients groups.

The RF of the statistically significant lipids in the CD4⁺ T-cells **(Figure 9)** showed the compounds that constitute the best predictor of low CD4⁺ T-cell count before the initiation of ART treatment, noting that the lipid with the most discriminatory between both groups was PC 30:0, along with SM 40:2 and PC 36:5.

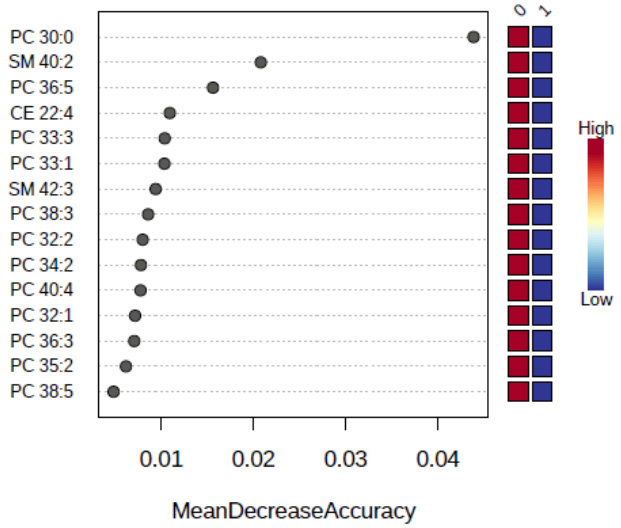

Figure 9. Random Forest analysis based in Mean Decrease Accuracy (MDA) of statistically significant lipids (p<0.05). The MDA range is 0.01-0.05. From the first lipid the prognostic significance of poor immune recovery decreases. Red means high concentration of the lipid in CD4⁺ T-cells, whereas blue means low concentration of the lipid compound. The group 0 (controls) had higher concentration of all lipids compounds than the group 1 (cases).

On the other hand, the statistical test of the $CD8^+$ T-cells showed little disparity between controls and cases, only differ in some types of triacylglycerol (TG) and SM **(Table 4; Annex 1)**. As with the analyses performed with $CD4^+$ T-cells data, those lipids with a P-value lower than 0.05 are represented on a PCA. In that case, the graphical representation **(Figure 10)** showed that differences between the two groups are minimal in $CD8^+$ T-cells. Thus, these results revealed that the difference in the lipid profile of the control and cases is greater in $CD4^+$ T-cells than in $CD8^+$ T-cells.

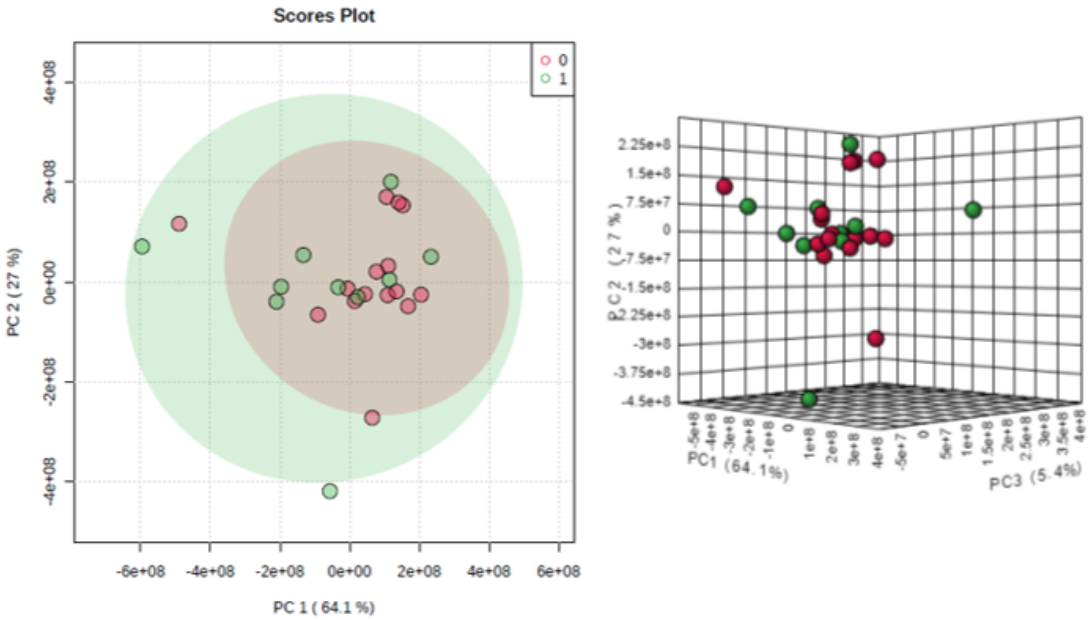

Figure 10. Score plot PCA in 2D and 3D. Representing the difference of statistical significant lipid compounds (p<0.05) in $CD8^+$ T-cells between controls (red dots) and cases (green dots).

Lipid differences between controls and IRs

Once the differences between controls and cases were stablished, we also examined if there were differences between the group of controls and the subgroup of cases with low $CD4^+$ T-cell count at the beginning of the antiretroviral treatment who showed an increased $CD4^+$ T-cells to ART (good immune recovery status), the IRs.

In the $CD4^+$ T-cells, the difference between the lipid profile showed in the statistical analysis is almost non-existent, only two lipids display significant distinction **(Table 5; Annex 2)**. As only two lipid species were statistically significant different (p<0.05) between controls and IRs in the $CD4^+$ T-cells, therefore the PCA graphical representation was not done due to the small amount of data to be represented.

