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Propionic acid as a potential biomarker to differentiate between immunological responders and immunological non-responders in HIV-1 infected patients

Final Degree Project

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The present Final Degree Project was done based on the External Practices carried out in the Infection and Immunity Group (INIM), located at the Pere Virgili Health Research Institute (IISPV) of Joan XXIII University Hospital, Tarragona, Spain, under the supervision of Dr. Anna Rull Aixa and Dr. Silvia Chafino Aixa.

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1. ABSTRACT

Human immunodeficiency virus (HIV) infected patients have different responses to the infection and/or treatment. The elite controllers (EC) are characterised by viraemic suppression and normal CD4+ T-cell count without antiretroviral therapy (ART). The HIV-infected people who initiate ART with low CD4+ T-cell count can be classified into two groups, immunological responders (IR) if they can recover their immune system under treatment or immunological non-responders (INR) if they cannot. The lipidome has an important role in the progression of HIV infection but the lipidomic profile in the specific groups has not been described. In this study, a complete lipid profile including, short-chain fatty acids (SCFA), bile acids, and hydrophobic lipids, was performed using GCM and HLCM in a cohort formed of 20 patients divided into 4 groups, 5 patients of each phenotype and 5 healthy. After analysing 200 lipids, 4 lipids showed significant differences in the EC group compared to the healthy including the decrease of 3 SCFA. In the INR, 12 lipids had significant differences comparing the IR and INR groups including TG and SCFA. Remarkably, the propionic acid (PA), which was increased in the INR group, obtained the best discriminatory power with AUC 1 to differentiate INR patient from IR before ART. In conclusion, we suggest that INR groups exhibit a higher incidence of lipidic changes. In particular, the PA emerges as a promising biomarker for predicting INR patients.

KEYWORDS: HIV, Lipidomics, Elite controllers, Immunological non-responders, Propionic acid.

2. ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
AUC	Area under the curve
BA	Bile acids
ChoE	Cholesterol ester
CRP	C-reactive protein
CVD	Cardiovascular disease
DG	Diglyceride
dsDNA	double-strain DNA
EC	Elite controllers
FA	Fatty acid
GC-MS/MS	Gas chromatography-Mass spectrometry/Mass spectrometry
GGT	Gamma-glutamyl transpeptidase
GPT	Alanine aminotransferase
HA	Heptanoic acid
HCV	Hepatitis C virus
HDL	High density lipoproteins
HIV	Human immunodeficiency virus
HPLC-MS/MS	High-precision liquid chromatography-Mass spectrometry/Mass spectrometry
HSC	Hematopoietic stem cells
IBA	Isobutyric acid
IL10	Interleukin 10
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-7R	Interleukin 7 receptor
INR	Immunological non-responders
INSTIs	Integrase strand transfer inhibitors
IR	Immunological responders
IVA	Isovaleric acid
LC-QTOF	Liquid chromatography-Quadrupole time of flight
LDL	Low-density lipoproteins
LPC	Lysophosphatidylcholine
LPC-O	Ether-linked Lysophosphatidylcholine
MG	Monoglycerides
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
PA	Propionic acid
PC	Phosphatidylcholine
PC-O	Ether-linked phosphatidylcholine
PE	Phosphatidylethanolamine

PI	Protease inhibitors
ROC	Receiver operating characteristic
SCFA	Short-chain fatty acids
SIV	Simian immunodeficiency virus
TG	Triglycerides
VA	Valeric acid

3. INTRODUCTION

The Human Immunodeficiency Virus (HIV) is a retrovirus that affected around 38.4 million people in 2021. Since the beginning of this pandemic, 84.2 million people have been diagnosed worldwide (1). The HIV infection destroys the CD4+ T cells, producing a progressive immunodeficiency which could end in acquired immunodeficiency syndrome (AIDS) (2). One of the consequences of this stage is the appearance of opportunistic diseases such as uncommon types of cancer and opportunistic infections (3), which have caused the death of nearly 48 million people worldwide (1).

3.1 HIV history

During the 80s, some cases of opportunistic pathologies in some homosexual young men were reported and the number of cases increased and helped to identify susceptible populations (4). These groups were defined as men who have sex with other men, heroin addicts and other injecting drug users, and people who needed blood transfusions (5). A few years later, this pathological status will be called AIDS (4).

In 1983, HIV was identified by Françoise Barré-Sinoussi and colleagues at the Institute Pasteur in Paris (6), although the origin of the virus was still unknown.

In 1985, a virus very similar to HIV named Simian Immunodeficiency Virus (SIV) was first identified in a New England primatology centre (7). Depending on the simian species, 2 different types of viruses were characterised. In 1989, a virus like HIV-2 was identified in grey mangabey (*Cercocebus atys*) (5) and a virus similar to HIV-1 was characterised in chimpanzees (*Pan troglodytes*) (8). The 2 types of viruses are indistinguishable in their clinical symptoms once it has progressed to the AIDS state. Despite that, the people infected with HIV-2 usually do not reach this state due to the HIV-2 viral loads being lower than the HIV-1 ones (5).

In the beginning, the HIV-1 infection had a very bad prognosis due to the lack of treatment. In 1996, the first protease inhibitors were introduced, but it was later when the protease inhibitor was combined with 2 nucleoside-analogue reverse

transcriptase inhibitors that produced a highly active antiretroviral therapy and changed the prognostic of HIV-infected patients (9).

Since the identification of the virus, there has been a lot of progress in understanding the disease, allowing scientists to find new treatments that have converted AIDS into a non-mortal chronic disease.

3.2 HIV prevention and diagnosis

While AIDS is currently considered a non-mortal chronic disease, a significant number of deaths occur each year worldwide due to the limited access to treatments or late diagnosis among some population groups. For this reason, the Joint United Nations Programme on HIV and AIDS (UNAIDS) launched the 90/90/90 strategy to try to control the AIDS epidemic. This strategy was composed by 3 main goals: 1) 90% of the people that are infected with HIV are aware of their infection; 2) 90% of those aware of their HIV status are able to receive the treatment; and finally, 3) 90% of the people that have access to treatment achieve viral suppression (9).

To perform a good diagnosis, serological tests based on the enzyme immunoassay are used (10). There are 5 generations of this type of essay being the 4^{th} and 5^{th} generations the most accurate ones. These 2 essays use the IgG, IgM, and antigen p24, which is a biomarker that permits early identification of the HIV-1 infection (11,12). These types of tests can diagnose a person infected with HIV-1 2 weeks after the infection with 99% of sensitivity and specificity (13). Nevertheless, the results obtained in these tests must be confirmed using a Western Blot (10).

3.3 HIV-1 genome

The HIV-1 is flanked by 2 long terminal repeat sequences and its RNA contains 9181 base pairs (bp) that are structured in 10 different coding genes. These genes are *Gag, Pal, Vif, Vpr, Vpu, Env, Antisese protein, Nef, Tat,* and *Rev* (14,15). The function of these 10 genes is related to HIV replication and the host cell's infection (16). The most important genes are the *Gag, Env,* and *Pol* genes. The *gag* gene encodes the structural proteins that form the capsid and *Env* encodes proteins that form the envelope of the virus (17). *Pol gene* encodes for

3 different enzymes that oversee DNA synthesis and integration. These 3 enzymes are a reverse transcriptase, an integrase, and a protease (16). The *Vpr* and *Vpu* are genes that determine the virus virulence and are one of the main differences between HIV-1 and HIV-2 because the gene *Vpu* is only found in HIV-, which is more virulent (18,19). The *Vif* gene's primary function is to counter the innate immune proteins that inhibit virus replication (20). The *Nef* gene encodes for a protein that is not essential for viral replication, but it has been seen that HIV-1 variants without this gene progress very slowly to the AIDS phase (21,22) (Figure 1).

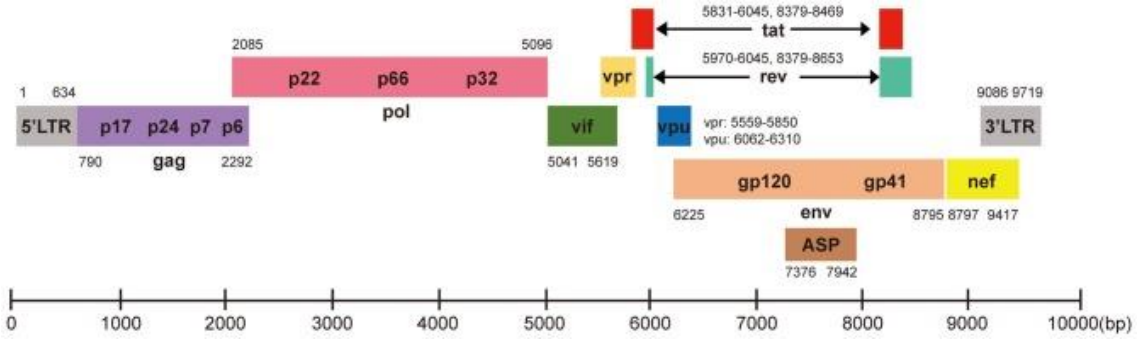

Fig. 1. HIV genome structure. The genome is composed of 10 genes that are involved in HIV replication and the host cell's infection (17).

3.4 HIV viral cycle

The HIV viral cycle is initiated by the entry of the virus into the host cells by binding its glycoproteins to the CD4+ receptor and the CCR5 or CXCR4 co-receptors. Depending on the virus variant, it will use the CXCR4, the CCR5, or both as co-receptors to infect the cell (23). The identification of the variant is crucial in determining the most effective treatment approach to inhibit the entry phase of the viral cycle preventing the virus to join its co-receptor (23). When the virus is attached to its receptors, the host cell membrane and the HIV membrane fuse producing the invasion of the host cell and the release of the viral RNA. After that, the viral RNA is transcribed to double-stranded DNA (dsDNA) by reverse transcriptase and the viral DNA enters the nucleus to integrate into the host DNA by the integrase. Then, the new viral RNA is used as genomic RNA to make viral proteins which will form immature viral particles on the cell surface. Finally, these

immature particles are released from the host cells, producing a viral protease forming the mature virion (Figure 2) (17).

