INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS

Blai Coll Crespo

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Influence of chemokine related polymorphisms in atherosclerosis and HIV-infection

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Blai Coll Crespo
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Dipòsit Legal: T.1044-2012

Summary

UNIVERSITAT ROVIRA I VIRGILI
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Dipòsit Legal: T.1044-2012

UNIVERSITAT ROVIRA I VIRGILI
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Dipòsit Legal: T.1044-2012

UNIVERSITAT ROVIRA I VIRGILI
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Blai Coll Crespo
Dipòsit Legal: T.1044-2012

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ApoE$^{-/-}$	Apolipoprotein E deficient mice
BCA	Bulb Carotid Artery
CAD	Coronary artery disease
CCA	Common Carotid Artery
CCR-2	CC Chemokine receptor 2
CCR-5	CC Chemokine receptor 5
CV	Cardiovascular
CVD	Cardiovascular diseases
CX3CR-1	Fractalkine receptor
CxCR4	CXC Motif, receptor 4
Env	Viral envelope glycoprotein
FH	Familiar Hypercholesterolemia
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ICA	Internal Carotid Artery
LTNP	HIV-infected patients considered long term non-progressors
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MIP-1α	Macrophage inflammatory factor-1 α
MIP-1β	Macrophage inflammatory factor-1 β
NNRTI	Non Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
PBMC	Peripheral Blood Mononuclear Cells
PI	Protease Inhibitors
R5	Macrophage (M)-tropic or non-syncytium inducing HIV
RANTES	Regulated on activation, normal T cell expressed and secreted
SDF-1	Stromal Derived Factor-1
SNP	Single Nucleotide Polymorphism
Tat	Trans-activator protein
X4	T cell line-tropic or syncytium inducing HIV

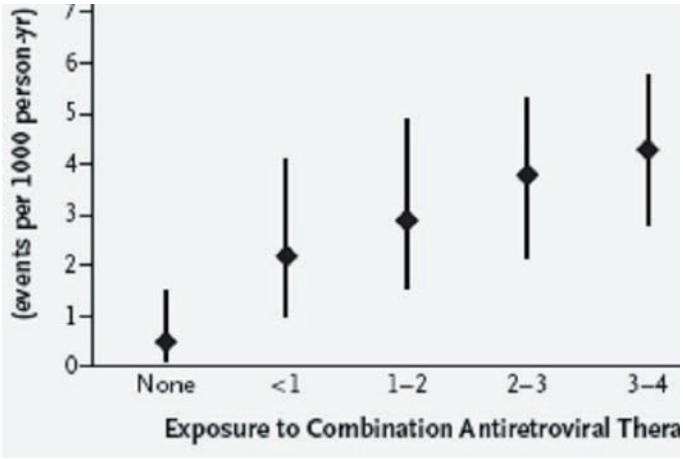
1.- Preface

The introduction of the Highly Active Antiretroviral Therapy (HAART) in HIV-infection has been associated with a marked decrease in morbidity and mortality from AIDS-related diseases[1]. However, the increase in the life expectancy and the side effects of HAART[2,3] are leading clinicians to challenges in the treatment of HIV-infected patients. Atherosclerosis is a systemic and progressive disease[4] that explains most of cardio-vascular events, and then the identification of either atherosclerosis risk factors or atherosclerosis itself is of great relevance. In recent years, the management of lipid abnormalities in HIV clinics[5], are a great focus of concern and the overall CV risk is increasing steadily in this population[6]. The reduction of the CV risk implementing conventional strategies (life style changes, lipid-lowering drug prescriptions) have been adapted, but we should take into account the influence of genetic variations on these processes and how, once identified, we could modify them, in order to minimize the incidence of CV events in this young population. This thesis deals with the early identification of atherosclerosis and these non-conventional markers associated with its development, in a cohort of HIV-infected patients.

2.- Cardiovascular diseases in HIV- infected patients

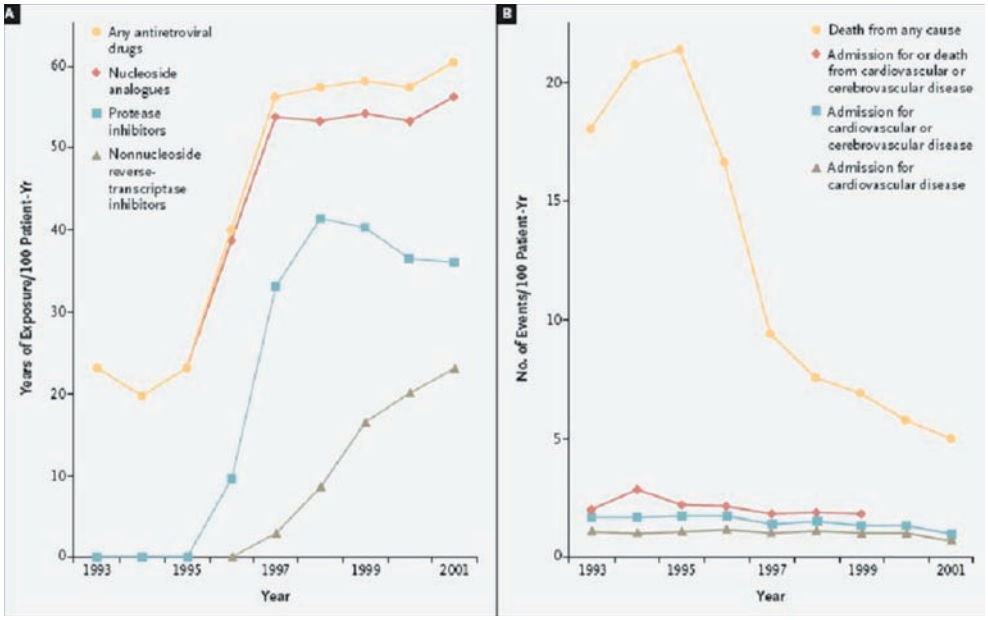
The incidence of CVD in HIV-infected patients has been addressed in several prospective and retrospective studies. In the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study[7], of 23,468 participants, 126 (0.5 percent) had a first MI, an incidence of 3.5 per 1000 person-years. Although there is no comparing data with non-infected population coming from prospective studies, one of the strongest conclusions of DAD was that the incidence of MI increased directly with longer exposure to antiretroviral therapy (relative risk, 1.26 [95 percent confidence interval, 1.12 to 1.41] per additional year of exposure; P<0.001) (Figure 1).

Figure 1. Incidence of myocardial infarction in HIV-infected patients according to time of exposure to antiretroviral therapy. (N Engl J Med. 2003;349:1993-2003.)



According to the DAD study, classical-known cardiovascular risk factors, such as, male sex, hypercholesterolemia, older age, smoking and prior history of cardiovascular disease, were also associated to a higher risk of developing MI. In a recent publication, investigators of the DAD study up-to-dated these results, collecting data of 23,437 HIV-infected patients, with an absolute number of MI of 345, resulting in an incidence of 3.65/1000 person-years. Further they found a significant increased relative rate of MI in those patients who had been treated with a PI-based HAART scheme [1.16 (1.10-1.23)] when compared to those with NNRTI [1.05 (0.98-1.13)][8]. However, these results have not been confirmed by other studies. Bozzette et al. analyzed retrospectively the rate of CVD since the generalization of HAART and evidenced that the rate of admission or death due to CVD did not increase with increasing exposure to HAART (Figure 2)[9].

Figure 2. Years of exposure to different types of antiretroviral therapies (Panel A) and the number of deaths and cardiovascular events in Panel B (N Engl J Med. 2003;348:702-10).



"the rate of CVD is higher in HIV-infected patients taking PI and the risk increases as the duration of treatment lengthens

An overview of the studies addressing cardiovascular incidence in HIV-infected patients are summarized in Table 1. Although these data indicate the great benefits of the HAART in terms of increased survival, they also suggest that the rate of CVD is higher in HIV-infected patients taking PI and the risk increases as the duration of treatment lengthens[10].

Table 1. Studies of CVD rates in HIV–infected patients.

	N	Follow-up	Events	Findings
RETROSPECTIVE		(person/years)		
Klein D. et al [11]	4,159	14,823	47	Greater risk in HIV than controls.
Mary-Krause M. et al [12]	19,795	88,029	60	Greater risk in PI treated patients.
Klein D. et al [13]	28,513	71,286	410	Greater risk in HIV treated patients
Bozette SA et al [9]	36,766	121,936	-	No difference in risk between ART groups
Barbaro G et al. [14]	1551	36 month	25	Greater risk of MI in PI vs non-PI.
PROSPECTIVE				
Holmberg SD et al. [15]	5,672	17,712	21	Greater risk of MI with PI. No multivariate association.
Friis-Moller N et al. [7]	23,468	36,199	126	Greater risk of MI with ART. 26% increased risk in the first 4-6y of ART.
Iloeje UH. et al [16]	7,542	-	-	Greater risk of MI with PI.
D:A:D study [8]	23,437	94,469	345	Greater risk of MI with PI.

Adapted from Stein JH. J Acquir Immune Defic Syndr 2005;38:115-123.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

It is of interest to remark that when compared the clinical appearance of coronary events between HIV-infected patients and non-infected population, those HIV-infected were predominantly younger males, with significantly lower HDL-cholesterol concentrations and with a higher rate of restenosis after coronary angioplasty or stenting (Table 2)[17,18,19,20,21]. Similarly, a pathologic study revealed, that the appearance of atherosclerotic lesions of coronary arteries of HIV-infected patients compared with non-infected population, were highly inflamed and resembled those observed in patients with a chronic cardiac transplant[22]. These results indicate that the inflammatory response of HIV-infected patients is particularly over expressed in the pathogenesis of atherosclerosis, and therefore, its inflammatory pathways are relevant in determining a different clinical presentation.

> **“ atherosclerotic lesions of coronary arteries of HIV-infected patients compared with non-infected population, were highly inflamed and resembled those observed in patients with a chronic cardiac transplant**

Table 2. Characteristics and follow–up of HIV–infected patients with MI.

	N	MI	Revascularization	Restenosis*	Comments
Matetzky S et al. [17]	24	24	21 (87.5)	43 % vs 11%	Higher incidence of re–infarction
Escaut L et al. [18]	17	11	17 (100)	45 %	No controls matched
Vittecoq D et al. [19]	51	34	45 (88.2)	NA	No difference in PI
Varíale P et al. [20]	29	29	13 (44.8)	NA	No controls matched
Hsue P et al. [21]	68	20	29 (42.6)	52 % vs 14 %	Higher incidence of restenosis

** Restenosis rates compared with non-infected population. Adapted from Circulation 2005:112:3947-3957[23].*

3.- Surrogate markers for atherosclerosis

The need of reliable data on cardiovascular studies lead to the design of large scale trials in which the end-points are cardiovascular events[24]. This strategy has several benefits but they need several years of follow-up, and it means that they are time and financially-consuming. Taking into account that HIV affects especially younger population, the need for a surrogate marker for atherosclerosis is of great value. The measurement of arterial intima-media thickness (IMT) (Figure 3) was first reported in 1986[25]. Since then, this non-invasive method has been validated with pathologic studies[26], and it has been correlated with both CVD risk factors and the prevalence of CVD[27,28,29]. The measurement of the IMT as an end-point ,in intervention studies, met the set of criteria to be considered a surrogate marker for atherosclerosis[30]: it is efficient; the linkage between the values of carotid IMT and cardiovascular events has been repeatedly assured; it is congruent and the therapeutic intervention impact independently in the course of IMT and in the rate of CVD. Further, IMT constitutes a direct method to detect atherosclerosis, and then a way to assess disease and not only risk factors for the disease. The measurement of the IMT has been proposed as an initial test, similar to the determination of the coronary calcium score, to stratify the risk of individuals to suffer from a CV event[31].

“IMT constitutes a direct method to detect atherosclerosis, and then a way to assess disease and not only risk factors for the disease

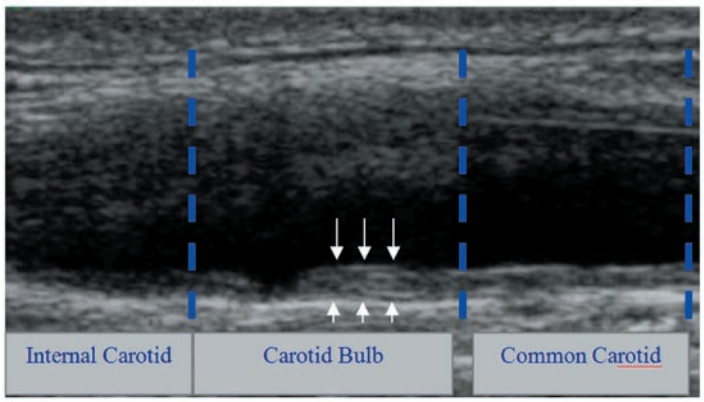


Figure 3. Overview of carotid intima-media thickness.

