Human Immunodeficiency Virus infection, asymptomatic atherosclerosis, and inflammation: A candidate gene study

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HUMAN IMMUNODEFICIENCY VIRUS INFECTION, ASYMPTOMATIC ATHEROSCLEROSIS, AND INFLAMMATION:

A CANDIDATE GENE STUDY

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Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada *"Human Immunodeficiency Virus Infection, Asymptomatic Atherosclerosis, and Inflammation: A Candidate Gene Study"* han estat realitzats per na Laura Ibáñez Lladó sota la seva direcció i considera que és apte per a ser presentat per a optar al grau de Doctor en Biomedicina per la Universitat de Barcelona.

I per tal de que en quedi constància, signa aquest document.

Terrassa, Desembre del 2014.

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TABLE OF CONTENTS

List of Tables

Background

Study I

Study II

Study III

Study IV

List of Figures

Background

Study III

Study IV

SUMMARY

HIV-infected individuals have an increased risk of death due to the high prevalence of age-related diseases in spite of antiretroviral therapy. It is thought that HIV-related co-morbidities are caused by a persistent inflammatory state induced by the HIV virus. Among these co-morbidities, HIV-infected patients show increased cardiovascular risk, more prevalence of traditional cardiovascular risk factors and early onset of the atherosclerotic disease. However, the underlying mechanisms are not known yet.

Atherosclerosis is known to be a complex disease potentially related to genetic variations. Inflammatory protein levels are altered in HIV-infected subjects. Although antiretroviral therapy diminishes levels of the inflammatory proteins, they do not reach the levels observed in non-infected subjects.

The purpose of this study was to investigate the possible association between genetic variants and gene expression of genes involved in inflammation and cardiovascular risk parameters in a cohort of HIV-infected subjects.

In the present study, several genetic variants have been found associated with atherosclerosis in HIV-infected patients, however, the associations are modest. The gene expression of several inflammatory genes has been found associated with cardiovascular risk factors and HIV parameters. This study shows for the first time that the 5-lipoxigenase pathway is involved in HIV-related atherosclerosis at genetic and transcriptional levels. Moreover, 5-lipoxigenase pathway gene expression is reduced in treated patients, but not to the levels observed in uninfected individuals. In summary, the results obtained in this thesis show that there is an intricate relationship between HIV-infection, antiretroviral treatment, atherosclerotic disease and inflammatory pathways.

RESUM

Els pacients infectats pel VIH presenten un risc de mort més elevat que els pacients seronegatius degut a l'elevada prevalença de malalties relacionades amb l'envelliment. Les comorbiditats associades al VIH es deuen molt probablement a la inflamació persistent induïda pel virus. Entre aquestes comorbiditats, els individus seropositius tenen un risc cardiovascular més elevat, més prevalença de factors de risc cardiovascular tradicionals i la aparició de la malaltia arterioscleròtica es dona en individus més joves. Els mecanismes causals d'aquests fets encara són desconeguts.

La malaltia arterioscleròtica és una malaltia complexa potencialment influenciada per variants genètiques. Els individus seropositius presenten concentracions alterades de proteïnes inflamatòries. Tot i que el tractament antirretroviral disminueix els nivells d'aquestes proteïnes, aquest no assoleixen els nivells observats en individus seronegatius.

L'objectiu d'aquest estudi va ser investigar la possible associació entre les variants genètiques i l'expressió gènica de gens pertanyents a la ruta inflamatòria amb paràmetres relacionats amb el risc cardiovascular en una cohort de pacients seropositius.

En aquest estudi s'han identificat diverses variants genètiques associades amb l'arteriosclerosi en pacients seropositius, tot i així, aquestes associacions van ser modestes. L'expressió de diversos gens relacionats amb la resposta inflamatòria es van trobar associats amb factors de risc cardiovascular i amb paràmetres relacionats amb el VIH. Aquest estudi mostra per primer cop que la via de la 5-lipoxigenasa està involucrada en la malaltia arterioscleròtica associada al VIH a nivell genètic i transcripcional. És més, l'expressió gènica d'aquesta via es veu reduïda per la presencia de teràpia antirretroviral, però no fins als nivells

observats en individus seronegatius. En resum, existeix una relació complexa entre la infecció per VIH, el tractament antirretroviral, la malaltia arterioscleròtica i la via inflamatòria.

RESUMEN

Los pacientes infectados por el VIH presentan un riesgo de muerte mayor que los pacientes seronegativos debido a la alta prevalencia de enfermedades relacionadas con el envejecimiento. Es muy probable que las comorbilidades asociadas al VIH se deban a la presencia de una inflamación persistente inducida por el virus. Entre estas comorbilidades, los individuos seropositivos tienen un riesgo cardiovascular mayor, una prevalencia de factores de riesgo cardiovascular tradicionales más elevada y la aparición de la enfermedad arteriosclerótica en sujetos más jóvenes. Los mecanismos causantes de estos hechos son aún desconocidos.

La enfermedad arteriosclerótica es una enfermedad compleja potencialmente influenciada por variantes genéticas. Los sujetos seropositivos presentan concentraciones alteradas de proteínas inflamatorias. Aunque los niveles de proteínas infamatorias disminuyen con el tratamiento antirretroviral, éstos no llegan a los niveles observados en sujetos seronegativos.

El objetivo de éste estudio fue investigar la posible asociación entre las variantes genéticas y la expresión génica de genes pertenecientes a la ruta inflamatoria con parámetros relacionados con el riesgo cardiovascular en una cohorte de pacientes seropositivos.

En éste estudio se han identificado múltiples variantes genéticas asociadas con la arteriosclerosis en pacientes seropositivos, a pesar de ello, estas asociaciones fueron modestas. La expresión de varios genes relacionados con la respuesta inflamatoria se encontraron asociados con factores de riesgo cardiovascular tradicionales y con parámetros relacionados con el VIH. Éste estudio muestra por primera vez que la vía de la 5-lipoxigenasa está involucrada en la enfermedad arteriosclerótica asociada al VIH tanto a nivel genético cómo a nivel transcripcional. Es más, la expresión génica de ésta vía se reduce en presencia

de terapia antirretroviral pero no hasta los niveles observados en sujetos seronegativos. En resumen, existe una relación compleja entre la infección por VIH, el tratamiento antirretroviral, la enfermedad arteriosclerótica y la ruta inflamatoria.

Abbreviations

5-LO	5-Lipoxigenase
AIDS	Acquired Immunodeficiency Syndrome
ALOX5	Arachidonate 5-Lipoxigenase
ALOX5AP	Arachidonate 5-Lipoxigenase Activating Protein
AP	Atherosclerotic Plaque
ART	Antiretroviral Treatment
B2M	Beta 2 Microglobulin
BMI	Body Mass Index
bp	Base Pair
CC	Correlation Coefficient
CCL	Chemokine (C-C motif) Ligand
CCR	Chemokine (C-C motif) Receptor
CDC	Centers for Disease and Control
cIMT	Carotid Intima Media Thickness
CoRIS	Cohorte de la Red Española de Investigación en SIDA
CRP	C - Reactive Protein
CVD	Cardiovascular Disease
CVR	Cardiovascular Risk
CX3CL	Chemokine (C-X3-C motif) Ligand

CX3CR	Chemokine (C-X3-C motif) Receptor
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic Cell
DMSO	Dimethylsulfoxide
EC	Endothelial Cell
FBS	Fetal Bovine Serum
FC	Fold Change
GWAs	Genome Wide Association Study
HCV	Hepatitis C Virus
HDL	High-Density Lipoprotein
HERMES	Harmonización de las Ecuaciones de Riesgo en el Mediterráneo Sur de Europa
HIV	Human Immunodeficiency Virus
HWE	Hardy-Weinberg Equilibrium
IFN	Interferon
IL	Interleukin
IL1B	Interleukin 1, Beta
IL1RN	Interleukin 1 Receptor Agonist
IMIM	Institut Hospital del Mar d'Investigacions Mèdiques
IN	Integrase
IP10	Neopterin

LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharide
LTA	Lymphotoxin Alpha
maf	Minor Allele Frequency
MI	Myocardial Infarction
MONICA	Multinational Monitoring of Trends and Determinants in Cardiovascular Disease
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitor
NK	Natural Killer Cell
oxLDL	Oxidized Low-Density Lipoprotein
P	Protease
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PROCAM	Prospective Cardiovascular Münster
qPCR	Quantitative Polymerase Chain Reaction
Regicor	Registre Gironí del Cor
RQ	Relative Quantity Value
RT	Reverse Transcriptase

rtPCR	Real-Time Polymerase Chain Reaction
RYR3	Ryanodine Receptor 3
SCORE	Systematic Coronary Risk Evaluation
SMART	Strategies for Management of Anti-Retroviral Therapy
SNP	Single Nucleotide Polymorphism
TGF	Transforming Growth Factor
Th	T Helper response
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization

BACKGROUND

1. Introduction

Acquired Immunodeficiency Syndrome (AIDS) was identified more than 30 years ago (1). The challenges for the clinicians and Human Immunodeficiency Virus (HIV)-infected individuals have changed over the years. Nowadays, viral replication is suppressed with Antiretroviral Therapy (ART). ART prevents AIDS related complications and decreases morbidity and mortality in HIV-infected subjects (2, 3). However, mortality rates among HIV-infected individuals remain 3-15 times higher than those observed in the general population (4-6). The excess of mortality observed among HIV-infected subjects can be partly attributed to HIV-related illnesses (7). However, more than half of the deaths observed among HIV-infected patients treated with ART are due to co-morbid disorders related to aging (6-8).

Aging is defined as the decreased ability to face stress that leads to an increased susceptibility to diseases. The high prevalence and early onset of co-morbid disorders, especially chronic conditions related to aging such as Cardiovascular Disease (CVD), renal failure, neurodegenerative disorders and tumors in HIV-infected individuals are of high concern (9-12). HIV infection and its treatment are associated with a series of biological events (e.g. inflammation, immune dysfunction, telomerase inhibition and mitochondria dysfunction), clinical factors (e.g. polypharmacy and multimorbidity), and social factors (e.g. social isolation and poverty) that are related and influence the aging process (12).

It is nowadays considered that HIV-infected patients are aging prematurely. Multiple hypotheses have been made to explain this premature aging. Chronic systemic inflammation, reduced vascular endothelial reactivity, increased endovascular hypercoagulability and immune activation have been suggested as possible mechanisms (Figure 1). These processes are more prevalent in HIV-infected patients than in the general population. Therefore, HIV-infected

patients are prone to develop age-related diseases or co-morbidities. In fact, it is considered that HIV-infected individuals reach old age at 50 years of age whereas the general population reaches it at 65 years of age (9).

Among the many co-morbid conditions, CVDs have become of particular concern. It has been demonstrated that HIV-infected populations have higher rates of cardiovascular events or subclinical atherosclerosis after taking into account traditional Cardiovascular Risk (CVR) factors (13-26). Interestingly, Lorenz et al (2008) estimated higher vascular age for HIV-infected individuals (7 years older than non-infected) (25).

HIV-infected individuals are a subgroup of the general population, with greater prevalence of traditional CVR factors unrelated to HIV or ART (13, 27-29). However, HIV and ART may increase CVR favoring the occurrence of traditional risk factors (30), enhancing inflammation (31) and affecting directly the pathogenesis of atherosclerotic disease (Figure 1) (23, 32).

Early detection of atherosclerosis in HIV-infected individuals is very important for CVD prevention and follow-up (33). Inflammation is involved in both diseases, HIV infection and CVD, and it could act synergistically in HIV-infected individuals. CVD evolves differently in HIV uninfected individuals than in those with HIV infection; therefore it is plausible that genetic variants or variations in gene expression may explain at least in part these differences. Studying the inflammatory pathway may be of major interest to detect those individuals at higher risk of CVD, independently of traditional CVR factors.

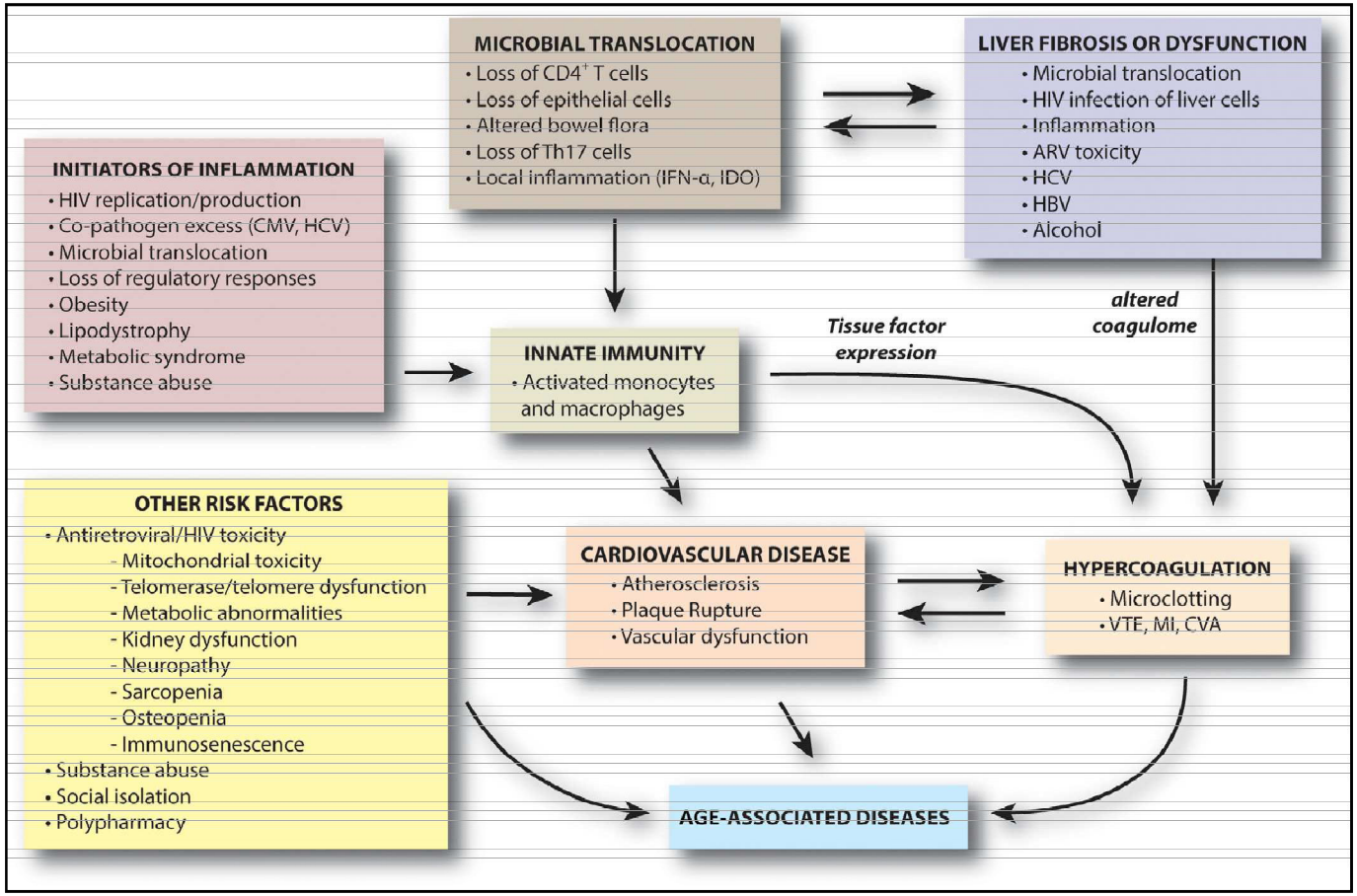


Figure 1. Impact of HIV on inflammation, coagulation and health. The diagram shows the different co-morbidities and risk factors associated with an increase in CVD and other age-associated disorders in HIV-infected individuals (12).

2. Human Immunodeficiency Virus Infection

The HIV is the viral agent responsible of AIDS (34). AIDS was reported for the first time in 1981 (1); posterior studies characterized it as an immunodeficiency syndrome where $CD4^+$ T-lymphocytes gradually decrease (35). Afterwards, this pathology was linked to an infectious agent transmitted by blood, blood products, intimate sexual contact, intravenous drug use and vertically from mother to child. This infectious agent was isolated from patient lymphatic nodes by Barre-Sinoussi, Cherman and Montagnier in the Pasteur Institute (Paris) in 1983 (34). It was identified as a retrovirus from the *Lentiviridae* family. Finally, the International Committee on Taxonomy of Viruses named it as HIV in 1986 (36).

2.1. Epidemiology of HIV-Infection

The number of HIV-infected subjects increases every year (40-fold increase since 2002[1]). ART treatments have succeeded in improving HIV-infected individual's quality of life, immunological conditions and consequently in reducing the number of deaths. However, the patients remain chronically infected. To date, 14 millions of people have died due to HIV globally (37). Approximately 35.0 million people were living with HIV according to estimates by World Health Organization (WHO) at the end of 2013[2] (Figure 2); however, half of them did not know they were infected and 7 millions had no access to treatment. Two point one million people were newly infected and 1.5 million died of AIDS-related causes[3] the same year. It has been a 33 % decrease in new HIV infections between 2001 and 2012[1] (Figure 3). Africa is the continent with the highest prevalence of HIV infection (4.5 %), with countries such as Botswana

[1] http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2013/JC2571 _AIDS_by_the_numbers_en.pdf
[2] http://www.who.int/hiv/data/epi_core_dec2014.png?ua=1
[3] http://www.unaids.org/sites/default/files/en/media/unaids/contentassets/documents/factsheet/2014/20140716_FactSheet_en.pdf

or Lesotho with more than 20 % of estimated prevalence (23 % and 23.1 %, respectively)[3] (Figure 2).

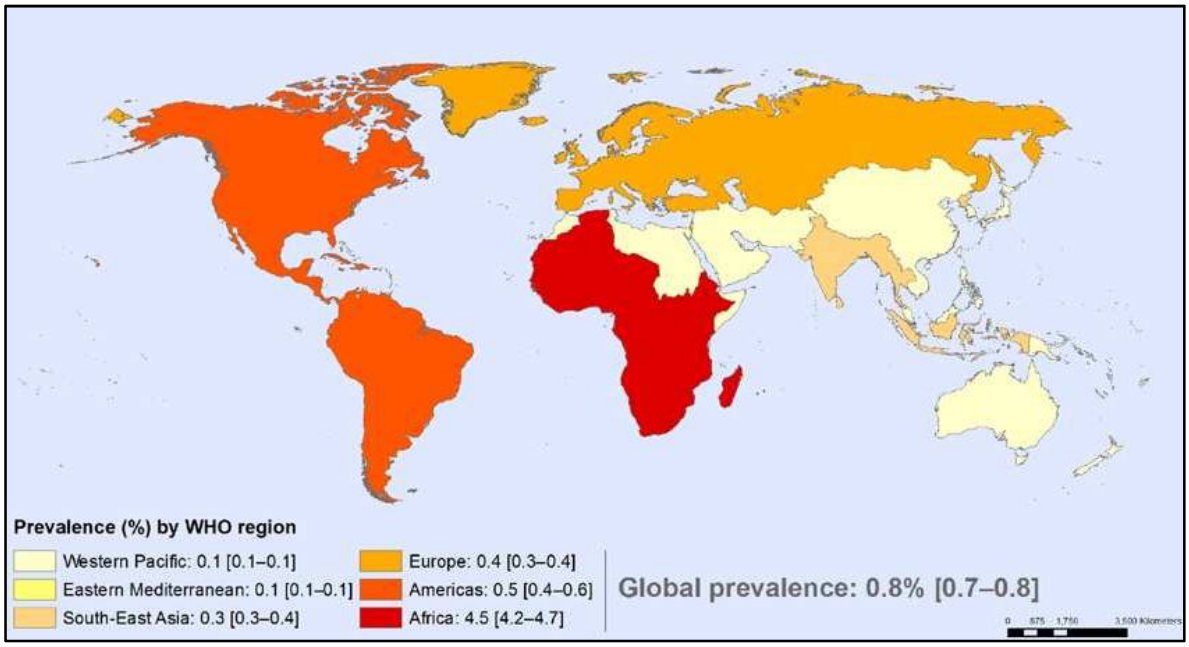


Figure 2. People living with HIV worldwide (Adult HIV prevalence by WHO region 2012)[4].

HIV infection is of major public health importance in Europe. The prevalence of HIV infection was 0.4 % and 29,157 new cases were reported[5] in 2013. Sexual transmission (70 %) and injected drug use (16 %) were the main transmission modes in the same year. The rate of new HIV diagnosis seems to have stabilized to approximately 6 per 100,000 new cases per year in Europe. The prevalence of AIDS has decreased since the apparition of ART to 0.9 cases per 100,000 habitants in 2013[5].

[4] http://www.who.int/gho/hiv/hiv_013.jpg?ua=1
[5]http://www.ecdc.europa.eu/en/publications/Publications/hiv-aids-surveillance-report-Europe-2013.pdf

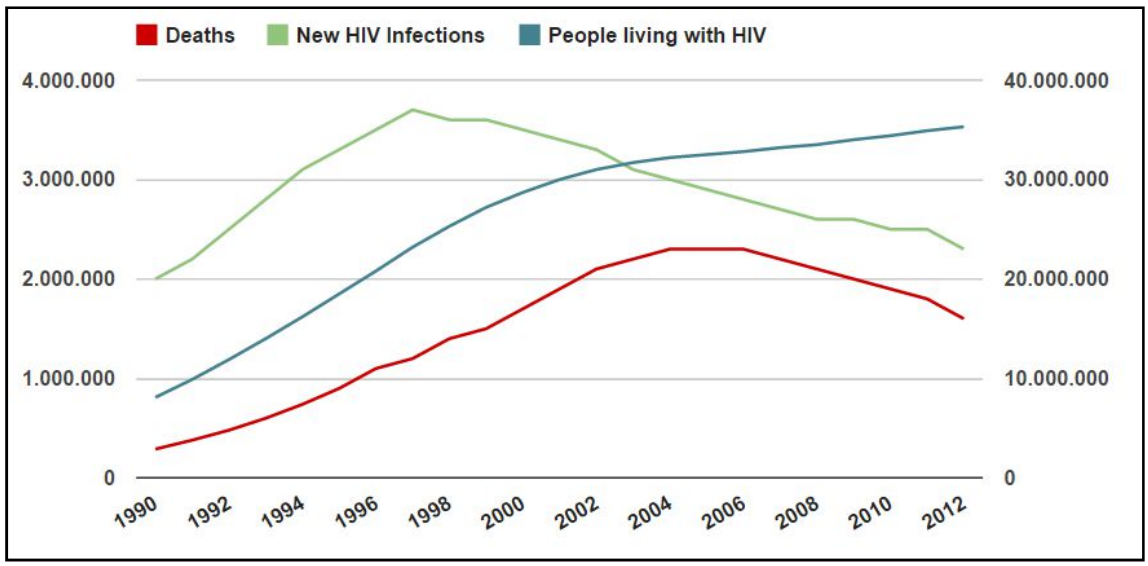


Figure 3. Global HIV trends (1990 - 2012). The annual number of deaths due to AIDS (red), the newly infected with HIV (green) and the number of people living with HIV (blue)[6].

The Spanish trends are not different from those observed globally or in Europe. However, the rate of HIV diagnosis was 2 points higher than the rate observed in Europe in 2011[7]. The initial expansion of HIV infection was due to the sharing of syringes and other items for drug injection. The virus was rapidly disseminated with the highest peak in 1994 with 190 HIV-infected subjects per million of habitants (Figure 4) and 7,354 new HIV-cases reported[8]. Since then, the rate of new infections has decreased to 3,210 new diagnosed infections in 2012 (8.5 / 100,000 habitants)[9]. HIV transmission mode has changed from infection through intravenous contaminated syringes to sexual transmission (37, 38). Catalonia is one of the Spanish communities with the highest number of

[6] http://www.ourworldindata.org/data/health/hiv-aids/
[7] http://www.isciii.es/ISCIII/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-alertas/fd-enfermedades/Informe_VIH-sida_Junio_2011.pdf
[8] http://data.unaids.org/publications/fact-sheets01/spain_en.pdf
[9] http://www.isciii.es/ISCIII/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-alertas/fd-enfermedades/fd-sida/Informe_VIH_sida_Junio_2013.pdf

new infections (22.4 % in 2012)[9] (38). Five hundred and sixty six new HIV cases were reported in the Catalan surveillance report from 2013[10].

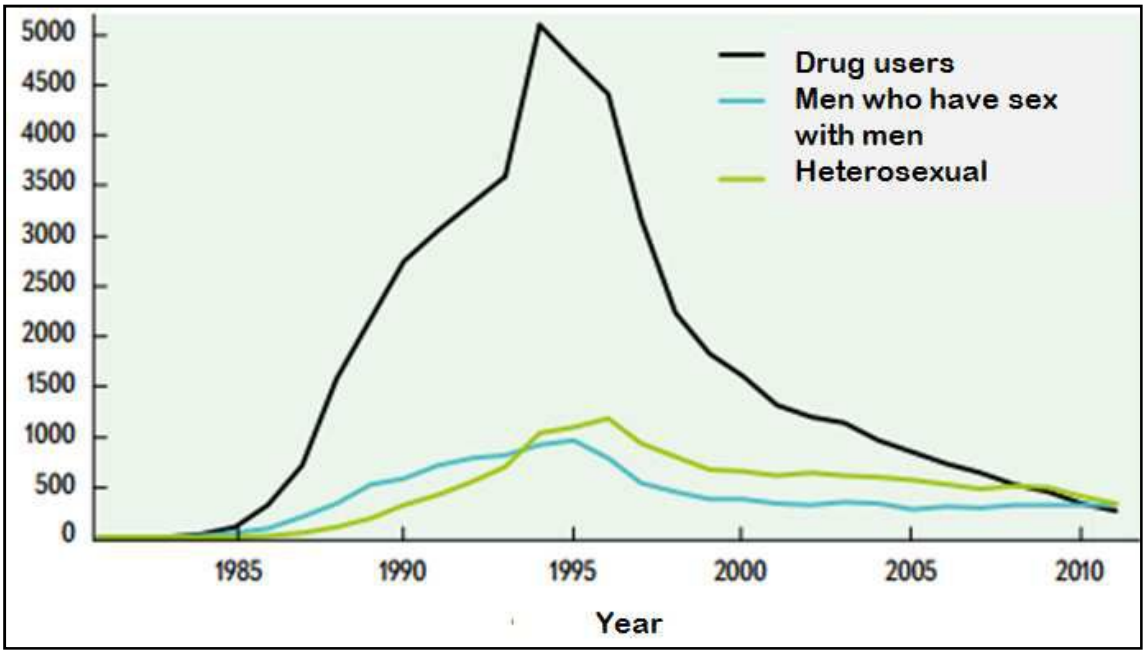


Figure 4. Spanish HIV-infection trends (1985 - 2010). Number of HIV-infected subjects classified by transmission type [adapted from (39)].