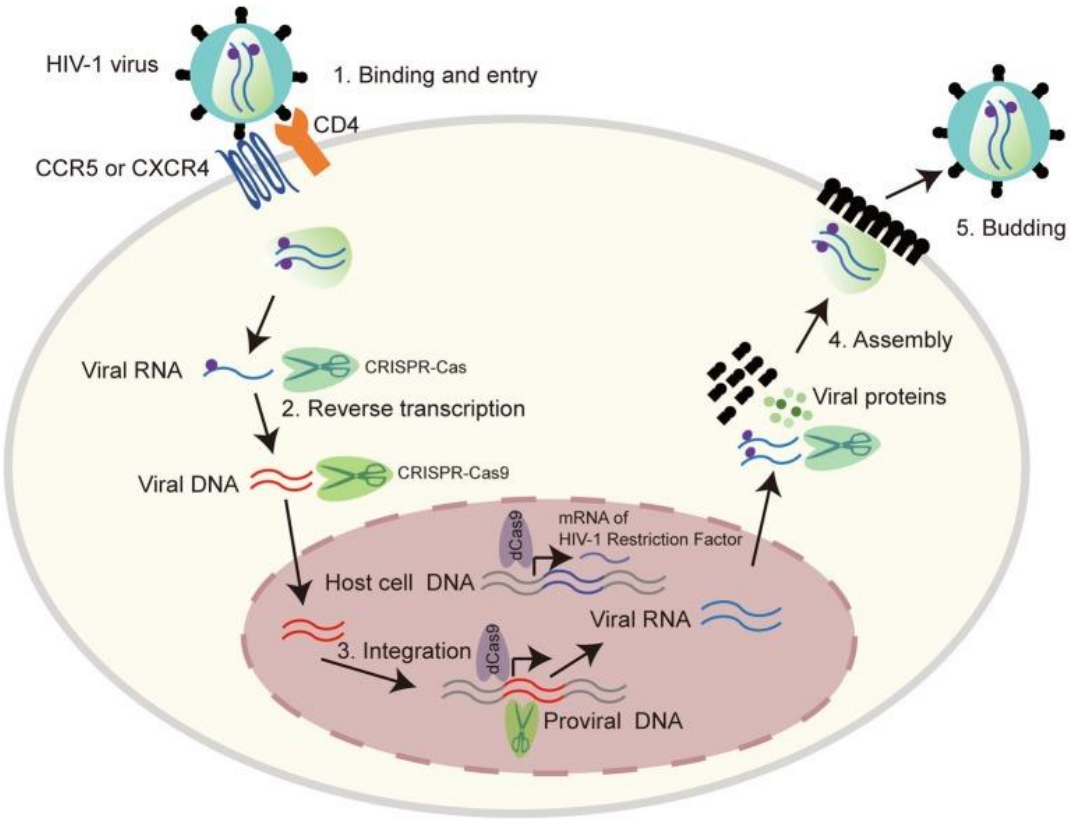

Fig. 2. HIV viral cycle. 1. Binding and entry: The virus binds its glycoproteins with the receptor and co-receptors CCR5 and CXCR4. 2. Reverse transcription: The reverse transcriptase transcribes the RNA into double-strain DNA (dsDNA). 3. Integration: The viral DNA is integrated into the host's DNA thanks to an integrase. 4. Assembly: The new viral RNA forms the viral proteins that will form the immature virion. 5. Budding: The immature particles are released, and the viral protease forms the mature virion (17).

3.5 HIV progression

Once a person is infected by HIV, if it is not treated, the disease has a poor evolution which usually ends up producing AIDS and eventually death.

HIV disease progression starts with a quick decrease of CD4+ T cells and an increase of viral count, this stage is called the acute phase. These changes can be accompanied by symptoms like fever or cough. Then, a latent phase starts, which is characterised by a gradual decrease or stable levels of CD4+ T cell counts while the viral RNA decreases and remains at low levels for years. The

latent phase varies greatly among individuals lasting for several years before progressing to the final stage of HIV infection, known as AIDS (24). The development of AIDS can be accelerated by different factors such as the consumption of drugs like heroin, crack, or alcohol intake (25,26). AIDS is characterised by a CD4+ T cell count lower than 200 cells/µL (24). This low CD4+T count is an immunodeficiency that can cause the death of the infected patient due to opportunistic diseases like Kaposi sarcoma or *Cryptococcal Meningitis* (27,28) (Figure 3).

The progression can be weakened with the help of various treatments, allowing HIV-positive patients to have almost a completely normal life.

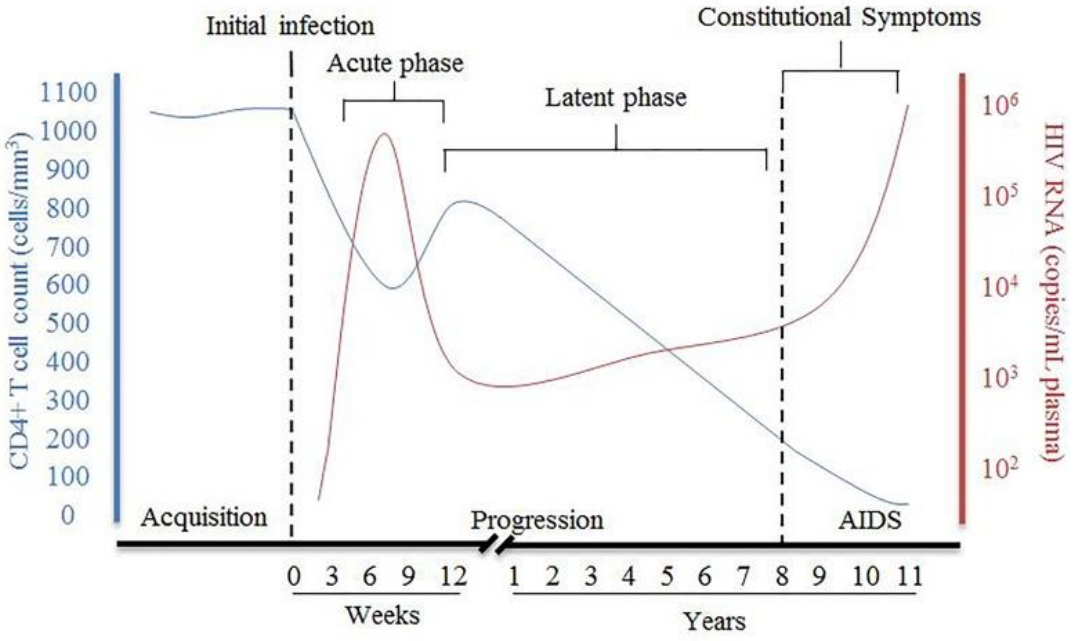

Fig. 3. HIV progression. The progression of HIV is marked by the viral load (red) and the CD4+ T cell count (blue). In the graph, the 3 phases of the infection Acute, Latent, and AIDS phase can be observed. The first one is characterised by a quick decrease in the CD4+ T cell count and a very important increase in the viral RNA. This phase can last for some weeks. The latent phase is characterised by a decrease in the viral load and a slow decrease in CD4. This phase can last for years. Finally, in the AIDS phase, the CD4+ T cell count is lower than 200 cells/µL, and the viral load increases (24).

3.6 Treatments for HIV infection

Nowadays, the HIV treatment's main goal is to effectively suppress the viral load using antiretroviral therapy (ART), as a cure for HIV is yet to be discovered (9). ART consists of a combination of different drugs like protease inhibitors (PIs), which are substrate-analogue inhibitors preventing the protease to do its function

(29). Nucleoside reverse transcriptase inhibitors (NRTIs) are reverse transcriptase inhibitors that mimic nucleosides, but this type of inhibitor must be combined with others due to the drug resistance that the HIV reverse transcriptase has acquired (30). Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are reverse transcriptase inhibitors that bind to the drug-binding pocket preventing the reverse transcriptase to form the complex with the viral RNA (31). These 3 ART are the most used, but there are more such as the CCR5 antagonists, fusion inhibitors, integrase strand transfer inhibitors (INSTIs), attachment inhibitors, post-attachment inhibitors, or pharmacokinetic enhancers (boosters) (32–35). Depending on the drug combination, the ART has shown different effectiveness, and some of the combinations had an increased risk of viral rebound (36,37).

Although ART has helped a lot of patients with their HIV evolution, the first antiretroviral drugs approved had a lot of secondary effects. Some examples were the tenofovir disoproxil fumarate, which had high renal and bone toxicity or the zidovudine, which was the first approved antiretroviral drug, but it had high haematological toxicity (9,38). Nowadays, ART has improved and reduced toxicity, but ART does not prevent the patients that are treated with ART to develop some comorbidities, such as cancer, metabolic syndrome, cardiovascular disease (CVD), or co-infections with other viruses like the hepatitis C virus (HCV). The appearance of comorbidities can significantly reduce the quality of life of HIV-positive patients and even cause them death (39,40).

3.7 EC, IR, and INR

Once individuals test positive for HIV, they can be classified into 3 groups based on their response to the infection and treatment: Elite controllers (EC), Immunological responders (IR), and Immunological non-responders (INR) (41,42).

3.7.1 Elite controllers

The Elite Controllers (EC) represents the 1% of the HIV-infected population and are characterised by maintaining their viral load at undetectable levels without the use of any ART (41). The EC group has been proposed as a good model to study different eradication strategies such as the HIV vaccine, or a functional cure (43).

A functional cure is a type of strategy that aims to suppress the replication of all proviruses maintaining the viral load at undetectable levels (44). The heterogeneity related to the clinical, virological, and immunological features of this group is a problem in finding a common therapeutic target to treat HIV infection (41).

3.7.2 Immunological responders

The immunological responders (IR) are a group of patients that, although they start ART with a low CD4+ T cell count, can restore their CD4+ T cell count and achieve viral suppression when they are treated with ART (42). The definition of IR is very variable depending on the study population and the research group. The most common definition is a total CD4+ T cell count > 500 cells/µL at 2-12 years after ART initiation and an undetectable plasma viral load (45). In addition to this definition, there are others that are also commonly used, for example, a total CD4+ T-cell count > 250 cells/µL at 48 weeks after ART initiation and an undetectable plasmatic viral load, which is used in the present study (46).

3.7.3 Immunological non-responders

The immunological non-responders (INR) are a group of patients that start the treatment with a low CD4+ T cell count, but in this case, they are characterised by failing to restore their CD4+ T cell counts despite achieving viral suppression by ART (42). The definition of INR, like in the case of the IR is very variable. The most common definition is a total CD4+ T cell count < 500 cells/µL at 2-12 years after ART initiation and an undetectable plasma viral load (45). Like in the case of IR, there are other definitions that are commonly used. In this study, the definition used was a total CD4+ T-cell count < 250 cells/µL at 48 weeks after ART initiation and an undetectable plasmatic viral load (46).

The incomplete immune reconstitution is the main problem of INR that increases the risk of suffering from some diseases, and the rate of morbidity and mortality (42). The INRs have worse prognostic than EC and IR, and for this reason, it is important to identify the differences between groups for finding specific immune reconstitution therapeutic targets (47).