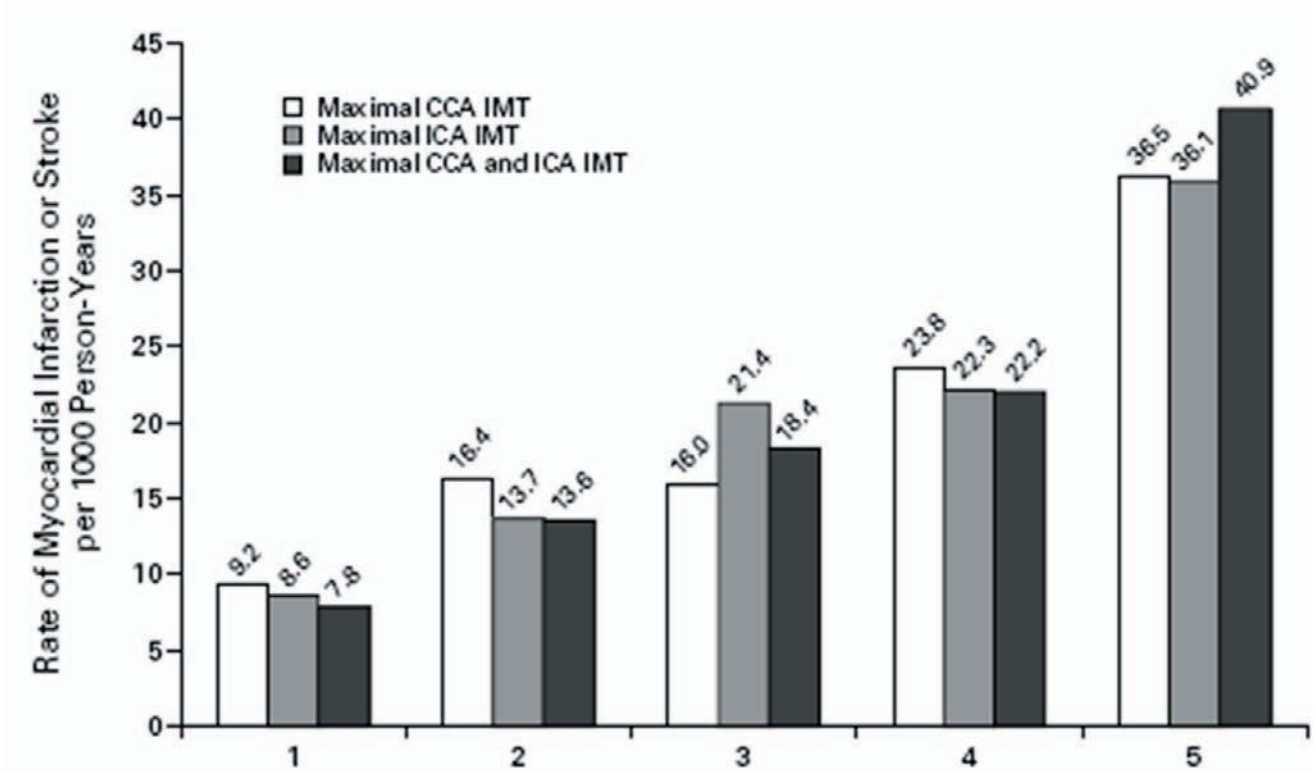
Footnote: the interphase lumen-intima is depicted by arrows and the interphase media-adventitia is depicted by arrow-heads. The distance between them constitutes the intima-media thickness (IMT).

IMT is an strong indicator of atherosclerosis in its early phases, because it is a reliable way to assess the artery wall, and not the consequences of atherosclerosis in the lumen [32]. The ability of IMT to predict future CV events

has been assessed in large observational studies. The increase of 0.3mm in the IMT of the common carotid artery was associated with a 78% higher risk of suffering from a coronary ischemic event and a 196% increased risk of having an stroke[33]. Similarly, prospective studies have shown that the higher the carotid IMT the higher the likelihood of suffering from MI or stroke[34]. O'Leary et al[35] in a population-based study of 5858 subjects found that the relative risk of having an MI/stroke of those participants in the fifth quintile of carotid IMT was 3.15 [(95% CI:2.19-4.52), Figure 4]. Further, the values in the carotid IMT are highly correlated with the extent of coronary lesions diagnosed by angiography [36]. All prospective studies reviewed are in accordance with the same conclusion, i.e.: the higher the carotid IMT the higher the likelihood of having a CV event in the future[37]. In a metanalyses of the main intervention studies in the course of IMT, for an absolute carotid IMT difference of 0.1 mm, the future risk of MI increases by 10% to 15%, and the stroke risk increases by 13% to 18%. Another interesting remark of this study, is that vascular events are rare in young individuals, which makes IMT particularly attractive as an end point in epidemiological and treatment studies in young populations (such as HIV-infected patients). This fact explains the non-linearity relationship between carotid IMT and cardiovascular risk: young individuals with increased IMT are at considerably lower absolute risk but higher relative risk of vascular events.

> **"the higher the carotid IMT the higher the likelihood of having a CV event in the future**

Figure 4. Incidence of myocardial infarction according to quintiles of carotid IMT.



From O'Leary DH et al. NEJM 1999;340:14-22.

3.1.-Which is the best carotid IMT segment as surrogate marker?

One of the critical points in comparing studies based on carotid IMT are the different protocols applied, and this may lead to a misinterpretation of results. Conditions of blood flow in the CCA make the presence of a focal IMT protrusion (atherosclerotic plaque) unlikely, and conversely the likelihood of a diffuse enlargement is higher than in the ICA [38]. The impact of assessing different anatomic regions in predicting CV events have been previously studied, and the values of the ICA are a better surrogate marker of atherosclerosis than those in the CCA. However, each image laboratory should check for the repeatability of the exams, since the variability in ICA tended to be higher than in the CCA [39].

Several manuscripts addressed the role of IMT in femoral arteries, with comparable results with those obtained in the carotid exams; higher values of femoral IMT were observed in older participants and with higher number of classical cardiovascular risk factors [40]. Further, the results of carotid and femoral IMT combined are highly related to coronary atherosclerosis [41].

Besides the use of IMT in cross-sectional designs, this technique has also been analyzed in follow-up studies, in order to know whether the rate of IMT increase/decrease yield stronger conclusions in terms of atherosclerosis risk than cross-sectional studies. de Groot E. et al [42], studied patients with familiar hypercholesterolemia and matched controls at different periods of their lives. They concluded that the rate of IMT progression was significantly higher in the group of FH-affected patients, i.e.: the carotid IMT observed at 40 years of age in the FH patients was the same which was at 80 years for the unaffected population (Figure 5).

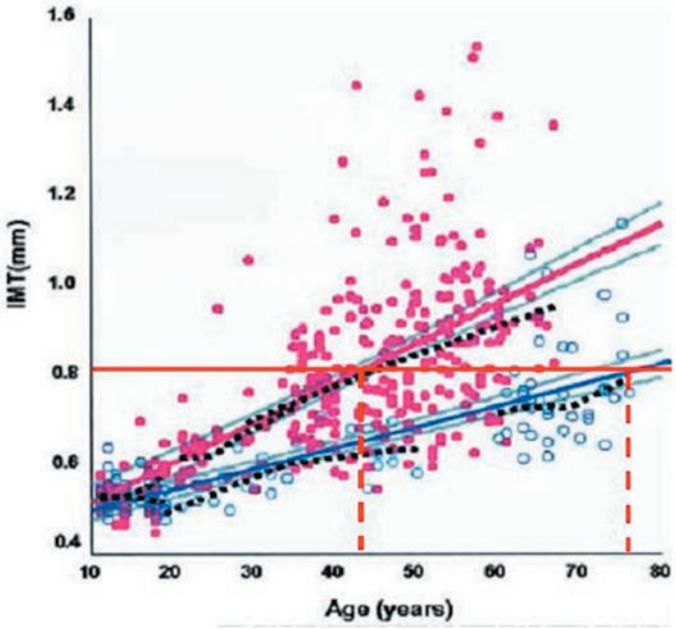
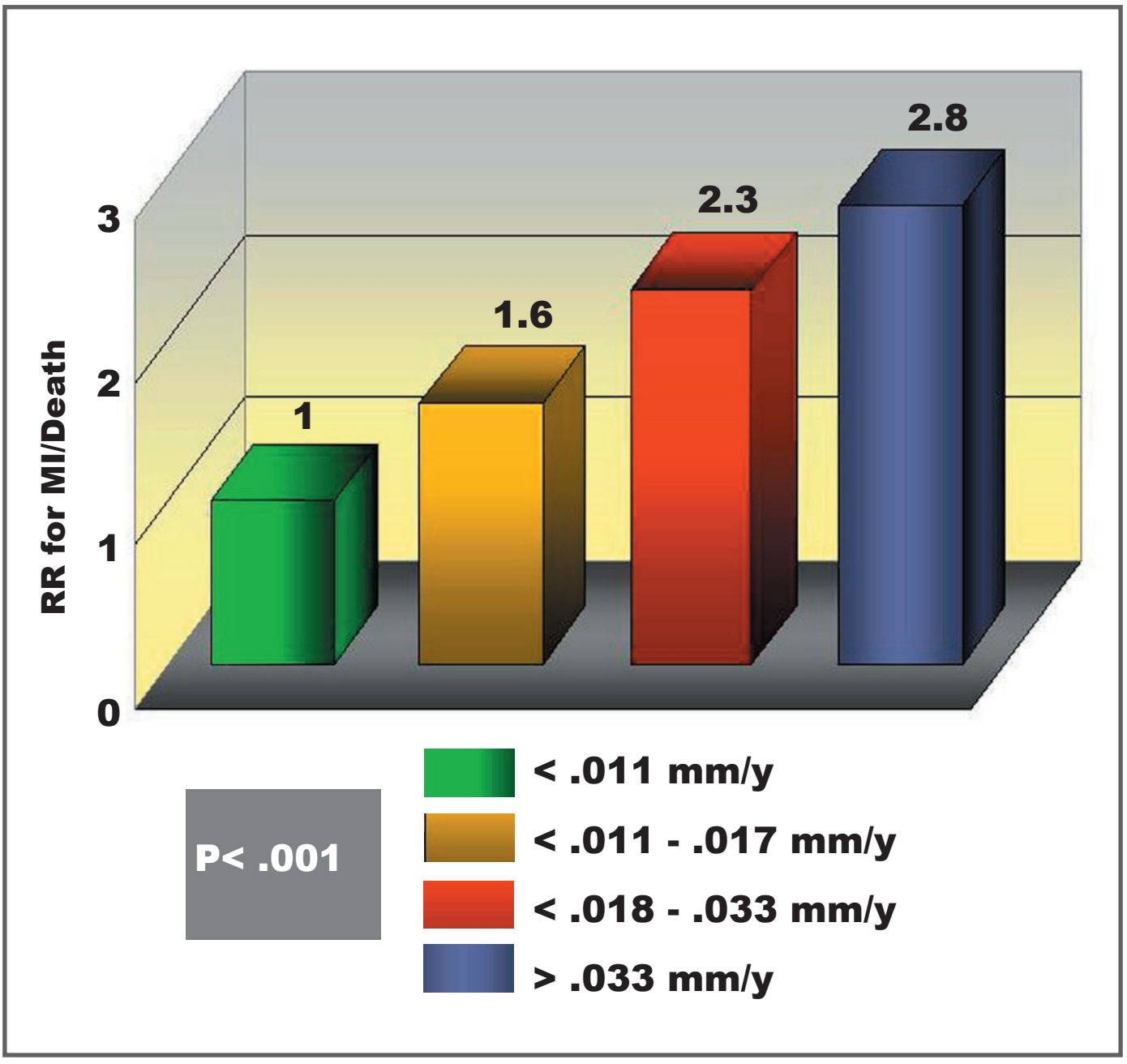


Figure 5. Progression of IMT in different populations; Familiar Hypercholesterolemic patients (red dots) compared with un-affected controls (blue dots). Circulation 2004;109:III: 33-38.

Most of the IMT progression studies have been performed in patients who had already had a CV event, and then, they are limited to secondary prevention. Hodis et al.[43] demonstrated that independently of the group of therapy (placebo or colestipol/niacin) a rate of IMT progression of >0.03mm/year was significantly related with a higher likelihood of having a CV event (Figure 6). These results were further confirmed in other studies [44,45], indicating that not only cross-sectional studies of IMT are highly informative in assessing the CV risk, but also longitudinal studies addressing the rate of IMT increase/decrease.

> **a rate of IMT progression of >0.03mm/year was significantly related with a higher likelihood of having a CV event**

Figure 6. Incidence of myocardial infarction/death according to the progression rate of carotid IMT.



4.- Intima-media thickness studies in HIV- infected patients

The availability of the ultrasound examination and the higher rates of dyslipidemia and other classical CV risk factors have pointed researchers to study atherosclerosis non-invasively in HIV-infected patients. Table 3 summarizes studies of carotid IMT in HIV-infected patients. According to these, they reach a definitive conclusion: the IMT of HIV-infected patients is significantly higher than non-infected population, and further, the carotid IMT progression of HIV-infected patients is also significantly faster than non-infected individuals [46]. However, the influence of antiretroviral therapy on the increase of carotid IMT is still a matter of debate. Preliminary results showed that, the control in classical cardiovascular risk factors, and the rationale use of PI as the mainstay of HAART is associated to a significant control in the progression rate of IMT [47].

“ the IMT of HIV-infected patients is significantly higher than non-infected population

Table 3. IMT studies in HIV-infected patients.

	N	Type of study		Mean Carotid IMT, mm		Plaque, %		Comments
		HIV +	HIV –	HIV +	HIV –	HIV+	HIV–	
Chironi G. et al [48]	36	√	√		8% lower IMT	NA	NA	HIV+ similar IMT values than those HIV- with metabolic disturbances
Depairon M. et al [49]	168	√	√	NA	NA	55.4	38.2	No influence of PI.
Maggi P. et al [50]	102	√	√	1.2	NA	16.5	0	PI related to plaques.
Maggi P. et al [51]	293	√	NA	>1.2	NA		NA	PI vs NNRTI and Naïve
Mercie P. et al [52]	423	√	NA	0.54	NA	NA	NA	Conventional CV risk factors associated with IMT
Seminari E. et al [53]	59	√	√	0.67	0.5	NA	NA	IMT higher in PI-treated patients than controls
Hsue P. et al [54]	148	√	√	0.91	0.74	NA	NA	Higher baseline and IMT progression than controls
Saint Martin L. et al [55]	154	√	NA	0.65	NA	NA	NA	PI related to IMT.
Jerico C. et al [56]	132	√	NA	0.59	NA	37.1	NA	HAART predictor for subclinical atherosclerosis.
Currier J. et al [57]	134	√	√	0.70	0.69	-	-	No influence of PI.
Mangili A. et al [58]	327	√	NA	0.65	-	13.5	NA	No influence of PI.
Boccara F. et al [59]	84	√	NA	0.68	NA	NA	NA	No influence of Pravastatin.