2.2. HIV Virus: Structure and General Characteristics

HIV virus is a non transforming cytopathic retrovirus from the *lentiviridae* family (40). It infects $CD4^+$ lymphocytes and it has an aggressive kinetic replication. HIV infection will destroy lymphocytes and interfere with the immune system leading to immunosupression. There are 2 virus types: HIV-1 and HIV-2. HIV-2 is different from HIV-1 in its genomic structure and antigenicity. Although HIV-2 is less aggressive, the syndrome caused by HIV-2 is similar to the one caused by HIV-1 (41, 42). HIV-1 is spread in Asia, Europe, Oceania, America and Africa whereas HIV-2 is prevalent in central and occidental Africa (43). HIV-1 is the most prevalent in Europe and Spain and it will be the focus of this thesis.

[10] http://www.ceeiscat.cat/documents/Informe_semestral_VIH.pdf

HIV-1 is classified in 4 phylogenetic groups M (major), O (outlier), N (non-M or non-O) and P (pending), each of which resulted from an independent cross-species transmission event (44, 45). The M group was the first to be discovered and represents the pandemic form of HIV-1 (> 98 % prevalence) (44). HIV-1 M group it is divided in 11 subtypes (A1, A2, B, C, D, F1, F2, G, H, J and K), an increasing number of circulating recombinant forms (CRFs) and unique recombinant forms (URFs). Each subtype has a different transmission origin and a different capacity for epidemic expansion (44-46).

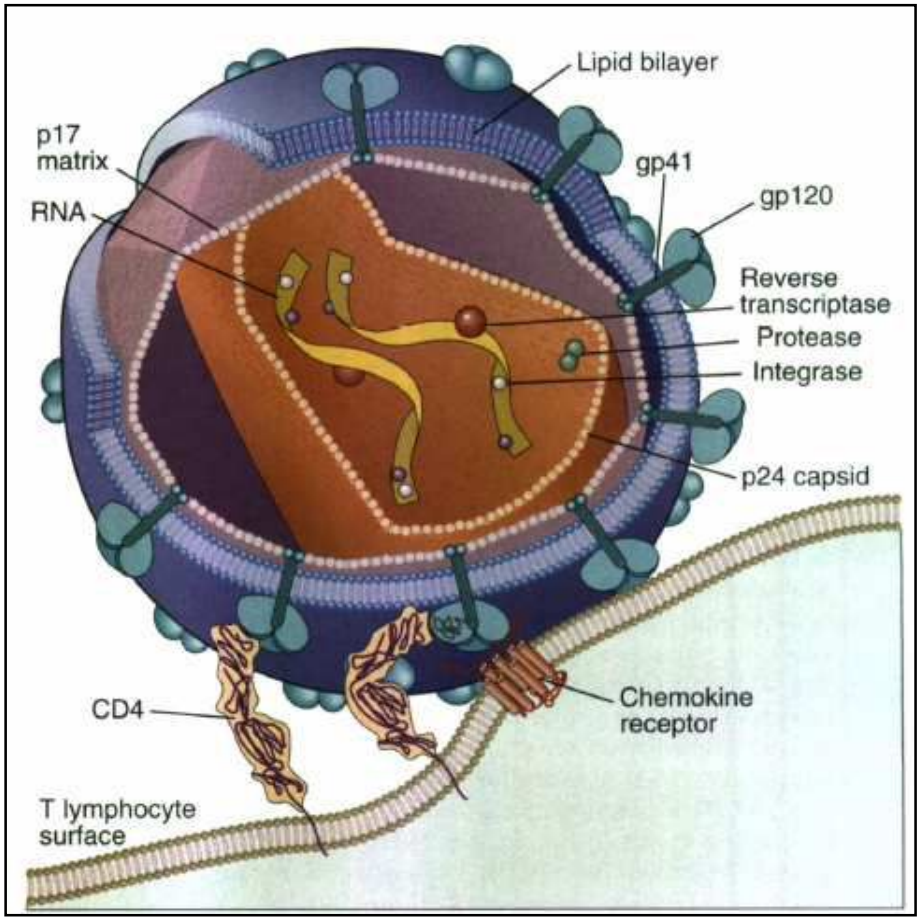


Figure 5. HIV structure (47).

The structure of HIV-1 and HIV-2 has been elucidated by electron microscopy. The infectious HIV is a spherical particle (virion) of 80 - 120 nm. It consists of 2 identical 9.8 kb strands of RNA packaged within a core of viral proteins to ensure RNA integrity and surrounded by a phospholipids bilayer (Figure 5). The

RNA strands are enclosed by a conical capsid conformed by 1200 to 2500 copies of the viral protein p24 to which it is also bound by the viral proteins p6 and p7. It also contains the necessary enzymes to develop new virions (Reverse Transcriptase (RT), Protease (P) and Integrase (IN)). A matrix composed of the viral protein p17 surrounds the capsid. The matrix is surrounded by a phospholipids bilayer derived by gemmation from the host cell membrane. It also contains the viral proteins gp41 and gp120 organized in trios (spikes) that will sustain viral structure and allow viral cell entry by interacting with CD4 and chemokine receptors (48).

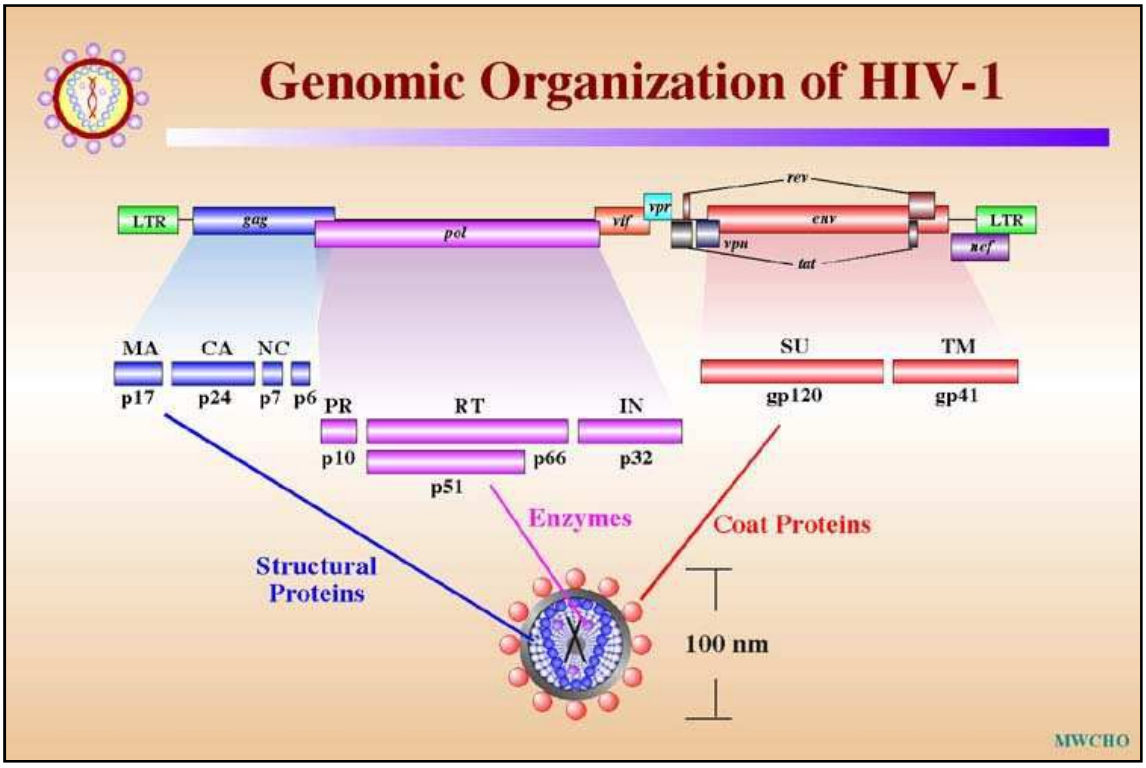


Figure 6. Genomic organization of HIV-1[11].

HIV-1 genomic RNA has 9 genes (Figure 6). Three of them are common in all retroviruses: *Gag* encode for the core structural proteins (p17, p24, p7, and p6), *pol* encode for the viral enzymes (P (p10), RT (p66), and IN (p32)), and *env* encode for envelope structural proteins (SU/gp120 and TM/gp41). The other 6 genes are unique to HIV and encode for regulatory proteins (*tat* and *rev*) and

[11] http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV-1b.jpg

accessory proteins (*vpu*, *vpr*, *vif* and *nef*). These genes control the virus capacity to infect cells, to generate new virus copies and to induce pathogenesis (48).

2.3. HIV Pathogenesis

The viral and immunological kinetics of HIV infection are shown in Figure 7. HIV virus has an aggressive replication kinetic. It will produce between 10^9 and 10^{11} viral particles every day. HIV life cycle *in vivo* can be divided in 3 stages: 1) the acute infection, 2) the establishment of the chronic infection (asymptomatic phase) and 3) AIDS; each one characterized by different phenomena (48, 49).

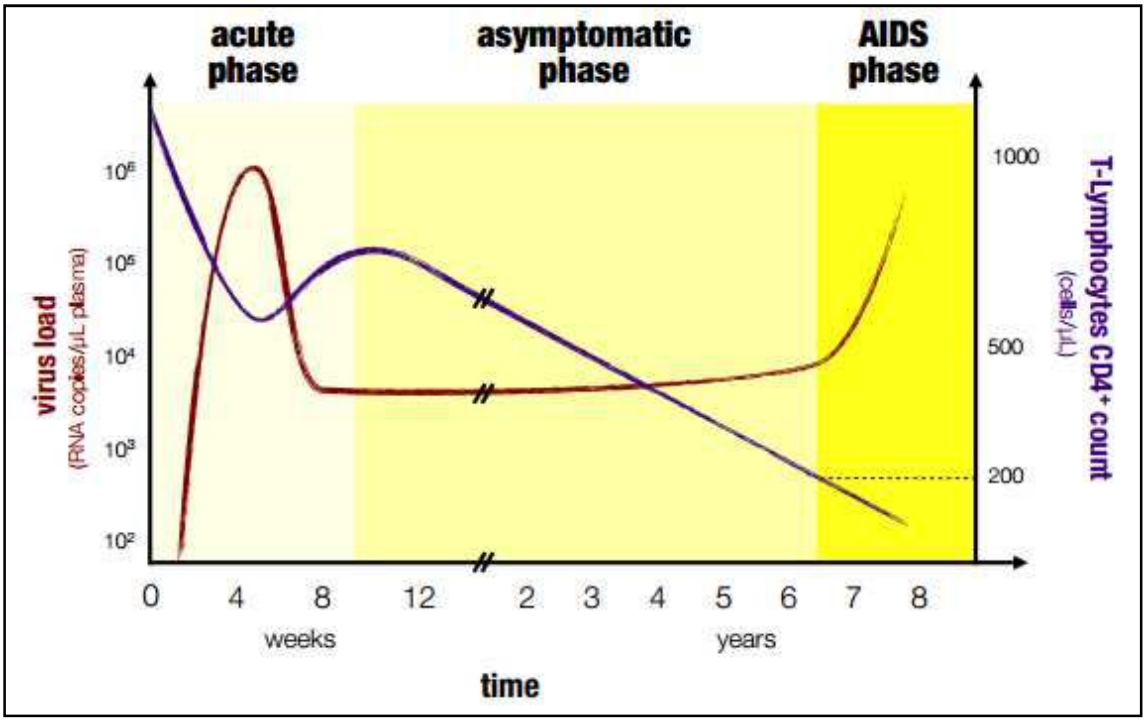


Figure 7. Immunological and viral kinetics of HIV infection. The figure shows the dynamics of the viral load (red), and the evolution of $CD4^+$ T-cell counts (blue). The three phases of an HIV infection are marked with different colors (adapted from (50)).

The Centers for Disease and Control (CDC) classify HIV infection and AIDS depending on the clinical conditions associated with HIV-infection and $CD4^+$ T-cell counts (51) (Figure 8). The CDC system is based on three ranges of $CD4^+$ T-

cell counts (> 500; 200 to 499 and < 200 cells/µl) and three clinical categories (A-Asymptomatic HIV infection; B-Symptomatic HIV infection; and C-AIDS defining condition). AIDS defining conditions are listed in the Figure 9. The majority of them are considered opportunistic infections.

1993 CDC HIV Classification System in Adults: Summary Table			
CD4 Categories	Clinical Categories		
	(A) Asymptomatic	(B) Symptomatic, Not A or C Conditions	(C) AIDS-Indicator Conditions
(1) ≥500 cells/mm^3	A1	B1	C1
(2) 200-499 cells/mm^3	A2	B2	C2
(3) <200 cells/mm^3 or CD4% <14	A3	B3	C3

Figure 8. 1993 CDC HIV infection classification system[12].

1) Acute HIV Infection

Acute infection lasts from 2 to 6 weeks after infection and ends when HIV antibodies are detectable (seroconversion). During the first days (7 to 21 days) of infection HIV is still not detectable in plasma (eclipse - phase). Plasma viremia increases exponentially and $CD4^+$ lymphocytes are depleted during this phase. The immune system responds firstly with a cellular immune response (after 1 to 2 weeks upon infection) and then with a humoral response (4 to 8 weeks after infection). HIV viral load will be partly controlled by immune response. Meanwhile, HIV will spread to the lymphoid organs where it will establish the persistent infection leading to the loss of $CD4^+$ T-cells and to epithelial injury.

[12] http://depts.washington.edu/hivaids/initial/case2/discussion.html

2) Chronic HIV Infection

The anti-viral immunity and cytokine production started in the acute phase of HIV infection continues into the chronic phase. The cellular and humoral immune responses keep viral load to constant levels maintaining a rapid turnover of infected cells for several years. This leads to chronic T-lymphocyte activation, persistent cytokine production and increased inflammation. The virus will increase its diversity and the number of $CD4^+$ T-cells will decrease progressively during this phase. Consequently, there is loss of immune cell replicative capacity and immune senescence (52). However, no symptoms can be observed. There are several viral and host factors such as host genetics and viral tropism that determine the time span and the rate of disease progression during the asymptomatic phase of HIV-infection. After initial HIV infection, the evolution of the disease depends on factors such as early treatment interventions and good immune control of viral replication.

The apparition of ART (1996) changed drastically the epidemic of HIV / AIDS increasing the survival of HIV-infected patients and transforming HIV infection into a chronic disease (53). ART suppresses viral replication and reduces viral load to undetectable levels in the blood of most patients leading over time to improved immune function and the near elimination of any risk for developing an AIDS-defining complication. Although ART increases the time span of the asymptomatic phase, it does not eliminate the virus from the infected individual and does not fully restore health. Quiescent virus reservoirs remain in protected compartments for many years. Viral replication may restart upon cessation of ART or the apparition of resistance mutations.

3) AIDS

The immune system is depleted and the viral replication increases leading to the development of AIDS. The AIDS phase is characterized by a dramatic loss in $CD4^+$

T-cells and increase of viral load. The accelerated replication allows the generation of mutants that will increase the probability of viral evasion and the generation of more cytopathyc variants. AIDS is defined as the time point when $CD4^+$ T-cell counts are below 200 cells / µL or when an opportunistic disease or HIV-related neoplasia appears due to the depleted immune system (Figure 9). It takes several years from primary infection to the development of symptoms of advanced AIDS, immunosuppression, and death even in untreated individuals (54).

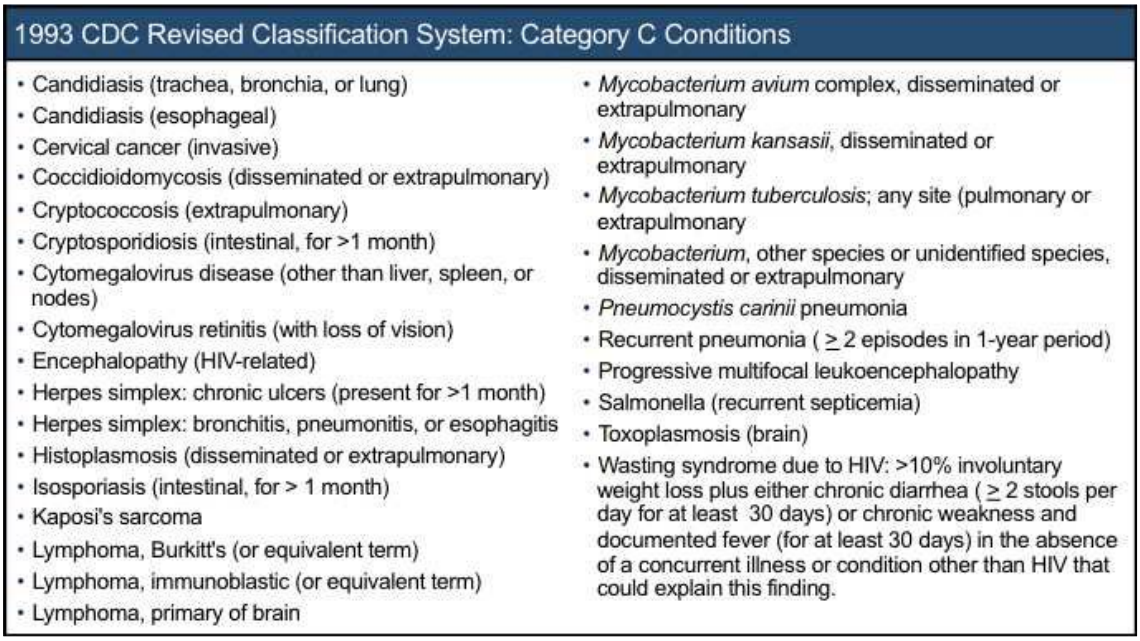


Figure 9. AIDS defining conditions[13].

2.4. Chronic HIV Infection and Chronic Inflammation

Chronic inflammation plays a central role in the pathogenesis of untreated HIV infection (12). It causes chronic activation of immune system, the adaptive and the innate. Chronic activation of the immune system lead to low but sustained inflammatory state that persists even after the virus is controlled with ART (12, 54).

[13] http://depts.washington.edu/hivaids/initial/case2/discussion.html

There are several potential causes for chronic inflammation in chronic HIV infection:

1) HIV replication contributes directly to T-cell activation.

2) Other pathogens that are activated when the immune system is depleted by HIV-infection contribute to increase the levels of T-cell activation.

3) HIV infection destroys the gut mucosa. This leads to chronic exposure to gut microbial products like Lipopolysaccharide (LPS).

4) HIV infection alters the normal function of the immune system leading to the release of dysfunctional immunoregulatory factors.

The chronic inflammatory environment causes fibrosis in lymphoid tissues, which in turn causes $CD4^+$ T-cell regenerative failure and disease. ART partially reverses many if not all of these pro-inflammatory pathways, but the effect is incomplete, and the inflammation persists indefinitely (12, 55). Chronic immune activation and chronic inflammation have been proposed as the underlying mechanisms of HIV-associated co-morbid diseases.

3. Atherosclerotic Disease

Atherosclerosis is a complex multifactorial disease of the medium and large arteries characterized by the accumulation of inflammatory cells, lipoproteins and fibrous tissue in the wall of arteries (56, 57). It is the most frequent underlying cause of CVD such as coronary artery disease, carotid artery disease and peripheral arterial disease (33). These diseases might cause cardiovascular events such as myocardial infarction (MI) or stroke. CVDs are the first public health concern and the first cause of death globally (Figure 10). Three out of every 10 deaths were caused by CVDs in 2012 (17.5 million people)[14]. Although

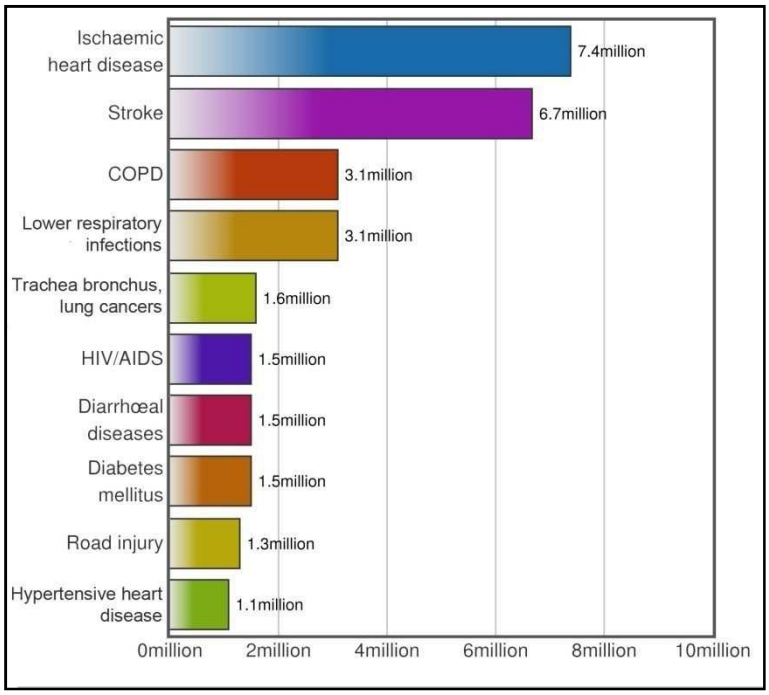


Figure 10. Ten leading causes of death in the world (2012)[15].

[14] http://www.who.int/mediacentre/factsheets/fs310/en/index2.html
[15] http://www.who.int/mediacentre/factsheets/fs310/en/

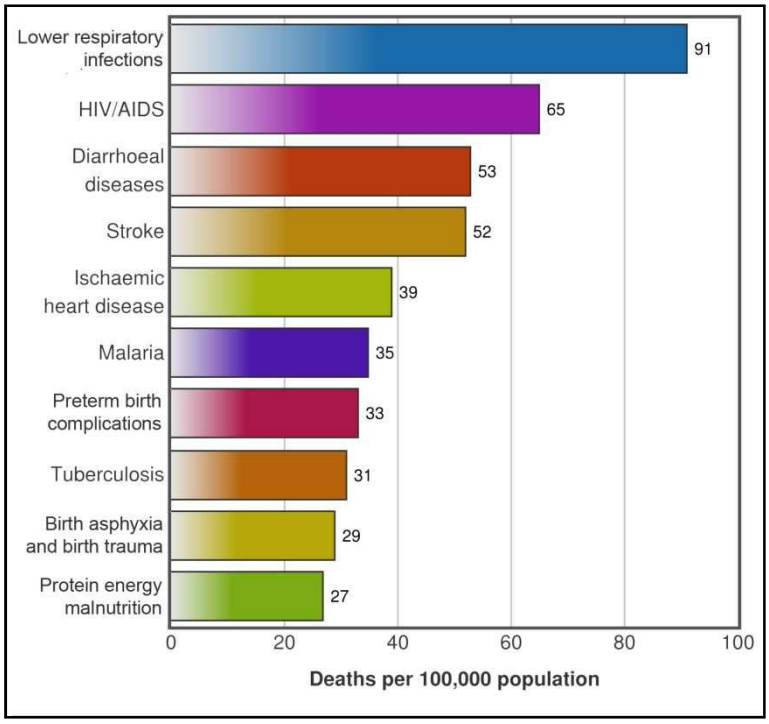


Figure 11. Ten leading causes of death in the low-income countries (2012)[16].

respiratory infections and HIV are the leading causes of death in low-income countries, death from CVDs is rapidly increasing (58) (Figure 11). Adverse cardiovascular events are expected to be the cause of death of more than 23 million people yearly by 2030 (59, 60)[17].

Atherosclerotic disease was considered a continuous process in which cholesterol accumulates in the vessel wall historically. Clinical manifestations were explained by the stenosis degree. Atherosclerosis is viewed as a chronic inflammatory disease with active and complex processes nowadays. The immune cells (macrophages, T-cells, Natural Killer (NKs) T-cells, Dendritic Cells (DCs), and mast cells) infiltrate atherosclerotic lesions in a process tightly regulated by several molecules (especially cytokines) at all stages of the disease. The final clinical manifestations (plaque rupture, thrombosis, and acute

[16] http://www.who.int/mediacentre/factsheets/fs310/en/index1.html
[17] http://www.who.int/cardiovascular_diseases/en/

ischemic symptoms) are explained by the interaction of multiple cell types, systemic inflammation and mediators located at the sites of plaque formation as well as mediators secreted in distal organs (57, 61, 62).

3.1. Atherosclerotic Plaque Formation

Atherosclerosis is a progressive disease. It has multiple stages beginning with early fatty streaks evolving to advanced atherosclerotic lesions (Atherosclerotic Plaque - AP) and ending with plaque rupture. Each stage is characterized by different cellular and molecular components (57, 61).

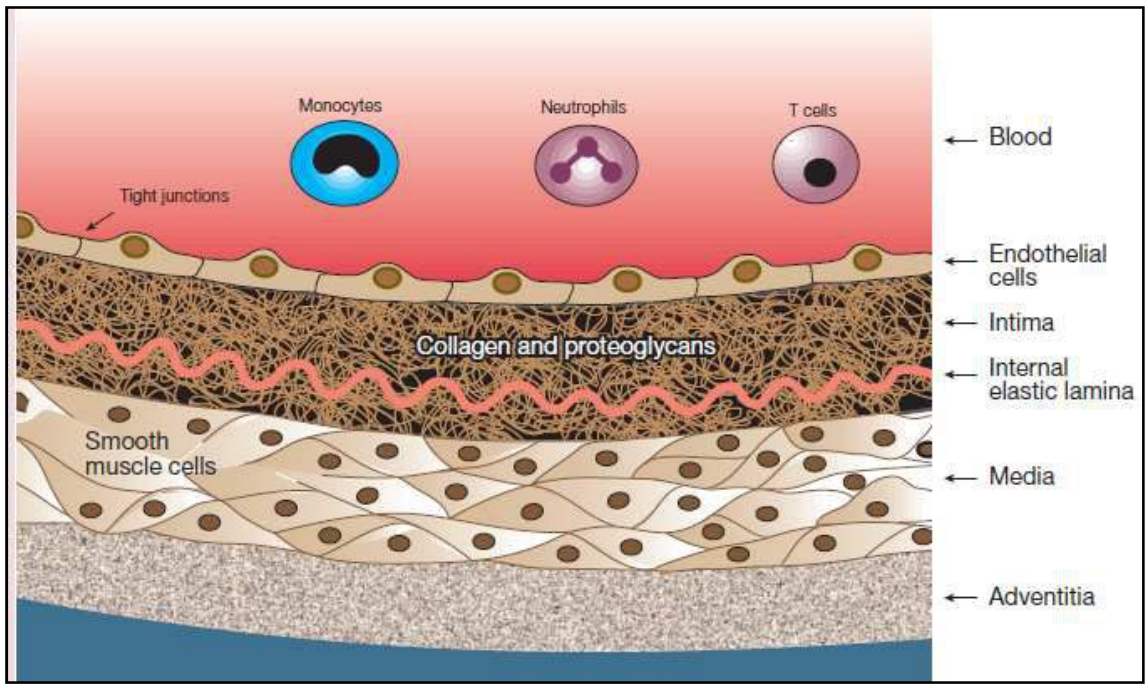


Figure 12. Healthy Vessel Structure (56).