3.8 Factors associated with the different phenotypes

Several factors can influence the immune response of a patient to HIV-1 infection, playing a crucial role in determining the phenotype and recovery of the individual.

3.8.1 Polymorphisms

Polymorphisms are genetic variants that cannot be explained only by mutations due to the prevalence of these variations in the population (48).

In the case of the ECs, they can exert control of HIV infection through different mechanisms, one of them is the prevalence of the HLA-B*57 (41,49).

Some polymorphisms have been related to a better CD4+ T cell recovery. For example, the interleukin 7 receptor IL-7R subunit alpha rs6897932 CT/TT has been related to a better CD4+ T cell recovery compared to the CC polymorphism (50). On the contrary, some polymorphisms have been associated with a poorer CD4+ T cell recovery, like the toll-like receptor 9 1635AA genotype (51).

3.8.2 Cytokines

Cytokines are mediators that regulate the immune and inflammatory responses through complex pathways (52).

In EC, the expression of some cytokines like the CCL14, CCL21, CCL27, or XCL1 are elevated producing a suppression of the viral replication as a consequence of downregulation of the CXCR4 on the CD4+ T cells (53).

In the case of the INR, some cytokines like the interleukin-7 (IL-7) and its receptor IL-7 (IL-7R) are necessary for the CD4+ T-cell homeostasis promoting its proliferation and survival. Some studies demonstrated that IL-7 responsiveness is impaired in INRs and can be related to the downregulation of IL-7R (54). Other important interleukin is the interleukin-6 (IL-6) which is increased in the INR compared with the other groups of HIV-positive patients. The high levels of IL-6 are related to a higher mortality risk (55).

3.8.3 CD34+ hematopoietic stem cells infection

One of the identified problems in INR is a reduction of haematopoiesis in bone marrow caused by the HIV-1 infection of CD34+ hematopoietic stem cells (HSCs)

which are the T cells precursors HIV-1 can infect HSCs, because they can express the CD4+ receptor and the CXCR4 and/or CCR5 co-receptors (56–58).

So, the infection of this type of cell is related to incomplete immune reconstitution because by destroying the precursor cells, this prevents them from differentiating or dividing, and therefore they cannot replenish the damaged cells (42).

3.8.4 Bacterial dysbiosis

Bacterial dysbiosis is a decrease in microbial diversity that produces an expansion of some specific bacterial taxa. This state is usually produced by stress conditions like an infection or bad nutrition (59).

The HIV-1 infection produces a depletion of the CD4+ T-cells in the gut producing and increase of gut permeability. Factors, such as reduced production of tight junction proteins, increased gastrointestinal inflammation, and/or enterocyte apoptosis (60,61) produce dysbiosis in the gut microbiota that is accompanied by the release of bacterial products into the bloodstream (bacterial translocation). This release of bacterial products induces chronic immune activation and increases inflammation (62).

It has been demonstrated that HIV-1-positive patients had lower bacterial diversity than healthy people (63). In the case of EC patients, they had a richer microbial diversity than the other infected groups and it was similar to the uninfected population (64). In the case of INR patients, there are more alterations in their microbiota. Some genera like the *Fusobacterium* and some species like the *Faecalibacterium prousnitzii, Subdoligranulum sp.,* and *Caprococcus comes* were increased in this group. The high abundance of these bacteria was associated positively with a higher CD4+ T cell activation and a poorer CD4+ T cell recovery (65,66).

3.9 Importance of omics sciences in the study of HIV

The omics sciences are a set of different biologic disciplines that try to study, characterize, and quantify different biological molecules from genes to several metabolites to understand their role in different cellular pathways. The omics sciences are used to understand the pathways involved in different diseases and

find possible biomarkers to improve the diagnosis and to use it as a target to find new treatments (67).

There are different types of omics, such as genomics, epigenomics, transcriptomics, proteomics, and finally metabolomics. Metabolomics characterizes a specific set of metabolites from different types of samples that can be cellular or plasmatic by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to identify metabolic pathways associated with specific phenotypes. On the other hand, lipidomics is part of metabolomics, but it focuses on the study of different types of lipids, such as short-chain fatty acids (SCFA), bile acids (BA), triacylglycerols, phospholipids, and sterols to understand their function and their structure (68) (Figure 4).

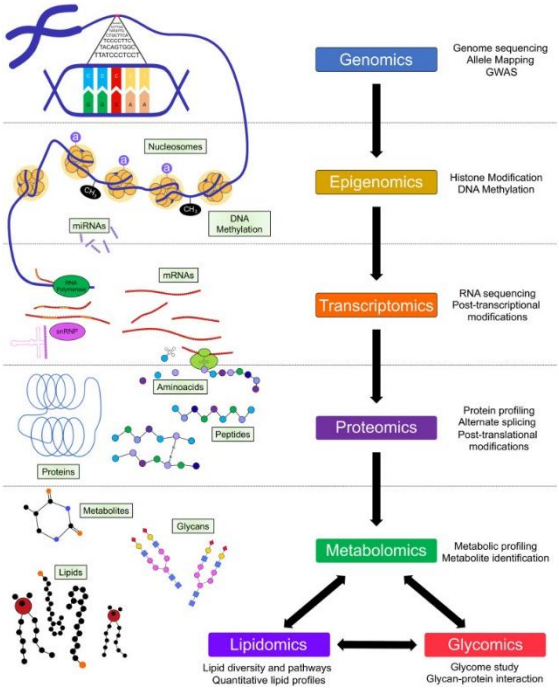

Fig. 4. The omics cascade. The omics cascade starts with the study of the genome, followed by the epigenome, and after that, the transcriptome, the next step is to study the proteomes, and finally, the metabolome that can be untargeted or can be targeted to a specific type of metabolites like the lipids (67).

3.9.1 Lipidomics in HIV Infection

Lipidomics in virus infection diseases play a key role to understand how the virus controls and reprograms the host's lipid metabolism (69,70). Several studies showed that virus infections produce drastic changes in the host lipidome (71).

It has been demonstrated in other virus infections that the changes in the plasmatic lipidome can be related to the severity of the disease (72). For example, it has been seen in other infections like the Ebola infection, that the triglycerides (TGs) are increased in the bloodstream, when the patient is critically ill (73).

Viruses encode proteins that can modify the host's lipid signalling and synthesis machinery and use the host lipids to establish protected sites of replication and generate the envelopment (74). Moreover, viruses are able to modify the host's metabolism to facilitate its replication, such as increasing the flux of metabolites channelled into fatty acid biosynthesis (75) or using lipid droplets as assembly sites and using the very low-density lipoproteins secretion machinery (76,77).

On the other hand, the SCFA have an important role in the inflammation produced by the HIV-1 infection. These types of lipids are produced by some of the species in microbiota, such as *Ruminococcus productus,* (78) *Caprococcus eutractus* (79), or *Prevotella copri* (80). The detection of the SCFA in plasma is used as biomarker for a dysbiosis in the gut microbiome, which is one of the complications associated with HIV infection (81).

Finally, the BA have an immunomodulatory function where they are able to stimulate the T cells to produce pro-inflammatory cytokines like IL-17 (82,83). Moreover, the BA have been recently related to microbiota (84). Some of the BAs reach the colon where they are metabolized to secondary hydrophobic BAs by microbes (85). So, it has been suggested that the BAs composition and the gut microbiome composition are connected (86).

In conclusion, lipids play a key role in viral infections, including the infection by HIV-1. Therefore, lipidomic studies are essential in order to identify new pathways and therapeutic targets to have a better understand the evolution of the infection and the immune response in the face of it.

4. HYPOTHESIS & OBJECTIVES

Since lipids have an important role in viral replication, bacterial translocation, and immune activation in HIV-1 infection, we hypothesise that there are differences

in the lipidome which are associated with the different phenotypes of HIV-1 infected patients.

To accomplish this hypothesis the next objectives were planned:

- Study of the different types of lipids dynamics among different groups.

- Study the specific differences in the lipidomic profile between the healthy (control) and the EC patients because the last one is a model of a functional cure.

- Study the specific differences in the lipidomic profile between the IR and INR groups due to the INR group being the most vulnerable one.

5. MATERIALS & METHODS

5.1 Study cohort

The study cohort included 20 patients, including 5 healthy individuals acting as a control group and 15 HIV-1-positive patients from 2 different hospitals. The hospitals are the Hospital Universitari Joan XXIII in Tarragona and the Instituto de Biomedicina de Sevilla (IBIS). Blood was obtained at baseline before the initiation of the ART from all the patients. To perform the lipidomic assay, plasma from blood samples was used. Next, the patients were divided into 4 groups. The first group was formed by healthy people acting as a control, the second group was formed by 5 EC that had a normal CD4+ T-cell count at baseline, the third one was formed by 5 INR, and finally, the last group was formed by 5 IR. In the case of the IR and INR groups, it was formed by late presenters, which means that at the moment of the diagnosis, the CD4+ T cell count was lower than 200 cells/ µL. The criteria to include the patients in the IR or INR groups was depending on the CD4+ T cell counts after 48 weeks of initiation of the ART therapy. If the patient's CD4+ T cell count is > 250 cells/ µL it will be classified as an IR, and if the CD4+ is lower than 250 cells/ µL it will be classified as an INR. (44) (Figure 5).