NA: not available.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

5.- Causes for higher atherosclerosis rates in HIV- infected patients

5.1.- Classical cardiovascular risk factors

Several variables exert a detrimental influence in the risk of HIV-infected patients to suffer from atherosclerosis. First, the life expectancy of HIV-infected patients has increased [60] and then, they are also under the detrimental influence of the most powerful and non-modifiable risk factor for the development of atherosclerosis, age. Further, most of HIV-infected patients are or used to be heavy smokers, a factor that might induce the premature development of atherosclerosis. Dyslipidemia, as in non-infected population, is highly correlated with atherosclerosis in HIV-infected patients [61]. It is interesting to highlight however, that HIV has direct influences in several metabolic parameters. Newly diagnosed HIV-infected patients generally presented with lower HDL cholesterol and higher triglyceride concentrations [62,63]. Further, HIV-infected patients under antiretroviral therapy experienced lower HDL cholesterol concentrations with hypertriglyceridemia compared with non-infected patients [64,65], and these changes yields to a more pro-atherogenic lipid profile. The characterization of different lipoproteins in HIV-infected patients, showed that HDL subpopulation profiles resembled those of coronary heart disease affected patients. Moreover, the HDL subpopulation profile changed unfavorably after a PI-based HAART, characterized by increased concentrations of the small, lipid-poor pre-beta-1 HDL and decreased concentration of the large, cholesterol-rich alpha-1 HDL [66]. According to these data, the lipid profile of HIV- infected subjects is deteriorated after receiving a PI-based treatment, which may cause increased risk of suffering from atherosclerosis. Although controversial, several works have assessed the efficacy of switching these therapies to a PI-sparing regimen, based on the use of efavirenz or nevirapine, and these results showed a favorable effect in decreasing total and LDL cholesterol, and increasing HDL cholesterol [67]. However, these studies may be biased by uncontrolled

HDL subpopulation profiles resembled those of coronary heart disease affected patients.

“ patients receiving HAART may develop a lipotoxicity due to mitochondrial dysfunction resulting in the excess release of free fatty acids

technical difficulties in measuring HDL concentration (i.e. HIV-infected patients with hyperglobulinemia, which is relatively common due to chronic hepatic disorders, presented falsely low HDL levels) [68]. Further, no study has addressed the effect of these therapies on changes in carotid IMT, and as such we have data on classical cardiovascular risk factors, and not in the impact on atherosclerosis.

The prevalence of metabolic syndrome is significantly higher in HIV-infected patients than in age-and-sex matched uninfected population[69]. Several causes have been hypothesized [70] but, as opposed to the “traditional” metabolic syndrome, patients receiving HAART may develop a lipotoxicity due to mitochondrial dysfunction resulting in the excess release of free fatty acids [71] , which in turn might be associated to the development of insulin resistance [72], one of the first key mechanisms in metabolic syndrome. The increase in free fatty acid concentration results in increased production of VLDL and small, dense LDL as well as low plasma levels of HDL. All these deleterious consequences have lead researchers to test the efficacy of acipimox, a nicotinic acid derivate that mainly blocks lipolysys. Acipimox resulted in significant sustained reductions in lipolysis, improved glucose homeostasis, and significant but modest reductions in triglycerides in HIV-infected individuals with abnormal fat distribution and hypertriglyceridemia [73] (Figure 7).

However, all these metabolic alterations may be the source of an overproduction of adipokines and inflammatory cytokines, that in turn, may increase the likelihood of suffering from CVD [74], due to an exceedingly inflammatory response located in the artery wall. The relationship between cytokines and metabolic variables will be further developed in chapter 6.

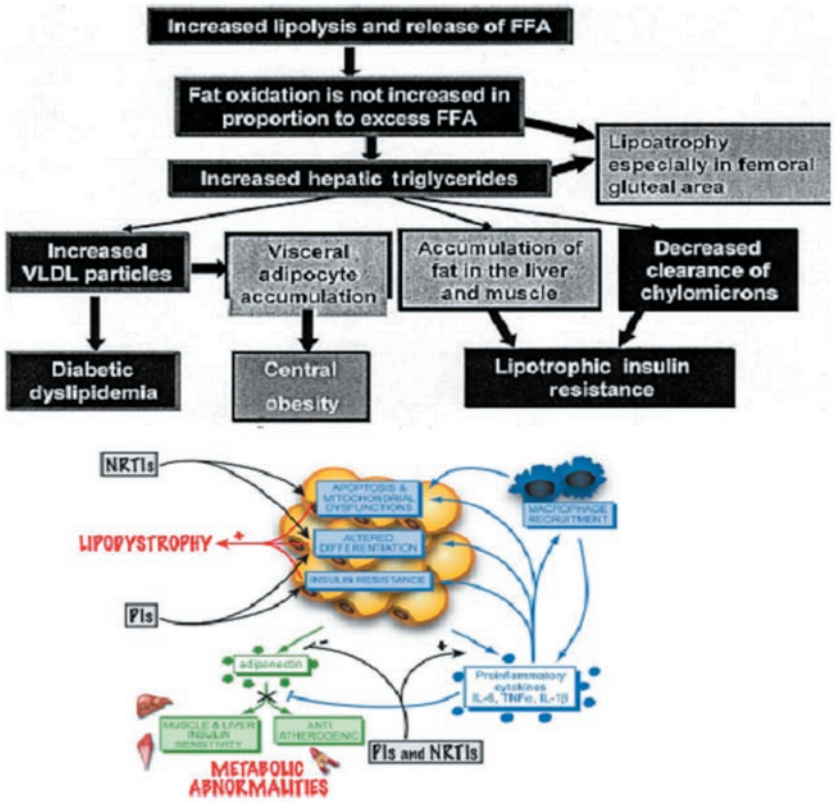


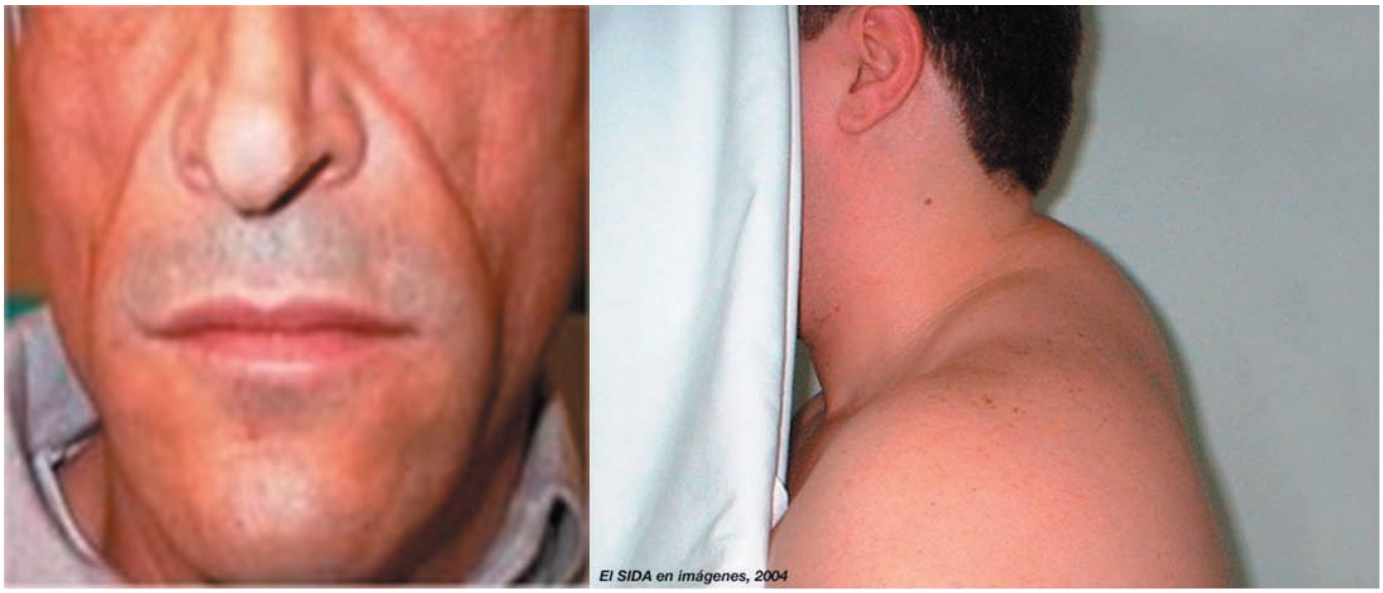
Figure 7. Pathophysiology of dyslipidemia and lipodystrophy in HIV-infected patients and the derived inflammatory response. Adapted from Biochimie 2005;87:65-71 [75].

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

5.2.- Lipodystrophy and atherosclerosis

Lipodystrophy has been characterized as a syndrome of fat redistribution. Several variables have been involved in its origin, some HAART drugs, the HIV itself and immune variables [76], that in conjunct lead those patients to have persistent metabolic disturbances [77]. These effects have not only physical and emotional consequences (Figure 8) but also are commonly associated with a greater cardiovascular risk profile.

Figure 8. Clinical appearance of HIV-infected patients with lipodystrophy.



Footnote: note the loss of subcutaneous fat in both cheeks and the abnormal accumulation in the dorso-cervical pad; these changes exemplify two different phenotypes in lipodystrophy.

In fact, lipodystrophy resembles the definition of the metabolic syndrome: abdominal obesity, hypertriglyceridemia and low concentrations of HDL cholesterol, high blood pressure and insulin resistance [78,79]. However, there is much controversy in its definition, and in fact, some authors consider the lipodystrophy in just those patients who develop lipohypertrophy, and not those with features of lipoatrophy [80,81]. Eventually, HIV-infected patients with lipodystrophy may be under the influence of several inflammatory, immunologic and metabolic alterations that may boost the development of early atherosclerosis. Although prospective observational studies in the incidence of CVD have not been conducted, Mercié P. et al [47] found that the presence of lipodystrophy was significantly associated with higher carotid IMT.

“ lipodystrophy resembles the definition of the metabolic syndrome: abdominal obesity, hypertriglyceridemia and low concentrations of HDL cholesterol, high blood pressure and insulin resistance

5.3.- Role of CD4+ T-cells in atherosclerosis

Atherosclerosis is an inflammatory disease[82] and several inflammatory-related variables have been not only identified in the atheromatous plaque[83], but also implicated in the risk of suffering from cardiovascular events[84]. HIV infection is associated with accelerated T-cell proliferation, heightened T-cell activation, and high levels of inflammatory markers[85,86]. This immune activation has been independently associated with the number of CD4+ T-cells at the moment of HIV infection diagnoses[87], which, in turn, was a predictor of atherosclerosis progression (being diagnosed of HIV infection with less than 200 CD4+T cells/mm3, was nearly significantly (p=0.08) correlated with a higher IMT progression)[46]. These data indicate that both immunodeficiency and immune reconstitution may be atherogenic. T lymphocytes, of which CD4 cells constitute the major population, play a key role in atherogenesis[88,89]. Among the cellular components of the atherosclerotic plaque, endothelial cells, monocytes and smooth muscle cells are the main players. However, T lymphocytes are also present and promote atherosclerosis through elaboration of pro-inflammatory cytokines, including tumor necrosis factor and interleukins[90]. The progression of atherosclerosis is also modulated by the immune system in which the role of CD4 cells is of great relevance [91]. The presence of oxidized LDL are considered the main antigenic stimuli in the atheromatous plaque among others such as heat shock proteins, beta-2-glycoprotein I and microbial antigens. It is well known the influence of Cytomegalovirus (CMV) and Chlamydia in atherosclerosis [92] , but this is specially relevant in HIV-infected patients, because they are under the influence of a higher T-cell activation and in whom the thicker carotid IMT has been related to the presence of CMV-specific T-cell responses [54] .These data support the results of Tabib et al. [22] in which an extensive, inflammatory activity are observed in atheromatous plaques of HIV-infected people. Accordingly, Barbaro G et al [93], reported the presence of HIV particles in endothelial cells, supported by an intense infiltration of T lymphocytes, in HIV-infected people who suffered from a myocardial infarction (Figure 9). Interestingly, studies in vitro revealed that the HIV replication rate is far more increased in the presence of endothelial cells in the medium of culture [94].

All these data may indicate that the HIV infection itself and the direct influence on CD4+T cells are able to promote an intense immune-inflammatory reaction in the artery wall, which might lead HIV-infected patients to be highly susceptible to known CV risk factors, and then they are prone to develop a highly inflammatory atheromatous plaques.