Healthy vessels have 3 morphologically different layers (Figure 12). The intima layer is the middle layer. It is attached to the Endothelial Cells (ECs) monolayer luminally and to the internal elastic lamina in the periphery. A healthy intima is a very thin layer formed by connective extracellular tissue matrix (proteoglycans and collagen as its major components) (56). Blood flux causes shear stress to ECs that affects ECs morphology. ECs have ellipsoid form and are orientated in

flux direction in the tubular vessel regions where the blood flux is laminar and more uniform. ECs have polygonal forms and do not have any special orientation in regions where vessels are curved or divided where the flux is more turbulent. The latest presents higher expression of adhesion molecules and inflammatory molecules and are more permeable to macromolecules such as Low-Density Lipoproteins (LDLs). These zones are more prone to atherosclerotic lesion initiation (56, 57, 62, 63).

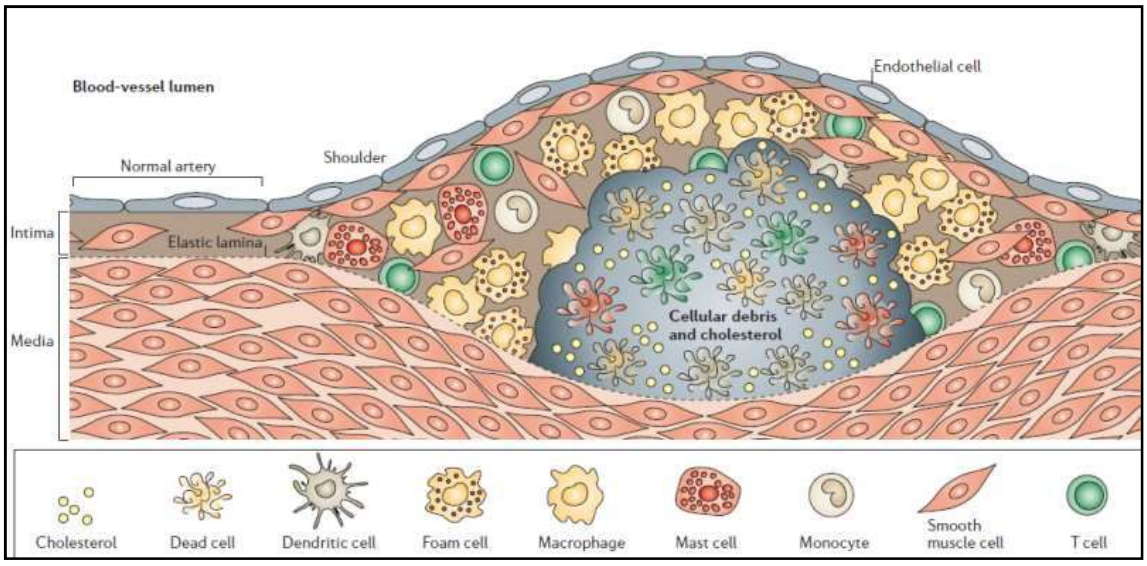


Figure 13. Atherosclerotic Plaque Structure (64).

AP formation begins with focal endothelial activation. Cholesterol-rich Very Low-Density Lipoproteins (VLDLs) and LDLs infiltrate the arterial wall when their plasma levels are elevated. This process continues until the accumulation of lipoproteins exceeds the capacity for its elimination. Lipoproteins are retained and oxidized in the extracellular matrix of the intima layer. The oxidized LDLs (oxLDLs) lead to the release of bioactive phospholipids that activate ECs. Activated ECs express several types of adhesion molecules which promote the migration of monocytes and T-cells into the forming lesion (56, 57, 62, 63, 65).

Monocytes differentiate into macrophages in the intima layer. Macrophages engulf and remove oxLDLs particles. OxLDLs accumulate as cytosolic droplets

when they are not removed to a sufficient extent transforming the macrophage in a foam cell (56, 57, 61-63). This process enlarges the lesion and encroaches the arterial lumen (Figure 13). Although that event could block the blood flow, the most important clinical complication (symptomatic atherosclerosis) is the plaque rupture resulting from inflammatory activation and the ensuing thrombosis that will cause an acute occlusion, resulting in MI or stroke (56, 57, 63).

3.2. Cardiovascular Risk Evaluation

Atherosclerotic lesions do not cause symptoms in early stages; however, the cardiovascular events resulting from plaque rupture are life-threatening. The identification of subjects at increased CVR is important to improve their prognosis (66). Traditional risk factors for atherosclerosis and CVD include older age, male gender, elevated lipid levels, smoking and high blood pressure. They are incorporated in risk algorithms that are used to predict an individual's absolute risk for CVD in the general population (67).

Atherosclerosis is a heritable disorder, with a heritability of 40 - 60 %. Although the majority of cardiovascular disorders are influenced by interactions between multiple genes and environmental factors, the association between genes and atherosclerosis is complex. Nevertheless, single-gene alterations (e.g in lipid metabolic pathways) account for a small proportion of familial and sporadic atherosclerosis. Molecular genetic studies have identified genes and genetic polymorphisms that influence atherosclerosis disease initiation and progression (58, 68).

The French paradox is the observation that French people have a relatively low incidence of coronary heart disease while their diet is rich in saturated fats (69). This is also true in other Mediterranean countries including Spain. Although the prevalence of CVR factors is higher in these countries than in other European

countries, the rate of MI is unexpectedly low (70). Individual CVR estimates provided by the Framingham charts overestimate three-fold and two-fold the individual risk in Spanish men and women respectively (71). Lifestyle, regional factors and the interaction between genes and environmental factors may play a crucial role in the explanation of the French paradox (69).

3.2.1. Cardiovascular Algorithms

There are several scoring systems to determine an individual's CVR. The scoring systems give an estimate of the probability that a person will develop CVD within a specified amount of time, usually 10 to 30 years. However, their accuracy in predicting CVR in individuals varies across populations (72). The most used equations are Framingham (73), PROCAM (Prospective Cardiovascular Münster) (74) and SCORE (Systematic Coronary Risk Evaluation) (75, 76). Each of them have their limitations: the PROCAM is only applicable to men (77); the SCORE equation only to individuals from 40 to 65 years of age (75) and the Framingham risk score (73) has been validated only in European American and African American populations (71, 78, 79). Framingham risk score is the most used to calculate CVR in the general population. Because the Framingham risk score overestimates risk in Mediterranean populations (71), it has been adapted and validated for the Spanish population (algorithm Regicor (Registre Gironí del Cor)) (80-82).

Framingham risk score includes the majority of the traditional CVR factors. The formula includes the following variables: age, gender, smoking status, diabetes status, cholesterol levels, and blood pressure values (76). However, it does not include risk factors such as left ventricular hyperatrophy, family history of premature coronary heart disease, increased waist circumference, body mass index, inflammatory markers, or triglyceride levels. Other CVD risk calculation algorithms (such as Reynolds Risk Score (83) or Q-RISK 2 (84)) have been

designed and include CVD risk factors not included in the Framingham risk score (33, 73, 85).

3.2.2. Vascular Wall Imaging

More recently, vascular wall imaging, such as carotid ultrasound and coronary calcium score, have been incorporated to better predict cardiovascular risk estimates in the future (86). Carotid ultrasonography allows the measurement of carotid Intima-Media Thickness (cIMT) and detects AP using ultrasound. It is an accepted method validated to determine the presence of subclinical atherosclerosis, that reflects structural and functional changes in the vascular tree (87), and to perform the follow-up of the disease (88, 89). It has been widely applied and defined as a surrogate marker for predicting cardiovascular events (90). Carotid ultrasonography technique is non-invasive, non-expensive, and gives better visualization of the changes of the arterial wall than other imaging modalities.

cIMT measures equal or greater than 0.9 mm or carotid plaque presence have been used as indicators of subclinical atherosclerosis (66). AP has been defined as a focal structure invading into the arterial lumen by at least 0.5 mm or 50 % of the surrounding cIMT value (90). cIMT measures correlate with pathological measures and are predictors of MI and stroke, even after adjusting by other CVR factors (91). The progression value is 0.01 mm / year in the general population. Progression over 0.03 mm / year is indicative of increased risk of suffering from cardiovascular events (92). Despite the widespread use of cIMT, it has limitations. Above all, the cIMT threshold value above which the risk is increased needs to be clearly defined; it is affected by multiple factors, being age the most influential (66) and its measure is highly subjective. However, imaging the arterial wall improves CVD risk prediction (86).

4. Atherosclerotic Disease in HIV-Infected Individuals

The first cases of acute MI in HIV-infected patients on ART were described in 1998 (93-95). Since then, the HIV-associated high risk for cardiovascular events has become increasingly evident (13-15, 18-26, 96). A 1.5 - 2 fold increased risk for coronary heart disease in HIV-infected individuals has been suggested by several observational studies (13, 20, 97-100). However, the relative contribution of HIV infection *per se* and the potential adverse effects of ART to coronary heart disease risk remain unclear. The high prevalence of CVR factors and the growing evidence of HIV-accelerated inflammatory processes (known to promote atherosclerosis) may explain the increased risk observed in HIV-infected subjects (101, 102).

Clinical presentations of HIV-related coronary heart disease tend to be different from the ones observed in the general population. Pathologically, HIV-related atherosclerosis lesions show features different from those observed in non-HIV atherosclerotic patients (101, 103). In general, HIV-infected subjects present non-calcified plaques that have inflammatory, necrotic, lipid-rich cores and are more vulnerable to rupture (16, 17, 32, 104). Demographically, HIV-infected subjects suffering from cardiovascular events have been shown to be more than a decade younger (105). They also present more prevalence of traditional CVR factors and co-morbidities than uninfected subjects (13, 105, 106).

4.1. HIV and ART Effect on Cardiovascular Risk

Several studies conclude that HIV is a CVR factor *per se* (91, 107). However, other studies have been unable to demonstrate a role for HIV (16, 108-110) or ART (108, 111, 112) in CVDs.

4.1.1. Direct Viral Effects

HIV non-controlled viremia seems to be related to CVD (4, 11, 52, 113). The inflammatory and endothelial activation pathways are down-regulated in treated aviremic HIV-infected individuals (4, 101, 114, 115). The SMART study (Strategies for Management of Anti-Retroviral Therapy), one of the largest HIV / AIDS treatment trials, tested the strategy of intermittent ART in patients with chronic HIV infection. Continuous ART was associated with reduced risk of CVD suggesting that HIV replication may increase the risk for heart disease (116).

Peripheral $CD4^+$ T-cell count has been consistently associated with risk of cardiovascular events, even under therapy (87, 105, 115, 117). Low $CD4^+$ T-cell counts were independently associated with increased prevalence of carotid lesions and greater cIMT (91, 117). Moreover, the $CD4^+$ T-cell count nadir predicts subclinical carotid atherosclerosis (91) and worse endothelial function (87). Additionally, the ratio $CD4^+$ / $CD8^+$ and the duration of known HIV infection were related to AP volume and coronary atherosclerosis respectively (26). Chronic immune activation persists during HIV infection despite effective treatment with ART (115, 118). Frequency of activated $CD4^+$ T-cell counts but not $CD8^+$ T-cell counts was associated with ultrasonographic measures of increased risk of carotid plaques and carotid artery stiffness (4, 119).

HIV might trigger and exacerbate atherosclerosis as a consequence of its inflammatory effect. Increases in inflammatory molecules such as Interleukin (IL) 6, Tumor Necrosis Factor (TNF) α, adhesion molecules, LPS and C-Reactive Protein (CRP) are observed during HIV infection (120, 121). Elevated CRP levels and IL6 levels are both independent predictors of cardiovascular events in the general population (115, 122-124). However, no concluding results have been reached in HIV-infected subjects (15, 120, 125).

HIV Nef protein seems to increase foam cell formation by impairing cholesterol efflux in macrophages (104, 122). Nef-induced foam cell transformation alone might be sufficient to increase inflammatory cytokines production from macrophages (126). This process along with immune activation has been proposed as the main pathogenic mechanism of HIV-related atherosclerosis (115, 122).

4.1.2. ART Treatment

The involvement of ART in the increase of CVR among HIV-infected subjects has not been elucidated (127). Although side-effects induced by certain antiretroviral drugs such as Protease Inhibitors (PIs) or Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (NRTIs) have been related to HIV-related atherosclerosis (21, 23, 98, 128-139), the real effect on HIV-related atherosclerosis development is not clear (27, 97, 109, 140, 141). High prevalence of traditional CVR factors and PI-induced dyslipidemia may partly explain the association between ART and HIV-related atherosclerosis (13, 27, 139). Nevertheless, ART is associated with better cardiovascular outcomes when compared with intermittent therapy (116, 141).

4.3. Measuring Cardiovascular Risk in HIV Subjects

4.3.1. Carotid Intima Media Thickness

HIV impact on the cIMT is similar to that of smoking or suffering from diabetes (142). Furthermore, both age and smoking seem to interact with HIV infection to promote a greater increase in cIMT (143, 144). Although the impact of HIV infection in cIMT progression is not clear, it seems to progress faster in HIV-infected subjects than in uninfected, suggesting that HIV-infected individuals are at increased CVR. There is no consensus on the rate of cIMT progression in HIV-infected individuals (91, 92, 113).

4.3.2. Scoring Systems

As mentioned, Framingham risk score is the most used scoring system to determine an individual's chances of developing CVD. However, it is not adapted to HIV-infected or Spanish populations. The Regicor score (80-82) even though is not adapted to the Spanish HIV-infected populations, is a better predictor than the Framingham score in the HIV-infected population (145). The DAD score is the HIV-adapted Framingham risk formula (146). However, its informative value is similar to the value obtained with other scores such as Framingham or PROCAM (147).

4.4. Inflammatory Molecules and HIV-Related Atherosclerosis

Inflammation is thought to be critical at all stages of atherosclerotic disease. Inflammation is a protective response to infection by the immune system. Communication between immune cells is important to give the correct response. Inflammation is an important part of the immune response, but chronic inappropriate inflammation can lead to destruction of tissues in autoimmune disorders and perhaps neurodegenerative or cardiovascular disease. Inflammatory markers have been observed to be elevated in both diseases. It is plausible that the alterations induced by HIV infection could aggravate atherosclerotic disease in HIV-infected subjects (4, 101).

The inflammatory molecules included in this thesis are classified as follows:

Non-specific inflammatory markers: One of the most studied surrogate markers of inflammation is the acute phase reactant CRP. It has been found elevated in HIV-infected individuals (148) even in those under ART (149-151). CRP protein levels have been associated with CVD (4, 58, 150) and with cIMT progression (111). Moreover, CRP levels have prognostic values for CVD in HIV-infected adults and in the general population (4, 58, 150).

Cytokines: Cytokines are a family of more than 50 secreted factors involved in intercellular communication. Cytokines are especially important in the tightly regulation of the inflammatory and the immune responses (Figure 14). They have crucial functions in controlling innate and adaptive immunity (152-154). Cytokines are classified as class I or class II cytokines, IL1 family, TNF family and Transforming Growth Factor (TGF) β superfamily depending on the structural homology of their receptors (152).

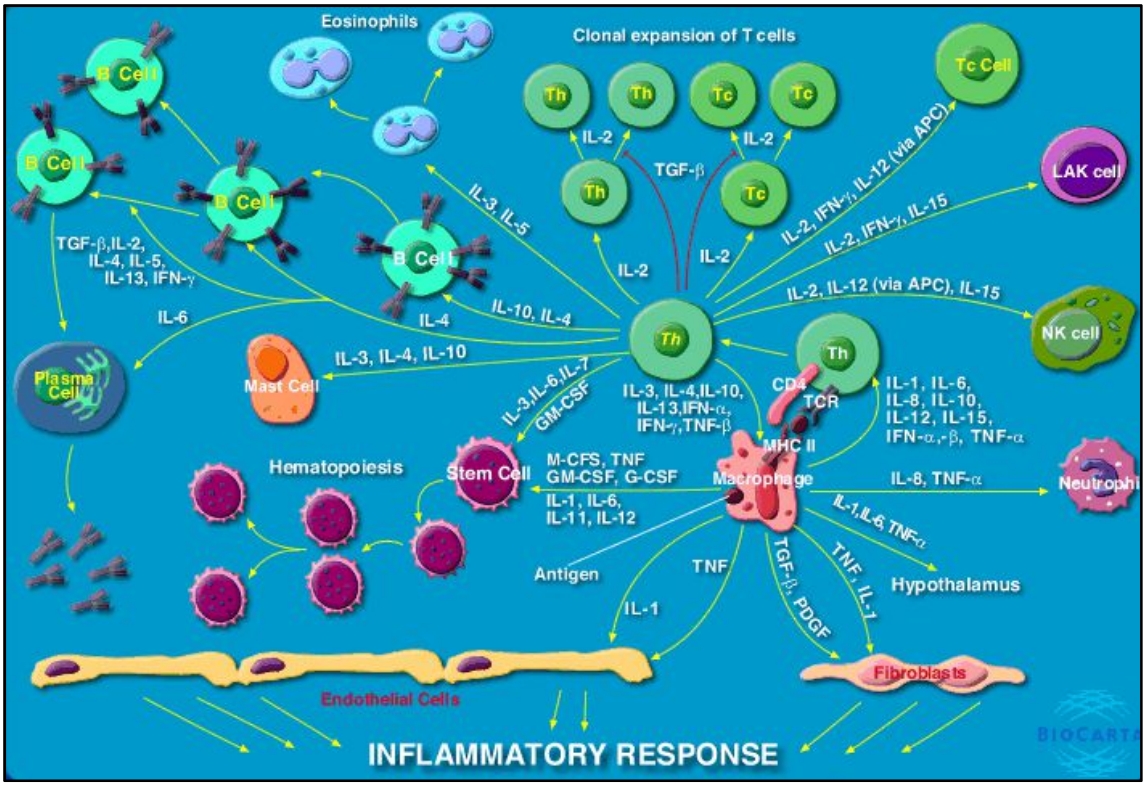


Figure 14. Cytokines and inflammatory response[18]. The figure shows the inter-relationship between specialized immune cells that secrete and respond to different cytokines.

Cytokine communication is often local, within a tissue or between cells in close proximity. Each of the cytokines is secreted by one set of cells and provokes a response in another target set of cells, often including the cell that secretes the cytokine (Figure 14). IL1 and TNF are secreted by macrophages to stimulate the

[18] http://www.biocarta.com/pathfiles/h_inflamPathway.asp

inflammatory responses of neutrophils, fibroblasts, and ECs that will recruit more immune cells to the site of infection. IL2 stimulates the proliferation and activation of B and T-cells and the cytotoxic activity of NK cells and lymphokine activated killer cells toward viral infected cells. T-cells secrete a variety of factors to coordinate and stimulate immune responses to specific antigen. IL12 and IL18 are involved in helper T-cell differentiation. IL10 and IL4 among others are secreted by T-helper (Th) cells to stimulate B cell responses as well as the clonal selection and differentiation of antigen-specific B cells to form antibody-secreting plasma B cells and memory cells. T-helper cells also secrete IL3 and IL5 to stimulate eosinophil proliferation and activation, whereas IL8 attracts neutrophils, basophils and T-cells to sites of inflammation. IL10 apparently acts to repress secretion of pro-inflammatory cytokines. The complex interplay of these different cytokine functions with immune cells is essential for correct immune function. In addition, cytokines stimulate the proliferation and differentiation of hematopoietic stem cells into the full range of immune cells[19] [20].

HIV infection results in decreased production of Th1 immune response cytokines (IL2 and Interferon (IFN) γ) and in an increased secretion of Th2 immune cytokines (IL4, IL10), pro-inflammatory cytokines (IL1, IL6, IL18) and TNF. Cytokines modulate infection and replication of HIV in CD4 T-lymphocytes and in cells of the macrophage lineage. IFNα, IFNβ, IL10, IL13 and IL16 inhibit viral replication, whereas TNF, IL1 and IL6 stimulates it (155). Macrophages produce inflammatory (TNFα, IL6, or IL18) and anti-inflammatory (IL10 and TGFβ) cytokines under hyperlipidemic conditions (such as in HIV infection) (154).

[19] http://www.biocarta.com/pathfiles/h_cytokinePathway.asp
[20] http://www.biocarta.com/pathfiles/h_inflamPathway.asp

Several cytokines such as IL1, IL2, IL6, IL8, IL10, IL12, IL18, TNF or IFNγ have been identified in human APs obtained by endarterectomy (152, 154).

Chemokines: They are low-molecular-weight proteins that coordinate leukocyte trafficking and activation. Their receptors trigger the assembly of the actin cytoskeleton and cellular movement after cytokine interaction, (65, 156, 157). Chemokines and their receptors play critical roles in directing leukocytes into atherosclerotic-prone vessels. The increase of pro-inflammatory chemokine and chemokine receptors within the aortas have been correlated with atherosclerotic lesion progression (65, 154, 156). Moreover, HIV virus uses chemokine receptors as co-receptors to enter into the targeted cell.

5-Lipoxygenase Pathway: The 5-lipoxigenase (5-LO) pathway is part of the eicosanoid pathway. The 5-LO pathway generates leukotriens using arachidonic acid as substrate. They are short lived - lipid mediators produced and excreted in response to various immune stimuli (158). Leukotriens have been involved in the atherosclerotic process (159) and in HIV pathogenesis (160, 161).

The inflammatory related genes included in this thesis are shown in Table 1.

Table 1. Genes related to inflammation studied in this thesis arranged by its chromosomic localization.

Full Name	Symbol	Synonyms	Location	Gene Size
C-reactive Protein, pentataxin-related	*CRP*	*PTX1*	1q23.2	2,301 bp
Interleukin 1, beta	*IL1B*	*IL-1, IL1F2, IL1-BETA*	2q14	7,020 bp
Interleukin 1 receptor agonist	*IL1RN*	*DIRA, IRAP, IL1F3, IL1RA, MVCD4, IL-1ra, IL-1ra3, ICIL-1RA*	2q14.2	16,124 bp

Full Name	Symbol	Synonyms	Location	Gene Size
Chemokine (C-C motif) receptor 2	*CCR2*	*CKR2, CCR2A, CCR2B, CD192, CKR2A, CKR2B, CMKBR2, MCP-1-R, CC-CKR-2*	3p21.31	7,179 bp
Chemokine (C-C motif) receptor 5	*CCR5*	*CKR5, CD195, CKR-5, CCCKR5, CMKBR5, IDDM22, CC-CKR-5*	3p21.31	6,065 bp
Chemokine (C-X3-C motif) receptor 1	*CX3CR1*	*V28, CCRL1, GPR13, CMKDR1, GPRV28, CMKBRL1*	3p21.3	18,242 bp
Interleukin 12A	*IL12A*	*P35, CLMF, NFSK, NKSF1*	3q25.33	7,178 bp
Chemokine (C-X-C motif) ligand 8	*CXCL8*	*IL8, NAF, GCP1, LECT, LUCT, NAP1, LYNAP, MDNCF, MONAP*	4q13 - q21	3,159 bp
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	*CXCL1*	*FSP, GRO1, GROa, MGSA, NAP-3, SCYB1, MGSA-a*	4q21	1,850 bp
Chemokine (C-X-C motif) ligand 2	*CXCL2*	*GRO2, GROb, MIP2, MIP2A, SCYB2, MGSA-b, CINC-2a*	4q21	2,246 bp
Chemokine (C-X-C motif) ligand 10	*CXCL10*	*C7, IFI10, INP10, IP-10, crg-2, mob-1, SCYB10, gIP-10*	4q21	2,380 bp
Interleukin 2	*IL2*	*TCGF, lymphokine*	4q26 - q27	5,026 bp
Lymphotoxin alpha	*LTA*	*LT, TNFB, TNFSF1*	6p21.3	2,225 bp
Tumor necrosis factor	*TNF*	*DIF, TNFA, TNFSF2, TNF-alpha*	6p21.3	2,769 bp
Interleukin 6	*IL6*	*HGF, HSF, BSF2, IFNB2*	7p21	4,856 bp
Interleukin 33	*IL33*	*DVS27, IL1F11, NF-HEV, NFEHEV, C9orf26*	9p24.1	16,305 bp

Full Name	Symbol	Synonyms	Location	Gene Size
Chemokine (C-X-C motif) ligand 12	*CXCL12*	*IRH, PBSF, SDF1, TLSF, TPAR1, SCYB12*	10q11.1	14,941 bp
Arachidonate 5-lipoxygenase	*ALOX5*	*5-LO, 5LPG, LOG5, 5-LOX*	10q11.2	71,937 bp
Interleukin 18	*IL18*	*IGIF, IL-1g, IL1F4*	11q22.2 - q22.3	20,865 bp
Arachidonate 5-lipoxygenase-activating protein	*ALOX5AP*	*FLAP*	13q12	28,888 bp
Ryanodine receptor 3	*RYR3*	-	15q14 - q15	555,127 bp
Chemokine (C-X3-C motif) ligand 1	*CX3CL1*	*NTN, NTT, CXC3, CXC3C, SCYD1, ABCD-3, C3Xkine, fractalkine, neurotactin*	16q13	12,543 bp
Chemokine (C-C motif) ligand 2	*CCL2*	*HC11, MCAF, MCP1, SCYA2, GDCF-2, SMC-CF, HSMCR30*	17q11.2 - q12	1,927 bp
Chemokina (C-C motif) ligand 5	*CCL5*	*SISd, eoCP, SCYA5, RANTES, TCP228, D17S136E, SIS-delta*	17q12	8,883 bp
Chemokine (C-C motif) ligand 3	*CCL3*	*MIP1A, SCYA3, G0S19-1, LD78ALPHA, MIP-1-alpha*	17q12	1,905 bp
Chemokine (C-C motif) ligand 4	*CCL4*	*ACT2, G-26, HC21, LAG1, MIP1B, SCYA2, SCYA4, MIP1B1, AT744.1, MIP-1-beta*	17q12	1,795 bp

HYPOTHESIS

HYPOTHESIS

HIV-infection has been suggested as a potential contributor to many chronic illnesses, including atherosclerosis (104). Given the unique effects of HIV on the immune system, it is plausible to consider that HIV infection itself could be involved in atherosclerosis. The link between both may be the result of a generalized increase in the activity of the inflammatory pathways (19), especially the cytokine network (155, 162). It is possible that these alterations in the inflammatory system may modulate the risk of atherosclerosis in HIV-infected individuals. These alterations may be caused by DNA sequence variants and / or by changes in the expression patterns of inflammatory pathway genes induced by the HIV infection or by ART.