The contrast between the two groups in the $CD8^+$ T-cells was reflected by the results of the statistical analysis, where discrepancies can be observed in lipids as several TG and PC **(Table 7; Annex 2)**. But PCA did not exhibit the same pattern **(Figure 11)**, the compounds profile of the two groups seemed alike.

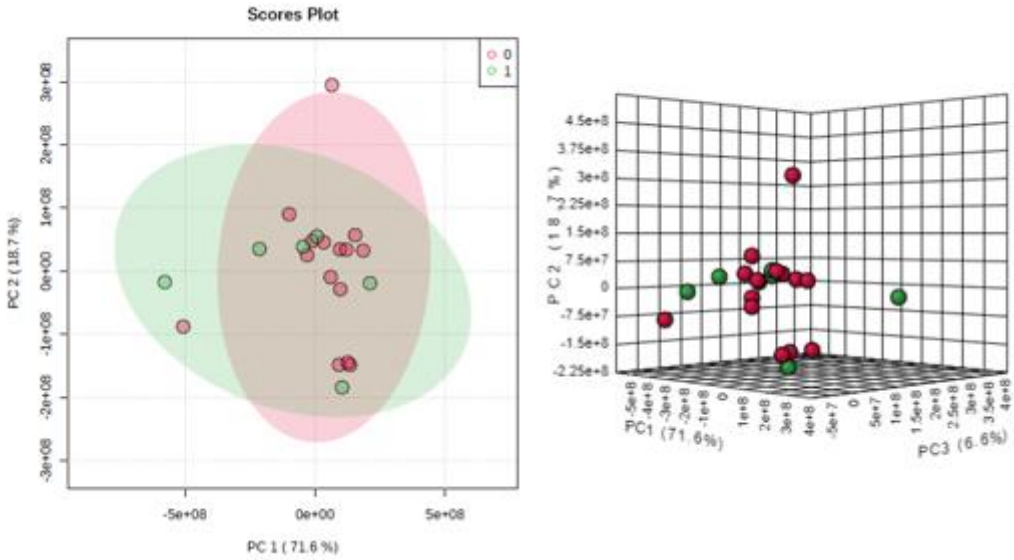

Figure 11. 2D and 3D score plot PCA of significant lipid species (p<0.05) in the CD8⁺ T-cells. Differentiating the groups, Immunological responders (green dots), Immunological non-responders (blue dots) and controls (red dots) by the concentration of the lipid compounds.

Lipid differences between controls and INRs

As in the last section, we also evaluated if there was a contrast in lipid composition between the controls and the HIV-patients with poor immune recovery after initiating ART treatment, the INRs, in $CD4^+$ T- and $CD8^+$ T- cells.

The statistical test revealed that the INRs and controls groups had a significantly distinction ($p<0.05$) on the PC composition in the $CD4^+$ T-cells **(Table 6; Annex 2)**. Hence, some lipid compounds had a big dissimilarity expression in these two groups and could be used to distinguish them.

Again, PCA and RF analyses were performed using statistically significant data to confirmed differences among groups. In that case, the graphic representation of the PCA and RF analyses of the data related to $CD4^+$-T cells reaffirmed the data given by the statistical test. In the PCA, one of the INRs cases was similar to the controls, these could be an outlier but we were not able to know this information due to the small size of the cohort **(Figure 12a)**. What is more, the RF described 4 predominant lipids that would allow the two groups to be distinguished, these were generally phosphatidylcholines, PC 30:0, PC 38:3 and PC 36:3, and a cholesterol ester, CE 22:4 **(Figure 12b)**.

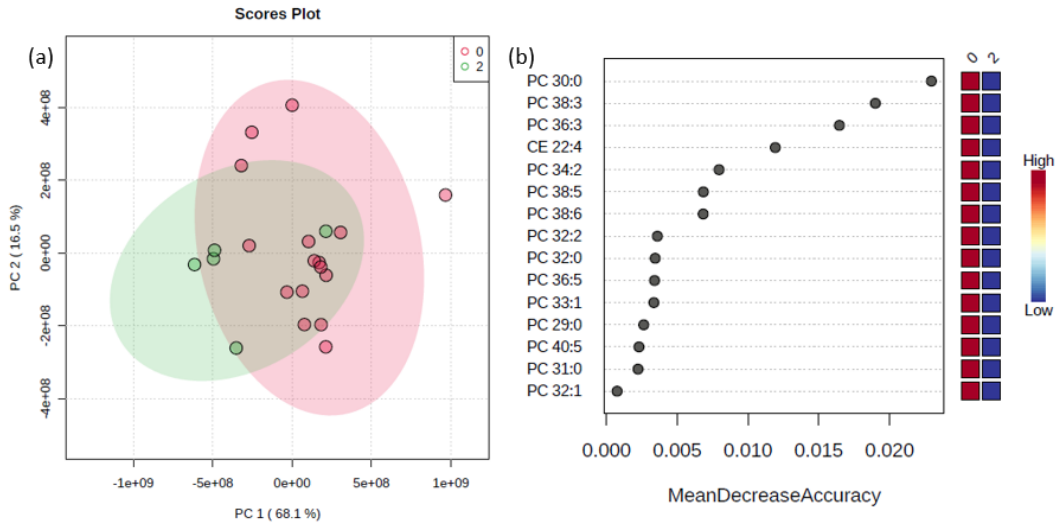

Figure 12. CD4⁺ T-cell lipid profile difference graphical representation. (a) Score plot PCA in 2D of representative lipid species ($p<0.05$) when analysing the difference between controls (red dots) and poor immune recovery patients (green dots). (b) The ranking of lipid species based on RF analysis. The MDA (range 0.000-0.025) marked high concentration (red) of compounds in controls (group 0), unlike the low concentration (blue) in INRs (group 2).