“ T lymphocytes, of which CD4 cells constitute the major population, play a key role in atherogenesis

“ HIV infection itself and the direct influence on CD4+T cells are able to promote an intense immune-inflammatory reaction in the artery wall

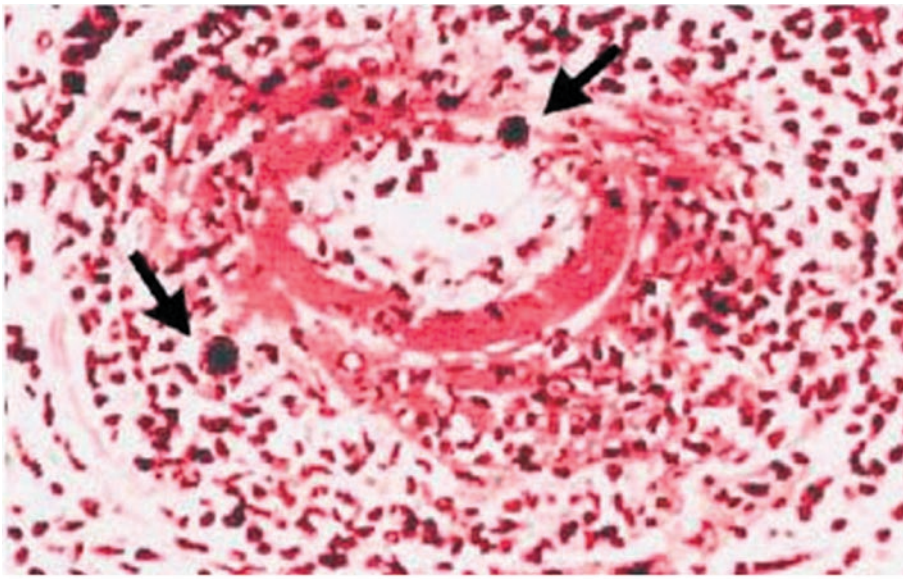
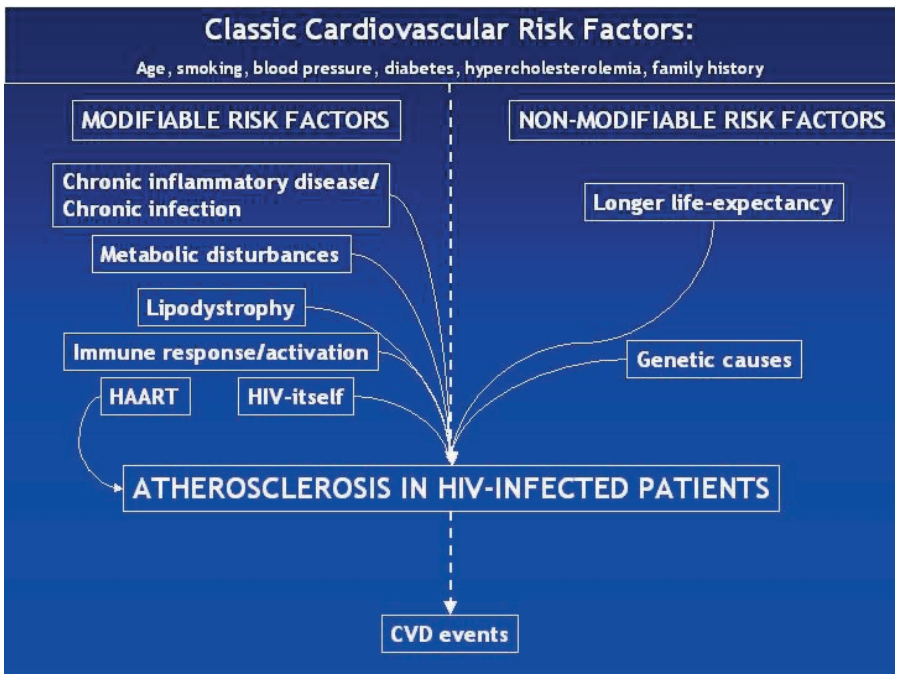


Figure 9. In Situ Hybridization of an HIV-1 RNA Probe in a Transverse Section of a Branch of the Left Anterior Descending Coronary Artery, of an HIV-infected patient with a myocardial infarction. Intense staining indicates the presence of HIV-1 sequences within the intima and the media (arrows). There is a dense infiltration of lymphocytes within the media and necrosis of the intima, which is covered with swollen endothelial cells.

Supporting these observations, recent data on the efficacy of interrupting HAART schemes, showed that the programmed interruption of HAART is associated with a higher risk of having a major cardiovascular event [95], and therefore, this may enhance the hypothesis that atherosclerosis in HIV-infected patients might be influenced by the immunologic and virologic status. These mechanisms should explain the arteritis in large-medium arteries observed in some HIV-infected patients [96].

In summary, HIV-infected patients presented a CV risk profile that should be encouragingly managed because of the clustering of risk factors which are boosted by the HIV infection itself and by the activation of the immune system, that might enhance the development of CVD in the future (Figure 10).



> **HIV-infected patients presented a CV risk profile that should be encouragingly managed because of the clustering of risk factors which are boosted by the HIV infection itself and by the activation of the immune system, that might enhance the development of CVD in the future**

Figure 10. Summary of the known CV risk factors and those related with the HIV infection, that may accelerate the development of atherosclerosis in HIV-infected patients.

6.- Pathogenesis of atherosclerosis: the role of chemokines

6.1.- Introduction

In the atheromatous plaque several types of inflammatory cells have been identified [97]. These cells reached the arterial wall proceeding from the arterial lumen and in these processes several molecules are implicated (Figure 11).

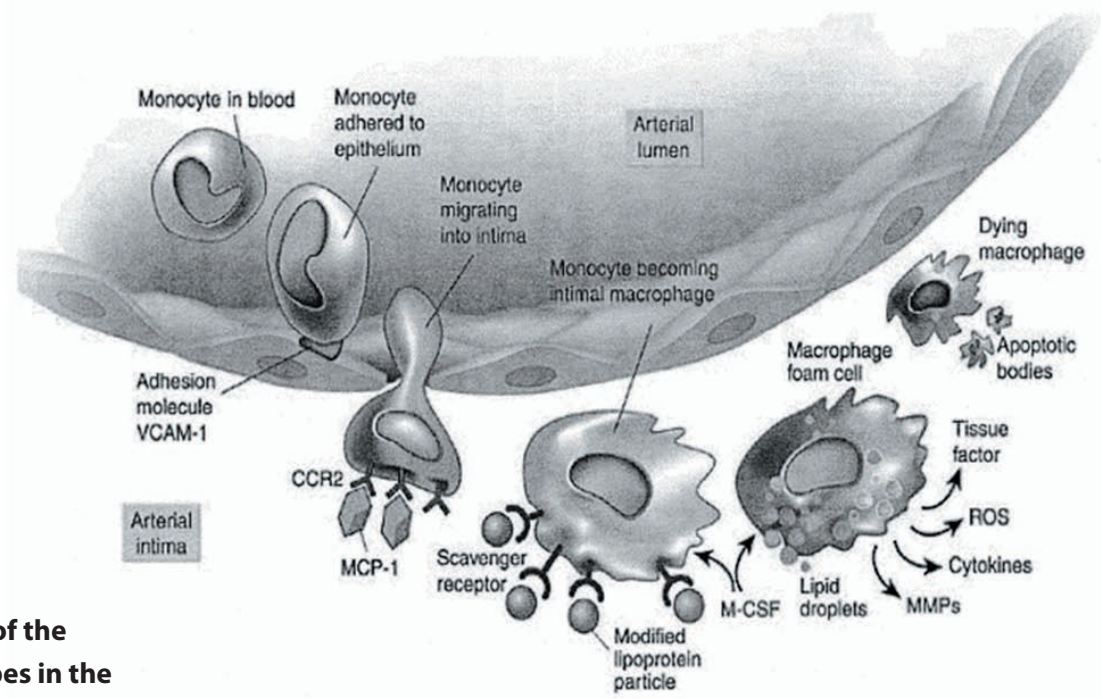


Figure 11. Overview of the inflammatory cell types in the arterial wall and processes mediated by chemokines.

Chemokines are cytokines whose main function is to direct the migration of circulating leukocytes to sites of inflammation [98]. Whether a leukocyte responds to a particular chemokine is determined by its complement of receptors. Chemokine binding activates a signal transduction cascade that activates phosphatidylinositol-3 kinase, increases levels of inositol triphosphate and intracellular calcium, activates Rho and mitogen-activated protein kinases, and eventually leads to actin re-arrangement, shape change, and cell movement [99,100].

There are four main families of chemokines, classified according to structure and function. The largest family is the CC chemokines, named because the first two of the four cysteine residues are adjacent to each other [101]. The

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most studied chemokine in the CC family is monocyte chemoattractant protein-1 (MCP-1) and its natural receptor is CCR-2. However, other CC chemokines (MIP-1α, MIP-1β, RANTES) also attract mononuclear cells to sites of chronic inflammation [102]. A second family of chemokines consists of CXC chemokines, with a single aminoacid interposed between the two cysteines. Among them, interleukin-8 (CXCL8) has been strongly related to the attraction of polymorphonuclear leukocytes to sites of acute inflammation[103]. The third family is characterized by having three aminoacids between cysteines, and so they are named CX_3C family. The unique chemokine in this group is fractalquine (CX_3CL1), and it is of particular interest because it may act either as a direct adhesin or as a soluble chemoattractant with the participation of TNF-α converting enzyme [104].

Insults to endothelial or smooth muscle cells, such as hypercholesterolemia or flow shear stress, stimulate the production of leukocyte chemoattractants that are both displayed on the luminal surface of endothelial cells and also secreted into the sub-endothelium [105]. When these factors activate their receptors on rolling leukocytes, this induces firm integrin-dependent adhesion to the endothelium, followed by diapedesis into the sub-endothelium [106].

6.2.- MCP-1/CCR-2 and atherosclerosis

6.2.1. Introduction

In the artery wall, monocytes differentiate into macrophages and take up cholesterol to become foam cells, a major component of the fatty streak [107]. The target cell specificity of MCP-1 for monocytes makes this chemokine a key candidate for the signal that brings circulating monocytes into the vessel wall [108, 109] . This may be the reason why MCP-1 is strongly expressed in macrophage-rich regions of human and rabbit atherosclerotic lesions[110] . Further, the induction of flow shear stress (in an animal model of hypertension) or the presence of oxidized LDL cholesterol have been associated with higher MCP-1 expression [111] , and interestingly, the sole interaction between monocytes and endothelial cells resulted in the increased expression of MCP-1[112]. The expression of MCP-1 is also influenced by pro and anti-inflammatory cytokines. Interleukin 4 has the

ability to promote inflammation in the vascular endothelium, by inducing the genes coding for vascular cellular adhesion molecule-1 and MCP-1[113]. TNF-α also modulates the expression of MCP-1, mediated by reactive oxygen species[114] and through a NF-kappa B-dependent distal enhancer[115]. The role of enhancers in MCP-1 expression is specially relevant, since the presence of activators, which bind the enhancers of the MCP-1 gene, accelerate the rate of gene transcription, indicating that the promoter region is highly influenced by these distal regions. One of these activators is CCAAT/enhancer-binding protein (C/EBP), that stimulate transcription of MCP-1, interact with HIV particles [116] and intervene in adipocyte differentiation [117]. In summary, all these evidences indicate that MCP-1 may be the mediator of known cardiovascular risk factors (hypercholesterolemia, hypertension) in the inflammation of the artery wall, a process in which a complex chemokine interaction takes place.

“ MCP-1 may be the mediator of known cardiovascular risk factors (hypercholesterolemia, hypertension) in the inflammation of the artery wall

The recruitment of monocytes/macrophages and migration to sites of inflammation may be performed by a number of different signaling molecules, but MCP-1 is probably the most important. Curiously, increased MCP-1 is detected in atherosclerotic lesions but not in normal arteries[118]. Mice rendered genetically deficient for MCP-1 have significant defects in the recruitment of monocytes to sites of inflammation[119], and, when crossed with well-known models of atherosclerosis (LDL receptor or ApoE$^{-/-}$), showed a decreased lesion formation [120] (Figure 12).

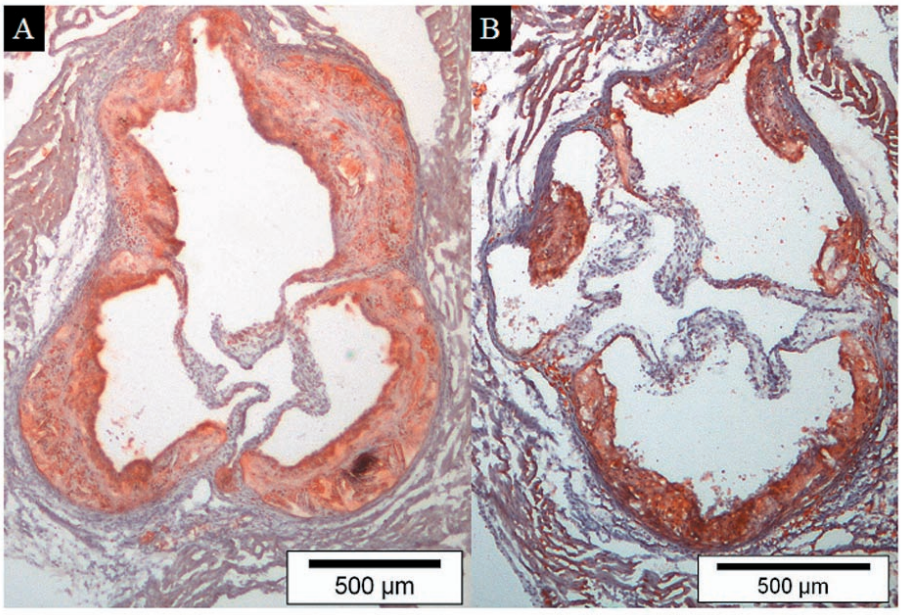

Figure 12. Aorta photomicrographs from mice deficient in LDL receptor (LDLr) (Panel A) and in double knockout (LDLr and MCP-1, Panel B) animals, showing a significant reduction in the area of atheromatous plaque in those animals deficient in MCP-1.

The effect of CCR-2 deficiency has been also tested in a model of femoral arterial injury [121] in which it was observed that a significant reduction in intimal hyperplasia occurs after arterial injury in CCR-2 $^{-/-}$ mice compared with CCR-2 $^{+/+}$ littermates. Conversely, the over-expression of MCP-1 accelerates atherosclerosis in ApoE-deficient mice by increasing the number of macrophages in the artery lesion and oxidized lipid accumulation as reported in irradiated Apo E $^{-/-}$ mice [122]. All these data suggest that MCP-1 and/or CCR-2 play a pivotal role in critical and early stages of atherosclerosis development.