AIMS

AIM

To assess the implication of genetic factors (DNA sequence variants and gene expression patterns) in several genes from the inflammatory pathway in subclinical atherosclerosis in HIV-infected individuals.

SPECIFIC AIMS

1. To describe epidemiologically the cardiovascular characteristics of the HIV-infected population attending the Hospital Universitari MútuaTerrassa.
2. To investigate the possible relationship between the selected genetic variants and subclinical atherosclerotic disease in HIV-infected individuals.
3. To establish whether there might be a relationship between the expression levels of selected genes and subclinical atherosclerosis in HIV-infected subjects.
4. To study if the gene expression levels of selected genes is affected by traditional cardiovascular risk factors.
5. To investigate the gene expression levels of selected genes and the possible relationship with HIV infection characteristics.
6. To assess the impact of ART introduction on the gene expression of selected genes in HIV-infected subjects as compared with uninfected individuals.

STUDIES

1. STUDY I:

Population Characteristics

1.1. Background

As HIV infection and AIDS have evolved into a chronic disease without a cure, lifelong ART has become the norm. ART-related immune reconstitution reliably prevents AIDS-related morbidity and mortality. Furthermore, ART allows discontinuation of primary and secondary prophylaxis for AIDS-related opportunistic infections (53). Consequently, introduction of ART has transformed HIV infection to a chronic, long-term survival disease. However, improved longevity has been associated with the emergence of new challenges and it has produced a shift of HIV-associated disorders from infection manifestations to other complex co-morbid disorders such as CVD (163).

The first cases of acute MI were described in HIV infected patients on ART in 1998 (93-95). Since then, several studies have shown that individuals with HIV infection are at 61 % higher relative risk for suffering a cardiovascular event when compared with HIV-uninfected individuals (13, 23, 96, 97, 100, 112, 164, 165). HIV-infected individuals suffering from cardiovascular events are younger and present more prevalence of traditional CVR factors such as smoking, hypertension, diabetes and dyslipidemia than the general population (106, 166).

HIV infection confers a progressive increase in the levels of viremia (viral load) and a drop in $CD4^+$ T-cell counts. Both of them influence CVD (87, 105, 113, 115, 117). Low peripheral $CD4^+$ T-cell count has been associated with increased risk of a cardiovascular event, even under therapy (105) and with a higher prevalence of carotid lesions and greater cIMT (91, 117). Moreover, the nadir $CD4^+$ T-cell count nadir predicts subclinical carotid atherosclerosis (91). Additionally, it has been suggested that non-controlled viremia is related to CVD (4, 11, 52, 113). The SMART study, one of the largest HIV / AIDS treatment trials ever conducted, tested the strategy of intermittent ART, guided by the $CD4^+$ T-cell count, in patients with chronic HIV infection. The overall rate of death and

the higher-than-expected rate of major CVD in the group assigned to intermittent ART exceeded predictions in the SMART study (116).

The involvement of ART in the increase of CVR among HIV-infected subjects is unclear. Several studies have found that toxicity induced by certain antiretroviral drugs such as PIs or NRTIs influence atherosclerosis development (21, 23, 98, 128-139). However, not all studies found significant associations (27, 97, 109, 140, 141). Nevertheless, ART seems to be associated with better cardiovascular outcomes when compared with intermittent therapy (105, 116).

HIV infection results in an altered lipid profile (4). The predominant lipid changes are hypertriglyceridemia, low HDL and low LDL values in the early stages of HIV infection before treatment (101, 122, 167). ART has complex direct effects on lipid levels (4). The LDL and total cholesterol concentrations increase with little changes in HDL cholesterol (especially under PI treatment) after initiation of ART (101, 115). Notwithstanding, ART initiation has global beneficial effects on CVR despite all the alterations in lipid profiles (141).

CVD is the leading cause of mortality worlwide. Early detection of CVR is important to reduce mortality from cardiovascular causes. Framingham risk score estimates the 10-year risk of developing coronary heart disease. However, it overestimates the risk in Mediterranean (71) and understimates the risk in HIV-infected populations (146, 168). It was adapted and validated for the HIV-infected population (DAD Score) (146) and also for the Spanish population (algorithm Regicor) (80-82). Regicor algorithm, even though it has not been adapted to the Spanish HIV-infected population, is a better predictor than the Framingham score (145).

cIMT is the most widely used surrogate marker of atherosclerosis (169). It relies on the fact that the presence of atherosclerosis in one vascular bed will correlate with the presence of atherosclerosis in any other vascular bed. High-

resolution B-mode ultrasound of the carotid arteries has been well validated among non HIV-infected population as a surrogate marker of CVR (170). Several studies have analyzed cIMT evolution in HIV-infected individuals. It is accepted that HIV-infected individuals have greater cIMT values and it progresses faster than in uninfected subjects (91, 92).

The aims of this study were to describe the CVR of the MútuaTerrassa HIV-Cohort and to test the possible association between subclinical atherosclerosis (measured by cIMT mean values and AP presence) and the collected clinical variables.

1.2. Materials and Methods

This is a cross-sectional study that includes 250 out of the 300 HIV-infected individuals followed in Hospital Universitari MútuaTerrassa cohort (Terrassa, Barcelona, Spain) (response rate 83.3 %). This project was approved by the local ethics committee (Approval number: EO / 0915). All participants gave written informed consent. Demographic, clinical and biochemical variables were collected at the time of enrolment. Data was collected by patient interview and medical notes review. Simultaneously ultrasonographic measures (cIMT and AP presence) were performed.

1.2.1. Carotid Artery Ultrasound

cIMT measurements and AP presence were obtained for each patient using a B-mode ultrasound recording with a 7 to 14-MZ transductor. All measures were performed by the same operator to reduce experimental variability. Patients were placed in the supine position with their head in the midline position and tilted slightly upwards, and the heart in systole for imaging the arterial wall. A total of eight measures were obtained for each participant: left and right common carotid and bulb region in both proximal and distal walls. The cIMT

value was defined as the mean of all values excluding those corresponding to AP thickness. According to Mannheim cIMT consensus, AP presence was defined as a focal structure encroaching into the arterial lumen by at least 0.5 mm or 50 % of the surrounding IMT value (90). It was recorded as a bimodal variable (yes / no).

1.2.2. Variable Definition

The dependent variable subclinical atherosclerosis was defined as having the cIMT value ≥ 0.9 mm and / or AP presence (171). It was recorded as bimodal variable (yes / no). The clinical variables waist circumference, height, weight and blood pressure (172) were measured following standard guidelines at the time of enrolment. Concomitant medication and smoking and drinking habits were collected using an adapted form of the HERMES-IMIM (Harmonización de las Ecuaciones de Riesgo en el Mediterraneo Sur de Europa - Institut Hospital del Mar d'Investigacions Mèdiques) questionnaire based on the MONICA study (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) (173). HIV infection parameters (nadir $CD4^+$ T-cell count, ART history, CDC stage, duration of known HIV infection and HIV-transmission group), demographic (gender, date of birth and ethnicity) and clinical variables (Hepatitis C Virus (HCV) coinfection, hypertension, diabetes and dyslipidemia) were collected by medical notes review.

Biochemical variables were collected from laboratory test reports performed in fasting conditions within the first month of enrolment. Fasting glucose, lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides), $CD4^+$ T-cell count and HIV viral load were collected.

Traditional CVR factors were defined as follows:

- Body Mass Index (BMI) was defined by the individual's weight divided by the square of their height. BMI was recorded as a continuous variable and then

stratified in 4 groups as follows[21]: 1) Low weight: BMI < 18.5 Kg / m^2; 2) Normal weight: BMI between 18,5 and 24.9 Kg / m^2; 3) Overweight: BMI between 25 and 29.9 Kg / m^2; and 4) Obesity: BMI ≥ 30 Kg / m^2.

- Abdominal obesity was defined as having waist circumference > 102 cm in men and > 88 cm in women (174).

- Hypertension was defined as having systolic blood pressure ≥ 140 mmHg and / or diastolic blood pressure ≥ 90 mmHg and / or being under antihypertensive treatment (174).

- Diabetes was defined as having fasting glucose levels ≥ 126 mg / dL, and / or having diabetes symptoms and glucose levels ≥ 200 mg / dL in a random determination and / or being under antidiabetic drugs (175).

- Dyslipidemia was defined as having total cholesterol ≥ 240 mg / dL, and / or HDL cholesterol ≤ 35 mg / dL, and / or triglycerides ≥ 200 mg / dL, and / or being under hipolipemiant treatment (174).

- Smoking status was collected as a bimodal variable (yes / no). Smoking was defined as being current smoker or former smoker of less than a year.

- Significant alcohol consumption was defined as a daily consumption greater than 60 grams of alcohol. It was collected as a bimodal variable (yes / no).

- Framingham and Regicor risk scores were calculated including the following variables: age, gender, systolic blood pressure, antihypertensive therapy (yes / no), total cholesterol value, HDL cholesterol value, and smoking status (yes / no). The 10-year risk of CVD was classified as low (< 10 %), moderate (10 % to 20 %) or high (> 20 %) (71, 146). DAD risk score was not considered because it had not yet been published at the time of the study.

[21] http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi

1.2.3. Statistical Analysis

Statistical analyses were performed using the SPSS version 20 (IBM, Chicago, IL) statistical package. Normal distribution of the collected clinical variables was determined using Kolmogorov-Smirnov tests. Homogeneity of variances between subclinical atherosclerosis and non subclinical atherosclerosis groups was checked using the Levene test. Chi square analyses or fisher exact tests (for those variables with less than 5 counts in any group) were used to test the possible association between subclinical atherosclerosis and the categorical independent variables. T-Test was used to test the possible association between subclinical atherosclerosis and normally distributed continuous variables. Finally the Mann-Whitney U-test was used to test the possible association between subclinical atherosclerosis and non-normally distributed continuous variables.

1.3. Results and Discussion

Baseline characteristics of the sample population are shown in Table 1. Atherosclerotic plaque was present in 36.1 % of the individuals (n = 90) and mean cIMT was 0.88 mm (Standard Deviation (SD) ± 0.21). Subclinical atherosclerosis was present in 55.2 % of the population (n = 138) and agree with data presented in previous studies (26). The mean cIMT was 1.0 mm (SD ± 0.19) and 65.2 % (n = 90) had atherosclerotic plaque in the group with subclinical atherosclerosis. The mean cIMT was 0.72 mm (SD ± 0.1) in the group without subclinical atherosclerosis.

Male prevalence was 75.5 % similar to the prevalence observed in the Spanish AIDS network cohort (Cohorte de la red española de investigación en SIDA - CoRIS) (176). The median age of patients was 44.9 years (Interqualtile Range: 25 % - 75 % (IQR): 40.1 - 49.3) in this cohort, 5 years older than the last CVR report of 2011 from CoRIS (176). However, patients with subclinical atherosclerosis had a median age of 47.5 years, similar to studies performed in proximal

geographic areas (137, 177). Being Spanish was associated with more subclinical atherosclerosis in comparison with other origins. However, it must be highlighted that they were significantly older than non-Spaniards (p = 0.018).

The studied cohort presented elevated rates of traditional CVR factors similar to the ones observed in other studies in HIV-infected subjects (13, 28, 176, 178). Dyslipidemia was present in 27.2 % of HIV-infected subjects, 9.4 % suffered from hypertension, 7.0 % from obesity and 3.2 % from diabetes (Table 1). All these values were similar to those described in the last CoRIS report (176) and other studies in Spanish Caucasians (13, 28, 178). However, smoking was more prevalent in the studied population (61 %) than in the CoRIS cohort (46 %) or in a meta-analysis with Spanish HIV-infected subjects (41.1 % in men and 24.3 % in women) (70, 176).

The meta-analysis by Islam et al. (2012) found 2 fold greater risk of CVR in HIV-infected patients under ART than in treatment naïve HIV-infected patients (23). However, the association between higher CVR and ART is unclear (29). Being under treatment was not associated with subclinical atherosclerosis in the studied population. The high prevalence of treated patients (81.1 %) may explain the lack of association between ART and subclinical atherosclerosis. When analyzing the possible association between lipid profile and ART treatment we only found triglycerides marginally associated (p = 0.043) with being under ART as previously described (179).

HIV infection and ART treatment modify the lipid profile. These changes might influence the CVR in the HIV-infected population. All lipid parameters were associated with atherosclerosis ($p < 0.05$ in all cases) except for HDL cholesterol. Lipid values were not different from the ones described in the last CoRIS report (176) and in the DAD study (179). Taking into account the cut-off values described in the general population (174), the studied population was within normal lipid values, even among those with subclinical atherosclerosis.

Table 1.1. Baseline characteristics of the sample population (Values are expressed as: Mean ± Standard Deviation, Median (Interquartile Range: 25 % - 75 %) or Frequency (%)).

Variable	Population (n = 250)	Subclinical Atherosclerosis		
		Yes (n = 138)	No (n = 112)	*p* value
Age, years	44.9 (40.1-49.3)	47.5 (42.5-51.3)	41.1 (36.3-44.8)	0.000
Males, n	188 (75.2)	102 (73.9)	86 (76.8)	ns
Spanish Caucasians, n	213 (85.2)	124 (89.9)	89 (79.5)	0.021
Body Mass Index, Kg/m^2	24.4±3.8	24.6±4.1	24.1±3.4	ns
Body Mass Index				
Low weight, n	8 (3.3)	4 (3.0)	4 (3.6)	
Normal weight, n	167 (68.4)	91 (68.4)	76 (68.5)	ns
Overweight, n	52 (21.3)	25 (18.8)	27 (24.3)	
Obesity, n	17 (7.0)	13 (9.8)	4 (3.6)	
Abdominal Obesity, n	32 (13.0)	23 (16.7)	9 (8.0)	0.043
Hypertension, n	23 (9.4)	20 (14.5)	3 (2.7)	0.001
Diabetes, n	8 (3.2)	8 (5.8)	0 (0)	0.009
Dyslipidemia, n	67 (27.2)	43 (31.2)	24 (21.4)	ns
Smokers, n	162 (66.1)	89 (64.5)	73 (65.2)	ns
Significant Alcohol Consumption, n	48 (20.2)	27 (20.8)	21 (19.4)	ns
Cardiovascular Risk Scores				
Framingham Risk Score, risk	3.5 (1.5-6.3)	4.0 (1.7-7.6)	3.05 (1.3-5.2)	0.002
Low, n	218 (87.9)	110 (80.9)	108 (96.4)	
Moderate, n	25 (10.1)	22 (16.2)	3 (2.7)	0.001
High, n	5 (2.0)	4 (2.9)	1 (0.9)	

Variable	Population (n = 250)	Subclinical Atherosclerosis		
		Yes (n = 138)	No (n = 112)	*p* value
Regicor Risk Score, risk	1.4 (0.7-2.4)	1.6 (0.8-3.3)	1.1 (0.6-1.9)	0.000
Low, n	243 (98.0)	131 (96.3)	112 (100.0)	
Moderate, n	5 (2.0)	5 (3.7)	0 (0.0)	0.048
High, n	0 (0.0)	0 (0.0)	0 (0.0)	
Lipid profile				
Total Cholesterol, mg/dL	175.1 (154.6-210.0)	179.0 (155.0-218.0)	171.7 (153.5-194.4)	0.004
LDL Cholesterol, mg/dL	100.5 (84.6-131.4)	104.4 (81.2-135.3)	96.9 (81.2-116.0)	0.018
HDL Cholesterol, mg/dL	44.7 (35.6-56.1)	45.6 (34.8-56.1)	43.1 (36.5-56.1)	ns
Triglycerides, mg/dL	121.6 (90.6-175.9)	135.6 (97.1-182.0)	110.7 (83.1-168.6)	0.021
HIV Characteristics				
Duration of Known HIV-infection, years	10.8 (3.5-17.4)	12.2 (3.6-17.7)	9.8 (3.2-17.0)	ns
Hepatitis C Coinfection, n	105 (42.5)	61 (44.2)	44 (39.3)	ns
Antiretroviral Therapy, n	203 (82.5)	114 (64.5)	89 (79.5)	ns
NRTIs, n	193 (80.1)	109 (83.2)	84 (76.4)	
NNRTIs, n	124 (51.5)	69 (52.7)	55 (50.0)	
IPs, n	67 (27.9)	37 (28.2)	30 (27.5)	ns
Entry Inhibitors, n	2 (0.8)	1 (0.8)	1 (0.9)	
Integrase Inhibitors, n	13 (5.4)	8 (6.2)	5 (4.5)	
Previous Antiretroviral Therapies, n	191 (77.6)	104 (75.4)	87 (77.7)	ns
CD4+ cell count, cells/ L	546.5 (336.5-753.0)	516.0 (319.0-760.0)	546.5 (331.5-727.8)	ns
CD4+ nadir cell count, cells/ L	265.5 (135.8-441.0)	245.0 (124.0-439.0)	282.0 (145.0-441.0)	ns
Undetectable Viral Load, n	133 (53.2)	75 (54.3)	58 (51.8)	ns
Viral Load, copies/mL (only detectable)	3,760 (54-63,000)	309 (45.1-42,400)	18,000 (65.9-87,075)	ns

Variable	Population (n = 250)	Subclinical Atherosclerosis		
		Yes (n = 138)	No (n = 112)	*p* value
CDC Stage				
A, n	170 (70.2)	98 (71.0)	72 (64.3)	
B, n	14 (5.8)	6 (4.3)	8 (7.1)	ns
C, n	58 (24.0)	28 (20.3)	30 (26.8)	
AIDS Criteria, n	105 (43.0)	60 (43.5)	45 (40.2)	ns
Risk Group				
Drug Users, n	79 (38.0)	48 (34.8)	31 (27.7)	
Sexual Transmission, n	119 (57.2)	63 (45.7)	56 (50.0)	ns
Others, n	10 (4.8)	7 (5.1)	3 (2.7)	

*Body Mass Index (BMI): the individual's body mass divided by the square of their height.
†Abdominal Obesity: waist circumference > 102cm in men and > 88cm in women.
§Hypertension was: systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥90 mmHg and/or antihypertensive treatment.
||Diabetes: fasting glucose levels ≥ 126 mg/dL, and/or having diabetes symptoms and glucose levels ≥ 200 mg/dL in a random determination and/or diabetes treatment.
#Dyslipidemia: Total Cholesterol ≥ 240mg/dL, and/or HDL Cholesterol ≤ 35 mg/dL, and/or Triglycerides ≥ 200 mg/dL and/or lipid lowering drugs.

While several studies have reported a higher risk of CVD in HIV-positive subjects with a low $CD4^+$ T-cell count (105, 180, 181), others have reported modest association or no association (98, 182-186). Other immunological or HIV-related parameters such as nadir $CD4^+$ T-cell count (91, 187), ratio $CD4^+$ / $CD8^+$ T-cell count (26), duration of known HIV infection (26) or HCV coinfection (188) have been found associated with increased CVR. None of these associations were replicated in this study. Differences in the end points might explain the discrepancies between studies.

Nearly all patients were classified as having low CVR using the Framingham or Regicor algorithms. Although the differences in Framingham and Regicor scores were statistically significant between HIV-infected individuals with or without subclinical atherosclerosis, the median relative risk was considered low in both groups. Additionally, none of the HIV-included subjects were classified as having high CVR when using the Regicor algorithm. Imaging the vascular wall permitted a better classification of individuals at higher CVR in this study and according with Peters et al. (86). Moreover, the CVR was probably underestimated because these scores are not adapted to HIV-infected populations. A prospective study of our cohort recording the cardiovascular events would be helpful to adapt the Regicor score to the Spanish HIV-infected population.

The studied population was not at high CVR when analyzing traditional CVR factors. Notwithstanding, vascular wall imaging showed that the studied HIV-infected population had a high prevalence of subclinical atherosclerosis (55.2 %). Mean cIMT of the whole population was near pathological values and 36 % presented AP. This population was young (median age nearly 50 years old) and presented 55 % prevalence of subclinical atherosclerosis indicating that HIV infection may be the explanation to this high prevalence of subclinical atherosclerosis at younger ages. More studies are needed to adapt the CVR calculation algorithms and to elucidate the causes of the high rates of subclinical

atherosclerosis observed in HIV-infected populations. While these causes are not known, active interventions to reduce CVR factors such as smoking, dyslipidemia, hypertension or sedentary life are needed to expand life-span and ameliorate quality of life in HIV-infected subjects.

2. STUDY II:

Single Marker Study

2.1. Background

CVD has been identified as a major cause of death in HIV-infected people (20, 101, 189). HIV-infected individuals have accelerated atherogenesis. This is associated to a high risk of suffering a cardiovascular event such as coronary artery disease, peripheral vascular disease and stroke. The prevalence of these events is higher in HIV-infected subjects than in the general population and have an earlier onset (23, 190). The biological mechanisms underlying such risk among HIV-infected people are unclear (4).

Several studies implicate inflammation processes in CVD (4, 58, 122, 191). Studies in non HIV-infected cohorts have demonstrated that markers of inflammation are strongly predictive of CVD events and mortality (191). Inflammatory markers are elevated in HIV-infected patients in comparison with non-infected individuals (4, 19). It has been hypothesized that this increased inflammation may be the explanation for the elevated CVR in HIV-infected individuals (122). In addition, the high prevalence of CVD traditional risk factors and the presence of several events induced by HIV infection such as immunosupression, inflammation, HIV ability to induce foam cell transformation, cumulative exposure to antiretroviral drugs, and mitochondrial and metabolic dysfunctions have been hypothesized to be involved in HIV-associated atherosclerosis (19, 149, 192). In summary, HIV is thought to play a crucial role in the pathogenesis of HIV-related atherosclerosis (19, 193).

Studies on inflammatory pathways have found genetic variants associated with atherosclerosis and CVR in the general population (58). Variations in genes encoding for inflammatory proteins such as *CCR2, CCR5* (194), *CRP* (195, 196)*, CX3CR1* (157, 197), *CXCL8* (58), *IL1* gene cluster (198, 199), *IL6* (58)*, IL18* (58)*, LTA* (58), *TNF* (58)*,* and the 5-LO pathway genes (58, 200) have been associated with cardiovascular events. The Genome Wide Association Study (GWAs)

conducted by Shrestha et al. revealed two variants in the gene *RYR3* associated with greater cIMT, a surrogate marker of atherosclerosis (201). These findings have been replicated by the same authors in later studies (202, 203). However, no independent study has confirmed their results.

The inflammatory pathway is involved in the pathogenesis of atherosclerosis in HIV-infected individuals. The aim of this chapter was to assess the implication of genetic variants in relevant inflammatory genes in the atherosclerotic disease of HIV-infected subjects.

2.2. Material and Methods

2.2.1. Sample Study Population

Spanish Caucasians patients were selected for this study to reduce sample genetic heterogeneity. Therefore, 213 out of 249 HIV-infected individuals were selected for the candidate gene study.

Demographic, lipid profile and HIV baseline characteristics of the studied population are shown in Table 2.1. The median age of the sample population was 49.9 years (IQR: 41.0 - 49.2). Males constituted 77.9 % of the sample. The majority of the individuals were receiving ART at the time of the study (82.2 %). AP presence (indicator of atherosclerotic lesion (62)) and cIMT means (indicator of arterial remodeling (89)) were the dependent variables investigated as markers of CVD. The study population was classified in two groups according to the bimodal variable AP presence. Baseline characteristics are shown in Table 2.1. Patients with AP presence conformed 39 % of the cohort (n = 83), with 73.5 % of males and a median age of 48.5 years (IQR: 44.2 - 51.3). Patients without AP had a median age of 42.6 years (IQR: 38.4 - 47.0) and 80.8 % were males.

Table 2.1. Baseline characteristics of the sample population (Values are expressed as: Mean ± Standard Deviation, Median (Interquartile Range: 25 % - 75 %) or Frequency (%)).

Variable	Population (n = 213)	Atherosclerotic Plaque Presence	
		Yes (n = 83)	No (n = 130)
Age, years	44.9 (41.0 - 49.2)	48.5 (44.2 - 51.3)	42.6 (38.4 - 47.0)
Males, n	166 (77.9)	61 (73.5)	105 (80.8)
Body Mass Index[*], Kg/m^2	24.1±3.76	24.1±4.09	24.1±3.56
Abdominal Obesity[†], n	26 (12.2)	15 (18.3)	11 (8.6)
Hypertension[§], n	20 (9.4)	14 (17.7)	6 (4.7)
Diabetes[‖], n	8 (3.8)	7 (8.4)	1 (0.8)
Dyslipidemia[#], n	59 (27.7)	28 (35.0)	31 (24.0)
Atherosclerosis Characteristics			
cIMT mean, mm	0.89 ± 0.21	1.01 ± 0.21	0.81 ± 0.17
Atherosclerotic plaque presence, n	83 (39)	83 (100)	0 (0)
Smoking Habits			
Smokers, n	150 (70.4)	61 (74.4)	89 (70.1)
Non Smokers, n	59 (27.7)	21 (25.6)	38 (29.9)
Lipid profile			
Total Cholesterol, mg/dL	175.1 (154.6 - 208.8)	188.7 (162.4 - 228.1)	170.7 (151.5 - 196.3)
LDL Cholesterol, mg/dL	100.5 (85.1 - 131.4)	110.2 (98.9 - 203.9)	100.5 (81.2 - 119.8)
HDL Cholesterol, mg/dL	44.8 (35.6 - 56.1)	45.6 (36.7 - 58.4)	44.5 (35.6 - 54.5)
Triglycerides, mg/dL	126.0 (92.8 - 175.9)	140.9 (98.9 - 203.9)	113.8 (86.6 - 162.8)
HIV Characteristics			
Duration of Known HIV-infection, years	12.2 (4.7 - 18.3)	13.4 (7.6 - 20.0)	10.0 (3.9 - 17.6)
Hepatitis C Coinfection, n	102 (47.9)	45 (54.9)	57 (44.5)

Variable	Population (n = 213)	Atherosclerotic Plaque Presence	
		Yes (n = 83)	No (n = 130)
Antiretroviral Therapy, n	175 (82.2)	72 (87.8)	103 (80.5)
Previous Antiretroviral Therapies, n	167 (78.4)	68 (82.9)	99 (77.3)
$CD4^+$ T-cell count, cells/ L	526.0 (332.0 - 762.0)	535.0 (326.0 - 852.0)	517.0 (332.0 - 727.0)
$CD4^+$ nadir T-cell count, cells/ L	263.0 (140.0 - 441.0)	264.0 (142.0 - 456.0)	260.0 (139.0 - 441.0)
Detectable Viral Load (> 19 copies/mL), n	71 (33.3)	23 (27.7)	48 (36.9)
Viral Load, copies/mL (only detectable)	20,600.0 (309.0 - 89,100.0)	1,580.0 (142.0 - 42,400.0)	38,100.0 (3,000.0 - 107,000.0)
CDC Stage			
A, n	143 (67.1)	59 (72.8)	84 (67.2)
B, n	13 (6.1)	3 (3.7)	10 (8.0)
C, n	50 (23.5)	19 (23.5)	31 (24.8)
AIDS Criteria, n	92 (43.2)	37 (45.1)	55 (44.0)
Risk Group			
Drug Users, n	78 (36.6)	35 (50.7)	43 (40.6)
Sexual Transmission, n	91 (42.7)	32 (46.4)	59 (55.7)
Others, n	6 (2.8)	2 (2.9)	4 (3.8)

*Body Mass Index (BMI): the individual's body mass divided by the square of their height.