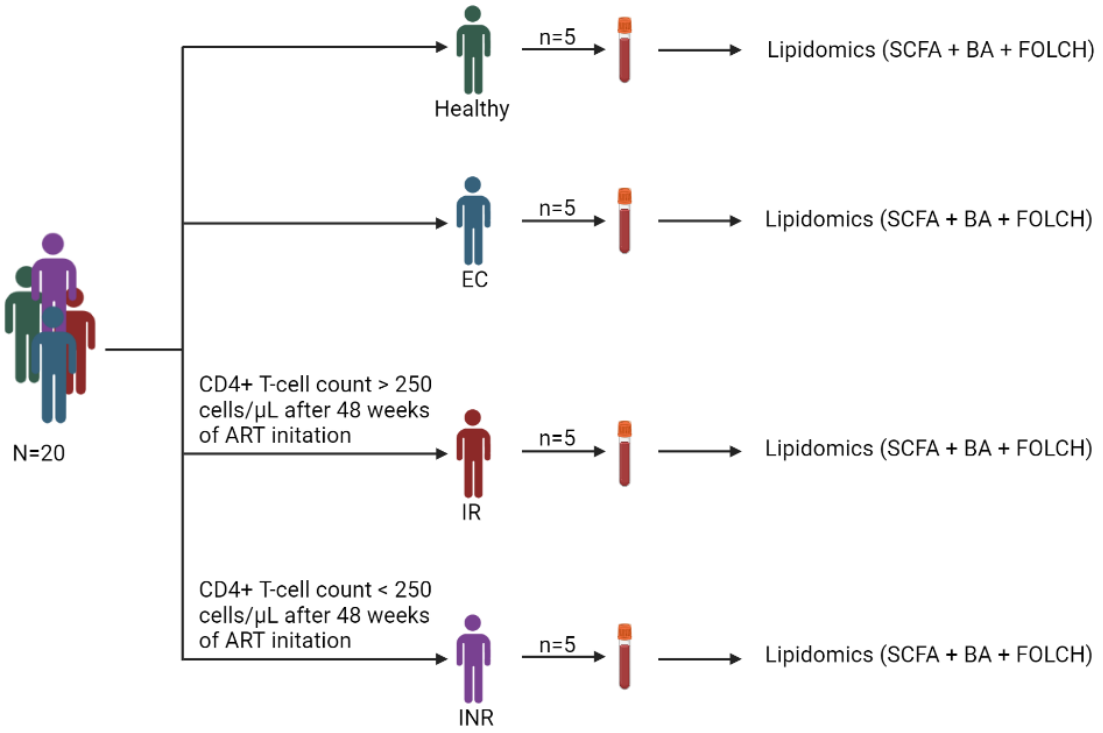

Fig. 5. Study cohort. The study consisted in 20 patients that were divided into 4 groups. One group of 5 healthy people will act as a control, one group of EC, one of IR, and one of INR of 5 people each. In all groups a lipidomic study was performed using plasma and, in the IR, and INR groups the medical records were used too. Patients with a CD4+ T-cell count lower than 250 cells/µL at 48 weeks after the initiation of ART were considered INR, while the patients with a CD4+ T-cell count higher than 250 cells/µL at 48 weeks after the initiation of ART were considered IR. Created with BioRender.com.

5.2 Lipidomics

The lipidomic analysis was performed by the Centro of Omics Sciences (COS) following the next protocols:

5.2.1 Determination of short-chain fatty acids by GC-MS/MS

Samples (100 microliters) were individually placed in 1.5 mL Eppendorf tubes. Afterwards, 10 µL of acidified water (15 % phosphoric acid v/v), and 10 µL of IS (AA-LAB at 300 µM, BA-LAB at 60 µM and IBA-LAB, VA-LAB, IVA-LAB, HA-LAB and PA-LAB of 30 µM) are added and vigorously mixed up. Consecutively, liquid-liquid extraction was performed using 150 µL of MTBE. The extraction was assisted by a vortex for 10 minutes. At this point, tubes were centrifuged at 15000 rpm for 10 minutes at 4 ºC. 100 µL were transferred into a vial with an insert. The

vials were centrifuged at 1000 rpm for 30 s at 4 ºC and 1.5 µL was injected into the GC-MS/MS. Briefly, SCFA were separated on DB-FFAP chromatographic column (30 m x 0.25 mm x 0.25 µm). The oven temperature was programmed as follows: (i) initial temperature 40 ºC, (ii) linearly raised at 12 ºC/min to 130 ºC (0 min), (iii) then linearly raised at 30 ºC/min to 200 ºC (0 min) and (iv) in the final step the temperature was ramped at 100 ºC/min to 250 ºC (4.5 min). The column flow was set at 1.5 mL/min using He as carrier gas. The injector was set at 250 ºC and the extracts were injected in a splitless mode. The Ionisation was carried out by electronic impart (70 eV) and the mass analyser operates on Multi Reaction Monitoring (MRM).

5.2.2 Determination of Bile Acids by HPLC-MS/MS

Samples (100 µL plasma) were aliquoted to a 1.5 ml Eppendorf tube and mixed with 400 µL of 100 ng/mL CA-d5 and 100 ng/mL TCA-d5 in ACN. Samples were vortexed for 1 min and centrifuged for 5 minutes at 15000 rpm and 4ºC. Supernatants were transferred to a new tube and were evaporated in a SpeedVac at 45ºC. Samples were reconstituted with 50 µL of Methanol: Water (1:1) and transferred to glass vials for their analysis. The chromatographic separation was performed with a mobile phase A of 0.1% ammonium hydroxide and 10mM ammonium acetate and B with Acetonitrile. The column temperature was set at 27ºC, and the injection volume was 2 µL.

5.2.3 Lipidomics analysis by Folch extraction (Lip-II) by LC-QTOF

For the extraction of more hydrophobic lipids, liquid-liquid extraction with chloroform: methanol (2:1) based on the Folch procedure was performed by adding four volumes of chloroform: methanol (2:1) containing internal standard mixture (Lipidomic SPLASH®) to plasma Then, the samples were mixed and incubated at -20ºC for 30 min. Afterwards, water with NaCl (0.8 %) was added and the mixture was centrifuged at 15,000 rpm. The lower phase was recovered, evaporated to dryness, and reconstituted with methanol:methyl-tert-butyl ether (9:1) and analysed by UHPLC-qTOF (model 6550 of Agilent, USA) in both positive and negative electrospray ionization modes. The chromatographic method consisted of elution with a ternary mobile phase containing water,

methanol, and 2-propanol with 10 mM ammonium formate and 0.1% formic acid. The stationary phase was a C18 column (Kinetex EVO C18 Column, 2.6 µm, 2.1 mm X 100 mm) that allows the sequential elution of the more hydrophobic lipids such as lysophospholipids, sphingomyelins, phospholipids, diglycerides, triglycerides, and cholesteryl esters, among others. The identification of lipid species was performed by matching their accurate mass and tandem mass spectrum, when available, to Metlin-PCDL from Agilent containing more than 40,000 metabolites and lipids. In addition, the chromatographic behaviour of pure standards for each family and bibliographic information was used to ensure their putative identification. The identification indicates the lipid family (LPC-lysophosphatidylcholine, PC-phosphatidylcholine, SM-sphingomyelin, DG-diglyceride, TG-triglyceride, and ChoE-cholesterol ester), the total number of carbons of the acyl chains and the number of double bonds.

5.3 Statistical analysis

The statistical analysis performed was based on the groups previously defined. First, the crude data obtained from lipidomics analysis was used in a Kruskal-Wallis analysis to determine which of the candidates had a significant difference between any of the groups. The lipidic species that did not have a significant difference were eliminated in the next analysis. In the second place, a U-Mann-Withney test was performed with the candidates that were selected using the Kruskal-Wallis test to find between which groups were the differences. To perform these analyses the software used was the IBM Statistical Package for the Social Sciences (SPSS) Statistics 15.0. This software is used to perform a large number of analyses like the Kruskal-Wallis or the U Mann-Withney (87). Next, a HeatMap showing the differences between the groups and Random Forest analysis was performed to study the impact that each compound had to differentiate between the IR and INR groups, using the Metaboanalyst 5.0. (88). The graphic representation was performed in Excel version 2208. Finally, receiver operating characteristic (ROC) curves to find which of the lipids had the most discriminatory power to differentiate between the IR and INR groups were performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics 21.0. (87). The figures were done by BioRender and PowerPoint version 2208.

6. RESULTS

6.1 Differences in the lipidome between the groups

First, to analyse the differences in the lipidome between the groups, a Kruskal-Wallis test was performed to find which of the 200 analysed compounds had a significant difference ($p<0.05$) between any of the groups. The results of the Kruskal-Wallis test were that 62 lipids showed a significant difference between any of the groups.

In the case of the SCFA and the BA, it was remarkable that the lipid expression profile of the IR and INR groups were similar between them as the healthy compared to EC but with some exceptions. The levels of propionic acid (PA) and valeric acid (VA) levels tended to rise, whereas isobutyric acid (IBA) levels tended to decrease in EC group. Finally, the healthy group's heptanoic acid (HA) levels were the lowest (Figure 6).

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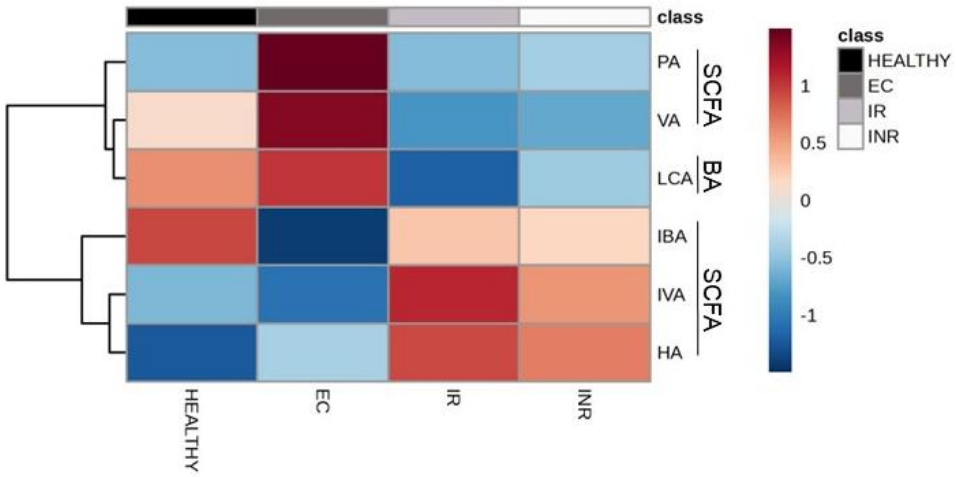

Fig. 6. Differences in the short-chain fatty acids and bile acids levels between the healthy, IR, INR, and EC patients. A) Heatmap showing significant SCFA and BA increasing or decreasing in accordance with different groups. Columns correspond to different phenotypes in this order (from left to right): healthy (control), EC, IR, and INR. Mean values for each type of lipid are colour-coded based on relative abundance, low in blue and high in red. Statistical differences ($p<0.05$) among the groups were determined by the nonparametric Kruskal-Wallis test. Abbreviations: SCFA: Short chain fatty acids, BA: Bile acids, EC: Elite controllers, IR: Immunological responders, INR: Immunological non-responders, PA: Propionic acid, VA: Valeric acid, LCA: Lithocholic acid, IBA: Isobutyric acid, IVA: Isovaleric acid, HA: Heptanoic acid.