Representing only these lipids, which appeared to be the ones with the greatest differences between groups in bar charts **(Figure 13)**, we could see that in all 4 cases the controls had a higher relative abundance of lipids in $CD4^+$ T-cells than the INRs.

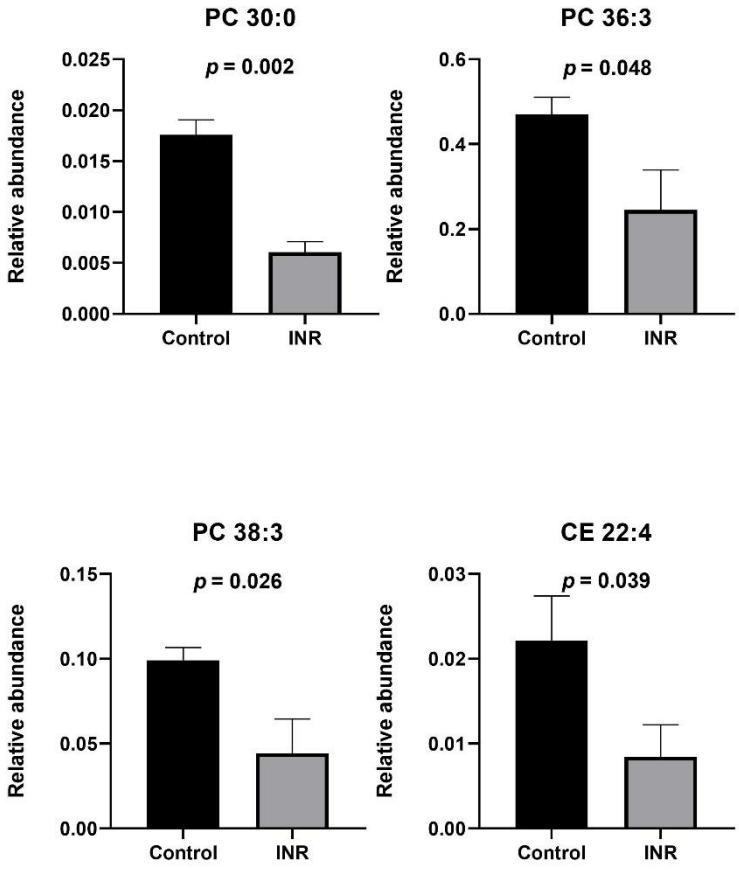

Figure 13. Bar chart comparison of lipid compounds relative abundance in CD4⁺ T-cells. Lipid compounds representation that distinguished controls from INRs patients in the RF analysis and statistical tests. The data was presented by mean ± S.E.M. The compounds MDA range is 0.010-0.025 and p-values are PC 30:0=0.002, PC 38:3=0.026, PC 36:3= 0.048 and CE 22:4=0.039.

On the other hand, in the $CD8^+$ T-cells the gap between the compounds profile was small, indicating that the control and INRs groups were quite similar **(Table 8; Annex 2)**. So the lipid profile could not be used to distinguish those two groups, controls and INRs.

Lipid profile differences between IRs and INRs

In order to identify de main lipid species related to poor immunologic non-responders, we compared lipid profile between INRs and IRs. The statistical analysis marked significant contrast between the two cases groups, hence the patients were able to be identified early by comparing the relative abundance of the lipid compounds on the $CD4^+$ T- and $CD8^+$ T-cells.

The statistical test only displayed one statistical significant lipid ($p< 0.05$) in $CD4^+$ T-cells, but we had analysed other 4 lipids $CD4^+$ T- and $CD8^+$ T-cells due to the small size of the study cohort. These lipids could had shown a tendency to be statistically significant **(Table 2)**. Thus, the lipid compounds were considered relevant for result interpretations. Hence, the analytical test displayed how the lipid profile can draw a distinction between IRs and INRs in both cellular types.

The principal lipid types which revealed this gap were PC and TG. Concretely, the lipids species are PC 36:0, TG 42:0, TG 60:0 and TG 60:4 resulted to be different in the $CD4^+$ T- cell composition between INRs ad IRS, whereas the PC 38:3 resulted to be different in the $CD8^+$ T-cell composition between INRs and IRs. But the only statistically significant was TG 60:4 (p=0.027) in the composition of $CD4^+$ T-cell, which could represent a good candidate as a lipid used to distinguish between the two groups of cases.

Table 2. Statistical significant (p<0.05) and relevant lipid species different between IRs and INRs patient groups in $CD4^+$ T- and $CD8^+$ T-cells.

$CD4^+$ T-CELLS: IR/INR	
Lipids	**Significance**
PC 36:0	0.086
TG 42:0	0.086
TG 60:0	0.086
TG 60:4	0.027

$CD8^+$ T-CELLS: IR/INR	
Lipids	**Significance**
PC 38:3	0.055

Only the relevant compounds were taken into account to perform PCA and RF analyses **(Figure 14)**. PCA graphical representation showed some similarities between the INRs IRs. The RF verified the information extract from the statistical test, the TG 60:4 was a lipid which could clearly differentiate two study groups, the INRs and the IRs.