†Abdominal Obesity: waist circumference > 102cm in men and > 88cm in women.

§Hypertension was: systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥90 mmHg and/or antihypertensive treatment.

||Diabetes: fasting glucose levels ≥ 126 mg/dL, and/or having diabetes symptoms and glucose levels ≥ 200 mg/dL in a random determination and/or diabetes treatment.

#Dyslipidemia: Total Cholesterol ≥ 240mg/dL, and/or HDL Cholesterol ≤ 35 mg/dL, and/or Triglycerides ≥ 200 mg/dL and/or lipid lowering drugs.

2.2.2. Genotyping

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Mini kit (Qiagen, Izasa, Barcelona, Spain) following the manufacturer's instructions. Genotyping was performed using Competitive Allele Polymerase Chain Reaction (PCR) methodology (KBioscience, Hoddesdon, United Kingdom). Genes from the inflammatory pathway were selected by bibliographic review and pathways analysis. A total of 113 Single Nucleotide Polymorphisms (SNPs) in 26 candidate genes were tested (Table 2.2). TagSNPs were selected from HapMap[22] for maximum coverage of the genes, using the parameters: $R^2 > 0.8$ and minor allele frequency (*maf*) > 0.05. We also included 8 relevant SNPs that were found associated with atherosclerosis in previous studies in HIV and non-HIV infected populations.

Table 2.2. Genotyped SNPs.

Chromosome	Gene	Genotyped SNPs
1	*CRP*	rs1205; rs1130864; rs180947
2	*IL1B*	rs55778004; rs1143634; rs1143633; rs1143627*; rs16944*
	IL1RN	rs3087263; rs380092; rs431726; rs452204; rs4252019; rs315952; rs4252041
3	*CCR2*	rs3762823; rs1799864
	CCR5	rs333*
	CX3CR1	rs17038663; rs11710546; rs3732378; rs2669850; 13088991; rs9862876; rs9868689; rs4423707; rs13062158; rs2853712; rs2853711; rs2669845
	IL12A	rs2243123; rs583911
4	*CXCL8*	rs4073*; rs2227306
	CXCL1	rs4074
	CXCL2	rs9131; rs3806792*
	CXCL10	rs8878; rs11548618*
	IL2	rs2069778; 2069777
6	*LTA*	rs1800683; rs2239704; rs909253; rs2229094; rs2229092

[22] http://hapmap.ncbi.nlm.nih.gov/

Chromosome	Gene	Genotyped SNPs
	TNF	rs4248160*; rs3093662
7	*IL6*	rs1800795; rs2069833; 2069840; rs1554606
9	*IL33*	rs4742170; rs7019575; rs7037276; rs1412420; rs7047921; rs1332290
10	*CXCL12*	rs1801157; rs2236533; rs2236534; rs2839693; rs10793538; rs3780891; rs7092453
	ALOX5	rs12783095; rs3824612; rs934187; rs7918542; rs7099684; rs2115819; rs10900213; rs11239523; rs12264801; rs1565096; rs1487562; rs3780914
11	*IL18*	rs3882891; rs5744258; rs5744247; rs795467; rs2043055
13	*ALOX5AP*	rs9579645; rs9579646; rs4075131; rs9578196; rs4293222; rs12429692; rs4769873; rs9315045; rs4503649; rs3885907; rs10162089; rs4254165; rs17245204; rs9579648; rs10507393; rs9315048; rs9315051; rs3935644; rs4769060
15	*RYR3*	rs2229116*
16	*CX3CL1*	rs170364; rs170361; rs4151117; rs614230
17	*CCL2*	rs1024611; rs3760396
	CCL5	rs3817655; rs2280789; rs2107538
	CCL3	rs1719134
	CCL4	rs1719147

* Selected from previous published studies.

2.2.3. Statistical analyses and quality control

Statistical analyses were performed using the Epi Info™, G*Power Calculator (204), SPSS version 20 (IBM, Chicago, IL) and Plink version 1.07 (205) statistical packages. The study had an 80 % power (considering $\alpha = 0.05$, two-sided) to capture the effect of SNPs with *maf* ≥ 0.1 and Odds Ratio (OR) ≥ 2. For rarer alleles (*maf* ≥ 0.05), the study had a 75 % power to capture genetic effects with OR ≥ 3. For continuous variables the study had a 95 % power (considering $\alpha = 0.05$, two-sided) to detect associations with small effect sizes ($f^2 = 0.05$).

Genotype call rate, Hardy-Weinberg Equilibrium (HWE) and allele frequencies were assessed for each SNP using Plink version 1.07. Those SNPs with call rates

< 95 % or *maf* < 0.01 and those individuals with more than 5 % missing genotypes were excluded from the analyses.

Chi square analyses or Fisher exact tests (for those alleles with less than 5 counts in any group) were used to investigate the possible association of genetic variants with the AP variable. cIMT was recorded as a continuous variable and it was normally distributed. Linear regression analyses were used to compare cIMT values with gene alleles.

Age, abdominal obesity, hypertension, diabetes, total cholesterol, LDL cholesterol and triglycerides were significantly associated with AP presence ($p < 0.05$ in all cases). Age, hypertension, diabetes, dyslipidemia, total cholesterol and LDL cholesterol were significantly associated with cIMT ($p < 0.05$ in all cases). Lipid values were highly correlated (correlation coefficient > 0.75 in all cases), thus dyslipidemia was included as a clinical adjusting variable representative of lipid profile in the regression models.

For AP presence, age, abdominal obesity, hypertension, diabetes and dyslipidemia were included in logistic regression analyses as adjusting clinical variables for all the findings. For cIMT, age, hypertension, diabetes, and dyslipidemia were included in the regression analyses as adjusting clinical variables. Despite a reduction in overall morbidity and mortality due to the prevention of AIDS-related events, new fears arose regarding the risk of long-term metabolic complications of ART, such as HIV-related CVD in the ART era. Therefore, ART was also included in the analyses of both clinical variables as an adjusting covariate.

2.3. Results and Discussion

2.3.1. SNP Quality Control

All SNPs were in HWE except for *IL1B* rs16944 (p = 0.044) and *IL1RN* rs380092 (p = 0.006) due to an underrepresentation of rare homozygotes; *CX3CR1* rs2853712 (p = 0.009), *CXCL12* rs2236533 (p = 0.040) and *ALOX5* rs3824612 (p = 0.025) due to an overrepresentation of heterozygotes and *CXCL12* rs2236534 (p = 0.029) due to an overrepresentation of rare homozygotes. Raw data were checked to detect possible genotyping errors. The genotyping of these SNPs needs replication and their results should be taken with caution.

Three SNPs had a call rate < 95% and were excluded from the analysis (*IL1RN* rs3087263, *CX3CR1* rs2669845 and *IL6* rs2069833). Two SNPs had a *maf* < 0.01 (*CXCL10* rs11548618 and *TNF* rs4248160) and two SNPs (*IL1B* rs55778004 and *IL1RN* rs4252019) were monomorphic in the sample population. The remaining 106 SNPs were included in the analysis. All individuals had call rates > 95 %, thus none of them was excluded from the analysis.

2.3.2. Single Marker Analysis

2.3.2.1. Atherosclerotic Plaque Presence Analysis

Single marker analyses by Chi-square or Fisher exact test revealed 12 associations with AP. Logistic regression analyses using age, abdominal obesity, hypertension, diabetes, dyslipidemia and ART as adjusting clinical variables showed 8 associations (Table 2.3).

IL-1B rs16944 has not been consistently associated with risk of MI (206-208) and susceptibility to atherosclerosis (209). No association was found between AP presence and this polymorphism. This finding adds evidence to the non-involvement of this genetic variant in AP development.

The associations between *IL1RN* rs4252041-T allele and AP presence (p = 0.027, OR = 2.40), and *CXCL2* rs9131-G allele, *CXCL2* rs3806792-T allele and *CX3CL1* rs170361-A allele with AP absence (p = 0.035, OR = 0.65; p = 0.026, OR = 0.63 and p = 0.040, OR = 0.57 respectively), were not confirmed in the logistic regression analysis when considering other contributing factors and were considered false positive discoveries.

The *ALOX5* rs2115819-C allele was associated with AP presence in the univariate analysis (p = 0.008, OR = 1.73) and in the logistic regression (p = 0.009, OR = 2.03). Three *ALOX5AP* SNPs were associated with AP absence and all of them were also significant in the logistic regression analyses: rs9578196-T (p = 0.014, OR = 0.45; p = 0.007, OR = 0.33); rs4769873-T (p = 0.011, OR = 0.40; p = 0.004, OR = 0.25) and rs9315051-G (p = 0.001, OR = 0.27; p = 0.0004, OR = 0.15). Previous studies have reported associations between *ALOX5* and *ALOX5AP* variants and CVD in non-HIV infected populations (200). However, these studies did not investigate the same SNPs. *ALOX5* and *ALOX5AP* encode for two important proteins of the 5-LO pathway which has been previously linked to atherosclerosis (200). Our results contribute to the growing evidence of the involvement of *ALOX5* and *ALOX5AP* in CVD and describe for the first time their involvement in HIV-related atherosclerosis.

The *CX3CL1* rs4151117-G and rs614230-C alleles were associated with AP absence (p = 0.040, OR = 0.52 and p = 0.015, OR = 0.54 respectively) in the logistic regression analyses. CX3CL1 has been implicated in the initial process of AP formation (157, 197). The functional effects of the *CX3CL1* rs4151117 and rs614230 variants are unknown. However, the rs4151117 SNP is located near a frame shift mutation that may decrease protein functionality. The *CX3CL1* rs614230 polymorphism is located in the 5' untranslated region. This variant may have regulatory functions as a result of its location. The role of these genetic variants needs further functional studies. However these results seem

Table 2.3. Summary of single marker analyses in relation to atherosclerotic plaque presence.

Gene	SNP	ALLELE COUNT Minor allele (*maf* Aff/*maf* Unaff)*	Unadjusted			Adjusted**		
			p value	OR	CI 95%	p value	OR	CI 95%
IL1RN	rs4252041	T (0.10 / 0.04)	0.027	2.40	1.08 - 5.30	*ns*	-	-
CXCL2	rs9131	G (0.37 / 0.47)	0.035	0.65	0.43 - 0.97	*ns*	-	-
CXCL2	rs3806792	T (0.36 / 0.47)	0.026	0.63	0.42 - 0.95	*ns*	-	-
ALOX5	rs2115819	C (0.50 / 0.37)	0.008	1.73	1.15 - 2.58	0.009	2.03	1.19 - 3.47
ALOX5AP	rs9578196	T (0.08 / 0.16)	0.014	0.45	0.23 - 0.86	0.007	0.33	0.14 - 0.73
ALOX5AP	rs4769873	T (0.06 / 0.14)	0.011	0.40	0.19 - 0.83	0.004	0.25	0.10 - 0.65
ALOX5AP	rs9315051	G (0.04 / 0.14)	0.001	0.27	0.12 - 0.63	0.0004	0.15	0.05 - 0.43
CX3CL1	rs170361	A (0.13 / 0.20)	0.040	0.57	0.33 - 0.98	*ns*	-	-
CX3CL1	rs4151117	G (0.15 / 0.24)	0.024	0.55	0.33 - 0.93	0.040	0.52	0.28 - 0.97
CX3CL1	rs614230	C (0.27 / 0.39)	0.013	0.59	0.38 - 0.89	0.015	0.54	0.33 - 0.88
CCL5	rs3817655	A (0.23 / 0.15)	0.035	1.72	1.04 - 2.85	0.018	1.96	1.12 - 3.44
CCL5	rs2107538	T (0.23 / 0.15)	0.033	1.71	1.04 - 2.80	0.018	1.93	1.12 - 3.31

**maf*: minor allele frequency / Aff: Affected / Unaff: Unaffected.

** Adjusted by clinical variables: Age, Abdominal Obesity, Hypertension, Diabetes, Dyslipidemia and ART.

to agree with the reported atheroprotective effects of CX3CL1 reduced activity (210).

Finally, two *CCL5* alleles were associated with AP presence in both Chi Square and logistic regression analyses: rs3817655-A (p = 0.035, OR = 1.72; p = 0.018, OR = 1.96) and rs2107538-T (p = 0.033, OR = 1.71; p = 0.018, OR = 1.93). The *CCL5* rs2107538 has been previously associated with higher CCL5 plasma concentrations and with increased risk of MI in Korean CAD patients (211) and Han Chinese MI patients (196) in agreement with the results of the present study. The *CCL5* rs3817655 finding is a novel association. There are two mutations located near this SNP that encode for new stop codons resulting in truncated proteins. This finding may reflect the functional effect of these mutations but further studies are required to confirm it.

2.3.2.2. Carotid Intima Media Thickness Analyses

Single marker analysis by linear regression showed 9 associations with cIMT whereas analyses using age, hypertension, diabetes, dyslipidemia, and ART as adjusting clinical variables revealed 4 significant associations (Table 2.4).

The *CRP* rs1130864-T allele was associated with smaller cIMT values in both, univariate and multivariate analyses (p = 0.006, β = -0.06; p = 0.0003, β = -0.07, respectively) whereas rs1800947-C allele was associated with greater cIMT in both analyses (p = 0.028, β = 0.09; p = 0.008, β = 0.10). These results are in the opposite direction that those reported in previous studies. The *CRP* rs1130864-T allele was found associated with increased CRP plasma levels (195), which are considered a marker of CVR (212, 213), and has been linked to a faster cIMT progression in HIV-infected individuals (111). The *CRP* rs1800947-C allele has been previously linked to decreased CRP plasma levels (196, 214), which are known to be atheroprotective. Although the functional consequences of these SNPs are not known, both of them are in regulatory regions. The cellular

alterations induced by HIV may disguise the functionality of these genetic variants explaining the discrepancy between these findings and those published previously in non-HIV infected populations. Another possible explanation is the different allele frequency between the Spanish and the Asian populations.

The *IL1RN* rs380092-T allele was found associated with smaller cIMT in both types of analyses (p = 0.019, β = -0.06 and p = 0.002, β = -0.07 respectively). However, previous studies have found this allele associated with increased risk of MI (198) and ischemic stroke (199). The *IL1RN* rs380092 variant is located approximately 200 bp up-stream of the rs121913161, which encodes for a new stop codon that significantly reduces IL1RN plasma levels. IL1RN has been suggested to be a proatherogenic cytokine (215) and diminished levels of IL1RN might be atheroprotective (216). This would agree with the findings of this study. However, our results should be considered with caution because the rs380092 variant was not in HWE. Other *IL1RN* variants investigated, rs431726 and rs315952, were not found associated with cIMT values, and this agrees with previous reports (217).

The *CX3CR1* rs2669850, rs13088991 and rs9868689 variants were found associated with cIMT, but none of these associations remained significant after adjustment by clinical variables suggesting that they may be false positive results. The *CX3CR1* rs3732378 (T280M) and rs3732379 (V249I) variants have been linked to faster AIDS disease progression (218, 219) and reduced atherosclerosis progression in HIV population (92). They have also been linked to reduced risk for coronary artery disease (220) and reduced cIMT in the general population (221). Although the majority of the studies describe protective associations for these polymorphisms, others have found deleterious or no association (197, 222). The rs3732378 (T280M) variant has not been associated with cIMT values or atherosclerotic plaque presence in the studied cohort. CX3CL1 is the natural ligand of CX3CR1. The *CX3CL1* rs614230-C allele

Table 2.4. Summary of single marker analyses in relation to cIMT.

Gene	SNP	ALLLELE COUNT - MAF	Unadjusted			Adjusted**		
			p value	β	CI 95%	p value	β	CI 95%
CRP	rs1130864	Minor Allele T - 0.34	0.006	-0.06	-0.10 - -0.02	0.0003	-0.07	-0.11 - -0.03
CRP	rs1800947	Minor Allele C - 0.06	0.028	0.09	0.01 - 0.17	0.008	0.10	0.03 - 0.17
IL1RN	rs380092*	Minor Allele T - 0.25	0.019	-0.06	-0.11 - -0.01	0.002	-0.07	-0.12 - -0.03
CX3CR1	rs2669850	Minor Allele C - 0.45	0.024	-0.07	-0.14 - -0.01	*ns*	-	-
CX3CR1	rs13088991	Minor Allele C - 0.48	0.041	0.06	0.003 - 0.12	*ns*	-	-
CX3CR1	rs9868689	Minor Allele T - 0.17	0.040	0.09	0.004 - 0.17	*ns*	-	-
IL18	rs2043055	Minor Allele G - 0.42	0.040	0.04	0.002 - 0.08	*ns*	-	-
ALOX5AP	rs3885907	Minor Allele C - 0.41	0.030	0.05	0.005 - 0.09	0.02	0.05	0.01 - 0.08
CX3CL1	rs170361	Minor Allele A - 0.17	0.049	-0.05	-0.10 - -0.001	-	-	-

* Not in HWE.

** Adjusted by clinical variables: Age, Hypertension, Diabetes, Dyslipidemia and ART.

has been previously associated with smaller cIMT in German non-HIV infected subjects (CAPS cohort) although this finding was not replicated in a French cohort (3C cohort) (197) or in the studied cohort. The *CX3CL1* rs170361-A allele showed a trend towards association with smaller cIMT (p = 0.049, β = -0.05) in the univariate analyses only. Thus, it was considered a false positive result.

Finally, the *ALOX5AP* rs3885907-C allele showed association with greater cIMT in both analyses (p = 0.030, β = 0.05; p = 0.002, β = 0.05). Genetic variations in *ALOX5AP* have been associated with increased risk of atherosclerosis in uninfected populations (200). However, this SNP has not been previously associated with subclinical atherosclerosis or cardiovascular events. This novel association adds importance to the involvement of the 5-LO pathway to the atherosclerotic development in HIV-infected patients.

An association was found between the *RYR3* rs2229116 polymorphism and cIMT in previous studies performed by Shrestha et al. on American HIV-infected patients (201). However, this finding was not replicated in the present study. Aside from a false negative result, the discrepancy could be explained by the methodological differences in cIMT measures and / or different allele frequencies between American and Spanish populations.

In summary, in this study we have found modest associations between genetic variants in several inflammatory genes and CVR markers in an HIV-infected population. However, none of the findings remained significant after Bonferroni correction. They need to be confirmed in a larger population and functional studies are needed to elucidate the consequences of these genetic changes. Nevertheless, our study adds evidence to the association between genetic variants in inflammatory pathways and the atherosclerotic disease in HIV-infected individuals.

3. STUDY III:

Gene Expression Study

3.1. Background

Side-effects associated with HIV infection and ART remains a matter of concern (101). CVD is one of the most prevalent co-morbidities in HIV-infected populations (29, 91), and constitutes a major cause of death in people living with HIV infection (101, 189). HIV-infected people have accelerated atherogenesis, higher prevalence of cardiovascular events such as coronary artery disease, peripheral vascular disease and stroke, as well as an earlier onset of atherosclerotic disease (23, 100, 190).

The underlying mechanisms behind the increased CVR observed in HIV-infected individuals are not well understood (4). Inflammation has been hypothesized to be one of the main players in the development of atherosclerotic disease in HIV-infected and uninfected populations (4, 31, 191). HIV infection alters serum levels of several inflammatory proteins. Although ART introduction reduces serum levels of inflammatory proteins, they remain elevated in comparison to those observed in non-infected individuals (12, 52). HIV-infection alters the cytokine network from the earliest stages to the chronic infection (12, 52, 223). Increased serum levels of several cytokines have been described during the acute HIV infection: β_2-microglobulin (B2M), CCL2, CXCL8, CXCL10, IL1B, IL6, IL10, IL18, IFNα, IFNγ, neopterin (IP10) and TNFα (12, 52, 223). At the same time, increased protein levels of CX3CR1, IL1β, IL6, IL10 and TNFα have been observed during chronic infection (12, 52, 162, 224). Alterations of several cytokines have been implicated with CVR in HIV infection: higher levels of CCL2, CX3CR1, IL1α, IL1β, IL6, IL18 and TNFα proteins are associated with increased CVR whereas lower levels of CXCL12 protein are associated with reduced CVR (107, 225, 226).

Atherosclerosis and CVD involve a complex inflammatory process. The acquisition of HIV infection in the same individual adds further complexity to

this process independently of traditional CVR factors (107, 122). The inflammatory pathway has many genes that may interact in the pathogenesis of both, atherosclerosis and HIV infection. Most of the published studies describe the association between serum levels of inflammatory proteins and CVR and / or HIV infection. Protein levels are the additive effect of the protein production in all tissues and may not accurately reflect the alteration in the individual cell or in a unique tissue. Moreover, the transcriptional involvement of blood cells in HIV-related inflammation is still uncertain. Analyzing gene expression levels in circulating cells may be more informative in HIV-infected individuals because $CD4^+$ T-cells constitute the HIV infection main target.

The aim of this study was to assess the implication of the gene expression of 9 relevant genes of the inflammatory pathway (*ALOX5, ALOX5AP, CCL5, CX3CR1, CXCL2, CXCL8, IL6, IL18* and *LTA*) in circulating peripheral blood mononuclear cells (PBMCs) from HIV-infected individuals with asymptomatic atherosclerosis (measured by cIMT and AP), traditional CVR factors and HIV characteristics. The possible relationship between gene expression and the SNPs related to cIMT or AP in the study 2 has also been tested.

3.2. Material and Methods

3.2.1. Sample Study Population

This was a cross-sectional study with 173 Spanish Caucasians HIV-infected individuals attended at Hospital Universitari MútuaTerrassa (Terrassa, Barcelona, Spain). The 173 HIV-infected individuals were selected from the Spanish Caucasian cohort analyzed in the study 2 (n=213) depending on PBMCs sample availability. At the time of enrolment, demographic, clinical, and biochemical variables were collected from each patient by interviews and from medical notes (Study 1). Simultaneously, ultrasonographic measures (cIMT and AP presence) were performed (described in the study 1).

Baseline characteristics are shown in Table 3.1. The median age was 45.17 years (IQR: 40.7 - 49.1). Males were 77.5 % of the sample population. The most prevalent traditional CVR factor was smoking (65.3 %). Although dyslipidemia prevalence was 28.9 % lipid profile was within normal values (triglycerides: 126.00 mg/dL (IQR: 90.6 - 171.5), total cholesterol: 178.20 mg/dL (IQR: 158.9 - 210.7), HDL cholesterol: 45.60 mg/dL (IQR: 36.4 - 56.4) and LDL cholesterol: 104.40 mg/dL (IQR: 85.1 - 135.3)).

The median time of known HIV infection was 11.08 years (IQR: 4.4 - 17.9). Patients under ART were the 82.1 %, of which 4.1 % were undergoing their first line of treatment. Patients who meet AIDS criteria represented the 38.2 % of patients and 21.4 % of them were in the CDC stage C of the disease. Detectable HIV viral load was found in 42.8 % of the patients, of which 58.1 % were not receiving treatment (n=18).

3.2.3. Gene Selection

Gene selection was based on the results from the single marker study (Study 2). Briefly, *ALOX5, ALOX5AP, CCL5, CRP, CX3CL1, CX3CR1, CXCL2, IL1RN* and *IL18* genetic variants were associated with AP and/or cIMT. The expression levels of these genes were measured by quantitative PCR (qPCR). *CRP, CX3CL1* and *IL1RN* gene expression was not detected in PBMC's samples in the preliminary experiments and were excluded from the study. Three additional inflammatory genes involved in CVD were selected: IL6 (124, 227, 228) and *LTA* (229-231) for its relation to MI and *CXCL8* for the relation described between its gene expression levels and coronary artery disease (232, 233).

Table 3.1. Baseline Characteristics of the sample population (Values are expressed as: Mean ± Standard Deviation, Median (Interquartile Range: 25 % - 75 %) or Frequency (%)).

Variable	**Population (n = 173)**
Age, years	45.2 (40.7 - 49.1)
Males, n	134 (77.5)
Body Mass Index, Kg / m^2	24.0 ± 3.72
Abdominal Obesity, n	21 (12.1)
Hypertension, n	16 (9.2)
Diabetes Mellitus, n	5 (2.9)
Dyslipidemia, n	50 (28.9)
Significant Alcohol Consumption, n	39 (22.5)
Smoking Habits	
Smokers, n	113 (65.3)
Non Smokers, n	58 (34.7)
Atherosclerosis Characteristics	
cIMT mean, mm	0.89 ± 0.21
Atherosclerotic Plaque Presence, n	66 (38.2)
Lipid profile	
Total Cholesterol, mg / dL	178.2 (158.9 - 210.7)
LDL Cholesterol, mg / dL	104.4 (85.1 - 135.3)
HDL Cholesterol, mg / dL	45.6 (36.4 - 56.4)
Triglycerides, mg / dL	126.0 (90.6 - 171.5)
HIV Characteristics	
Time of Known HIV-Infection, years	11.1 (4.4 - 17.9)
Antiretroviral Therapy, n	142 (82.1)
Previous Antiretroviral Therapies, n	135 (78.0)
CD4+ cell count, cells / L	532.0 (331.5 - 767.0)
CD4+ nadir cell count, cells / L	266.0 (143.0 - 453.0)
Detectable Viral Load (> 19 copies) / mL, n	74 (42.8)
Viral Load, copies / mL (only detectable)	2,170.0 (62.4 - 42,100.0)
CDC Stage	
A, n	118 (68.2)
B, n	12 (6.9)
C, n	37 (21.4)
AIDS Criteria, n	66 (38.2)
Risk Group	
Drug Users, n	63 (36.4)
Sexual Transmission, n	75 (43.4)
Others, n	6 (3.5)

*Body Mass Index (BMI): the individual's body mass divided by the square of their height.
†Abdominal Obesity: waist circumference > 102cm in men and > 88cm in women.
§Hypertension was: systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥90 mmHg and/or antihypertensive treatment.
||Diabetes Mellitus: fasting glucose levels ≥ 126 mg/dL, and/or having diabetes symptoms and glucose levels ≥ 200 mg/dL in a random determination and/or diabetes treatment.
#Dyslipidemia: Total Cholesterol ≥ 240mg/dL, and/or HDL Cholesterol ≤ 35 mg/dL, and/or Triglycerides ≥ 200 mg/dL and/or lipid lowering drugs.