In the case of the other lipids, the lipid profile of the IR and INR groups was similar between them as happens between Healthy and EC groups. Focusing on the IR and INR lipidic profiles, the monoglyceride 16:0 (MG 16:0) tended to increase, whereas the ether-linked phosphatidylcholine 38:6 (PC-O 38:6) was decreased in the IR group. On the other hand, the TG 52:0 was increased in the INR, whereas the ether-linked lysophosphatidylcholines 16:1p and 18:1p (LPC-O 16:1p and LPC-O 18:1p), and the lysophosphatidylcholine 18:3 (LPC 18:3) were clearly increased in the INR group. On the other hand, some lipids like phosphatidylcholine 38:6 (PC 38:6), PC-O 36:5, TG 52:5, and PC-O 36:4 were decreased in the INR group. Moreover, the phosphatidylethanolamine 38:4 (PE 38:4), PC-O 36:4 and cholesterol ester 20:5 (ChoE 20:5) were increased, whereas the TG 52:0, LPC 16:0, MG 17:1, and TG 54:0 were decreased in the healthy group compared with other groups. In the case of the EC group, the diglyceride 32:0 (DG 32:0), PC 35:4, and TG 50:4 were increased and MG 18:0, MG 18:1, and PE 38:4 were decreased (Figure 7).

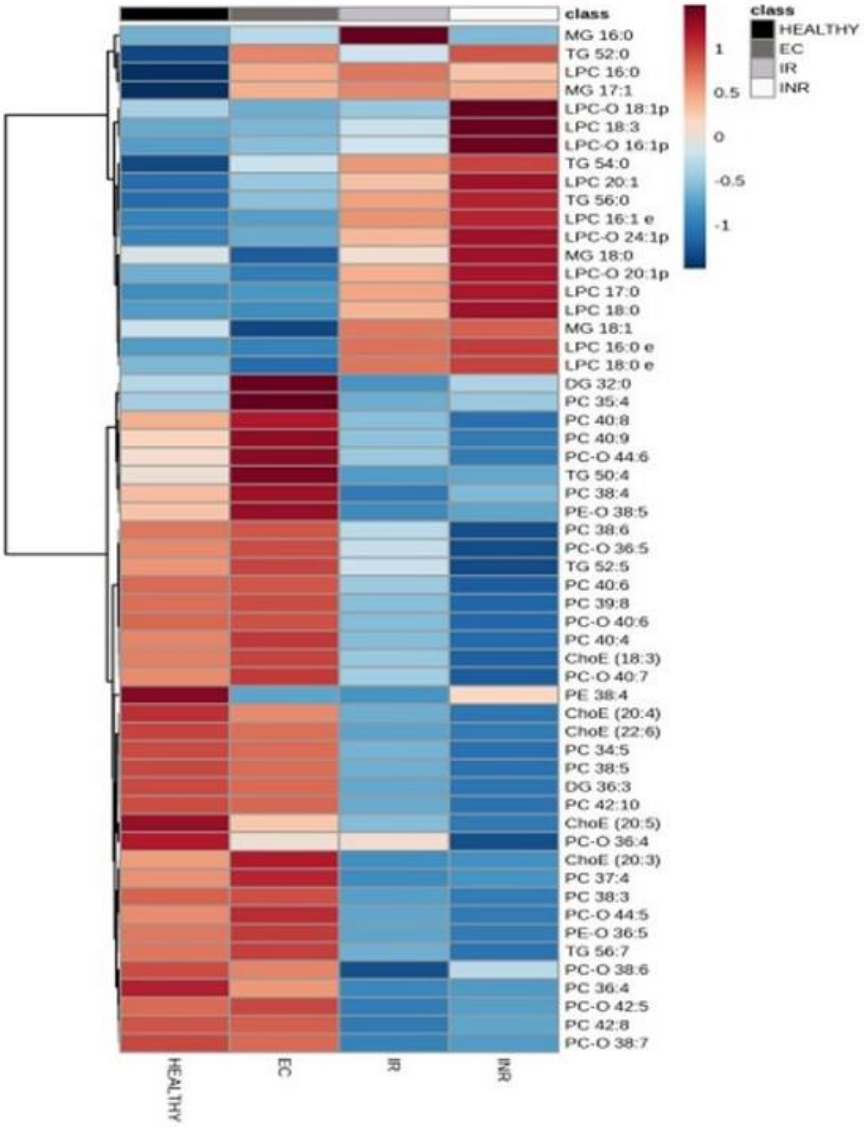

Fig. 7. Differences in the lipidomic profile between the healthy, IR, INR, and EC groups. Heatmap represents the significant relative abundance of different types of lipids increasing or decreasing in accordance with different groups. Columns correspond to different phenotypes in this order (from left to right): healthy (control), EC, IR, and INR. Mean values for each type of lipid are colour-coded based on relative abundance, low in blue and high in red. Statistical differences ($p<0.05$) among the groups were determined by the nonparametric Kruskal-Wallis test. Abbreviations: EC: Elite controllers, IR: Immunological responders, INR: Immunological non-responders, MG: Monoglycerides, TG: Triglycerides, LPC: Lysophosphatidylcholines, LPC-O: Ether-linked lysophosphatidylcholines, DG: Diglycerides, PC: Phosphatidylcholine, PC-O: Ether linked phosphatidylcholine, PE-O: Ether linked phosphatidylethanolamine, PE: Phosphatidylethanolamine, ChoE: Cholesterol esters.

6.2 Differences between the Healthy and the EC groups

Next, a more exhaustive study was performed in order to identify the molecules involved in EC phenotype. The Healthy groups and the EC groups were compared using the Mann-Withney U-test to find the lipids that had a significant difference between these 2 groups.

After performing the statistical test, only 4 lipids showed significant differences between both groups. The DG 32:0 levels were increased in the EC group compared to the healthy (Figure 8A). Besides that, IBA, isovaleric acid (IVA) and HA were decreased in the EC compared to the controls (Figure 8B).

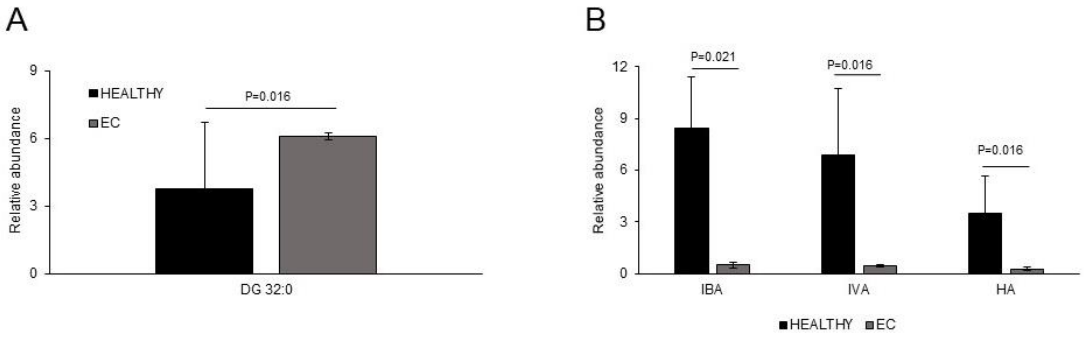

Fig. 8. Levels of DG and SCFA in the healthy vs EC groups. A) Relative abundance levels of DG 32:0 increased in EC patients compared to controls (healthy group), and B) Relative abundance levels of IBA, IVA, and HA decreased in EC patients compared to controls (healthy group). Bar graphics represent the mean with the S.E.M of the group. Statistical differences ($p<0.05$) among the groups were determined by nonparametric Mann-Whitney U-test and indicated in each graph. Abbreviations: EC: Elite controllers, DG: Diglyceride, IBA: Isobutyric acid, IVA: Isovaleric acid, HA: Heptanoic acid.

6.3 Differences between the Healthy, IR, and INR groups

Healthy, IR, and INR groups were analysed with more detail, putting special attention to the differences between the IR and INR groups. These 3 groups were compared using Mann-Withney U-test to find the lipids that had a significative difference between these groups and then, the lipids were represented in bar graphs. In this case, 15 lipids showed a significant difference.

In the case of the SCFAs, the IBA, VA, and PA showed significant differences between the IR and INR groups. In all of them, the highest relative abundance was in the INR group and the lowest in the healthy group (Figure 9 A-C).

Remarkably, non-significant difference between IR or INR related to IBA levels were found compared to Controls (Figure 9 A), whereas VA and PA levels showed a significant increase in INR group compared to IR and the Healthy group (Figure 9 B and C).

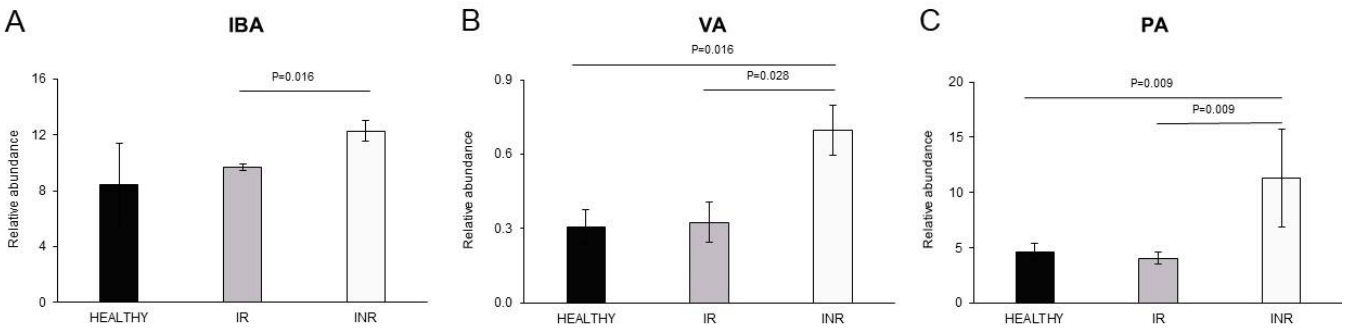

Fig. 9. Levels SCFA in the healthy, IR, and INR groups. A) Significant differences in relative abundance levels of IBA, B) VA, and C) PA in healthy, IR, and INR patients. Bar graphics represent the mean with the S.E.M. of the group. Statistical differences ($p<0.05$) among the groups were determined by nonparametric Mann-Whitney U-test and indicated in each graph. Abbreviations: IR: Immunological responders, INR: Immunological non-responders, IBA: Isobutyric acid, VA: Valeric acid, PA: Propionic acid.

In the case of the LPCs, the relative abundance of LPC 20:1, LPC-O 18:1p, LPC-O 20:1p, and LPC-O 24:1p, were higher in the INR group, while the lowest one was in the healthy group (Figure 10 A, C and D). In the LPC 20:1, LPC-O 20:1p, and LPC-O 24:1p there were significant differences between the IR and INR groups and between the healthy and INR groups. On the other hand, the LPC-O 18:1p showed significant differences between all the groups (Figure 10 B). On the contrary, in the case of the PC 36:4 and the PC 39:8, the highest relative abundance was in the healthy group and the lowest one was in the INR group. In both cases, there were significant differences between all the groups (Figure 10 E and F).