“ MCP-1 and/or CCR-2 play a pivotal role in critical and early stages of atherosclerosis development

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

6.2.2. MCP-1-based strategies in the management of atherosclerosis

Recent research has investigated MCP-1 as a potential therapeutic target in animal models of atherosclerosis. It has been described that an N-terminal deletion mutant of MCP-1 (7ND) gene, which lacks N-terminal amino acids 2 to 8, produce a modified MCP-1 molecule that blocks the MCP-1/CCR-2 pathway and completely inhibits MCP-1-mediated monocyte chemotaxis in vitro [123]. Similarly, in ApoE$^{-/-}$ mice, it inhibited the formation of atherosclerosis lesions without significant effects on serum lipid concentrations, and it also limited the progression of preexisting lesions [124]. Moreover, the lesion composition showed fewer macrophages, less lipids, more smooth muscle cells and more collagen [125]. Furthermore, the use of 7ND gene therapy is associated with a significant reduction in the intima hyperplasia of the vein graft [126], and rats and monkeys transfected with the mutant gene were more resistant to restenosis after balloon injury [127].

In summary, these studies showed that MCP-1 is a promising therapeutic target to reduce not only the development of atherosclerosis but also the restenosis of coronary artery by-pass grafting or stent implantation in atheromatous plaques. The lack of MCP-1 is followed by impaired monocyte recruitment to the inflamed or injured artery wall, and then the development or instability of atheromatous plaques is less likely. Although these investigators found no adverse effects associated with 7ND transfection (studies lasted for 2 to 8 weeks), it is unlikely that a continuous blockade of MCP-1 function would have no major consequences. However, if limited to short periods of time, i.e. to prevent restenosis after coronary manipulation, this gene therapy may prove to be useful, and consequently, the search for products which may transiently inhibit the MCP-1 response to inflammation seems to be an strong strategy [128].

MCP-1 is a promising therapeutic target to reduce not only the development of atherosclerosis but also the restenosis of coronary artery by-pass grafting or stent implantation

6.2.3. MCP-1 is an insulin-responsive gene; implications in metabolic alterations.

MCP-1 and its receptor CCR-2 have not only a direct effect in determining the impact of the inflammatory response in the atherosclerotic lesion, but they are also under the influence of metabolic variables and vice versa. The expression of CCR-2 is up-regulated in cell cultures enriched with LDL cholesterol, and these results have been confirmed in vivo. The CCR-2 expression was increased about 2-fold in monocytes isolated from hypercholesterolemic patients, compared to monocytes from normal controls, and there was a significant correlation between CCR-2 expression and plasma LDL concentration [129]. Similarly, Tous M et al. [130] described a significant increase in MCP-1 expression in aorta and in the liver that was mediated, at least in part, by the hypercholesterolemia experienced by ApoE$^{-/-}$ mice. It implies a higher likelihood for MCP-1/CCR-2 to interact and therefore a higher monocyte recruitment could be expected.

“there is a close link between the inflammatory response and some metabolic variables

MCP-1 is sensitive to insulin resistance states, as shown in obese mice. The hyperinsulinemia that accompanies obesity leads to an over-expression of MCP-1, which in turn modifies the function of adipocytes [131]. This may be important in the assessment of complications in type II diabetes and metabolic syndrome affected patients.

Recent evidence showed that CCR-2 exerts a significant influence in both the development and maintenance of obesity. When mice were fed a high-fat diet, CCR-2 modulated feeding behavior, the development of obesity, and the development of obesity-associated adipose tissue inflammation and insulin resistance [132].

“the influence of MCP-1/CCR-2 is beyond the monocyte chemotaxis regulation and they are implicated in the pathophysiology of complex metabolic disturbances

Obesity has been closely related with the over-expression of chemokines and the plasma concentration of MCP-1 is higher in obese subjects than in controls [133], an association that is significantly attenuated by a reduction in body weight [134]. These data indicate that there is a close link between the inflammatory response and some metabolic variables, a link that is bi-directional, and that it could become an interesting focus of research in years to come. It is of note that these findings suggest that the influence of MCP-1/CCR-2 is beyond the monocyte chemotaxis regulation and they are implicated in the pathophysiology of complex metabolic disturbances.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

6.2.4. Determinants of plasma MCP-1 concentration

The variables implicated in the plasma concentration of MCP-1 were first reported in a cohort of Japanese participants [135]. Age was significantly associated with higher plasma concentrations of MCP-1, even when participants with any atherosclerosis-related events were excluded.

Several polymorphisms in the MCP-1 gene also have effects on MCP-1 expression and, consequently, on its plasma concentration. The substitution of adenine for guanine at the position –2518 of the promoter region is the most studied, and influences the plasma concentration of MCP-1[136]. Those individuals who bear the mutated allele may be more responsive to any inflammatory stimuli, and then, more monocytes can be recruited to sites of inflammation. This boosted inflammatory response was probably a selective factor when infections were the main cause of death among population. This concept may well be exemplified with the deletion of 32 base pairs in the CCR-5 gene, a mutation that protects against the consequences of *Yersinia pestis* infection [137]. Nowadays, however, it may be a predisposing factor to a detrimental inflammatory response in the artery wall and in particular may become a strong atherosclerosis promoter.

The oxidation of lipids, specially the presence of ox-LDL cholesterol in the artery wall, is a highly efficient trigger in the inflammatory cascade, which leads to the development of atherosclerosis [138]. The presence of these oxidized particles increases the expression of MCP-1 [139], and again, these data confirm that MCP-1 may be the driver of the inflammatory cascade originated by oxidized particles in the artery wall.

There are several drugs that may modulate both the expression and concentration of MCP-1. Statins, by inhibiting HMG-CoA reductase, are potent inhibitors of MCP-1 expression in several cell culture lines [140]. Studies in humans have confirmed a beneficial influence of lipid-lowering therapy, with statins and/or fibrates, in the reduction of plasma MCP-1 concentration, an effect that is not solely a consequence of the significant reduction in LDL cholesterol concentration but is a direct effect on the gene expression of MCP-1[141, 142]. This reduction was further incremented when patients with hypercholesterolemia and hypertension were treated with a combination of statins and an angiotensin II receptor antagonist [143].

> **“ individuals who bear the mutated allele may be more responsive to any inflammatory stimuli, and more monocytes can be recruited to sites of inflammation**

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

6.2.5. MCP-1 and atherosclerosis in humans.

MCP-1 has been assessed in several studies addressing its influence in atherosclerosis and results are summarized in Table 4. Serum concentrations of MCP-1 are higher in salt-sensitive high blood pressure patients [144], and have also been related to a worse metabolic control in diabetic patients [145], suggesting that persistently higher concentrations of plasma glucose are associated with an up-regulation of MCP-1 and CCR-2. In another study, the serum concentration of MCP-1 did not show an independent predictive value in the vascular risk assessment, although the presence of the mutated allele in the promoter region (-2518G) was associated with a higher risk of subclinical atherosclerosis [146].

Several studies have addressed the relationship between MCP-1 and the occurrence and severity of cardiovascular diseases. Studies combining the measurement of IMT or the coronary calcium score and the systemic inflammatory activity revealed that the higher the plasma MCP-1 concentration, the higher the carotid IMT or the CAC score [147, 148] (Table 4). Results from a large prospective study showed a significant association between plasma MCP-1 concentration and clinical outcomes in patients with acute coronary syndrome [149]. The association was also strong with cardiovascular risk factors such as age, smoking, family history of coronary artery disease, hypertension, diabetes, hypercholesterolemia and higher concentration of C-reactive protein. MCP-1 has also been studied in patients after receiving coronary angioplasty, and the findings indicate that the higher the plasma concentrations of MCP-1, the higher the likelihood of having a restenosis, which highlights the potential of using plasma MCP-1 as a biomarker to assess the prognosis of the acute coronary syndromes[150]. Although, most of these studies showed a clear relationship between plasma MCP-1 concentration and the presence of the disease, it is not clearly stated whether MCP-1 represents an independent risk factor for its development . More recently, Van Mieghem CA. et al [151] have published their results concerning the use of non-standard laboratory and image-related variables for the prediction of coronary events. The concentrations of C-reactive protein, interleukin-6 and MCP-1 were not associated with changes in novel invasive imaging modalities, such as palpography or virtual histology of the coronary arteries, adding further controversies to the feasibility of using MCP-1 as a biomarker for CV diseases.

“ **significant association between plasma MCP-1 concentration and clinical outcomes in patients with acute coronary syndrome**

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

6.2.6. MCP-1 as a potential biomarker for atherosclerosis and concluding remarks.

Biomarkers are generally considered to be plasma measurements of molecules, proteins, or enzymes that provide independent diagnostic or prognostic value by reflecting an underlying disease, state or condition. Several issues should be accomplished by the biomarker to emerge in the clinical settings [152]:

1. It should be able to account for a significant proportion of the disease being evaluated.
2. It should be accurate and reliable.
3. It should provide good sensitivity, specificity, and predictive value.
4. It should be available for widespread application.

Moreover, the clinical application ultimately requires that the biomarker substantively must add new information to traditional risk factors, such as those used in the Framingham risk score. Among relatively new biomarkers, these attributes have been partly accomplished by C-reactive protein [153]. Only three studies provide data to assess plasma MCP-1 as a biomarker for atherosclerosis. McDermott et al [136] measured the serum concentration of MCP-1 in the Framingham cohort. There was a significant correlation with age, cigarette smoking, triglycerides, body mass index, and waist-to-hip ratio. However, these covariates explained only 6% of the variability in serum MCP-1, which in turn were not associated with the prevalence of myocardial infarction in multivariate models. Similarly, Iwai N. et al [154] reported that MCP-1 concentration is highly correlated with carotid IMT, but this association lacked significance in the multivariate model. In this study, however, there was no data on the plasma MCP-1 concentration for patients with MI. Finally, Mosedale DE et al.[155] assessed the role of plasma MCP-1 concentration in predicting atherosclerotic burden and the correlation with the Framingham risk score. They did not find any significant relationship.

All these results suggest no value for circulating MCP-1as a biomarker for atherosclerosis. It may be argued, however, that because MCP-1 acts in the inflamed sites, including the artery wall, the measurement of its circulating concentrations is not reliable. Technical limitations (due to the inherent circulating nature of MCP-1) should not overshadow the relevant implications of this chemokine in atherosclerosis. The role of MCP-1 in atherosclerosis is beyond discussion, and chemokine research specially focused in MCP–1/CCR–2 supports the role of this chemokine axis in the pathophysiology of atherosclerosis.

“ The role of MCP-1 in atherosclerosis is beyond discussion, and chemokine research specially focused in MCP-1/CCR-2 supports the role of this chemokine axis in the pathophysiology of atherosclerosis.

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Table 4. Summary of human studies assessing the relationship between circulating MCP-1 and atherosclerosis.

Reference	N	Sample	End Point
Larrouse M. et al [144]	43	Serum	High Blood Pressure (HB
Mine S. et al [145]	106	Serum	Glucose control
Kim CS. et al [133]	100	Serum	Obesity
Kim MP. et al [146]	255	Serum	Framingham Risk Scor
Deo R. et al [148]	3499	Plasma	Coronary Risk Factors CAC score
Mosedale DE. et al. [155]	446	Serum	Coronary Heart Diseas Carotid Atherosclerosi
Alonso-Villaverde C. et al. [i]	183	Plasma	Carotid IMT
Coll B. et al. [147]	129	Plasma	Carotid IMT
Iwai N. et al. [154]	2180	Serum	Carotid IMT
Pawlak K. et al. [ii]	81	Plasma	Carotid IMT
De Lemos J. et al. [149]	2549	Plasma	Death or Myocardial Infarction
Cipollone F. et al. [150]	50	Plasma	Restenosis after PTCA

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

	Results
BP)	Higher CCL2 in salt-sensitive HBP patients
	Higher CCL2 associate with worse metabolic control in DM
	Higher CCL2 in obese subjects than controls
e	No significant associations with CCL2 concentrations
	CCL2 associated with coronary risk factors and higher CAC score
e s	No differences in CCL2 concentration between patients with and without atherosclerosis
	Higher CCL2 was associated with higher carotid and femoral IMT in HIV-infected patients
	Higher CCL2 was associated with higher carotid IMT in Lipodystrophic HIV-infected patients
	Higher CCL2 was associated with higher IMT using univariate analysis
	Higher CCL2 was associated with higher IMT in patients with chronic renal insufficiency
	CCL2 was associated with the occurrence of myocardial infarction
	Higher baseline CCL2 associated with higher risk of restenosis

DM indicates diabetes mellitus. IMT: intima-media thickness; CAC: coronary calcium score; PTCA is percutaneous coronary angiography.