3.2.4. Sample Processing

A whole blood sample from each participant was obtained by venopuncture at the time of enrolment. The sample was processed immediately to obtain PBMCs using the ficoll standard protocol (Ficoll-Paque ^{TM} PLUS - GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBMCs were stored in liquid nitrogen with Fetal Bovine Serum (FBS) and 10 % Dimethylsulfoxide (DMSO) until RNA extraction.

Total RNA was extracted from stored PBMC's using a commercial kit and following manufacturer's instructions (High Pure RNA Isolation kit - Roche, Mannheim, Germany). RNA was treated with DNAse-I (RQ1 RNase-Free DNase - Promega, Madison, WI) to avoid genomic DNA contamination and immediately retrotranscrived to cDNA (Transcriptor First Strand cDNA Synthesis Kit - Roche, Mannheim, Germany). cDNA amplification of the 9 selected genes was performed with SYBR Green (FastStart Universal SYBR Green Master (ROX) - Roche, Mannheim, Germany) using one set of primers for each gene (Primer sequences in Table 3.2) and an annealing temperature of 60ºC. Real-time PCR (rtPCR) was performed in a QuantStudio 12K Flex Real-Time PCR system (Life Technologies, Carlsbrad, CA). Amplicons were sequenced as a quality control of primer specificity. Ten randomly selected samples were checked for integrity on an Agilent Bioanalyzer (Life Technologies, Carlsbrad, CA). No contamination or degradation of RNA was detected.

Table 3.2. Primer Sequences (* Base Pairs (bp)).

Gene		Primer Sequence	Amplicon
ALOX5	Forward	5' - GCC ATC AGG ACG TTC ACG G - 3'	114 bp*
	Reverse	5' - CAT GCC GTA CAC GTA GAC ATC G - 3'	
ALOX5AP	Forward	5' - TGC AGC CAA GTT CCT GCT G - 3'	119 bp
	Reverse	5' - CGT TTC CCA AAT ATG TAG CCA GG - 3'	
CCL5	Forward	5' - CCT CCC CAT ATT CCT CGG AC - 3'	121 bp
	Reverse	5' - ACG ACT GCT GGG TTG GAG C - 3'	
CX3CR1	Forward	5' - CAC CAT GGA TCA GTT CCC TG - 3'	163 bp
	Reverse	5' - CAC TAC CAA CAA ATT TCC CAC C - 3'	
CXCL2	Forward	5' - GTG GTC AAC ATT TCT CAT GTT GAA G - 3'	146 bp
	Reverse	5' - GAA ACC TCT CTG CTC TAA CAC AGA GG - 3'	
CXCL8	Forward	5' - CTC TGT GTG AAG GTG CAG TTT TG - 3'	158 bp
	Reverse	5' - CAG AAA GCT TTA CAA TAA TTT CTG TGT TG - 3'	
IL6	Forward	5' - GAT TCA ATG AGG AGA CTT GCC TG - 3'	145 bp
	Reverse	5' - GGA TCA GGA CTT TTG TAC TCA TCT GC - 3'	
IL18	Forward	5' - GAC TGA TTC TGA CTG TAG AGA TAA TGC AC - 3'	154 bp
	Reverse	5' - CAG GAG GAT TCA TTT CCT TAA AGG - 3'	
LTA	Forward	5' - GCC CAG GGG CTC CCT - 3'	122 bp
	Reverse	5' - CTG GGG TCT CCA ATG AGG TG - 3'	
ACTB	Forward	5' - CCA ACC GCG AGA AGA TGA - 3'	97 bp
	Reverse	5' - CCA GAG GCG TAC AGG GAT AG - 3'	
GAPDH	Forward	5' - AGC CAC ATC GCT CAG ACA C - 3'	66 bp
	Reverse	5' - GCC CAA TAC GAC CAA ATC C - 3'	

3.2.5. Quantitative PCR Analyses

Quantitative real-time PCR reactions were performed in triplicate to reduce variability. ExpressionSuite Software version 1.0.2, (Life Technologies, Carlsbad, CA) was used for melting curve quality control. The Ct value mean was calculated for each sample and transcript (for triplicates, SD < 0.2 in all cases). Relative gene expression was calculated using the ΔΔCt method with *ACTB* or *GAPDH* (Table 3.2) as housekeeping genes. ΔΔCt was elevated to the power of 2 to obtain the Relative Quantity value (RQ).

3.2.6. Statistical Analyses

Statistical analyses were performed using the statistical packages G*Power Calculator (204), SPSS version 20 (IBM, Chicago, IL) and Plink version 1.07 (205). The sample had > 95% statistical power (considering $\alpha = 0.05$, two-sided) to capture the effect of variations in mRNA levels (variance = 0.042; standard error = 0.016) on cIMT values and AP presence. Mann-Whitney U, Kruskal-Wallis and Spearman correlation tests were performed to investigate the possible association between relative gene expression values and the dependent variables (AP presence, cIMT mean, traditional CVR factors, and HIV-infection characteristics). Linear regression analyses were performed to investigate the possible influence of SNPs on their gene expression.

3.3. Results and Discussion

This study investigated the possible association between gene expression levels of 9 genes related to inflammation (*ALOX5, ALOX5AP, CCL5, CX3CR1, CXCL2, CXCL8, IL6, IL18* and *LTA*) with CVR factors and HIV characteristics in 173 Spanish HIV-infected subjects. The results are summarized in Table 3.3 and Figure 3.1. The possible association between the genetic variants found associated with cIMT or AP (Study 2) and gene expression levels were also tested.

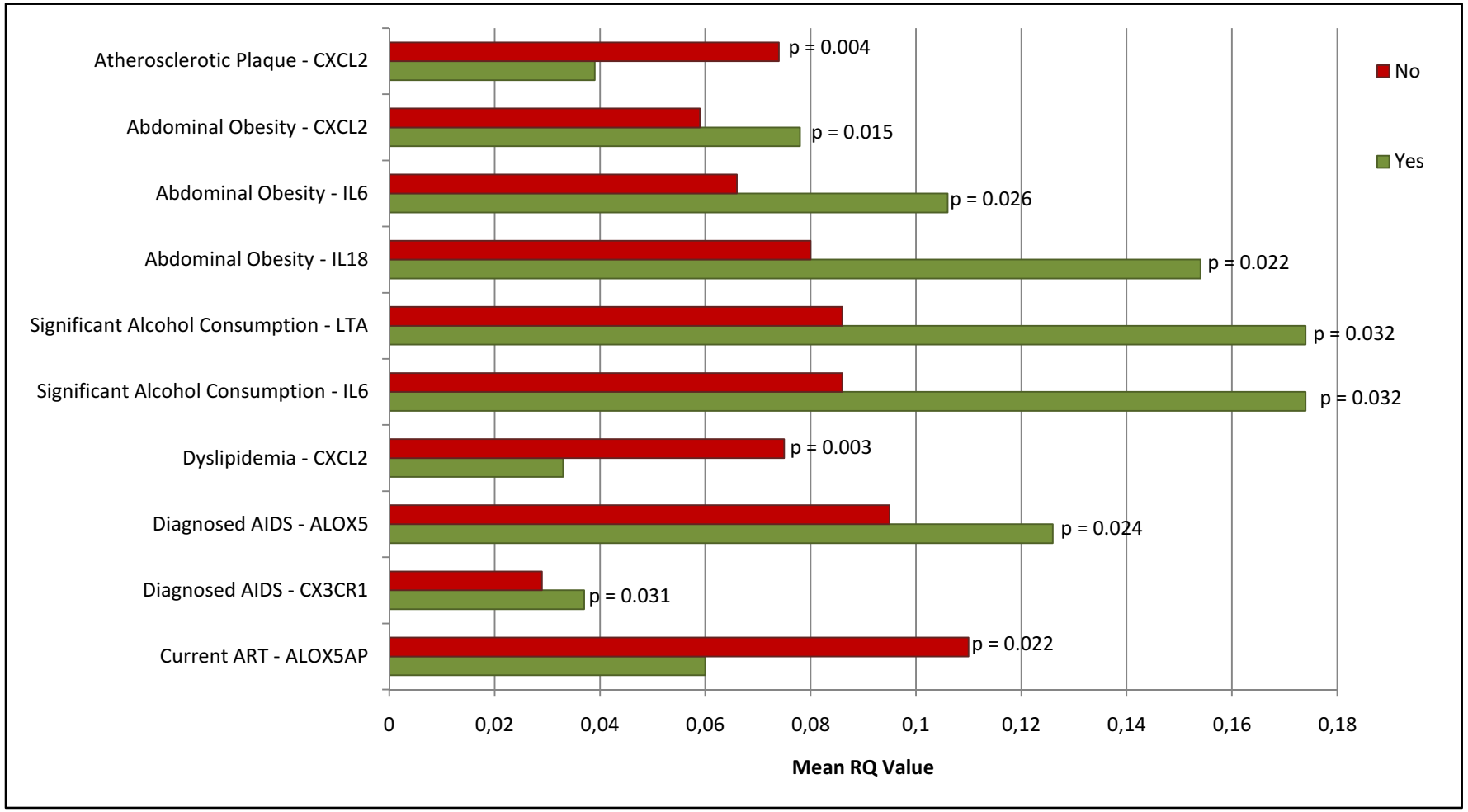
Table 3.3. Summary of gene expression analyses results.

VARIABLE	GENE	Categorical Variables				Continuous Variables	
		Mean RQ		*p* value	FC*	*p* value	CC**
		Yes	No				
Atherosclerotic Plaque Presence	*CXCL2*	0.039	0.074	0.004	1.90	-	-
cIMT	*CXCL2*	-	-	-	-	< 0.001	- 0.362
	CXCL8	-	-	-	-	< 0.001	- 0.373
	IL6	-	-	-	-	< 0.001	- 0.303
	CCL5	-	-	-	-	0.014	- 0.189
Abdominal Obesity	*CXCL2*	0.078	0.059	0.015	1.32	-	-
	IL6	0.106	0.066	0.026	1.61	-	-
	IL18	0.154	0.080	0.022	1.93	-	-
Alcohol Consumption	*LTA*	0.174	0.086	0.032	2.02	-	-
	IL6	0.174	0.086	0.032	2.02	-	-
Body Mass Index (BMI)	*IL18*	-	-	-	-	0.021	0.182
Dyslipidemia	*CXCL2*	0.033	0.075	0.003	2.27	-	-
HIV Viral Load	*IL18*	-	-	-	-	0.034	0.165
Time Infected	*IL18*	-	-	-	-	0.049	- 0.161
Diagnosed AIDS	*ALOX5*	0.126	0.095	0.024	1.33	-	-
	CX3CR1	0.037	0.029	0.031	1.28	-	-
Current ART	*ALOX5AP*	0.060	0.110	0.022	1.83	-	-

* Fold Change.

** Correlation Coefficient.

Figure 3.1. Summary of the results of gene expression analyses. The graphic shows the relative expression quantities within categorical variables and their significance p values.



The *CX3CR1* rs2669850 variant influenced *CX3CR1* gene expression levels (Table 3.4 and Figure 3.20). The figures show the mean value of gene expression for each categorical variable (RQ) and the *p* value and Fold Change (FC) between categories.

Variations in gene expression levels were found associated with AP (*CXCL2*) and cIMT (*CXCL2, CXCL8, IL6* and *CCL5*). Regarding CVR traditional factors, *CXCL2, IL6* and *IL18* gene expression levels were found associated with abdominal obesity, *LTA* and *IL6* expression levels with alcohol consumption, *IL18* levels with BMI, and *CXCL2* levels with dyslipidemia. Finally, regarding HIV-infection characteristics, *IL18* gene expression levels were found correlated with HIV viral load and time of known HIV infection. *ALOX5* and *CX3CR1* gene expression levels were found associated with meeting AIDS criteria. ALOX5AP gene expression levels were found associated with current ART.

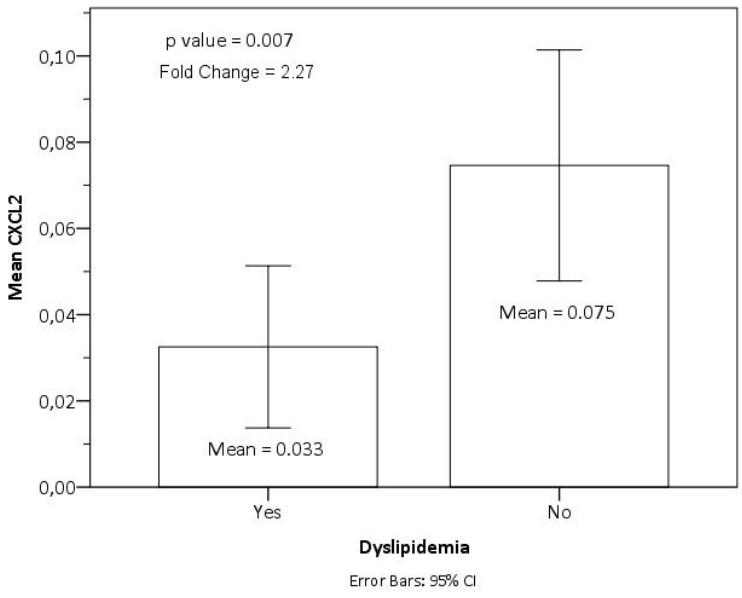


Figure 3.2. Graphic representation of *CXCL2* gene expression values (RQ) versus dyslipidemia.

The CXCL2 protein is stimulated by 5-LO pathway products promoting hyperlipidemia (234). However, 5-LO pathway is known to be functionally reduced in HIV-infected individuals (160, 235). Increased *CXCL2* gene expression was found associated with dyslipidemia absence (p = 0.003; FC = 2.27) (Figure 3.2) and increased *ALOX5* gene expression levels related to patients who meet AIDS criteria (p = 0.024, FC = 1.33) (Figure 3.3). Interestingly, *CXCL2* and *ALOX5* gene expression levels were correlated (p < 0.001, Correlation Coefficient (CC) = 0.32) (Figure 3.4). *ALOX5* mRNA production may be increased to compensate for the decreased functionality caused by the virus (160, 235) and stimulate *CXCL2* gene expression. This may explain the relation found between increased *CXCL2* gene expression and dyslipidemia absence. Although no correlation between lipid levels and *ALOX5* or *CXCL2* mRNA levels were found, this pathway is relevant in lipid metabolism and atherosclerosis (236). The association found between *ALOX5AP* gene expression levels and ART (p = 0.022, FC = 1.83) (Figure 3.5) may indicate its involvement in the lipid changes described for several ART drugs. Inhibitors of the 5-LO pathway are being developed to treat dyslipidemia in the general population (237). Its inhibition may be an alternative treatment to HIV- and ART-related lipid alterations. Their use could potentially avoid previously described interactions with lipid lowering drugs (238). Clinical trials testing the therapeutic effect of 5-LO inhibitors in HIV-infected populations may be of high interest.

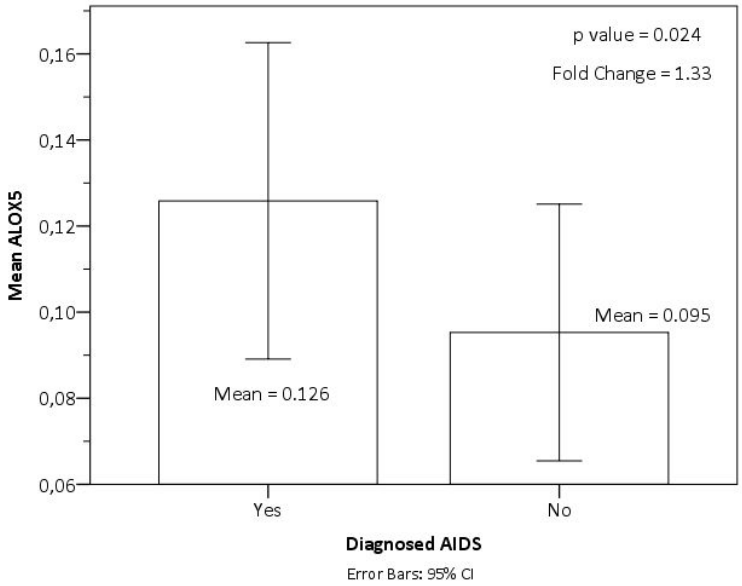


Figure 3.3. Graphic representation of *ALOX5* gene expression values (RQ) versus patients who meet AIDS criteria.

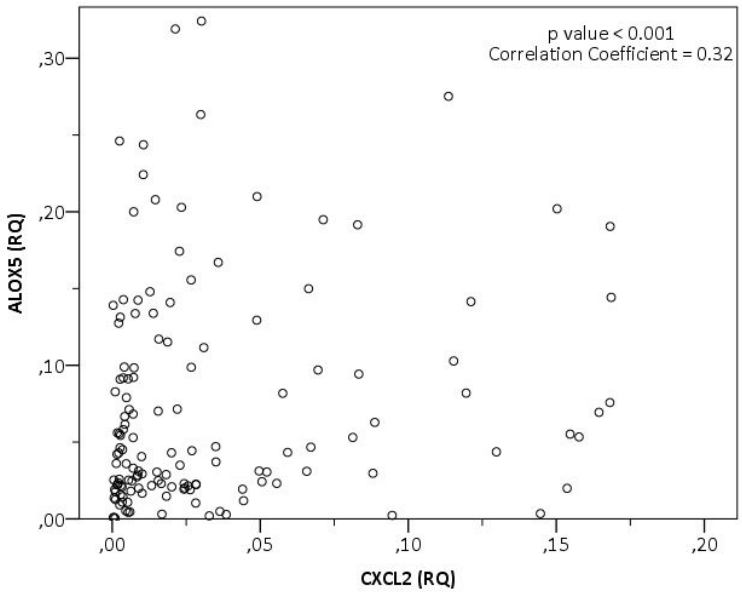


Figure 3.4. Graphic representation of the correlation between *CXCL2* and *ALOX5* gene expression values (RQ).

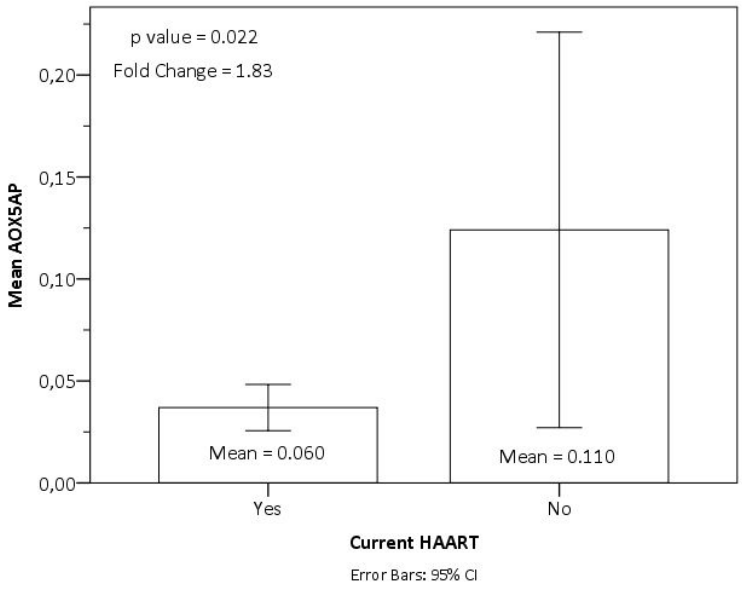


Figure 3.5. Graphic representation of *ALOX5AP* gene expression values (RQ) versus patients who are under current ART.

Increased *CXCL2* gene expression levels were found associated with abdominal obesity presence (p = 0.015, FC = 1.32) (Figure 3.6). Increased protein levels of CXCL2 and abdominal obesity were previously described in the general population (239). This finding together with the one described in this study suggests that CXCL2 protein involvement in abdominal obesity is also applicable to HIV-infected individuals. Furthermore, the source of CXCL2 protein might be PBMCs in both HIV-infected and uninfected individuals. Although no associations between CXCL2 gene expression and HIV-related clinical variables were found in this study, HIV-infection increases CXCL2 protein levels (240, 241). Increased *CXCL2* gene expression levels seemed to be atheroprotective in HIV-related subclinical atherosclerosis (AP (p = 0.004, FC = 1.90) (Figure 3.7), smaller cIMT (p < 0.001, CC = -0.362)) (Figure 3.8). However CXCL2 protein levels were not found associated with AP or cIMT in the general population (242). Although the associations of *CXCL2* gene expression with AP and/or cIMT are

novel findings, they may be exclusive to HIV-infected population due to HIV-related cellular alterations such as the one described for 5-LO pathway. This study showed protective effects of increased *CXCL2* gene expression levels in HIV-related subclinical atherosclerosis. However increased *CXCL2* gene expression levels were also associated with abdominal obesity, a known CVR factor. A replication study with a larger sample population may clarify the role of *CXCL2* in HIV-associated atherosclerosis.

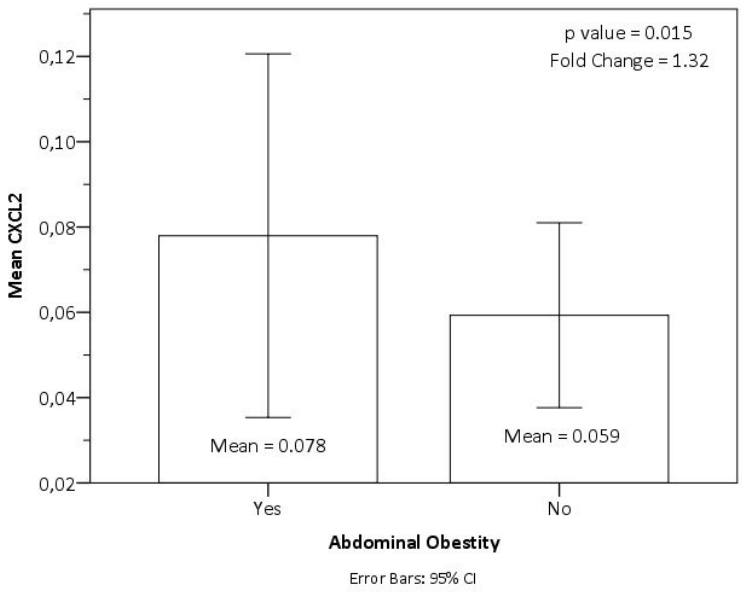


Figure 3.6. Graphic representation of *CXCL2* gene expression values (RQ) versus abdominal obesity.

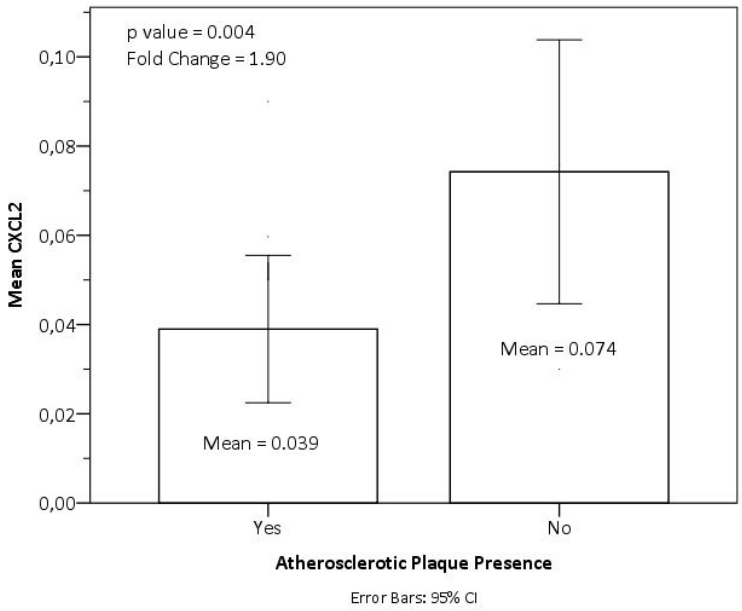


Figure 3.7. Graphic representation of *CXCL2* gene expression values (RQ) versus AP.

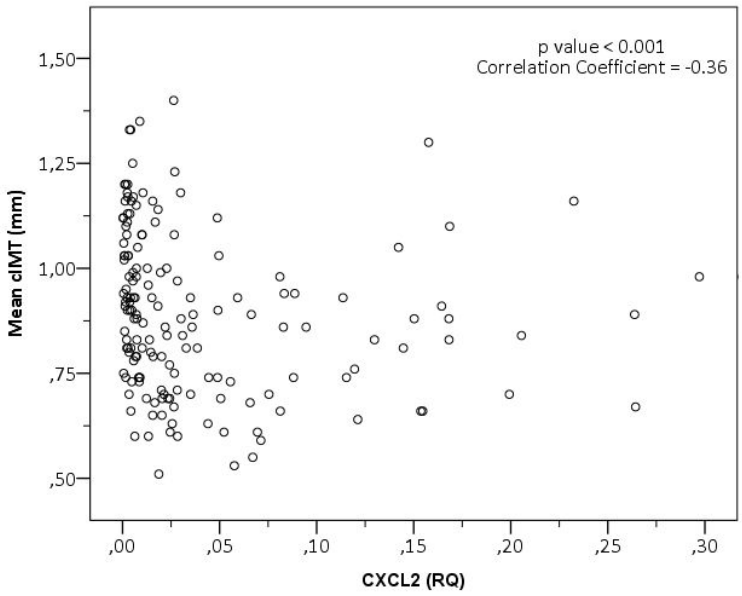


Figure 3.8. Graphic representation of the correlation between *CXCL2* gene expression (RQ) and mean cIMT values.

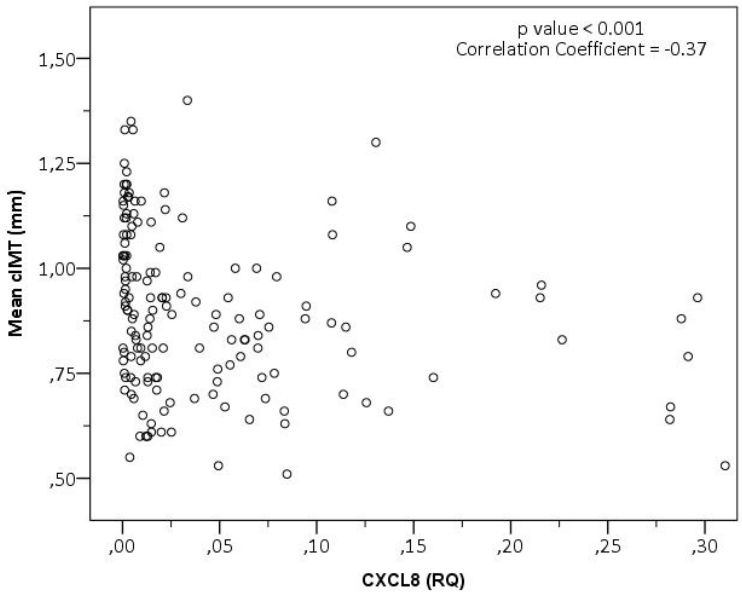


Figure 3.9. Graphic representation of the correlation between *CXCL8* gene expression (RQ) and mean cIMT values.