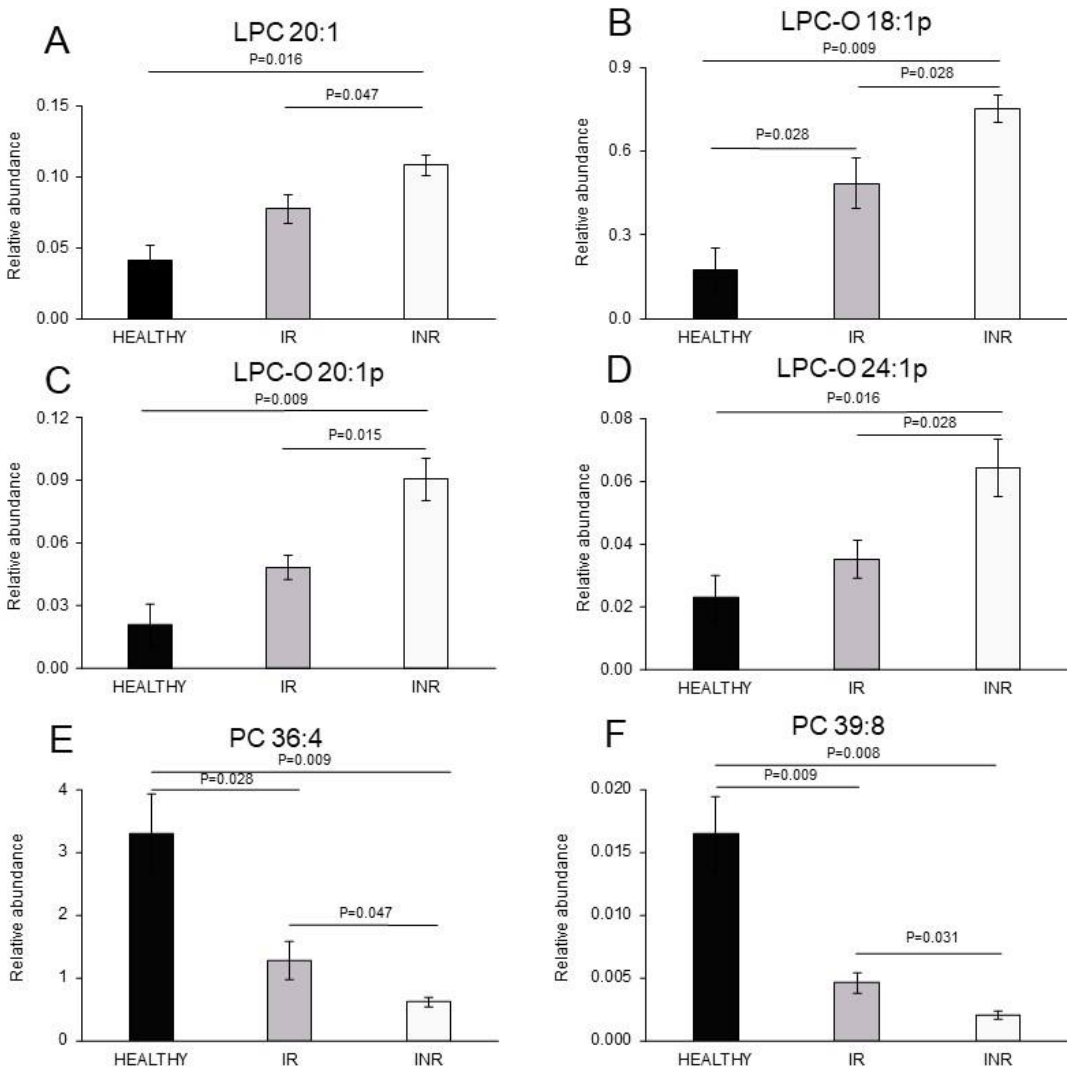

Fig. 10. Levels of LPC, LPC-O, and PC in the healthy, IR, and INR. A) Relative abundance levels of LPC 20:1, B) LPC-O 18:1p, C) LPC-O 20:1p, and D) LPC-O 24:1p increased in INR patients compared to IR and controls (healthy group). E) Relative abundance levels of PC 36:4 and F) PC 39:8 decreased in INR patients compared to IR and controls (healthy group). Bar graphics represent the mean with the S.E.M. of the group. Statistical differences ($p<0.05$) among the groups were determined by nonparametric Mann-Whitney U-test and indicated in each graph. Abbreviations: IR: Immunological responders, INR: Immunological non-responders, LPC: Lysophosphatidylcholines, LPC-O: Ether-linked lysophosphatidylcholines, PC: Phosphatidylcholine.

Finally, in the case of the TGs, some of them showed a significative difference between the IR and INR groups, while others had similar concentrations between the IR and INR groups, but they were elevated compared to the healthy group (Figure 11).

The levels of TG 50:4, TG 52:5, and TG 56:7 were significantly decreased in the INR compared to IR group (Figure 11 A-C). Remarkably, significant differences between all the groups were detected in TG 56:7 levels (Figure 11 C). On the other hand, the relative abundance of TG 52:0, TG 54:0 and TG 56:0 were higher in INR than the Healthy group. Of note, non-differences between IR and INR levels related to these TGs were found (Figure 11 D-F).

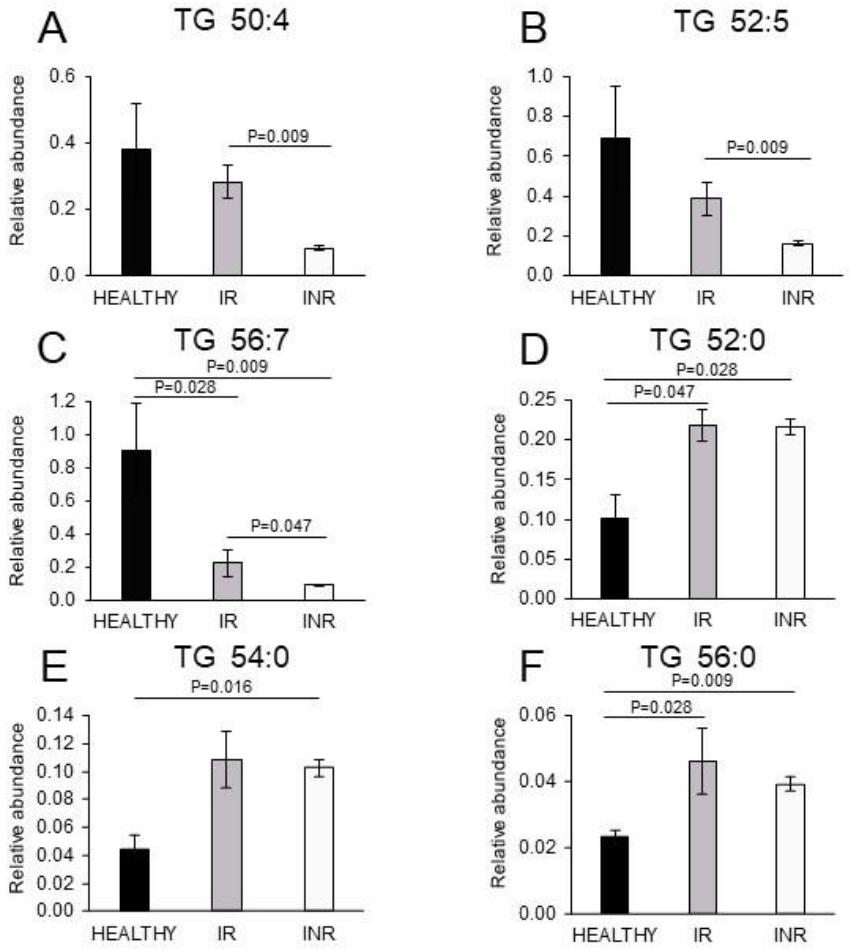

Fig. 11. Concentration of TGs in the healthy, IR, and INR. A) Relative abundance levels of TG 50:4, B) TG 52:5, and C) TG 56:7 decreased in INR patients compared to IR and controls (healthy group). D) Relative abundance levels of TG 52:0, E) TG 54:0, and F) TG 56:0 increased in INR and IR patients compared to controls (healthy group). Bar graphics represent the mean with the S.E.M. of the group. Statistical differences ($p<0.05$) among the groups were determined by nonparametric Mann-Whitney U-test and indicated in each graph. Abbreviations: IR: Immunological responders, INR: Immunological non-responders, TG: Triglycerides.

In conclusion, there are differences in the lipidome of the IR and INR groups where the IBA, VA, PA, LPC 20:1, LPC-O 18:1p, LPC-O 20:1p, and LPC-O 24:1p obtained a higher relative abundance in the INR group, whereas the PC 36:4, PC 39:8, TG 50:4, TG 52:5, and TG 56:7 obtained a lower relative abundance in the INR compared to the IR group.

6.4 Clinical characteristics of the IR and INR groups

Once, all the lipids with significant differences were identified, the study focused on the INR group. To achieve this, the clinical characteristics of the IR and INR groups were used.

The clinical characteristics of the 5 IR and the 5 INR patients included in this study were represented in Table 1. The patient's median age was significative different between the groups (53 in IR and 69 years in INR). In the case of the IR group, 60% of the patients were males and in the INR group 100%. 3 (60%) of the patients in the INR group arrived at the AIDS state. The same number of patients co-infected with hepatitis B and C viruses, 60% and 20% respectively. The homosexuality risk factor was 40% for the IR patients and 20% for the INR patients. Finally, in the case of comorbidities such as diabetes *mellitus* and hypertension, in both cases, 40% of the patients in the INR group were affected.

Table 1. Patients' characteristics. There are significative differences in some of the parameters presented in the table like in the age, if the patients have arrived at the AIDS state, the defining pathology and in the CD4 count at all the time points measured. Values are represented in n (%) for categorical variables or median (interquartile range) for continuous variables. The Mann-Whitney U-test was used and all p values < 0.05 were considered significant and are highlighted in bold.