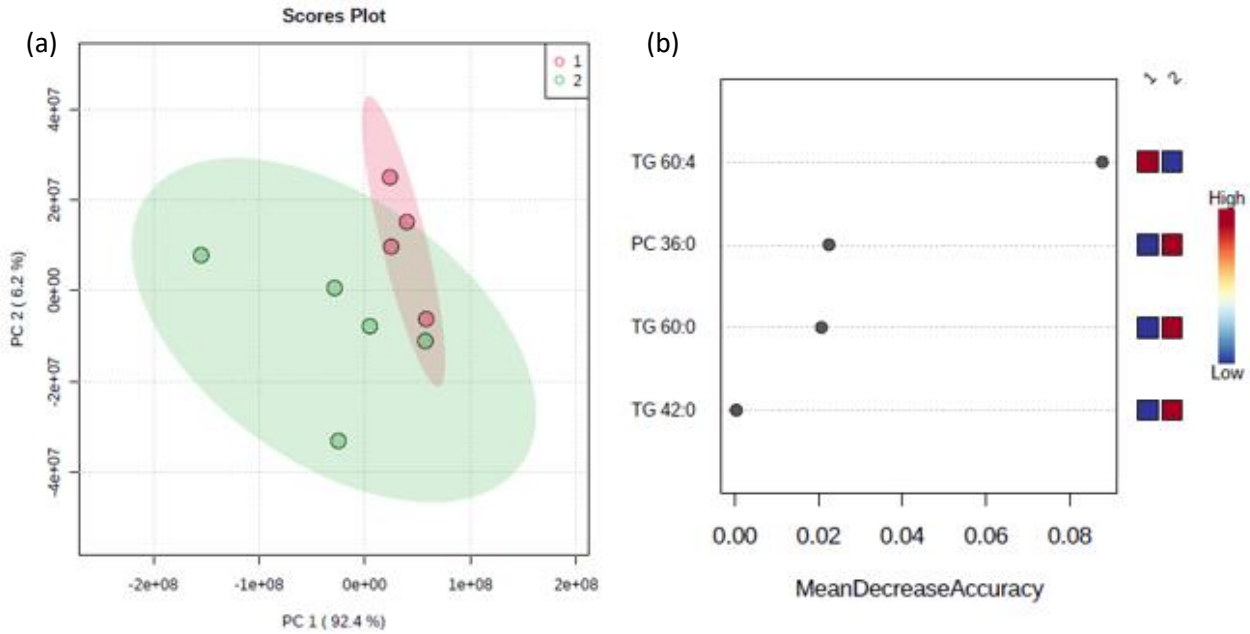

Figure 14. Graphical analyses of relevant lipid profile in $CD4^+$ T-cells. (a) 2D score plot PCA display the different lipid species between poor immune recovery (green dots) and good immune recovery status patients (red dots). (b) RF analysis of four relevant lipid compounds. Rank by MDA>0.00, marking high concentration (red) of the lipid compounds and lower concentration of compounds (blue) to compare the profile of controls IRs (group 1) and INRs (group 2).

To confirm the later facts about these lipids in $CD4^+$ T-cells, we plotted them in bar charts independently **(Figure 15)**, so that we were able to analyse the difference between the relative abundance of the compound between the study groups individually. In this case, we confirmed that the 4 lipids showed differences in their relative abundance. In fact, these graphs allowed us to identify which of the two groups has a higher relative abundance in each case. PC 36:0, TG 42:0 and TG 60:0 presented a higher abundance in the INRs than in the IRs, whereas TG 40:0 presented the opposite trend.

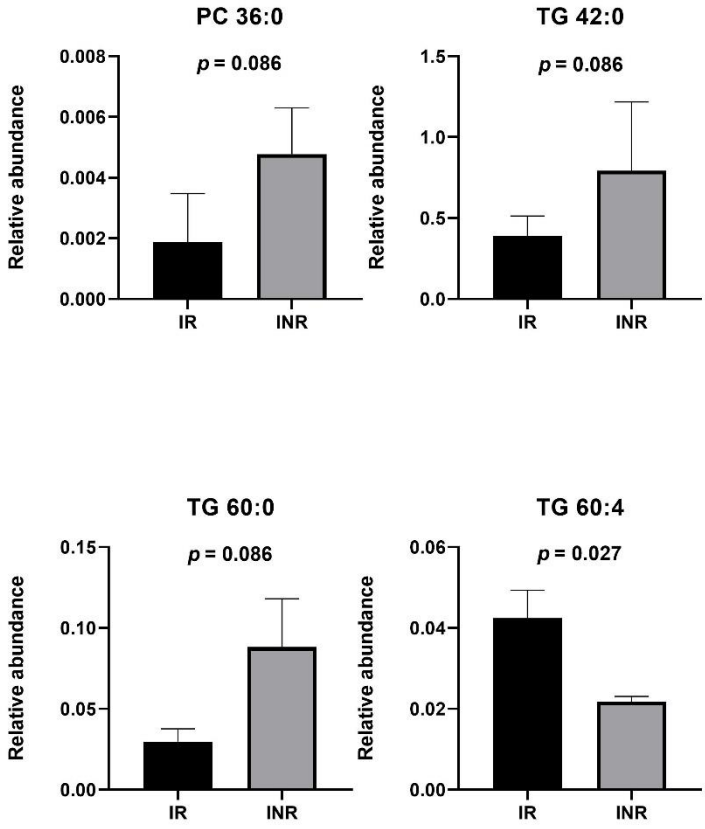

Figure 15. Bar chart comparison between IRs and INRs lipid compounds concentration in $CD4^+$ T-cells. The representation of relevant lipid compound that distinguished IRs and INRs. The data was presented by mean ± S.E.M. The p-values are PC 36:0=0.086, TG 60:0=0.086, TG 42:0= 0.086 and TG 60:4=0.027