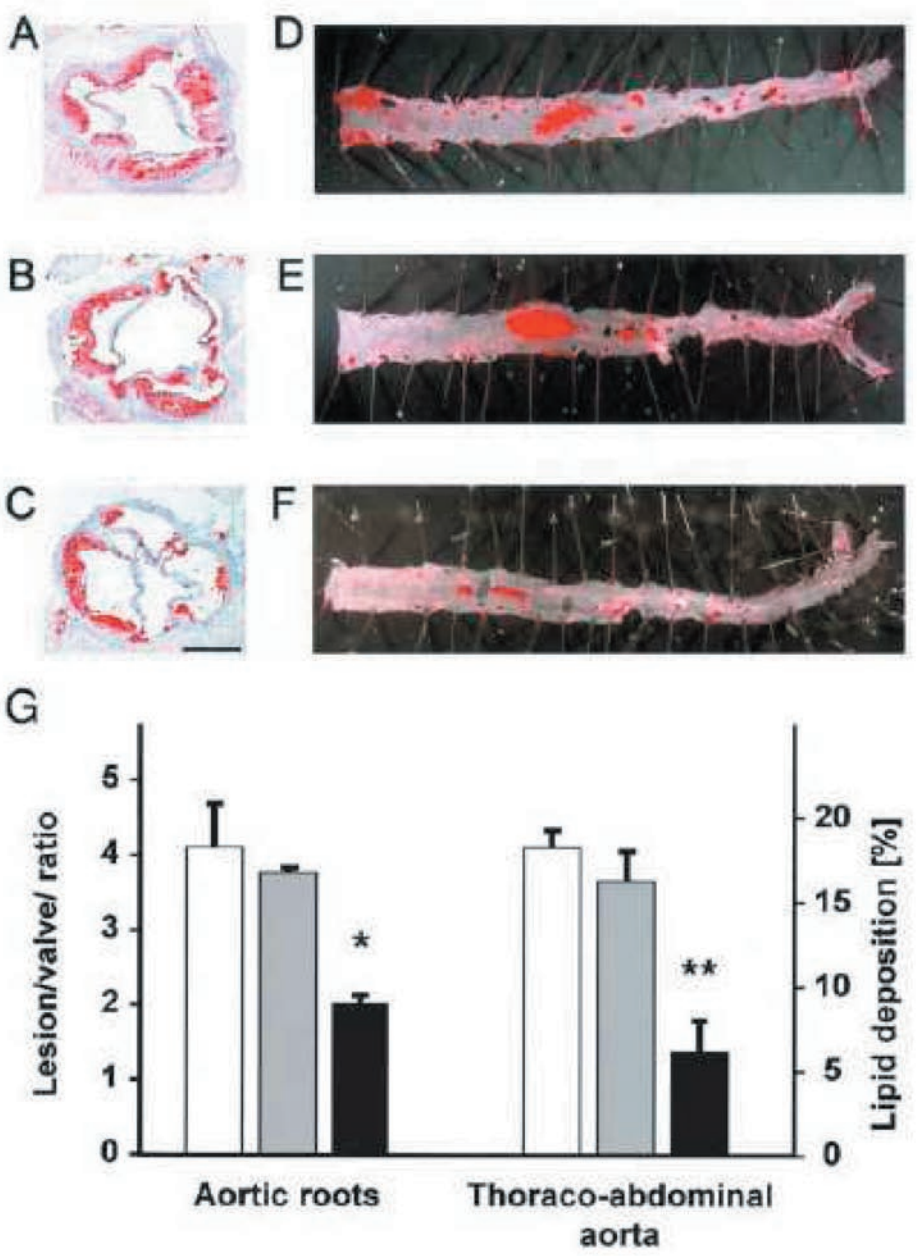
UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

6.3.- RANTES, MIP-1α, MIP-1β/CCR-5 and atherosclerosis

RANTES, MIP-1α, MIP-1β belong to CC group of chemokines, and their natural receptor is CCR-5, a trans-membrane protein[164]. They are also involved in the recruitment of monocytes to the inflamed arterial wall, although the implication of these chemokines in atherosclerosis is more controversial[165,166]. CCR-5 is present on human aortic smooth muscle cells, and its ligands mobilize intracellular calcium and induce the expression of tissue factor [167]. Thrombosis mediated by tissue factor is widely regarded as a key factor in the pathogenesis of acute coronary syndromes [168]. Thus, CCR-5 may transduce signaling pathways known to have clinical manifestations and to be associated with smooth muscle cell activation. Met-RANTES, a chemokine receptor antagonist that blocks CCR-1 and CCR-5, significantly reduced lesion progression in atherosclerotic mice[169]. Met-RANTES also inhibits intimal hyperplasia after arterial injury to ApoE $^{-/-}$ [170],supporting a role for these chemokines in mediating the response to arterial injury (Figure 13).

Figure 13. Effect of Met-RANTES (block of CCR-5) in the aorta of mice prone to atherosclerosis. (Circ Res. 2004;94:253-261).



Footnote: a significant reduction in plaque area was observed in those mice treated with Met-RANTES paralleled with a reduction in lipid deposition (black bars in figure G). C and F slides withdraw those treated with Met-RANTES. These research have led to the design and study of anti CCR-5 molecules that have been tested in mouse-model of atherosclerosis. TAK-779 is an anti-CCR5 agent that effectively decreased the development of atherosclerosis in mice, by decreasing the number of T lymphocytes in the atheroma plaque [171].

6.4.- SDF-1 and atherosclerosis

SDF-1 is a CXC chemokine that mainly chemoattracts lymphocytes and monocytes [172]. It has been associated with the aggregation and activation of platelets [173], it is highly expressed in atherosclerotic plaques [174], and it has been associated with the mobilization of progenitor cells in diabetic patients treated with insulin [175] . Interestingly, platelets express and release SDF-1 into the microcirculation upon activation, and platelet-derived SDF-1 is functionally involved in the recruitment of endothelial progenitor cells to arterial thrombi [176]. SDF-1 also induces tissue factor synthesis in smooth muscle cells, mediating a procoagulant state in the arterial wall [177]. Furthermore, neutralizing antibodies to SDF-1 inhibited intimal hyperplasia after carotid arterial injury in the ApoE $^{-/-}$ mice [178]. These data attribute a detrimental effect of SDF-1 in the development of atherosclerosis and in the destabilization of atheromatous plaques. However, data on animal models targeting SDF-1 in the development of atherosclerosis is lacking and studies in humans are scarce.

Interestingly, SDF-1 is also related with metabolic and immune variables. The SDF-1 gene is located on chromosome 10q11.1 near type 1 diabetes susceptibility locus IDDM10, that may suggest a contribution by SDF-1 to the induction of diabetes. A selected polymorphism in the SDF-1 gene is associated with the age-at-onset of diabetes mellitus, and this association is probably related with the activation of monocytes and naïve T cells mediated by SDF-1, that determine the degree of the insulitis before development of overt diabetes [179]. Indeed, Matin and coworkers [180] have reported that administration of anti-SDF-1 antibody to female diabetic mice resulted in the suppression of insulitis and diabetes onset.

Footnote: arrowheads indicate lymphocyte infiltration. Note the lymphocyte spread in the untreated diabetic mice(in slide C) when compared to anti-SDF-1 treated mice (B) and non-diabetic controls (A).

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

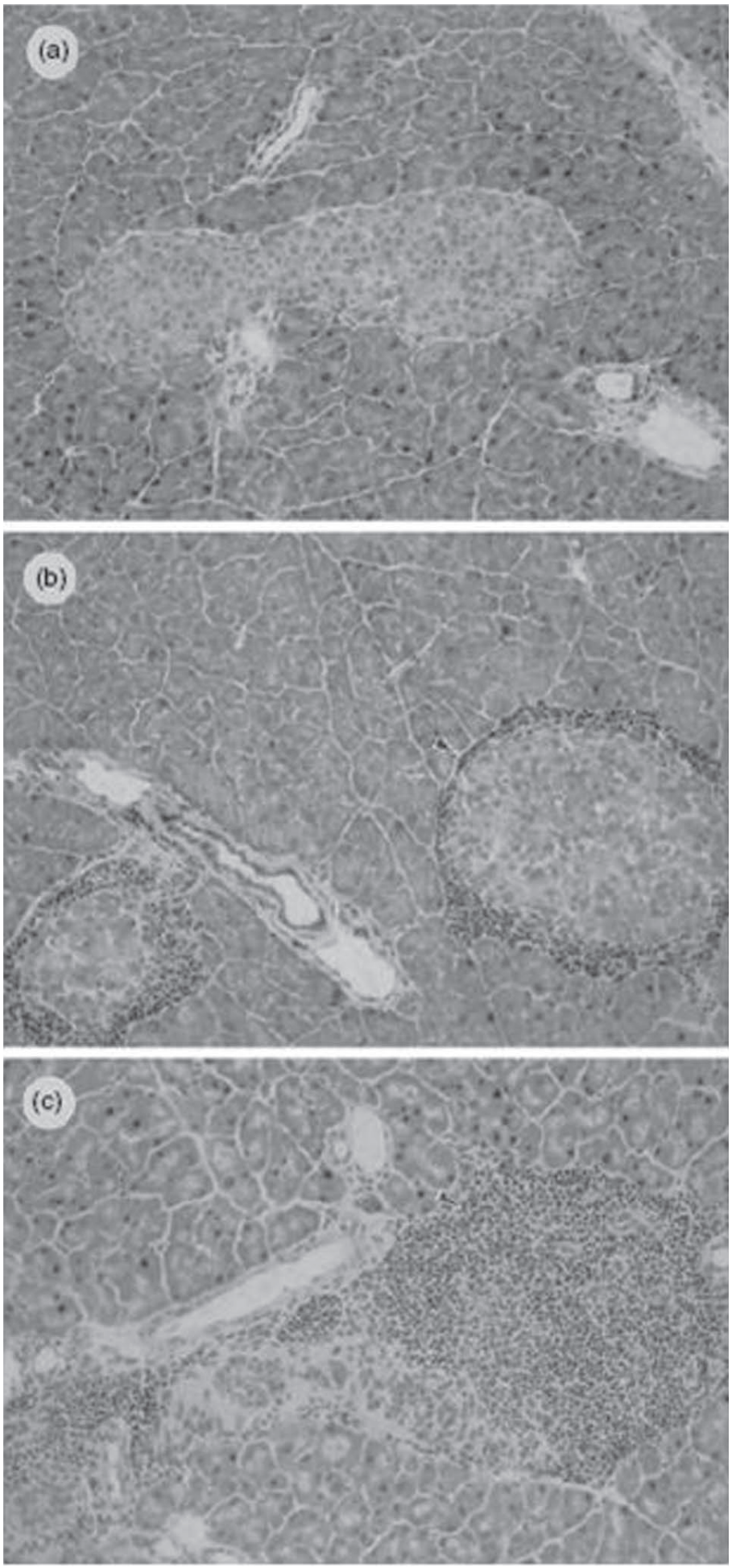


Figure 14. Photomicrographs of pancreatic islets in : A) non-diabetic mouse, B) anti-SDF-1 treated diabetic mice and C) untreated diabetic mice (Inmunology 2002; 107;222).

6.5.- CX3CR-1 and atherosclerosis

Fractalkine and its natural receptor, CX3CR-1, have a critical role in the chemotaxis of circulating monocytes to the subendothelial space [181]. Fractalkine contain multiple domains and is structurally distinct from other chemokines. The extracellular domain connects to an extended mucin-like stalk, followed by a transmembrane domain and an intracellular domain of 37 amino acids [182], and therefore, it functions not only as a chemo-attractant but also as an adhesion molecule [183], thereby obviating the need for both the association with proteoglycans and other adhesion molecules [184]. The CX3CR-1 gene is located in the chromosome 3q22 and fractalkine is in 16q. The expression of fractalkine can be markedly induced by inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1, and interferon γ (IFN) [185].

CX3CR1-expressing cells bind rapidly and with high affinity to immobilized fractalkine or fractalkine-expressing cells in both static and physiological flow conditions (Figure 15).

This particularity supports the active research concerning CX3CR-1/fractalkine and atherosclerosis [186]. Fractalkine expression is upregulated in atherosclerotic lesions and crossing CX3CR1$^{-/-}$ into the ApoE$^{-/-}$ mice, results in decreased atherosclerotic lesion formation with reduced macrophage accumulation [187, 188].