	IR (n=5)	INR (n=5)	*p-value*
AGE	53 (43.5-61.5)	69 (60-83)	**0.036**
MALES	3 (60%)	5 (100%)	0.134
AIDS	0 (0%)	3 (60%)	**0.05**
Defining pathology:			
-No-one	5 (100%)	0 (0%)	**0.003**
-Diverse	0 (0%)	2 (40%)	0.134
-Pulmonar TBC	0 (0%)	1 (20%)	0.317
-Toxo SNC	0 (0%)	2 (40%)	0.134
Hepatitis B coinfection	3 (60%)	3 (60%)	1
Hepatitis C coinfection	1 (20%)	1 (20%)	1
Risk factor:			
-Homsexual	2 (40%)	1 (20%)	0.513
-Heterosexual	3 (60%)	2 (40%)	0.549
-ADVP	0 (0%)	1 (20%)	0.317
-Unkwown	0 (0%)	1 (20%)	0.317
Smoke	1 (20%)	2 (40%)	0.513
DM	0 (0%)	2 (40%)	0.134
HTA	0 (0%)	2 (40%)	0.134
Viral load log basal	4.92 (4.47-5.29)	5.62 (4.96-5.8)	0.117
Viral load log 12 months	1.28 (1.28-2.65)	1.69 (1.28-2.24)	0.117
Viral load log 36 months	1.28 (1.28-1.28)	1.28 (1.28-1.9)	0.136
CD4 basal	157 (71-165.5)	19 (9-77.5)	**0.036**
CD4 12 months	317 (263.5-353)	114 (75.5-255.5)	**0.028**
CD4 36 months	369 (307.5-532.5)	186 (100.5-207.5)	**0.009**
Glucose basal	84.5 (81.5-98.75)	92 (81-106)	0.593
Glucose 12 months	87 (82.25-93.25)	88 (88-90)	0.463
Glucose 36 months	78 (73.25-95.5)	94.5 (92-97)	0.355
Cholesterol basal	123.5 (87-160.75)	171 (131-349)	0.157
Cholesterol 12 months	160 (134.5-193)	235 (146-320)	0.289
Cholesterol 36 months	151 (139.75-187.25)	259 (207-311)	0.064
LDL basal	60.5 (36-83.5)	107 (76-275)	0.157
LDL 12 months	80.5 (61-121)	140 (78-247)	0.289
LDL 36 months	78.5 (50.5-108)	176 (111-241)	0.165
GPT basal	28 (21.5-37.5)	29 (26-83)	0.48
GPT 12 months	21 (16.75-24.5)	26 (20-34)	0.157
GPT 36 months	21 (15-31.5)	34 (15-53)	0.623
GGT basal	38 (21.5-312.5)	41 (38-57)	0.724
GGT 12 months	37.5 (18.5-94)	56 (53-135)	0.289
GGT 36 months	36 (19-119.75)	107.5 (24-191)	0.355

Regarding the biochemical parameters, the viral load log was similar in both groups and decreased at 12 and 36 months, demonstrating that the treatment was effective. The CD4+ count was very low at baseline because the patients were late presenters (< 200 cells/µL at the moment of the diagnosis), but it was significantly lower in the INR group (19 cells/µL) than in the IR group (157 cells/µL). At 12 and 36 it can be observed that in the IR group, the CD4+ count increased over the 250 cells/µL (317 and 369 cells/µL respectively) whereas in the case of the INR patients, the CD4+ count did not surpass the threshold (114 cells/µL at 12 months and 186 cells/µL at 36 months) (Table 1 and Figure 12A). Finally, in the other biochemical parameters, there are no significative differences between the groups but in some of them, a trend can be observed. Changes in glucose levels cannot be observed either between the groups or over time (Figure 12B). The cholesterol and low-density lipoproteins (LDL) followed a similar trend where both increase in the INR group and this difference increased over time (Figure 12C and D). Finally, the alanine aminotransferase (GPT) and the gamma-glutamyl transpeptidase (GGT) followed a similar trend where they had similar concentrations at baseline, but the concentration in the INR group increases over time (Figure 12E and F).

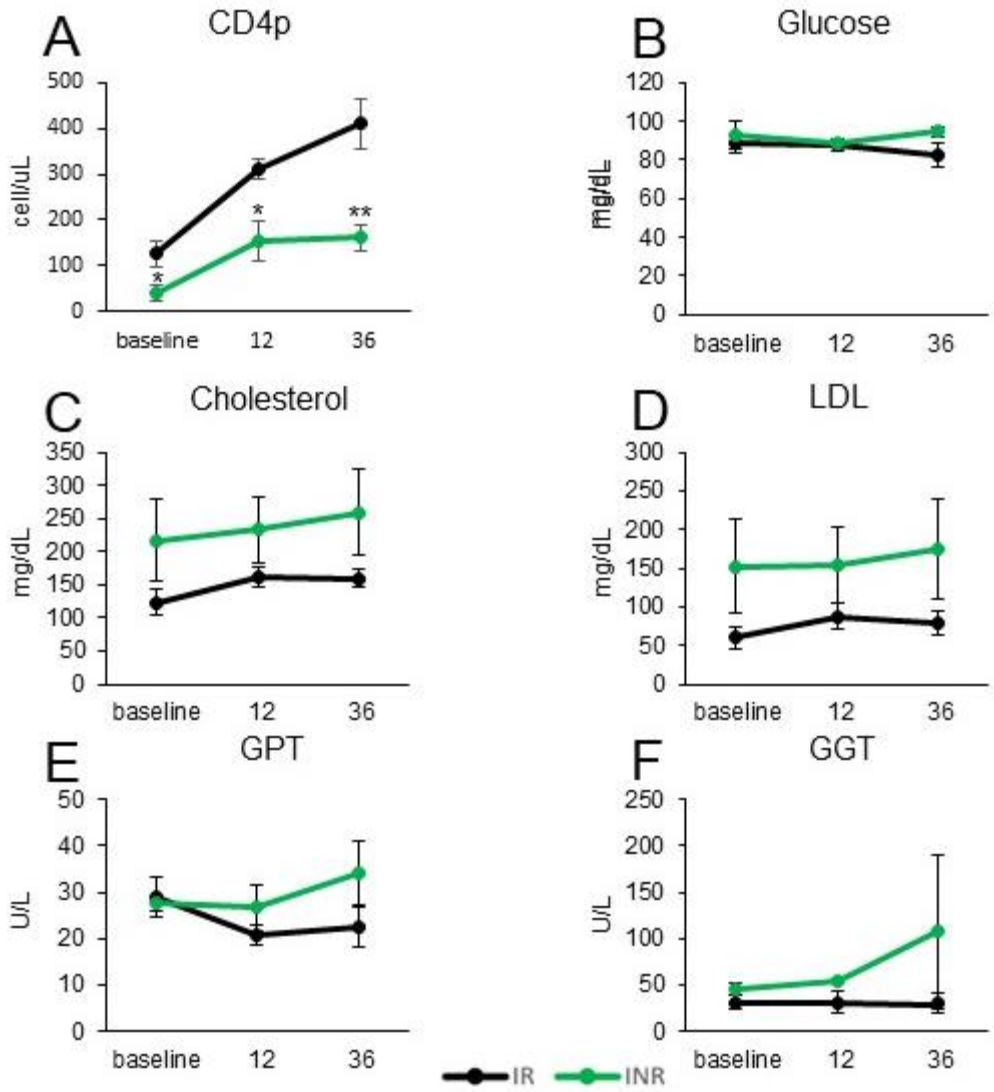

Fig. 12. Several biochemical parameters of the IR and INR patients at different time points. A) CD4+ T-cell count, B) Glucose levels, C) Cholesterol levels, D) LDL levels, E) GPT concentration, and F) GGT concentration of IR and INR patients at 3 different time points (baseline, 12, and 36 months). Graphics represent the mean with the S.E.M of the group and statistical differences ($p<0.05$) among the groups were determined by nonparametric Mann-Whitney U-test and are indicated in each graph with (*=$p<0,05$) or (**=$p<0,01$). Abbreviations: IR: Immunological responders, INR: Immunological non-responders, LDL: Low-density lipoproteins, GPT: Alanine aminotransferase, GGT: Gamma-glutamyl transpeptidase.

These results could suggest that the glucose metabolism is not affected by the HIV infection related to IR and INR, but considering the variations in the cholesterol and LDL, it can be seen that the lipidic metabolism was altered.

6.5 Potential biomarkers to differentiate IR and INR

Finally, the study focused on finding which lipidic molecules were better biomarkers to differentiate between the IR and INR groups. The LPC-O 24:1p, LPC-O 20:1p, IBA, and PA were the 4 lipids with more impact to differentiate between the IR and the INR, and all of them were associated with the INR group. Other lipids like the TG 50:4, TG 52:5, TG 56:7 or PC 39:8 had less impact and were associated with the IR group, so they were not selected (Figure 13A). The IBA, PA, LPC-O 20:1p, and LPC-O 24:1p presented an excellent discriminatory power to differentiate both groups (Figure 13B-E). In the case of the IBA, the area under the curve (AUC) was 0.960 (CI = 0.843-1.077) (Figure 13B). The PA showed a perfect AUC of 1 (CI= 1-1) so the specificity and sensibility were both 100% and the (Figure 13C). The best cut-off point obtained with the coordinates of the curve was 5.76 meaning that if the value obtained in an analysis is higher than this cut-off point the patient will be INR, whereas the value is lower than 5.76, the patients will be IR. The LPC-O 20:1p had an AUC of 0.920 (CI = 0.738-1.102) (Figure 13D). Finally, the AUC of the LPC-O 24:1p was 0.960 (CI = 0.843-1.077) (Figure 13E).