Comparison between $CD4^+$ T- and $CD8^+$ T- cells

Finally, the statistical analysis gave the information about the contrast between the lipid profile of the $CD4^+$ T- and $CD8^+$ T- cells. Therefore, analysis could be used to decide which of these cellular types is more useful to identify different groups, controls and cases.

The statistical analysis reflected the significant differences between these two cellular subtypes in terms of the relative abundance of lipid compounds in these cells. The gap was embodied in all lipid types, in fact, of the 127 compounds identified, 48 showed a significantly different relative abundance in the two cell types. Among the lipid groups showed in this statistical analysis were triacylglycerol, sphingomyelins, phosphatidylcholines, cholesterol ester, diacylglycerol and lysophosphatidylcholines.

As there were so much possible lipid compounds, we focused the analysis in the one's which statistical and RF analysis marked them as significantly different. These lipids were CE 22:4, PC 30:0, PC 36:0, PC 38:3 and TG 60:4 **(Figure 16)**. The bar charts revealed the differences in the lipid profile between the two cellular types. It allowed an individualised study of the relative abundance of each lipid. In this case, we were able to see that the distinction between the two cell types in PC 38:3 and TG 60:4 was almost non-existent. On the other hand, in the rest of the lipids, CE 22:4, PC 30:0 and PC 36:0, there was a significant contrast. In fact, whereas CE 22:4 and PC 36:0 showed the same pattern, being the concentration of these lipids higher in $CD4^+$ T- compared to $CD8^+$ T- cells, PC 30:0 showed the opposite trend.

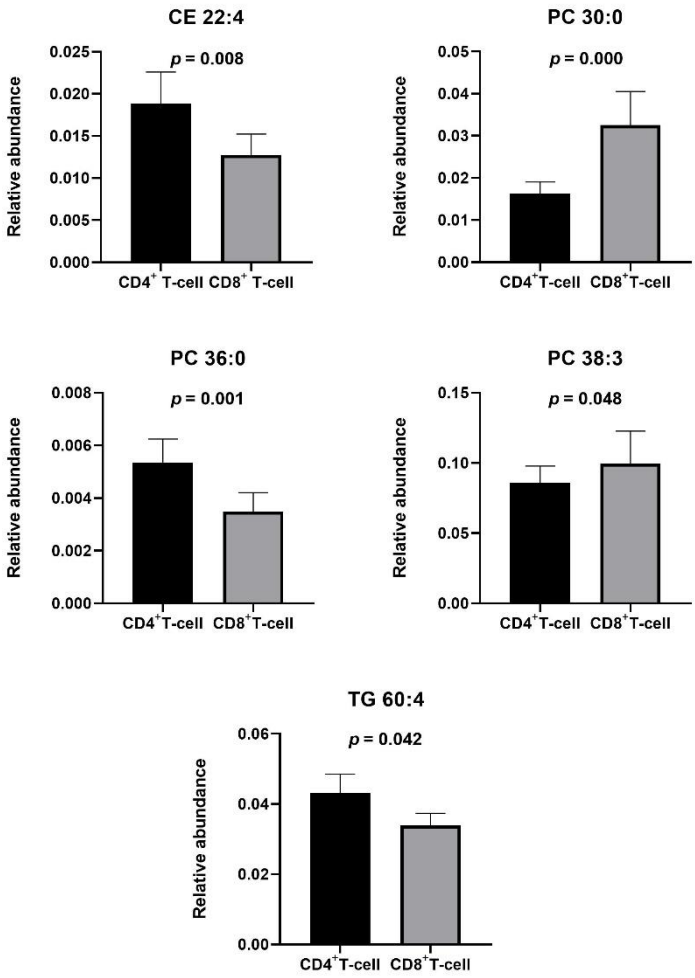

Figure 16. Graphical bar chart representation of the statistical significant lipid species which differ by cell type, $CD4^+$ T- and $CD8^+$ T-cells. The data was represented by mean ± S.E.M. The p-values are CE 22:4=0.008, PC 30:0=0.000, PC 36:0= 0.001, PC 38:3=0.048, and TG 60:4=0.042.

Discussion

The research of lipid profile in HIV-positive patients before and after the initiation of the suppression antiretroviral therapy is a novel study. The impact of the incomplete immune reconstitution on the lipid profile is not yet known. Lipidomic studies of these patients need to be developed in research studies to interrelate the role of certain lipid compounds in the non-recovery of $CD4^+$ T-cell counts.