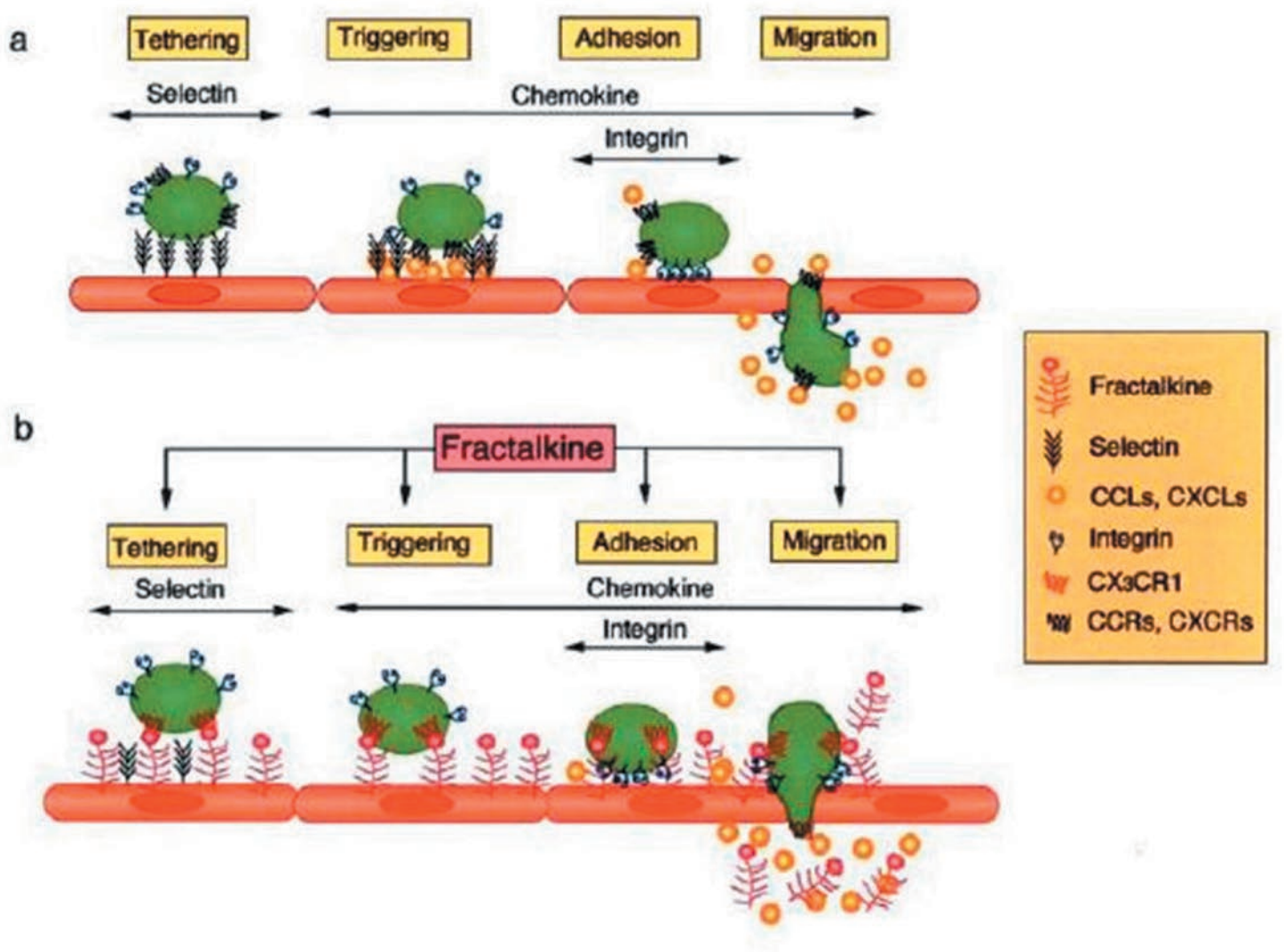


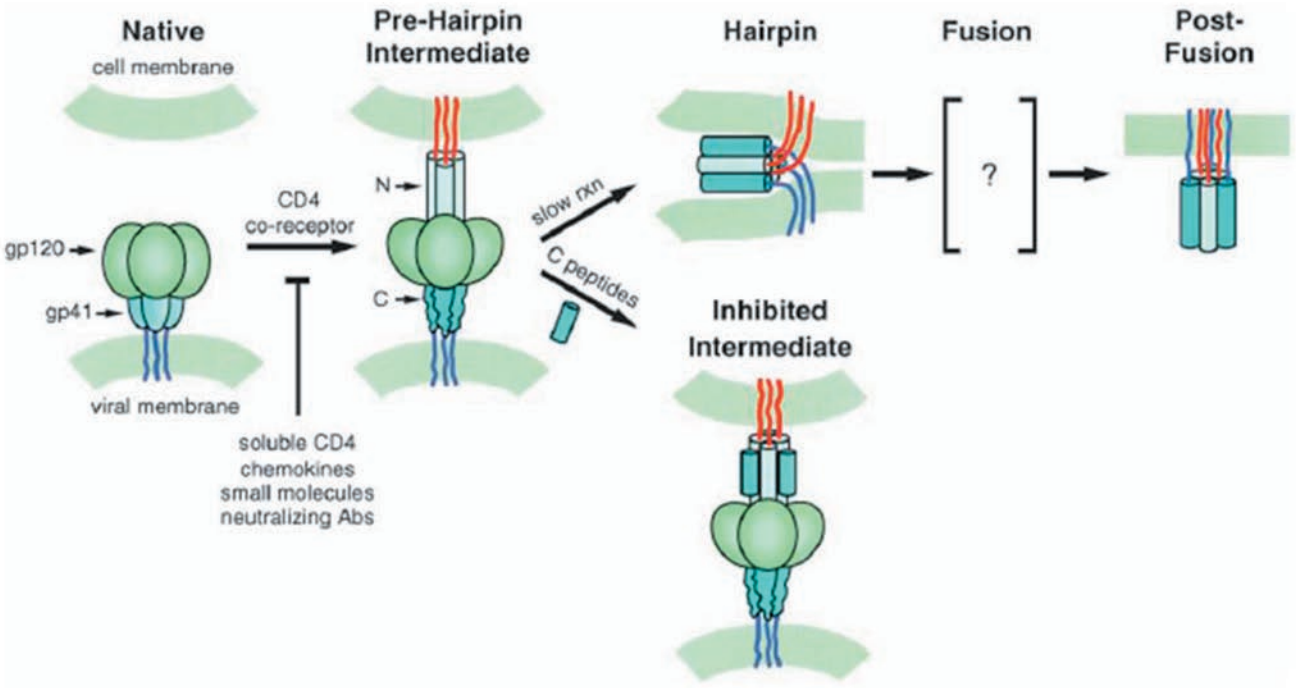
Figure 15. Schematic overview of monocyte chemotaxis, comparing chemokines (Panel A) and fractalkine (Panel B) (Arterioscler Thromb Vasc Biol. 2004;24:34-40). Interestingly, fractalkine is selectin and integrin-independent, which results in firmer adhesion of circulating leukocytes.

7.- Have these chemokines been involved in HIV pathogenesis?

The entry of HIV into the cell is highly influenced by molecular interactions, in which chemokines and chemokine-receptors have a relevant role [189]. HIV possess a single type 1 integral membrane glycoprotein, the viral envelope (Env), which is responsible for both receptor binding and mediating the membrane fusion between the virus particle and the host cell membranes. Env is initially synthesized as a monomeric glycoprotein precursor (gp160) which is processed into the gp120 surface subunit and gp41 transmembrane subunit domains [190]. The entry process is a two-step event where an initial association of Env with CD4+ cell surface elicits conformational changes in gp120 that expose the chemokine receptor binding site. Subsequent interaction of the gp120-CD4 complex with the relevant chemokine receptor molecule results in further conformational changes in the gp41 subunit which in turn facilitates the fusion event between the viral and host cell membranes [191] (Figure 16).

“The entry of HIV into the cell is highly influenced by molecular interactions, in which chemokines and chemokine-receptors have a relevant role

Figure 16. Schematic overview of the process of HIV-entry into the cell (Biochimica et Biophysica Acta 2003; 1614: 36– 50 [192]).



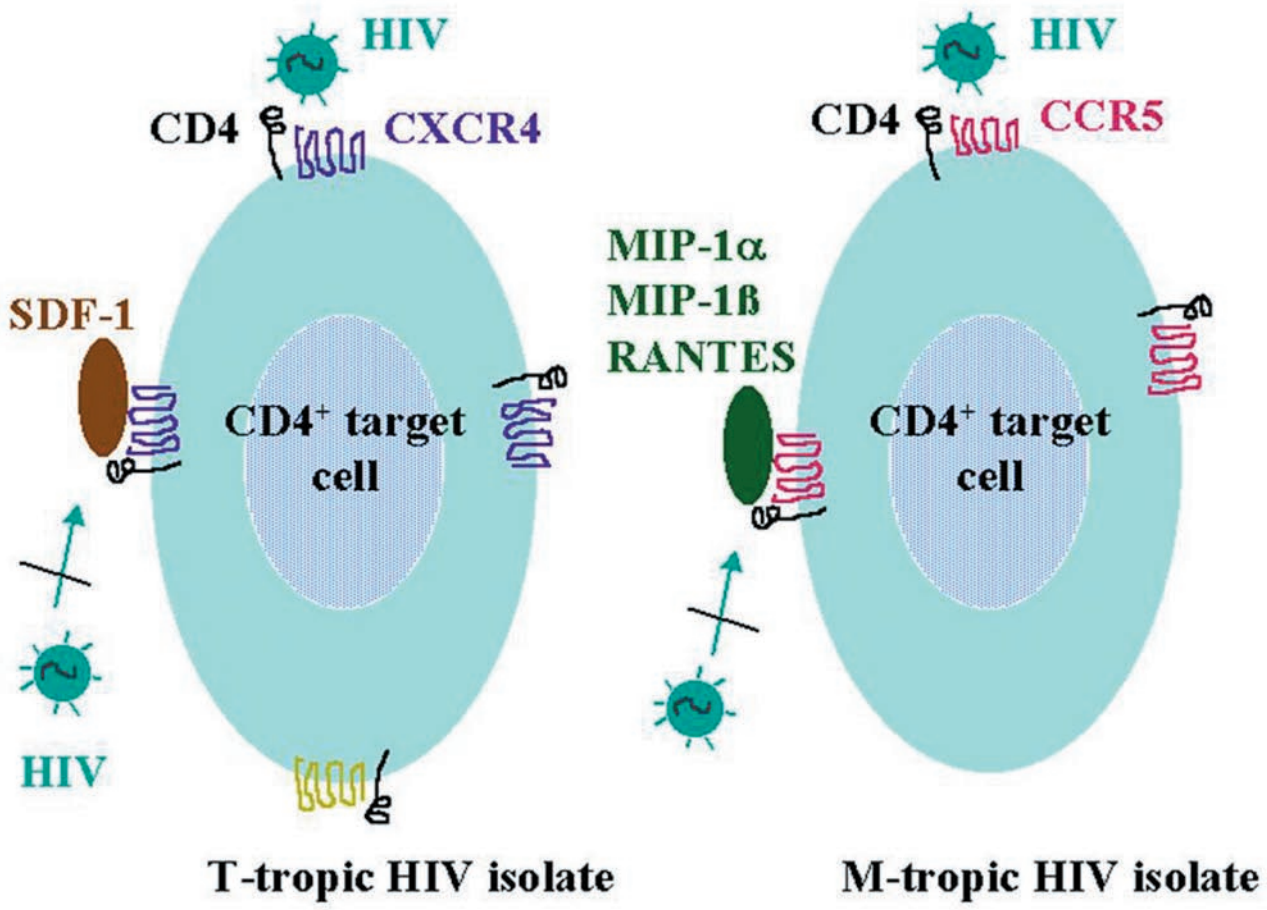
Different chemokine receptors (CCR-2, CCR-3, CCR-5, CXCR-4, STRL33) or orphan, chemokine receptor-like molecules (GPR1, GPR15, V28, APJ) may participate in HIV entry, but the initially described CXCR-4 and CCR-5 molecules remain the principal co-receptors for X4 (T cell line-tropic or syncytium inducing) or R5 (macrophage (M)-tropic or non-syncytium inducing) isolates, respectively [193].

In early stages of HIV-infection, HIV generally uses M-tropic co-receptors (CCR-5), and progressively the virus may use both ways of cellular entry (CCR-5 and/or CXCR-4)[194]. Thus, all the natural ligands of these chemokine receptors may interact and compete with HIV in the entry into the cell (Figure 17). The inhibitory effect of the CCR-5 natural ligands, MIP-1α, MIP-1β and RANTES, on the infection by R5 HIV strains in vitro is well established. Likewise, SDF-1 suppresses the entry of X4 HIV strains. Both blocking and down-regulation of chemokine receptors are ways by which their physiologic ligands or modified analogs can reduce HIV entry [195]. Besides, co-receptor dimerization is postulated as a powerful mechanism in avoiding the interaction between HIV-Env proteins and host cell co-receptors [196].

> **“ all the natural ligands of these chemokine receptors may interact and compete with HIV in the entry into the cell**

> **“ the entry of HIV into the cell and the migration of monocytes through endothelial cells in the first steps of atherosclerosis development, share molecular mechanisms**

Figure 17. Interaction of chemokines, chemokine co-receptors and HIV virus.



“Tat or its “chemokine-like” region induced rapid and transient calcium influx in monocytes and macrophages

In conclusion, the entry of HIV into the cell and the migration of monocytes through endothelial cells in the first steps of atherosclerosis development, share molecular mechanisms, in which chemokines and their receptors are highly involved. HIV needs these chemokine-based nets to reach the interior of CD4+ cells, and consequently both systems (i.e. HIV virus and host chemokine system) display mechanisms to interact with each other. In fact, the virus itself is able to modify the expression of both chemokine and chemokine-receptors in cells [197]. HIV infection may influence the intracellular signaling pathways [198] and potentially modify the cellular response to chemokines. For instance, HIV-1 transactivator (Tat) protein is produced by infected cells and released into the extracellular medium. Tat can mediate its effects either by penetrating cells and inducing gene transactivation, or binding to cell surface molecules such as integrins or chemokine receptors[199]. It was recently observed that incubation of PBMC with Tat increased surface expression of CCR-5 and CXCR-4 [200]. Analysis of the Tat protein sequence revealed conserved amino acids which corresponded to critical sequences seen in chemokines and remarkably, Tat was a potent chemoattractant for monocytes. Tat or its “chemokine-like” region induced rapid and transient calcium influx in monocytes and macrophages[199]. These “pleiotropic” activities of Tat constitute a defense mechanism run by HIV itself, that ultimately favors HIV replication, but simultaneously imply a major likelihood for mononuclear cells to interact with inflamed sites, such as the artery wall. For these reasons, the influence of HIV on chemokines should be considered in the study of atherosclerosis, since it might represent a key mechanism for its development (Figure 18).