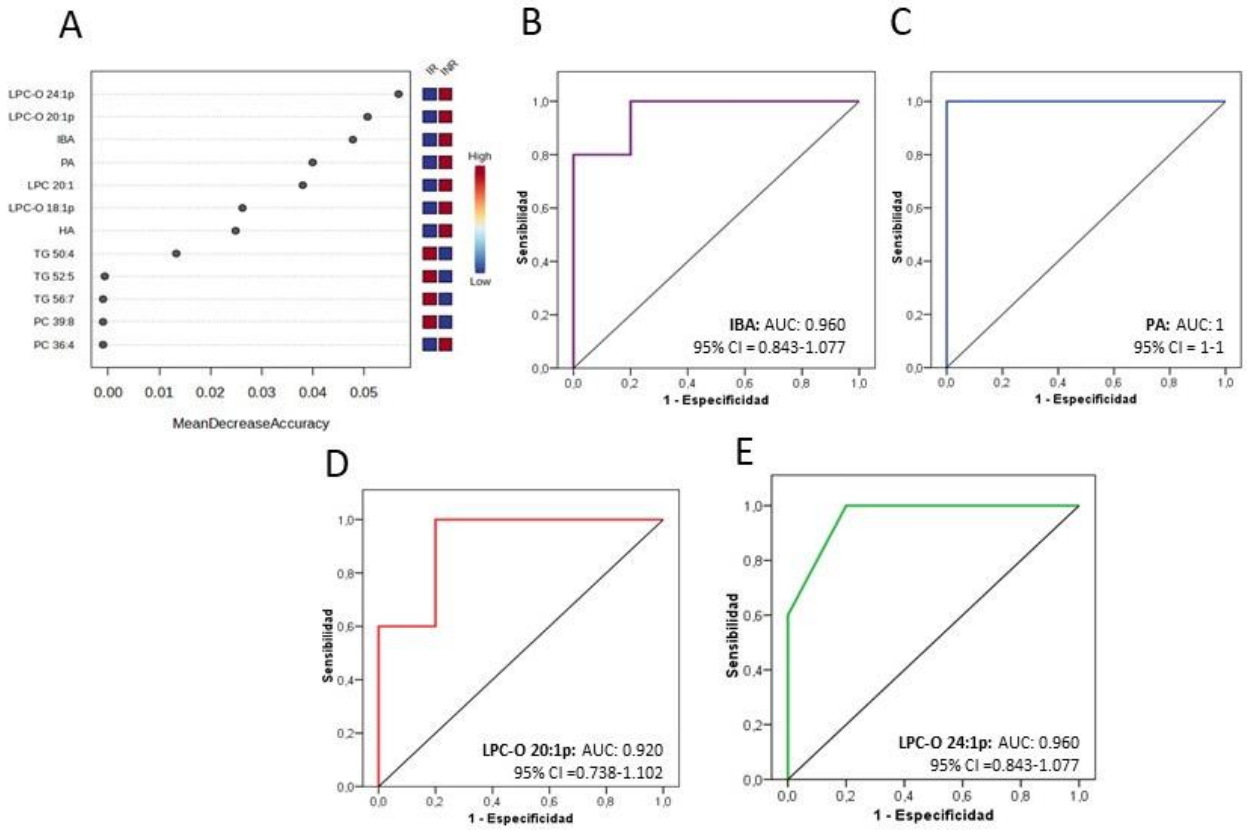

Fig. 13. Biomarker analysis for distinguishing INR from IR patients. A) Random forest of significant lipids comparing INR and IR outcomes by MetaboAnalyst 5.0. The intensity of colours indicates the significance of the compound in differentiating groups (high in red and low in blue). B) Receiver operating characteristic (ROC) curves of IBA, C) PA, D) LPC-O 20:1p, and E) LPC-O 24:1p to discriminate INR patients from IR using IBM SPSS Statistics 21.0. Abbreviations:AUC: Area under the curve, IR: Immunological responders, INR: Immunological non-responders, LPC-O: Ether-linked lysophosphatidylcholines, IBA: Isobutyric acid, PA: Propionic acid, LPC: lysophosphatidylcholines, HA: Heptanoic acid, TG: Triglycerides, PC: Phosphatidylcholine.

The 4 compounds had excellent AUCs. However, PA exhibited a remarkably high AUC, which suggests that could be considered a very good biomarker to differentiate between the IR and INR groups.

7. DISCUSSION

The study of the lipid profile of HIV-1-positive patients is still an emerging area of research which allow us to understand how the changes in the levels of different lipids can affect the development of the different HIV phenotypes such as EC, IR,

or INR. For this reason, the present study was focused on analysing several types of lipids species associated with the different HIV phenotypes.

The results showed that the lipid profile between the 3 HIV-1 phenotypes is different due to 18 different types of lipids that were significantly detected. Remarkably the major changes were observed in the INR group compared to other groups of patients. In the case of the EC group, these patients have a lipidic profile more similar to the healthy group than to the INR and IR groups. These variations in the lipidome are reflected in how the infection affects the patient, where the EC are almost like the healthy population while the IR and INR groups undergo several alterations caused by the HIV-infection (41,42).

First, the levels of DG 32:0 was elevated in the EC group, characterised by viraemic suppression without ART (89). The DG 32:0 is a lipid that is involved in *de novo* lipogenesis (90) and its concentration can be augmented in response to high glucose levels (91). It has been seen that the levels of glucose in EC patients are usually increased (92), which could be correlated with the increase in the DG 32:0 in the EC. The EC patients are characterized by having an increased risk of cardiovascular diseases (CVD) due to their inflammatory state (93). However, there is no link between the increase of the DG 32:0 and a higher risk of CVD, but other DGs like the DG (16:0/22:5) has been associated with CVD mortality (94). Nevertheless, it has been found that the DG 32:0 is augmented in other diseases like obesity or *diabetes mellitus* type 2 which are factors that increase the CVD risk (90,91).

On the other hand, in the INR, characterised by poor CD4+ cell recovery despite ART, 12 different lipids were significantly different among groups. In the case of the LPCs, all compounds were increased in the INR group compared with the IR and healthy groups. These types of lipids can be augmented or decreased in HIV-1-positive patients depending on the type of LPCs (95). The effect of the LPCs mostly depends on their fatty acid (FA) composition, some of the LPCs can promote inflammation, contributing to chronic immune activation and the development of some of the comorbidities that are associated with the HIV infection (95,96). Some studies suggest that the relative abundance of monounsaturated LPCs is inversely related to the production of IL-6 in HIV patients (97). In addition, an augment in the LPC concentration is related to a

higher risk of atherosclerosis due to the presence of this type of lipid in the LDL and oxidized LDL in the general population (97).

Related to TG levels, our results show that TG 50:4, TG 52:5, and TG 56:7 were decreased whereas TG 52:0, TG 54:0, and TG 56:0 were increased in the INR and IR group compared to the healthy group. Some studies showed that the TGs can be increased in viral infections due to a higher hepatic production of TG-rich lipoproteins and a lower clearance of them due to a reduction of the lipoprotein lipase activity (98). Previous studies from our group showed indeed, that the high-density lipoproteins (HDL) and the LDL were increased when compared with their baseline values (99). These augments in the TGs and the LDL could be produced by the alterations of the lipoprotein metabolism caused by cytokines that mediate the immune response (100). These changes have only been demonstrated in the general HIV-positive population, but these effects could be accentuated in the INR group. In the case of the TGs that were decreased, this decrease has been only seen before in older HIV-positive patients that had been in long-term treatment with Raltegravir (101). It should be noted that in our study the INR population was older than the IR population, so the decrease in the levels of some of the TGs could be produced by the age of the patients.

On the contrary, the PC 36:4 and PC 39:8 were decreased in the INR group compared to the IR and healthy groups. Dirajlal-Fargo et al. described in their study that polyunsaturated PCs, such as PC 36:4 and PC 39:8 are inversely associated with the CVD risk in HIV-infected children (102), which means that the lower the relative abundance of these lipids, the higher risk of suffering CVD. However, more correlation studies with clinical data need to be conducted to associate these levels of LPCs and PCs with specific comorbidities in the INR group.

Recent studies have highlighted the use of SCFA as markers of dysbiosis resulting from HIV-1 infection in the host's gut microbiota (60). Some studies have demonstrated that in HIV-positive patients, the SCFA relative abundance is usually higher due to this dysbiosis (103).

In this study, the IBA, IVA, and HA were decreased in the EC group. Some studies demonstrated that the relative abundance of SCFA is lower in the EC compared

to the healthy population as our results showed (104). The composition of the gut microbiota of the EC is different from other groups of healthy and HIV-1-positive patients and the altered composition has been related to the immune activation and inflammation in this group of patients (64). Remarkably, the IBA and IVA have an important anti-inflammatory role (105–107), and it has been demonstrated that patients with inflammatory intestinal diseases have a significant reduction in their IVA levels (108). Additionally, high HA levels have shown an important role in improving mitochondrial function under inflammatory conditions (109). The 3 SCFA have an important role in improving the inflammatory state, so, the decrease in their relative abundance may help to explain the chronic inflammation that can be seen in the EC. The IBA and IVA have been studied in HIV patients before (110), but this study is the first one that has found that IBA, IVA, and HA is associated with the EC group. Given the novelty of these findings, additional research is needed to explore the mechanisms by which these changes are manifested.

In contrast, IBA, VA, and PA were augmented in the INR group compared to the IR and healthy groups. Previous studies claimed that the relative abundance of IBA was increased in the IR group in faecal samples (110), by contrast in our study, IBA in the plasmatic samples was increased in the INR group. High levels of C reactive protein (PCR) levels have been described (16.3 mg/L) in INR patients (111), which indicates a high proinflammatory state that could be associated with the high levels of SCFA produced by intestinal dysbiosis although more factors may be involved in inflammatory status and activations of immune system.

Contrary to our study, Quing et al. claimed that the VA was decreased in faecal samples of VIH-1-positive patients whereas in our study VA plasmatic levels increased. The VA, in normal conditions, has a protective effect on the intestinal mucosa (112). One possible explanation is that the conditions of both studies had been different. One used faecal samples, whereas our study was realised using plasma, and the studies were realized in different cohorts.

Finally, in the case of the PA was increased in the INR group and obtained the best discrimination power using a ROC curve of AUC of 1 to differentiate the INR and IR groups. The PA is a SCFA that can produce an increase in the regulatory

T-cell count and in the production of interleukin 10 (IL-10) (113) through upregulation of the *Foxp3, IL10* and the regulatory T-cell receptor G protein-coupled receptor genes (114). One of the mechanisms through which the PA is able to produce the upregulation of these genes is the inhibition of some histone deacetylase (115). So, based on our results, we hypothesize that the increase in PA could be associated with poor immune CD4+ T cell recovery in the INR patients, promoting an excessive activation of the innate immune response that can lead to the depletion of the immune system over time (116).

Some studies showed that the INR group at *phylum* level, had a higher abundance in the *Proteobacteria, Fusobacteria, Tenricutes,* and *Saccharibacteria* (117). Some of these *phyla* are PA producers like the *Proteobacteria* (118) or the *Fusobacteria* (119). This increase in the abundance of these types of micro-organisms could explain the high levels of PA.

In conclusion, the different lipidomes of HIV-1-positive patients could be associated with the specific phenotype where the most significant changes were identified in the INR group. In fact, this preliminary study has characterised for the first time the levels of SCFA and other lipids in EC and INR highlighting the role of the PA in the prediction of the INR phenotype. Despite that, further investigations are needed to understand why the different phenotypes are produced and how to solve them.

Limitations

The principal limitations of this study were the size of the study cohort. Due to the low number of patients, there was a low sex and age variability, which could be important factors. This is a preliminary study, so the good results obtained, above all in the INR group, should be validated in a higher cohort with more variability of age and sex. The clinical records from the EC group were missed and consequently biochemistry parameters could not be analysed.

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