Generally, our findings pointed out the baseline difference between the lipid profile of the HIV-positive patients initiating first antiretroviral treatment and which were categorized as INRs or IRs after 2 years of successfully virological suppression under antiretroviral treatment. The research study also revealed lipidomic differences in the composition of both $CD4^+$ T- and $CD8^+$ T-cells in HIV-patient showing low $CD4^+$ T-cell count compared to those with greater values of $CD4^+$ T-cell count before starting the ART therapy.

First, our data showed that HIV-patients with low $CD4^+$ T-cell count at the initiation of ART treatment could be distinguished by the relative abundance of PC 30:0, SM 40:2 and PC 36:2 in the $CD4^+$ T-cell composition. The PC is a type of phospholipid species which is involved in several metabolic pathways essential in cells. They form the lipid bilayer of cellular membrane. Thus, an alteration in the concentration of these lipid species could be cause damage and unbalance in the composition of the cell membranes. In fact, the PC 30:0, the glycerophospholipids which revealed the greater statistically significant difference between the controls and cases in $CD4^+$ T-cells, is mostly found in the cellular membrane related with the biosynthesis of other phosphatidylcholines and cell signalling [34]. By contrast, PC is a known anti-inflammatory, and the alteration of its concentration or metabolism can lead to changes in the body's immune response against the virus. Like the previous one, SM play an important part in the maintenance of the cell membrane and are also a marker for the dysregulation of cholesterol metabolism.

The graphical test showed some discrepancies between cases and controls lipid profile in $CD4^+$ T-cells. The discrepancies could be explained by the results obtained comparing controls and IRs patients lipid composition. Hence, the baseline difference between the cases which had a greater $CD4^+$ T-cell count after initiation of the treatment and HIV-positive patients having high $CD4^+$ T-cell count before ART treatment was almost non-existent in $CD4^+$ T-cells and $CD8^+$ T-cells. The fact that the IRs patients recovered $CD4^+$ T-cell count may be implicated in the lack of differences in lipid profile between IRs and controls. As the lipidic signature could seem similar between IRs and controls, the IRs lipidomic profile should be healthier than the immunological poor recovery patients. Other research studies have revealed this same pattern in the metabolome of patients who increase their $CD4^+$ T-cell count after starting ART treatment, being the metabolomics signature similar to the control group [24,33].

The HIV-positive patients with INRs have worst prognosis and more possibilities to develop non-AIDS illness related. The INRs identification before initiating ART treatment using lipidomic profile could improve the situation of those patients. The metabolic profile of the poor immune recovery HIV-patients have been studied in the last several years in different research studies, showing that the alteration of the concentration of certain cellular metabolites in this type of patients [24,33]. However, the lipidomic approach is novel because there are not existing studies that address this issue. The novelty lies in the possible identification of biomarkers to distinguish

the lipid profiles of poor immune recovery patients and immune responders before initiating ART treatment. The lipidomic study provides a more specific picture of the lipid profile than previous metabolomic studies.

Unlike the IRs similar lipid signature to the control group in $CD4^+$ T-cell, our data displayed the statistical significant gap in the baseline lipid concentration between the poor immune recovery patients and the control group in the $CD4^+$ T-cells. The prognosis gap between IRs and INRs could be shown by the differences of the lipid profile. The most statistically relevant lipids were PC 30:0, PC 38:3 and PC 36:3, and a cholesterol ester (CE) 22:4. These lipid species of the phosphatidylcholine family reflected a decrease in the concentration of INRs. Due to the relationship of these lipid compounds to lipid metabolism and cell membrane formation explained above, it is possible that these processes may be altered. The alteration could affect processes that are indispensable for the cell. It may be possible that these changes in lipid metabolism are related to the poorer prognosis of INRs. The fatty acid and lipid metabolism and cellular membrane composition are the main processes where these lipids are involved [35,36]. The relevance of these lipids in the HIV infection on INRs patients should be studied further in the future.

Likewise, PC 30:0 was a lipid that had previously appeared in the results, as it allowed us to differentiate between controls and cases. Thus, we could confirm that this differentiation is due to the INRs and not to the IRs. Therefore, the PC 30:0 had appeared as a possible prognostic molecule of the cases. The data obtained showed that could identify the cases, specifically those patients who did not experience a recovery of $CD4^+$ T-cell counts after initiating treatment. Another study similar to the one reported in this thesis, also identified a decrease in the concentration of PC 30:0 in immunological non-responders. The other two types of PC, PC 38:3 and PC 36:3, have similar cellular roles as PC 30:0. In addition, some lipidomic studies have also shown a correlation between HIV infection and plasma PC 38:3 lipid. Furthermore, the PC 38:3 possible relationship with diabetes in HIV patients has also been studied [37].

On the other hand, the CE 22:4 belongs to the family of steryl esters. The CE 22:4 have been related with HIV-patients and decrease risk of diabetes in other research studies of the lipidomic profile [37]. The correlation between HIV infection and CE 22:4 could be explained by the lipid function in cell signalling, lipid and cholesterol metabolism. These data could show a tendency to impaired cholesterol and lipid metabolism in INRs [38,39].

The TG and PC were the two lipid types that mainly showed statistically significant differences between the two types of cases, the poor immune recovery and good immune recovery status, in the $CD4^+$ T- and $CD8^+$ T-cells. In $CD4^+$ T-cells, the PC 36:0 was one of the lipids which concentration increased in the INRs. These lipid species was involved in the same processes and metabolic pathways as the other PC described above [40]. In $CD8^+$ T-cells, the concentration of PC 38:3 which seemed to have an important role in the poor immune recovery, as discussed above, resulted also altered in $CD4^+$ T-cells. The alteration of the concentration of these two compounds showed how lipid metabolism was affected in INRs, indicating a less healthy lipid profile in this subgroup of patients. Further study of these facts could lead to an explanation of why a worse prognosis occurs in patients with poor immune recovery and how lipid metabolism was related.