Increased *CXCL8, IL6,* and *CCL5* gene expression levels showed association with smaller cIMT ($p < 0.001$, CC = -0.373 - Figure 3.9; $p < 0.001$, CC = -0.303 - Figure 3.10 and $p = 0.014$, CC = -0.189 - Figure 3.11 respectively). Although these results showed protective effects on cIMT in HIV-infected patients, increases in CXCL8, IL6 and CCL5 protein levels have been associated with CVR in the general population. Increased serum levels of CXCL8 and IL6 proteins have been related to atherosclerosis and cardiovascular events in uninfected individuals (227, 228, 232, 243-245). Moreover, Increased serum levels of CXCL8 and IL6 proteins have been described in HIV infection (121, 246, 247) but not consistently associated with HIV-related and unrelated atherosclerosis (15, 120, 232). Notwithstanding, only IL6 protein levels have been related to all-cause mortality in HIV-infected (248) and uninfected individuals (124). *CCL5* has been found expressed in human atherosclerotic plaques (194) and elevated in relation to atherosclerosis (211, 249). Studies with atherosclerosis mice models ($ApoE^{-/-}$) showed that knocking down *CCL5* reduced neointimal thickening (250). The results of the

present study were different from the ones described previously. The alterations of cytokine levels described in HIV infection (223) may mislead the importance and direction of the findings described in this study and justify the different findings. Studies with higher statistical power are required to elucidate the role of *CXCL8, IL6*, and *CCL5* gene expression levels in HIV-related atherosclerosis.

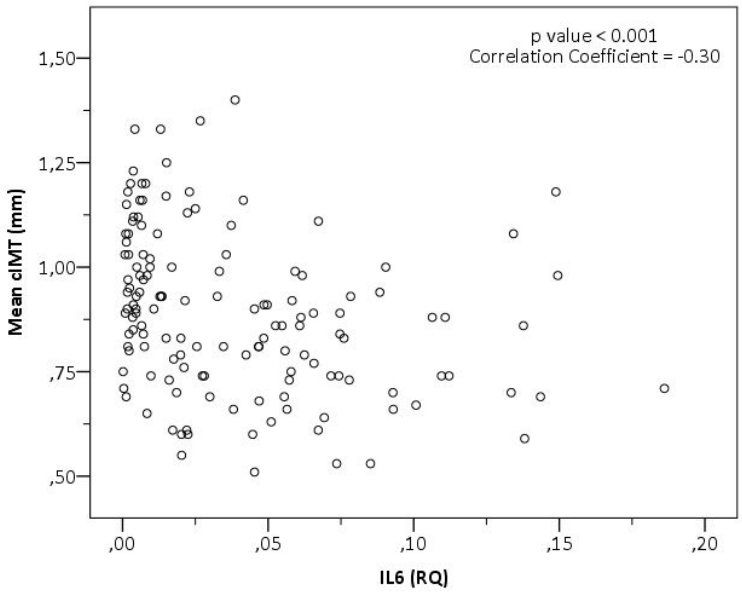


Figure 3.10. Graphic representation of the correlation between *IL6* gene expression (RQ) and mean cIMT values.

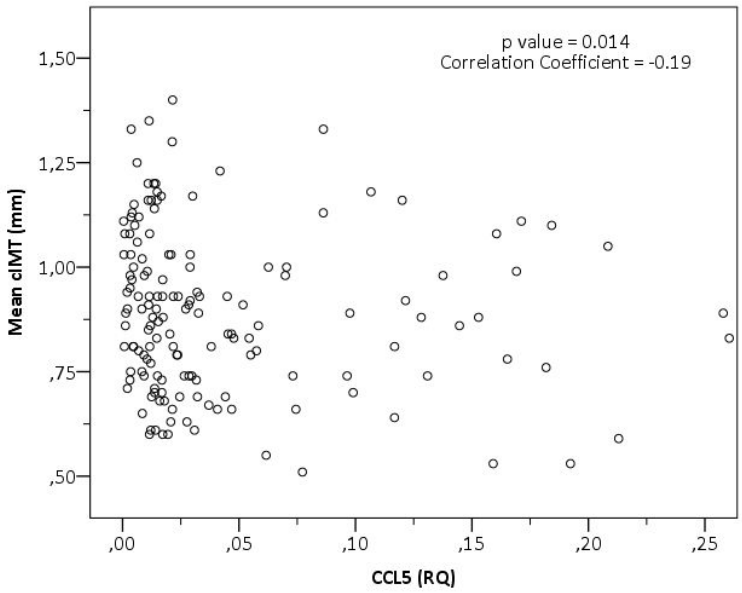


Figure 3.11. Graphic representation of the correlation between *CCL5* gene expression (RQ) and mean cIMT values.

Increased *LTA*, *IL6, IL18* gene expression levels were found associated CVR traditional factors such as alcohol consumption (p = 0.032, FC = 2.02 for both *LTA* and *IL6* - Figures 3.12 and 3.13 respectively), BMI (p = 0.021, CC = 0.182 for *IL18* - Figure 3.14) and abdominal obesity presence (p = 0.026, FC = 1.61 for *IL6*; and p = 0.022, FC = 1.93 for *IL18* respectively - Figures 3.15 and 3.16 respectively). Increased IL6 and IL18 protein levels have been previously associated with abdominal adiposity or obesity (251-253) and increased IL6 levels with alcohol consumption (254, 255). This suggests that the observations regarding CVR factors in non-HIV studies may be also applicable to HIV-infected individuals. However, the association between *LTA* gene expression levels and alcohol consumption is a new association. It has been suggested that alcohol consumption triggers the inflammatory pathway through Toll Like Receptors (TLRs) (256). The ethanol-dependent production of pro-inflammatory cytokines such as LTA and IL6 may explain these findings.

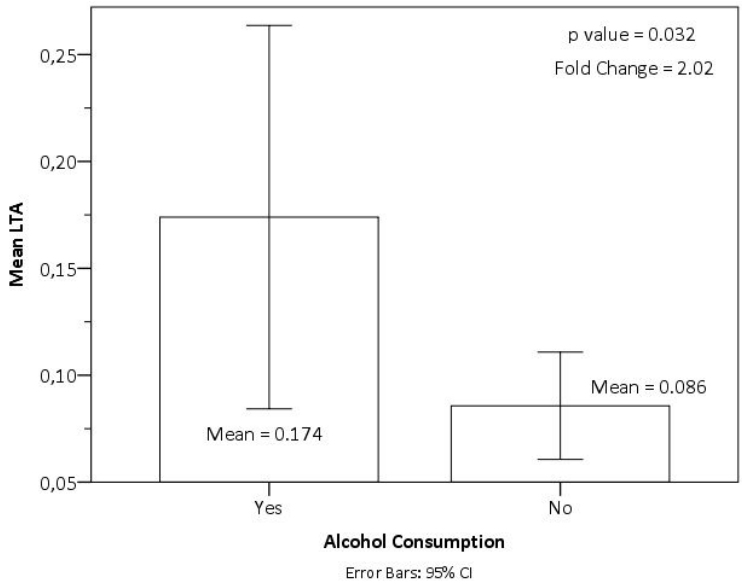


Figure 3.12. Graphic representation of *LTA* gene expression values (RQ) versus alcohol consumption.

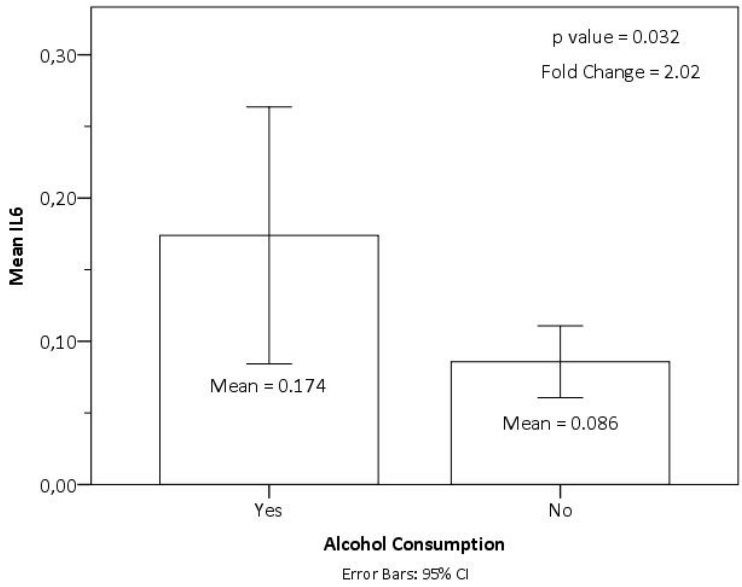


Figure 3.13. Graphic representation of *IL6* gene expression values (RQ) versus alcohol consumption.

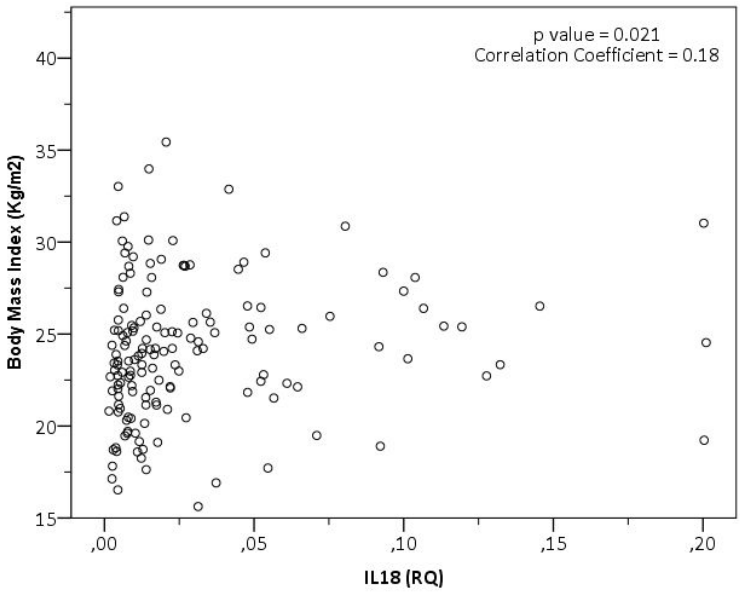


Figure 3.14. Graphic representation of the correlation between *IL18* gene expression (RQ) and Body Mass Index (BMI) values.

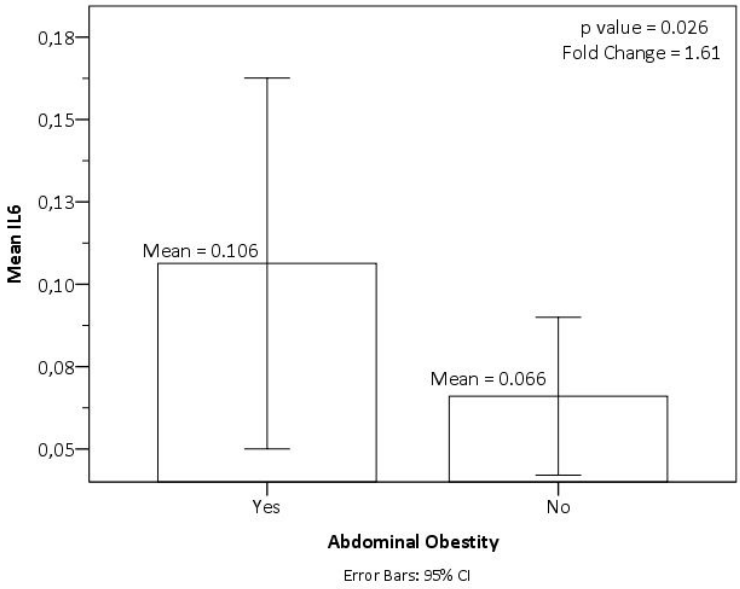


Figure 3.15. Graphic representation of *IL6* gene expression values (RQ) versus abdominal obesity.

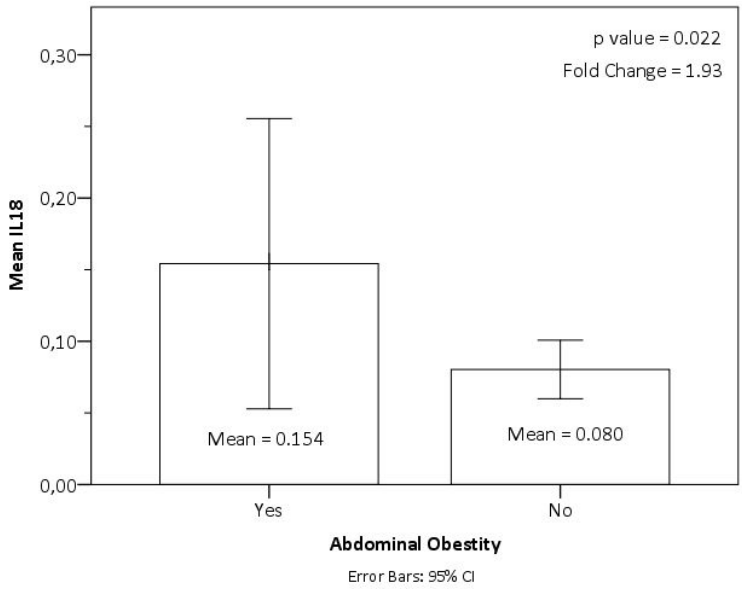


Figure 3.16. Graphic representation of *IL18* gene expression values (RQ) versus abdominal obesity.

Although *IL18* gene expression levels have been related to subclinical atherosclerosis in HIV-infected and uninfected patients (257, 258), no association was found in the present study. However, increased *IL18* gene expression levels were found correlated with HIV viral load (p = 0.034, CC = 0.165) (Figure 3.17) as previously described (259). Increased serum levels of IL18 protein have been described in HIV-infected individuals and related to progression to AIDS (259-261). A marginal association was found between decreased *IL18* gene expression levels and longer known HIV infection (p = 0.049, CC = -0.161) (Figure 3.18). The elevated prevalence of ART treated patients (81.2%) in this cohort and the reduction of IL18 protein levels by ART (260, 262) may explain the differences in *IL18* gene expression findings. The non involvement of PBMCs in HIV-associated IL18 protein production (261) may also explain these different findings.

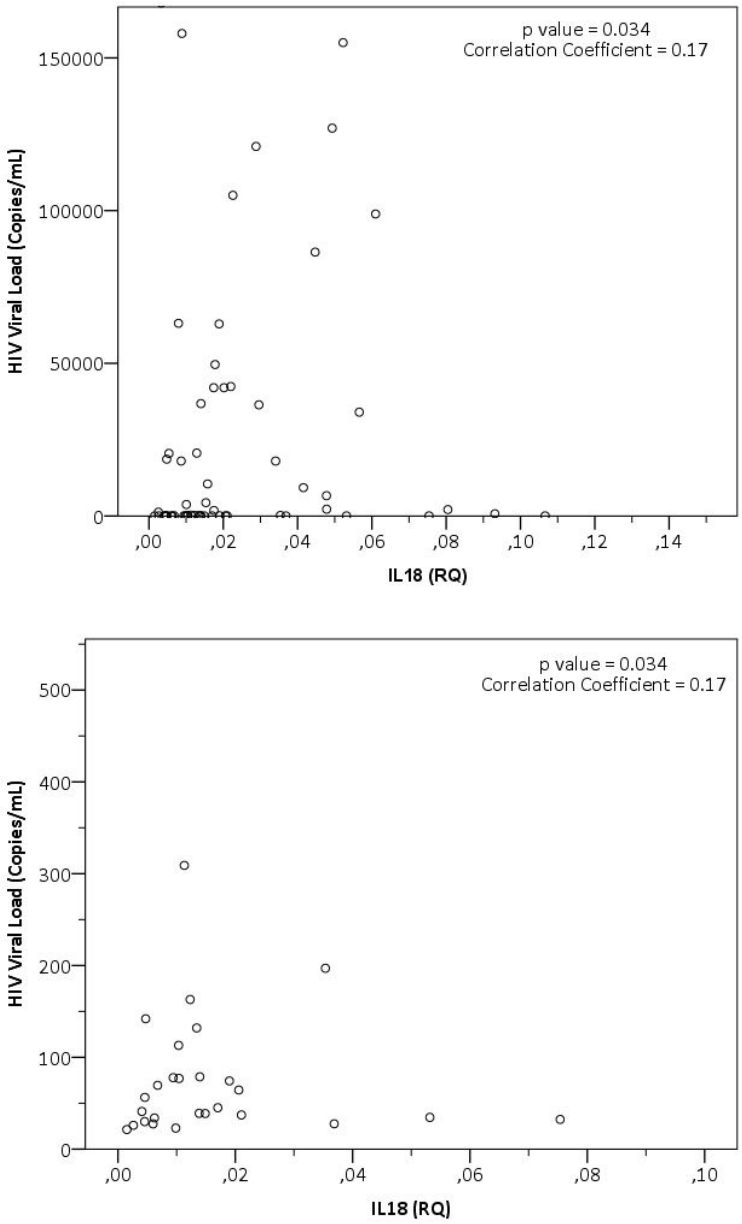


Figure 3.17. Graphic representation of *IL18* gene expression values (RQ) versus viral load. The graphic only show patients with detectable viral load. Two scales have been used for a better visualization of the data.

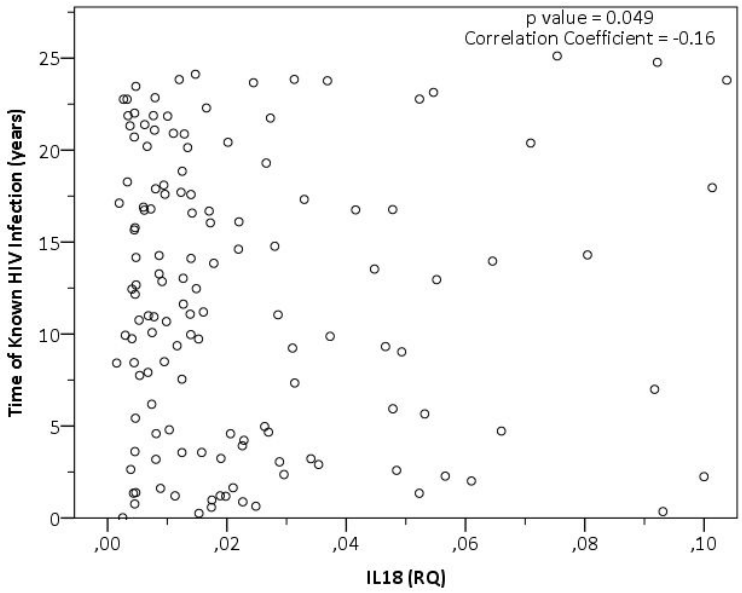


Figure 3.18. Graphic representation of *IL18* gene expression values (RQ) versus time of known HIV infection.

CX3CR1 genetic variants (rs3732378 and rs3732379) have been associated with faster progression to AIDS (218, 219). Increased *CX3CR1* expression levels were found associated with patients who meet AIDS criteria (p = 0.031, FC = 1.28) (Figure 3.19). Although the functionality of these SNPs is not known, and not all studies have found them associated with disease progression, they may be linked to causal variants. The association between *CX3CR1* gene expression levels and patients who meet AIDS criteria may explain the biological consequences of *CX3CR1* genetic variants. Although HIV infection alters *CX3CR1* gene expression (224), its levels are restored to normal after ART introduction (224). However, no association was found between *CX3CR1* gene expression and receiving ART.

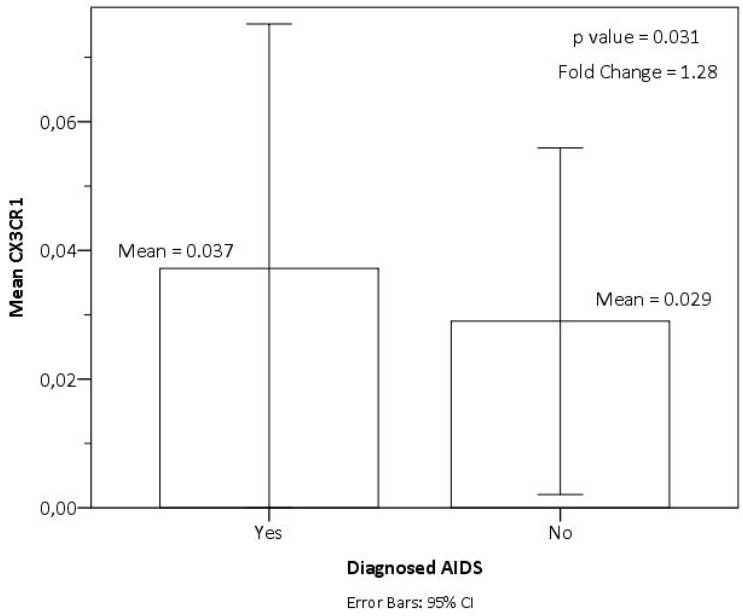


Figure 3.19. Graphic representation of *CX3CR1* gene expression values (RQ) versus patients who meet AIDS criteria.

The *CX3CR1* rs2669850-C allele was found associated with smaller cIMT in the study 2 (p=0.024). However, it may be a false positive result because the association did not remain statistically significant in the multivariate analyses when clinical adjusting variables were included in the model. The *CX3CR1* rs2669850-C allele was found associated with increased *CX3CR1* gene expression (Table 3.4 and Figure 3.20). Reduced expression or lack of CX3CR1 is associated with reduced lesion formation (263, 264) whereas high CX3CR1 levels are deleterious for cIMT (92, 263). We expected to find reduced *CX3CR1* gene expression levels for the rs2669850-C allele. However the opposite effect was observed, suggesting that this may be a false positive result. Although this SNP is not associated with HIV-related atherosclerosis, it may be linked to AIDS progression.

Table 3.4. Association between *CX3CR1* rs2669850 (minor allele C - *maf* = 0.47) and *CX3CR1* gene expression values.

Analysis	*p* value	β	CI 95%
Additive (Allele)	0028	0.035	0.004 - 0.066
Genotype	ns	-	-
C Dominant	ns	-	-
C Recessive	0.040	0.055	0.003 - 0.107

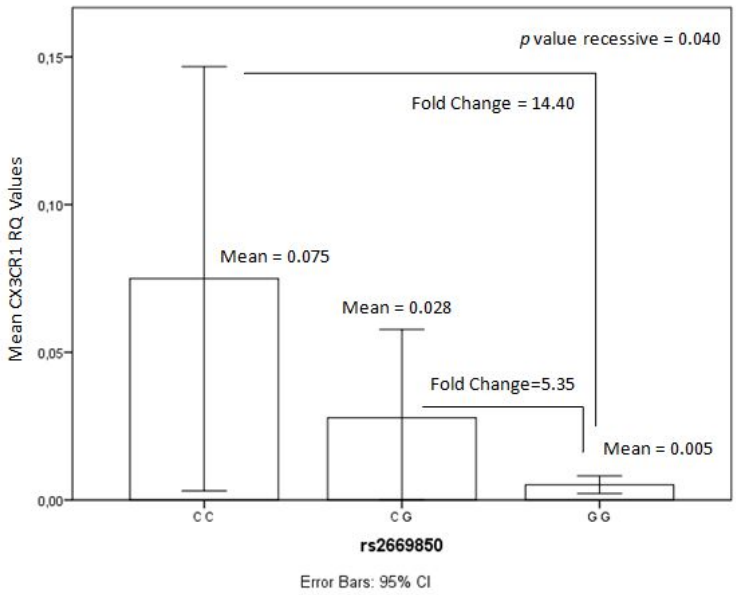


Figure 3.20. Graphic representation of *CX3CR1* gene expression values (RQ) versus rs2669850 genotypes.

In summary, altered cytokines levels were found associated with HIV- and CVR-related clinical variables. However, the relation between gene expression levels and AP or cIMT was modest. Interestingly, *CXCL2* gene expression levels were related to AP, cIMT and CVR variables, whereas *ALOX5* and *ALOX5AP* were associated with HIV-related variables. These three genes are part of the 5-LO pathway and may interact among themselves influencing both pathologies. Although the 5-LO pathway has been studied regarding CVD or HIV-infection, this study has related 5-LO pathway to HIV-related atherosclerosis for the first time.

4. STUDY IV:

Influence of ART in Gene Expression

4.1. Background

The use of ART has transformed HIV infection to a chronic long-term disease (9). However, HIV infection co-morbidities and ART related toxicity remain a matter of high concern (4, 101, 123). HIV infection alters the cytokine network from the earliest stages to the chronic infection (12, 52). After introduction of ART cytokine levels are restored, but not to those levels observed in non-HIV infected individuals (12). In the previous study reduced *ALOX5AP* and increased *IL18* gene expression levels were found associated with current ART and HIV viral load respectively.

IL18 is a pro-inflammatory cytokine produced by macrophages and other cells. It binds its receptor, located on T and NK cells, and induces cell mediated immunity. Persistent IL18 protein elevation has been described and linked with AIDS progression in HIV-infected subjects (259-262, 265), especially among those untreated (259, 260). However, IL18 serum protein levels are reduced after ART introduction (260, 262). Moreover, virologic treatment failure was associated with raised IL18 serum protein levels (260). IL18 has shown proviral activities in both maintaining and worsening HIV infection during different stages of the disease (266). It has been demonstrated that IL18 stimulates HIV replication *in vitro* (267).

Arachidonic acid metabolites are synthesized through the 5-LO pathway. The metabolites are potent lipid mediators of inflammatory reactions (236). They have been implicated in many inflammatory and allergic disorders such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, bronchial asthma and atherosclerosis (236). Alterations in the 5-LO pathway have been investigated and proposed to have a role in HIV pathogenesis (160, 161). HIV coat glycoprotein gp120 seems to activate the 5-LO pathway (268-270).

However, decreased activity of this pathway has been described in HIV-infected subjects (160, 235).

The aim of this study was to assess the variations of mRNA levels of *IL18* and *ALOX5AP* in treated or untreated HIV infected patients compared with those non-infected.

4.2. Material and Methods

4.2.1. Sample Study Population

This was a cross-sectional study with 173 (142 treated, 31 untreated) HIV-infected individuals and 19 uninfected Spanish Caucasians attended in Hospital Universitari MútuaTerrassa (Terrassa, Barcelona, Spain).

Baseline characteristics are shown in Table 4.1. The HIV-infected population median age was 45.2 years (IQR: 40.7 - 49.1). Males were 77.5 % of the population. Regarding HIV characteristics, the median time of known HIV infection was 11.1 years. The cohort was highly experienced; 82.1 % of patients were currently receiving ART, but only 4.1 % were on first line of ART. NRTIs were the most used drug types (95.1 %), followed by NNRTIs (59.9 %) and PIs (34 %). Patients who meet AIDS criteria were 38.2 %, whereas the CDC C stage prevalence was 21.4 %. A detectable HIV viral load was found in 42.8 % of patients; of them, 58.1 % were not under ART. The control group median age was 44.7 years (IQR: 33.5 - 51.7) and 25 % were males.

4.2.2. Sample Processing

Blood was obtained at the time of enrolment by venopuncture. PBMC's were obtained immediately using ficoll standard protocol (Ficoll-Paque ^{TM} PLUS - GE Healthcare Bio-Sciences AB, Uppsala, Sweden). They were stored in liquid nitrogen with FBS and 10 % DMSO until RNA extraction was performed.

Total RNA was extracted from stored PBMC's (High Pure RNA Isolation kit - Roche, Mannheim, Germany) and immediately treated with DNAse I (RQ1 RNase-Free DNase - Promega, Madison, WI) and retrotranscrived to cDNA (Transcriptor First Strand cDNA Synthesis Kit - Roche, Mennheim, Germany). The amplification was performed using one set of primers (Table 4.2) for each gene with SYBR Green (FastStart Universal SYBR Green Master (ROX) - Roche, Mannheim, Germany). SYBR Green's manufacturer's protocol was used with 60ºC as annealing temperature in a QuantStudio 12K Flex (Life Technologies, Calsbrad, CA). All amplicons were sequenced as specificity quality control. Ten randomly selected samples were checked for integrity on an Agilent Bioanalyzer (Life Technologies, Carlsbrad, CA). No contamination or degradation of RNA was detected.

4.2.3. Quantitative PCR Analyses

Quantitative real-time PCR (qPCR) reactions were performed in triplicate for each gene and sample. Melting curve quality control was performed using ExpressionSuite Software version 1.0.2 (Life Technologies, Carlsbad, CA).

Ct values for each gene and patient were calculated using the median of the 3 Ct values (SD < 0.2 in all cases). Relative gene expression was calculated using ΔΔCt method with ACTB as housekeeping gene. ΔΔCt values were elevated to the power of 2 to obtain the RQ.

Table 4.1. Baseline Characteristics of the sample population (Values are expressed as: Mean ± Standard Deviation, Median (Interquartile Range: 25 % - 75 %) or Frequency (%)).

Variable	HIV-infected	Uninfected
Age, years	45.2 (40.7 - 49.1)	44.7 (33.5 - 51.7)
Males, n	134 (77.5)	5 (25)
HIV Characteristics		
Time of Known HIV Infection, years	11.1 (4.4 - 17.9)	-
Hepatitis C Coinfection, n	81 (46.8)	-
Antiretroviral Therapy, n	142 (82.1)	-
NRTIs, n	135 (95.1)	-
NNRTIs, n	85 (59.9)	-
IP, n	48 (34.0)	-
Entry Blockers, n	2 (1.4)	-
Integrase Inhibitors, n	8 (5.7)	-
Previous Antiretroviral Therapies, n	135 (78.0)	-
CD4+ cell count, cells/ L	532.0 (331.5 - 767.0)	-
CD4+ nadir cell count, cells/ L	266.0 (143.0 - 453.0)	-
Viral Load > 19 copies/ml, n	74 (42.8)	-
Viral Load, copies/mL (only detectable)	2,170.0 (62.4 - 42,100.0)	-
CDC Stage		
A, n	118 (68.2)	-
B, n	12 (6.9)	-
C, n	37 (21.4)	-
AIDS criteria, n	66 (38.2)	
Risk Group		

Variable	HIV-infected	Uninfected
Drug Users, n	63 (36.4)	-
Sexual Transmission, n	75 (43.4)	-
Others, n	6 (3.5)	-

Table 4.2. Primer Sequences.

Gene		Primer Sequence	Amplicon length
ALOX5AP	Forward	5' - TGC AGC CAA GTT CCT GCT G - 3'	119 bp
	Reverse	5' - CGT TTC CCA AAT ATG TAG CCA GG - 3'	
IL18	Forward	5' - GAC TGA TTC TGA CTG TAG AGA TAA TGC AC - 3'	154 bp
	Reverse	5' - CAG GAG GAT TCA TTT CCT TAA AGG - 3'	
ACTB	Forward	5' - CCA ACC GCG AGA AGA TGA - 3'	97 bp
	Reverse	5' - CCA GAG GCG TAC AGG GAT AG - 3'	

Table 4.3. RQ values analyses results among treated, untreated and uninfected.

GENE	Mean RQ			Untreated vs Treated		Treated vs non-Infected		Kruskal-Wallis
	Untreated	Treated	Non-Infected	*p* value	Fold Change	*p* value	Fold Change	*p* value
ALOX5AP	0.11	0.06	0.02	0.022	1.83	ns	-	<0.0001
IL18	0.15	0.08	0.02	ns	-	<0.0001	4.19	<0.0001

4.2.4. Statistical Analyses

Statistical analyses were performed using G*Power Calculator (204) and SPSS version 20 (IBM, Chicago, IL). The study had more than 95 % power (considering $\alpha = 0.05$, two-sided) to capture the effect of variations in mRNA levels (variance = 0.042; standard error = 0.016). Mann-Whitney U and Kruskal-Wallis tests were used to compare RQ values with the dependent variables ART treatment (yes / no) and drug class.

4.3. Results and Discussion

This study tested the possible association between *IL18* and *ALOX5AP* mRNA variations and ART treatment in 173 HIV-infected individuals and 19 non-HIV infected subjects. Increased *ALOX5AP* and *IL18* mRNA levels were observed when comparing treated and untreated HIV-infected individuals, although only *ALOX5AP* mRNA levels reached statistically significant values. *IL18* and *ALOX5AP* mRNA levels were lower in uninfected controls compared with HIV-treated subjects. However, only *IL18* mRNA levels reached statistically significant values.

IL18 RQ levels were significantly lower in controls ($p < 0.0001$, FC = 4.19) (Table 4.3 and Figure 4.1) compared with treated HIV-infected subjects. However, the comparison between *IL18* RQ values between treated and untreated HIV-infected individuals did not reach statistically significant values, probably due to the small study sample. Nevertheless, Kruskal-Wallis test showed statistically significant differences when comparing treated, untreated and non-infected individuals ($p < 0.0001$) (Table 4.3, Figure 4.1).

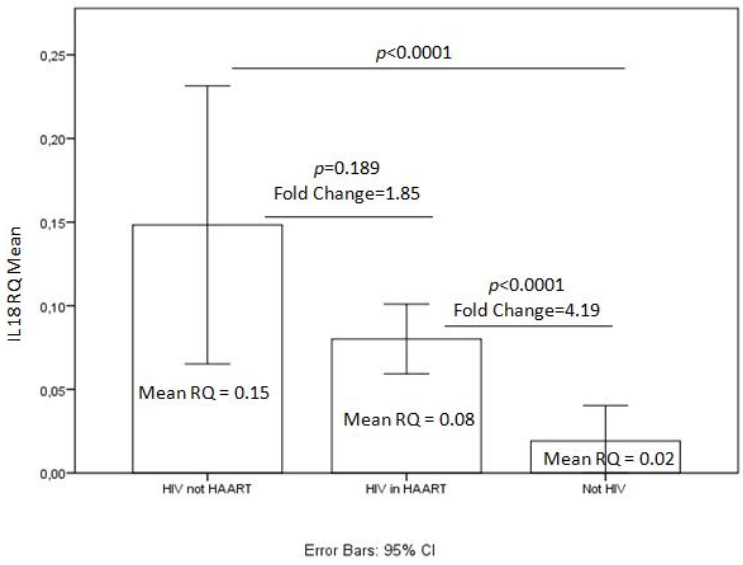


Figure 4.1. IL18 RQ mean values vs HIV treated, HIV untreated and uninfected.

Increased serum levels of IL18 protein have been widely described in HIV-infected subjects (259-261, 265) and correlated with HIV viral load (259, 261) (study 3). IL18 protein level reduction has been described in the serum of ART-treated HIV-infected subjects although not to the levels observed in non-infected individuals (259-261, 265). Ahmad et al described the non involvement of PBMC's in IL18 protein production (261). However the present study demonstrated that *IL18* mRNA is elevated in PBMC's *in vivo*. PBMC's may be the source of the increased IL18 protein in serum although the sources of IL18 protein may be many and increased *IL18* mRNA levels in PBMCs may not reflect the levels of protein in serum. Elevated serum levels of IL-18 may have an important role in non-HIV co-morbidities and in AIDS due to its pivotal role in the Th1 / Th2 immune response balance and it is association to AIDS progression.

Increased *ALOX5AP* gene expression values were observed when comparing HIV untreated and HIV treated subjects (p = 0.022, FC = 1.83) (Table 4.3 and Figure 4.1). Although the differences in *ALOX5AP* gene expression values of HIV-infected individuals receiving ART and non-infected controls did not reach

statistically significant values, they show a marked tendency. The reduction in *ALOX5AP* gene expression values when comparing the three groups (HIV-treated, HIV-untreated and non-infected) was statistically significant ($p < 0.0001$) (Table 4.3, Figure 4.2) suggesting that a larger sample may show significant differences.

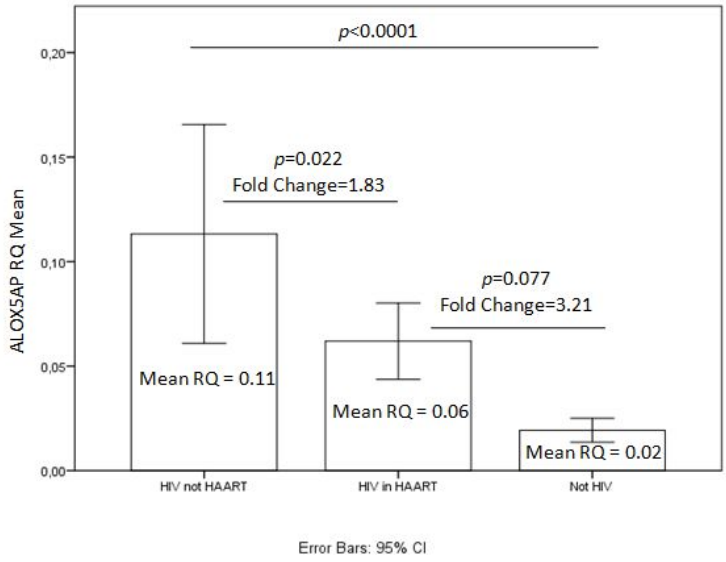


Figure 4.2. ALOX5AP RQ mean values vs HIV treated, HIV untreated and uninfected.

The 5-LO pathway has never been studied regarding ART although it has been related to HIV infection (160, 161). The results from this study showed that this inflammatory pathway is involved in HIV infection. *ALOX5AP* gene expression levels were reduced in HIV infected patients under ART, but not to those levels observed in uninfected subjects. The 5-LO pathway seems to be activated in PBMCs from HIV-infected individuals. The activation of this pathway in untreated HIV-infected individuals may be explained by the presence of the viral protein gp120, an inductor of this pathway (268-270). However, reduction in final 5-LO product production has been described in previous studies (160, 235). This apparent non-concordant finding may be explained by post-translational alterations such as blockade of the pathway or binding to other proteins. It is

also possible that the increased levels of mRNA may remain untranslated or be degraded by the cellular machinery. The 5-LO pathway needs further investigation to elucidate its real role in HIV-infection.

Stratification by ART families did not show differences among treated HIV patients in *IL18* or *ALOX5AP* gene expression values. Likewise, this study was unable to demonstrate which drug family is the responsible for the reduction in *IL18* or *ALOX5AP* expression levels. The involvement of NRTIs in lowering *IL18* or *ALOX5AP* mRNA levels is highly plausible. NRTIs have been linked to a reduction of the cytokines such as IL8, LTA or CCR5 (271, 272). Notwithstanding, the high prevalence of NRTI's treatment in the studied population (95.1 %), precludes the demonstration of its potential association.

In conclusion, alterations in mRNA levels of the *IL18* and *ALOX5AP* genes*,* both of them involved in the inflammatory pathway, were observed in HIV-treated subjects in comparison with uninfected controls. However, the mRNA levels in HIV-treated patients were markedly inferior to those observed in HIV-untreated subjects, highlighting the possible benefits of earlier introduction of ART. This study adds evidence of the involvement of the inflammatory pathway in HIV infection. Further studies are needed for a better understanding and control of the inflammatory pathway.

DISCUSSION

DISCUSSION

Clinical studies have demonstrated that HIV-infected individuals suffering from atherosclerosis are younger and present higher prevalence of traditional CVR factors such as smoking, male gender, diabetes and dyslipidemia than the general population (106, 166). This study investigates the genetic contribution to subclinical atherosclerosis in a well-characterized cohort of HIV-infected patients representative of our geographical area. The specific objectives were to test the possible association between genetic and transcriptional changes of several inflammatory genes with HIV-related subclinical atherosclerosis.

The possible association between clinical variables and subclinical atherosclerosis was investigated in the first study. The cohort presented elevated rates of traditional CVR factors similar to the ones observed in other studies in HIV-infected subjects (13, 28, 176, 178). Lipid values were not different from the ones described in previous studies (176, 179). Taking into account the cut-off values described in the general population the studied population was within normal lipid values, even among those with subclinical atherosclerosis. When analyzing the possible association between lipid profile and ART treatment we only found triglycerides marginally associated with being under ART as previously described (179). Although lipid parameters were found associated with subclinical atherosclerosis, the studied population was at low risk of suffering cardiovascular events, even using the Framingham or the Regicor scores. Moreover, the CVR was probably underestimated because these scores are not adapted to HIV-infected populations. However, imaging the vascular wall permitted a better classification of individuals at higher CVR (86).

A high prevalence of subclinical atherosclerosis was found when the arterial wall was imaged. Moreover, the mean cIMT of the studied population was near pathological values. CVR scores or lipid parameters may be less informative in HIV-infected than in uninfected populations. More studies are needed to

elucidate the causes of the high rates of subclinical atherosclerosis observed in HIV-infected populations. Clinical studies to adapt CVR prediction tools to the Spanish HIV-infected population such as the DAD equation (CVR prediction algorithm adapted to HIV-infected subjects (146)) would be useful. Meanwhile, systematic use of imaging tools may add important information for the management of HIV-related atherosclerosis. While the causes of HIV-related atherosclerosis are not known, active interventions to reduce CVR factors such as smoking, dyslipidemia, hypertension or sedentary life are needed to expand life-span and ameliorate quality of life in HIV-infected subjects.

Immunological and HIV-related parameters such as $CD4^+$ T-cell count (98, 105, 180-186), nadir $CD4^+$ T-cell count (91, 187), ratio $CD4^+$ / $CD8^+$ T-cell count (26), duration of known HIV infection (26) or HCV coinfection (188) have been found associated with increased CVR in HIV-infected individuals. None of these associations were replicated in this study. Differences in the end points might explain the discrepancies between studies.

The second study investigated the possible association between genetic variants in several inflammatory genes (see table 2.2, page 70)) and subclinical atherosclerosis measured by cIMT and AP. The associations identified between genetic variants in the studied genes and CVR measures were modest. However, several of the findings are novel associations such as *CCL5* rs3817655. Moreover, they are located in regulatory regions or near putative causal mutations. Most of the described associations could be explained by mutations in the nearby region. For example, there are two mutations located near the *CCL5* rs3817655 that encode for new stop codons resulting in truncated proteins. SNPs previously reported in association with CVR were also included in the second study. However, most of them were not associated with HIV-related subclinical atherosclerosis or were found associated in the opposite direction.

The cellular alterations induced by HIV may disguise the implication of these genetic variants explaining the discrepancies.

Several of the SNPs included in this study had been previously investigated with contradictory results. The *CX3CL1* rs614230-C allele has been previously associated with smaller cIMT in German non-HIV infected subjects (CAPS cohort) although this finding was not replicated in a French cohort (3C cohort) (197) or in this study. This suggests that the CAPS finding was a false positive result. Similarly, no association was found between AP presence and *IL-1B* rs16944 (206-209) adding evidence to the non-involvement of this genetic variant in AP development. Several *CX3CR1* variants have been linked to reduced risk for coronary artery disease (220) and reduced cIMT in the general population (221). They have also been linked to faster AIDS disease progression (218, 219) and reduced atherosclerosis progression in HIV population (92). None of these previous findings have been replicated in this study. Moreover, the majority of the studies describe protective associations for these polymorphisms, although others have found deleterious or no associations (197, 222) similarly to our results.

The results of the second study suggest that genetic variants in the investigated genes may play a minor role and may not be the underlying cause of the increased CVR observed in HIV-infected individuals. HIV-related CVD is probably influenced by non-genetic factors such as traditional CVR factors, the HIV-virus itself and by ART.

The third study investigated the possible implication of the gene expression levels of nine genes (*ALOX5, ALOX5AP, CCL5, CX3CR1, CXCL2, CXCL8, IL6, IL18* and *LTA*) on subclinical atherosclerosis and HIV-related and non-related clinical variables. Variations in gene expression levels were found associated with AP (*CXCL2*) and cIMT (*CXCL2, CXCL8, IL6* and *CCL5*). The *CXCL2* finding is a novel finding. Increased *CXCL2* gene expression levels seemed to be atheroprotective

in HIV-related subclinical atherosclerosis. However CXCL2 protein levels were not found associated with AP or cIMT in the general population (242). Although the associations of *CXCL2* gene expression with AP and/or cIMT are novel findings, they may be exclusive to HIV-infected population. The findings regarding CCL5, *CXCL8*, and *IL6* have been previously described, but in the opposite direction. This may be explained by the cellular alterations caused by the presence of the HIV.

Several gene expression levels were found associated with clinical variables. Most of the findings have been previously reported in other studies such as the association between *CXCL2, IL6* and *IL18* gene expression levels and abdominal obesity (239, 251-253) or *IL6* levels with significant alcohol consumption (254, 255). However, the association between *LTA* gene expression levels and alcohol consumption is a novel finding. Although significant alcohol consumption was not found associated with subclinical atherosclerosis in the first study, it is known to increase triglyceride levels. The increased *LTA* gene expression may be explained by the ethanol-dependent production of pro-inflammatory cytokines (256). However, *LTA* gene expression was not correlated with triglyceride values, probably due to the small sample size.

Gene expression alterations may be caused by alterations in the gene sequence. Therefore, we investigated if the genetic variants identified in the second study were correlated with the findings from the gene expression study. No association was found between the results of the second and third study. Although *CX3CR1* gene expression did not seem to have an effect on cIMT or AP, the *CX3CR1* rs2669850-C allele was found associated an increased *CX3CR1* gene expression. This SNP was found associated with reduced cIMT in the single marker analysis. Reduced expression or lack of CX3CR1 is associated with reduced lesion formation (263, 264) whereas high CX3CR1 levels are deleterious for cIMT (92, 263). We expected to find reduced *CX3CR1* gene expression levels

for the rs2669850-C allele. However the opposite effect was observed. Interestingly, increased *CX3CR1* gene expression was found in patients who met AIDS criteria. It seems that HIV-infection alters *CX3CR1* gene expression (224), but its levels are restored to normal after ART introduction (224). However, no association was found between *CX3CR1* gene expression and receiving ART.

No other correlation was observed between gene variants, gene expression levels and cIMT or AP suggesting that the sequence variants associated with HIV-related subclinical atherosclerosis may affect gene function rather than gene expression. However, this hypothesis should be checked with the corresponding functional studies.

To discern the direct effects of ART on the inflammatory state and indirectly on CVR, the relation between ART and the gene expression levels of *ALOX5AP* and *IL18* were investigated in the fourth study. Although *IL18* gene expression levels have been related to subclinical atherosclerosis in HIV-infected and uninfected patients (257, 258), no association was found in the present study. However, increased *IL18* gene expression levels were found correlated with HIV viral load (259, 261) and found elevated in untreated patients (260, 262) and reduced in ART-treated HIV-infected subjects although not to the levels observed in non-infected individuals (259-261, 265) as previously described. Elevated serum levels of IL-18 may have an important role in non-HIV co-morbidities and in AIDS due to its pivotal role in the Th1/Th2 immune response balance and it is association to AIDS progression.

The 5-LO pathway has never been studied regarding ART although it has been related to HIV-infection (160, 161). *ALOX5AP* gene expression levels were reduced in HIV infected patients under ART, but not to those levels observed in uninfected subjects. The 5-LO pathway seems to be activated in PBMCs from HIV-infected individuals. The activation of this pathway in untreated HIV-infected individuals may be explained by the presence of the viral protein

gp120, an inductor of this pathway (268-270). However, reduction in final 5-LO product production has been described in previous studies (160, 235). This apparent non-concordant finding may be explained by post-translational alterations such as blockade of the pathway or binding to other proteins. It is also possible that the increased levels of mRNA may remain untranslated or be degraded by the cellular machinery. The 5-LO pathway needs further investigation to elucidate its real role in HIV-infection.

This study was unable to demonstrate which drug family is the responsible for the reduction in *IL18* or *ALOX5AP* gene expression levels. The involvement of NRTIs in lowering *IL18* or *ALOX5AP* mRNA levels is highly plausible. NRTIs have been linked to a reduction of the cytokines such as IL8, LTA or CCR5 (271, 272). Notwithstanding, the high prevalence of NRTI's treatment in the studied population, precludes the demonstration of its potential association.

The most relevant finding of this thesis is the involvement of the 5-LO pathway in HIV infection and in HIV-related subclinical atherosclerosis. *CXCL2* gene expression levels were related to AP, cIMT and CVR variables, whereas *ALOX5* and *ALOX5AP* were associated with HIV-related variables. These three genes are part of the 5-LO pathway and may interact among themselves influencing both pathologies. Although the 5-LO pathway has been studied regarding CVD or HIV infection, this study has related 5-LO pathway to HIV-related atherosclerosis for the first time. The CXCL2 protein is stimulated by 5-LO pathway products promoting hyperlipidemia (234). Although no correlation between lipid levels and *ALOX5* or *CXCL2* mRNA levels were found, this pathway is relevant in lipid metabolism and atherosclerosis (236). 5-LO pathway is functionally reduced in HIV-infected individuals (160, 235) and HIV-infection increases CXCL2 protein levels (240, 241). *CXCL2* and *ALOX5* gene expression levels were found correlated. *ALOX5* mRNA production may be increased to compensate for the decreased functionality caused by the virus (160, 235) and stimulate *CXCL2* gene

expression. The association found between *ALOX5AP* gene expression levels and ART may indicate its involvement in the lipid changes described for several ART drugs. Inhibitors of the 5-LO pathway are being developed to treat dyslipidemia in the general population (237). Its inhibition may be an alternative treatment to HIV- and ART-related lipid alterations. Their use could potentially avoid previously described interactions with lipid lowering drugs (238). Clinical trials testing the therapeutic effect of 5-LO inhibitors in HIV-infected populations may be of high interest.

In summary, inflammation seems to be involved in HIV-related atherosclerosis. The 5-LO inflammatory pathway may play an important role in this disorder. This pathway is important in the metabolism of lipids, which is closely related to atherosclerosis. To our knowledge this is the first study describing the implication of the 5-LO pathway in HIV-related atherosclerosis. Genetic variants in two key genes of this pathway (*ALOX5* and *ALOX5AP*) were associated with HIV-related atherosclerosis. Although no differences were found in their gene expression, ART introduction reduced *ALOX5AP* gene expression levels, but not to the levels observed in uninfected controls suggesting the potential benefits of early ART initiation. Importantly, the 5-LO pathway interlinks lipid metabolism and inflammation and it is altered in HIV infection (160, 235). Thus, 5-LO pathway dysfunction may be critical in HIV-related atherosclerosis development.

The identified genetic associations may be a result of viral infection and / or treatment. However, they could be directly related to the development of atherosclerosis and need to be tested in an uninfected population. Similarly, changes in expression levels may be affected by treatment and infection in HIV-infected subjects and need to be investigated in the general population to discern their contribution to cardiovascular events. Important differences have been found between HIV-treated individuals and uninfected controls. Several of

the associations found between gene expression levels and atherosclerosis-related clinical parameters have been previously described in uninfected populations. However, most of the identified genes have also been found associated with HIV-related characteristics. This may reflect the inter-relationship between both diseases: HIV infection may alter genes directly involved in the development of atherosclerosis. This inter-relationship may be, at least partially, the cause of the increased CVR observed in HIV-infected subjects.

This study had several limitations. The studied cohort had a limited sample size, although it was very well characterized. High prevalence of cardiovascular risk traditional factors and the demographic and clinical bias observed in HIV-infected populations should also be considered as a limitation and a possible source of error in the findings. The lack of an uninfected control group prevented the study of the contribution of HIV infection to early atherosclerosis. However, studies have suggested that the biological processes involved in HIV-related atherosclerosis are different from the ones observed in non-infected individuals. Moreover, it seems that the atherosclerotic plaque structure and characteristics are also different (103). The results from this study support this hypothesis. The genes identified by this study were different when cIMT or atherosclerotic plaque variables were analyzed except for *ALOX5AP* genetic variants and *CXCL2* gene expression. The implication of different genes may be explained by the different biological processes involved in the widening of cIMT and atherosclerotic plaque formation although enlargement of the cIMT is the first step in atherosclerotic plaque formation (56). However, *CX3CL1* genetic variants were found associated with AP whereas *CX3CR1* genetic variants were found associated with cIMT. This may indicate that although cIMT engrossment and AP formation are different biological processes, they may be closely related. Finally, another limitation is that carotid ultrasonography measures are

conditioned by their inter - and intra-variability. However, all measures were performed by the same operator.

The findings reported in the present study provide substantial evidence on the involvement of the inflammatory pathways on HIV-related atherosclerosis. However, they need to be confirmed in larger samples. It would also be interesting to investigate and compare gene expression levels in atherosclerotic plaques and arterial walls from HIV-infected and uninfected individuals to further understand the different processes involved in HIV-related atherosclerosis.

CONCLUSIONS

CONCLUSIONS

1. Imaging the arterial wall is a better predictor of subclinical atherosclerosis than the Framingham or Regicor scores in HIV-infected individuals.

2. Although genetic variants in the inflammatory genes are implicated in HIV-related atherosclerosis, they do not appear to be the underlying cause of the increased cardiovascular risk observed in HIV-infected individuals.

3. There seems to be a synergistic relationship between atherosclerotic disease and HIV infection at a transcriptional level.

4. The results indicate that the inflammatory pathway 5-LO is involved in HIV-related atherosclerosis at genetic and transcriptional levels.

5. *IL18* and *ALOX5AP* gene expression levels are reduced in ART-treated patients, although not to the levels observed in non-infected individuals. Early ART initiation may have potential clinical benefits reducing inflammation and HIV-related co-morbidities.

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