The TG are lipid family related with the lipid and energy metabolism and inflammatory response. The TG can be used to mitochondrial oxidation as energy source, hence during the inflammatory response the biosynthesis of TG increase. During HIV infection the patients suffers a chronic inflammation process, the increase concentration of TGs had been analysed in recent studies [33]. The TG 60:0 and TG 42:0 displayed increase

concentration in IRs, and these lipids species are related with the immune inflammation response. Concretely, these two lipid species have been previously studied in relation to cancer and metabolic syndrome, two illnesses common between HIV patients [41,42].

Finally, the most statistical significant different lipid, TG 60:4, is implicated in lipid metabolism and biosynthesis, cellular signalling and inflammatory response as the other TGs. The decreased relative abundance of this lipid specie in the INRs could become a biomarker of prognosis in HIV-positive patients. For this, more specific studies would be needed as this is a screening study, so the results obtained in this study should be confirmed as this is a very new and underdeveloped field, and there is little information already available.

Some research studies displayed discrepancies with our results, generally point to sphingomyelins as another lipid species whose relative abundance is altered in patients with lower $CD4^+$ T-cell recovery. In addition, this study points to different lipid species of phosphoacylcholines from those highlighted by our results [43].

As we had discussed, the lipid profile gap is much more marked in $CD4^+$ T-cells than in $CD8^+$ T-cells, yet the abundance of some compounds is altered in the latter cell type. However, the lipid profile of $CD4^+$ T-cells is likely to be more prognostic than that of $CD8^+$ T-cells. Among the lipid groups showed in this statistical analysis are triacylglycerides, sphingomyelins, phosphatidylcholines, cholesterol ester, diacylglycerol and lysophosphatidylcholines.

The results showed that the lipid profile between these two cell types was very different, in fact the 5 most statistically significant lipids were lipids that already showed differences between the different study groups. In this way, metabolic alterations in the two cell types could be studied independently. The results showed that $CD4^+$ T-cells were more relevant to differentiate the lipidomic profile of patients with a low $CD4^+$ T-cell count after initiating ART therapy.

In conclusion, HIV the poor immune recovery patients could be differentiated by their lipid profile, and especially by lipid composition of $CD4^+$ T- cells of both controls and IRs. There have been some lipid species that showed significant differences in the concentration of immunological non-responders. This pilot study has made it possible to carry out a first screening of the possible lipids but further investigation of these lipids is needed to identify whether they might actually be biomarkers of incomplete immune recovery.

Limitations

The principal limitation of this research study was the size if the cohort. The number of HIV-patients, INRs and IRs after initiation of ART therapy and controls should be higher to validate the altered lipids as definitive biomarkers of INRs. In addition, human patients involved a great deal of variability due to the large number of factors that cannot be controlled. Further studies with a larger initial cohort of HIV-positive patients will be needed to confirm such definitive relationships between the lipidomic profile and the different case studies. The inability to obtain the results of metabolomics and proteomics analysis of these same patients was an important limitation, due to the delay in the delivery of the results of these analyses. In fact, a multi-omics analysis could provide more extensive and interrelated information on the differences between INRs and IRs.

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Annex 1

Table 3. U Mann Whitney results of significant (p<0.05) difference lipids between controls and cases in $CD4^+$ T-cells shown as p-value and Fold-Change (FC). The FC results < 1 were related with lower concentration of the lipid in cases; FC > 1 were related with a higher concentration of the lipid in cases.

$CD4^+$ T-CELLS		
Lipids	**P-value**	**Fold-Change**
CE 20:5	0.031	0.744
CE 22:4	0.017	0.625
LPC 16:0	0.042	0.338
LPC 20:0	0.027	0.340
PC 29:0	0.005	0.477
PC 29:1	0.042	0.586
PC 30:0	0.002	0.395
PC 31:0	0.002	0.346
PC 32:0	0.004	0.443
PC 32:1	0.002	0.445
PC 32:2	0.005	0.514
PC 33:0	0.031	0.512
PC 33:1	0.007	0.538
PC 33:2	0.017	0.580
PC 33:3	0.036	0.514
PC 34:1	0.036	0.526
PC 34:2	0.008	0.473
PC 35:0	0.024	0.459
PC 35:2	0.031	0.556
PC 36:3	0.020	0.529
PC 36:4	0.005	0.450
PC 36:5	0.007	0.339
PC 38:3	0.009	0.416
PC 38:4	0.048	0.507
PC 38:5	0.017	0.493
PC 38:6	0.006	0.403
PC 40:5	0.020	0.517
PC 40:6	0.024	0.486
SM 33:1	0.048	0.615
SM 36:0	0.031	0.520
SM 36:1	0.027	0.493
SM 36:2	0.042	0.542
SM 36:3	0.036	0.501
SM 38:2	0.013	0.442
SM 40:2	0.036	0.518
SM 42:3	0.042	0.527

Table 4. Significant lipids results comparing controls and cases study groups. p-value results and FC from the statistically significant lipids in $CD8^+$ T-cells. The FC results < 1 were related with higher concentration of the lipid in controls; FC > 1 were related wih higher concentration of the lipid in cases.

$CD8^+$ T-CELLS		
Lipids	**P-value**	**Fold-Change**
SM 43:1	0.027	1.392
SM 43:2	0.035	1.486
TG 52:3	0.020	1.286
TG 54:2	0.011	0.336
TG 58:2	0.006	1.858
TG 58:3	0.020	1.876
TG 60:2	0.046	1.572
TG 60:3	0.040	1.589

Annex 2

Table 5. Statistical analysis results of significant lipids, including p-value and FC, between controls and IRs in $CD4^+$ T-cells. The FC results < 1 were related with lower concentration of the lipid in IRs; FC > 1 were related with higher concentration of the lipid in IRs.

$CD4^+$ T- CELLS: CONTROL/IR

Lipids	P-value	Fold-Change
SM 38:2	0.023	0.288
TG 56:1	0.011	0.521

Table 6. Results of statistically significant lipids comparing controls to INRs, shown as p-value and FC in $CD4^+$ T-cells. The FC results < 1 were related with higher concentration of the lipid in controls; FC > 1 were related with higher concentration of the lipid in INRs.

$CD4^+$ T- CELLS: CONTROL/INR

Lipids	P-value	Fold-Change
CE 22:4	0.039	0.396
LPC 20:0	0.032	0.197
PC 29:0	0.005	0.450
PC 30:0	0.002	0.348
PC 31:0	0.002	0.290
PC 32:0	0.005	0.438
PC 32:1	0.002	0.432
PC 32:2	0.005	0.500
PC 33:1	0.008	0.586
PC 33:2	0.032	0.538
PC 34:2	0.013	0.444
PC 36:3	0.048	0.532
PC 36:4	0.005	0.453
PC 36:5	0.013	0.328
PC 38:3	0.026	0.460
PC 38:5	0.032	0.517
PC 38:6	0.010	0.389
PC 40:5	0.039	0.531
PC 40:6	0.048	0.507

Table 7. Statistical significant different lipids profile between controls and IRs in $CD8^+$ T-cells. Shown as p-value and FC. The FC results < 1 were related with higher concentration of the lipid in controls; FC > 1 were related with higher concentration of the lipid in IRs.

$CD8^+$ T- CELLS: CONTROL/IR

Lipids	P-value	Fold-Change
PC 29:0	0.043	0.640
PC 38:3	0.005	0.538
SM 43:2	0.043	1.464
TG 54:2	0.029	0.359
TG 58:2	0.010	2.005
TG 58:3	0.029	2.074
TG 60:3	0.029	1.776

Table 8. U Mann Whitney result of significant lipids comparing controls to INRs in $CD8^+$ T-cells, shown as FC and p-value. The FC results < 1 were related with lower concentration of the lipid in INRs; FC > 1 were related with higher concentration of the lipid in INRs.

$CD8^+$ T- CELLS: CONTROL/INR

Lipids	P-value	Fold-Change
TG 52:2	0.046	1.287
TG 52:3	0.046	1.155
TG 60:4	0.036	0.494

Annex 3

Table 9. Wilcoxon statistical analysis results of significant lipids between $CD4^+$ T- and $CD8^+$ T- cells shown as p-value and FC. The FC results < 1 were related with higher concentration of the lipid in $CD8^+$ T-cells; FC > 1 were related with higher concentration of the lipid in $CD4^+$ T-cells.

$CD4^+$ T- CELLS/$CD8^+$ T- CELLS		
Lipids	**P-value**	**Fold-Change**
CE 18:1	0.005	1.500
CE 18:2	0.000	2.066
CE 18:3	0.014	1.403
CE 20:4	0.006	1.454
CE 22:4	0.008	1.476
DG 32:0	0.042	1.368
DG 34:1	0.019	1.383
DG 36:1	0.034	1.340
LPC 16:0	0.021	1.318
LPC 18:0	0.028	1.250
LPC 18:1	0.009	1.291
PC 29:0	0.025	1.274
PC 30:0	0.000	0.500
PC 31:0	0.000	0.519
PC 32:1	0.000	0.552
PC 32:2	0.004	0.680
PC 33:0	0.000	0.618
PC 33:1	0.002	0.671
PC 33:2	0.015	0.829
PC 36:0	0.001	1.536
PC 37:1	0.033	1.246
SM 36:0	0.013	1.332
SM 36:1	0.014	1.308
SM 38:1	0.001	1.554
SM 38:2	0.007	1.372
SM 38:3	0.048	1.502
SM 40:1	0.001	1.586
SM 40:2	0.044	1.232
SM 41:1	0.001	1.519
SM 42:1	0.002	1.590
SM 44:1	0.048	1.682
TG 46:0	0.042	1.669
TG 46:2	0.031	1.732
TG 47:0	0.042	1.709
TG 48:2	0.050	1.652
TG 48:3	0.042	1.686
TG 50:1	0.048	1.444
TG 50:2	0.044	1.571
TG 50:3	0.022	1.651
TG 52:1	0.048	1.381
TG 52:2	0.03	1.396
TG 52:3	0.008	1.463
TG 52:4	0.042	1.433

TG 54:5	0.025	1.444
TG 54:6	0.027	1.393
TG 56:1	0.038	1.481
TG 58:2	0.048	1.324
TG 58:3	0.027	1.349
TG 60:4	0.042	1.272