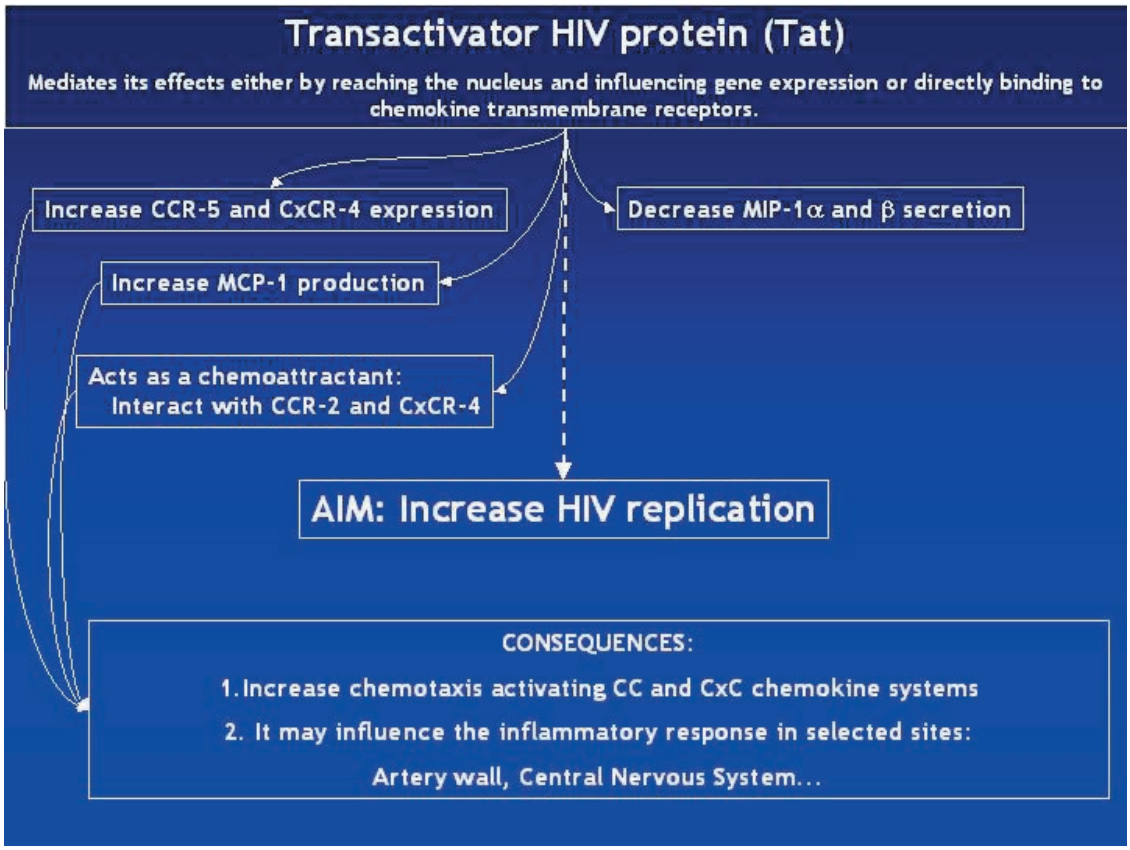
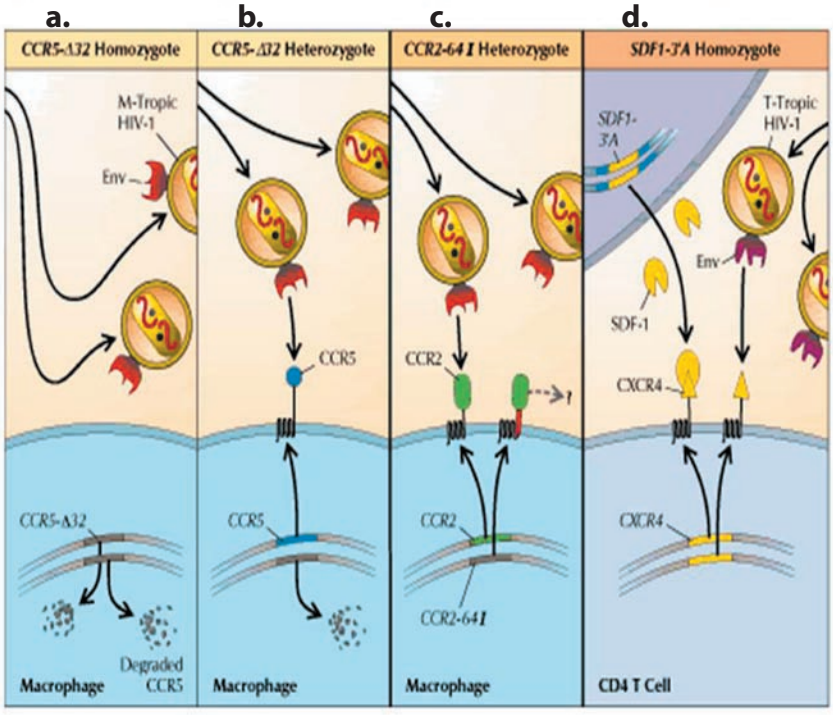


Figure 18. Overview of the influences of HIV on chemokines. Its potential implications in atherosclerosis are depicted, focusing on the effects of Tat.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

8.- Role of chemokines and chemokine-receptors polymorphisms in atherosclerosis and HIV

Single nucleotide polymorphisms (SNPs) in genes coding for these molecules have been identified. These SNPs cause a relevant alteration in the function or the quantity of the gene product, and therefore, different clinical phenotypes should be expected, both in HIV-infection and atherosclerosis [201,202] (Figure 19 and Table 5). The influence of selected polymorphisms in MCP-1 and CCR-2 genes in the development of atherosclerosis have been previously analyzed (see Pathogenesis of atherosclerosis: the role of chemokines, Chapter 6).



a. The homozygous deletion of 32 base pairs in the CCR-5 gene, alters the structural conformation of CCR-5, that makes this receptor unable to interact with HIV proteins.
b. The loss of 32 base pairs in one of the alleles in the CCR-5 gene, alters the expression of CCR-5 and makes the interaction of HIV with CCR-5 unlikely.
c. The substitution of Valine for Isoleucine in the position 64 of the CCR-2 gene alters the function of CCR-2. This change may difficult the interaction between CCR-2 and HIV.
d. The substitution of G to A in the untranslated region of the SDF-1 gene (801), named SDF1-3'A, is associated with changes in the expression of the gene product, that interacts with its receptor and block the entry of HIV into the cell.

“SNPs cause a relevant alteration in the function or the quantity of the gene product, and therefore, different clinical phenotypes should be expected

Figure 19. Schematic overview of the effects of selected SNPs in chemokines affecting the entry of HIV into the cell .

MCP-1—2518G

The substitution of Adenine for Guanine in the position 2518 of the promoter region of MCP-1 gene, confers a major MCP-1 expression in response to inflammatory stimulus [203]. HIV-infected patients have higher plasma concentrations of MCP-1 than non-infected population [204], and probably this is related to the effect of Tat protein of HIV [205]. Taking all these data into account, we could hypothesize that those HIV-infected patients who bear the mutated MCP-1 allele would be predisposed to an even higher MCP-1 expression than those individuals non-infected who bear the mutated allele.

“we could hypothesize that those HIV-infected patients who bear the mutated MCP-1 allele would be predisposed to an even higher MCP-1 expression than those individuals non-infected who bear the mutated allele.

Studies in HIV-infected patients and MCP-1 yield contradictory results. The presence of –2518G allele of the MCP-1 gene has been related to a lower risk of acquiring HIV infection. Conversely, it has been found that, in patients who are already infected and who carry the MCP-1mutated allele, the progression to AIDS is accelerated and the risk of suffering from HIV-associated dementia and Mycobacterium avium complex infection is higher than in patients with the common variant [206]. Conversely, in a Spanish cohort study, the allele frequency of MCP-1-2518 G was significantly higher in HIV-infected patients than 36 exposed-uninfected individuals and 100 controls [207]. Several reasons may explain the discrepancies, such as the different genetic background between populations or the different study design and eligibility criteria for the inclusion in the study. However, a wider approach should be addressed, probably studying haplotypes instead of SNPs. Modi WS et al.[208] studied the long arm of chromosome 17 along 33 kb, where MCP-1, MCP-3 and eotaxin are encoded. They find that three SNPs (-2136 T located in the MCP-1 promoter region, 767G in intron 1 of MCP-1, and -1385A in the Eotaxin promoter) were nearly always found together on a 31 kb haplotype (H7). Frequencies of the three variants and the H7 haplotype were significantly elevated (odds ratio, 0.6; P = 0.005) in uninfected European- Americans repeatedly exposed to HIV through high-risk sexual behavior or contaminated blood products, suggesting that genetic variation in the H7 region protects to acquire HIV-1 infection.

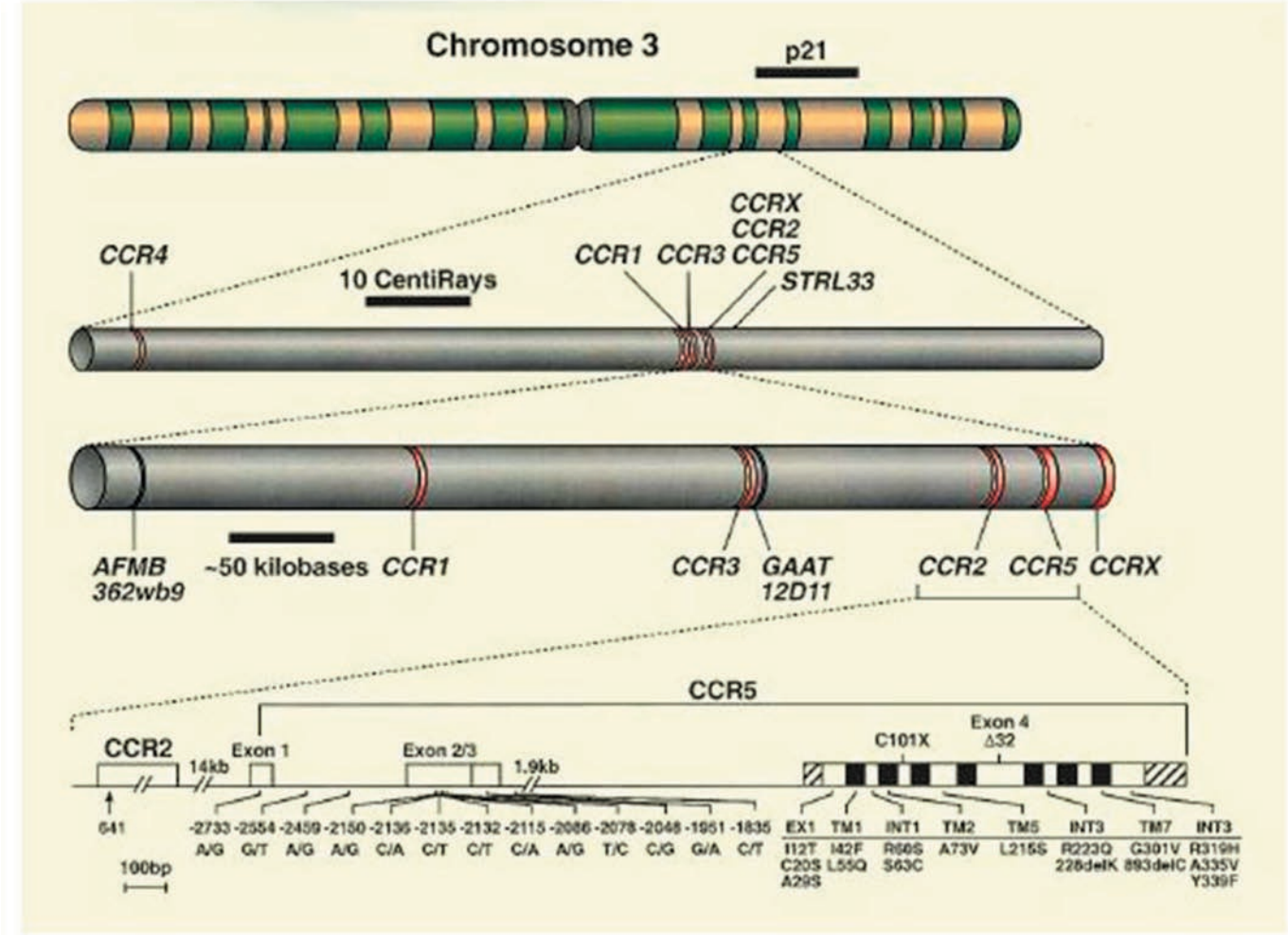
“genetic variation in the H7 region protects to acquire HIV-1 infection

Thus, MCP-1 expression had a two-edged role in HIV infection: it might afford partial protection from viral infection, but during infection, its pro-inflammatory properties collectively may contribute to accelerates disease progression and increases risk of dementia and other AIDS-associated diseases [206].

CCR2-64I

The substituion of Valine for Isoleucine in the position 64 (in the first transmembrane domain) of the CCR-2 gene has been associated to changes in receptor functions [209]. CCR-2 is a minor co-receptor for HIV infection, and it is CCR5's closest gen, based on chromosomal proximity (Figure 20).

Figure 20. Genetic map of chemokine receptor gene cluster on human chromosome 3p21. CCR2-64I, CCR5 delta 32 and single nucleotide variants of the CCR5 promoter region are indicated by their nucleotide position (Immunological Reviews 2000; 177: 99–111. [210]).



Although studies in human populations have yielded contradictory results, probably due to the low mutated allele frequency [211], this polymorphism has been associated to a delay in the onset of AIDS for homozygotes and heterozygotes, without effect on HIV transmission (Figure 21) [212]. Interestingly, HIV-infected patients with nadir CD4+T cells between 200 and 500 and who bear the CCR-2 mutated allele, achieved undetectable HIV viral loads significantly earlier than wild type individuals, although this beneficial response was not paralleled by a better rate of CD4+T cell recovery [213]. The mechanisms underlying these effects are

“this polymorphism has been associated to a delay in the onset of AIDS for homozygotes and heterozygotes, without effect on HIV transmission

not fully understood. The presence of the CCR2-64I allele is in complete linkage disequilibrium with CCR-5Δ32, thus, CCR5Δ32 and CCR2-64I had independent and potent additive effects on delaying AIDS [214]. Functional assays showed little difference in monocyte chemoattractant protein-1 ligand signaling or in the quantity of CCR-2 expressed on peripheral blood mononuclear cells of different CCR-2 genotypes, but they did show indirect evidence for heterodimerization between CCR-2 and the quantity of CXCR-4 and CCR-5 expressed [215, 216]. Similarly, Rodriguez-Frade et al. reported the beneficial effects of CCR-2 activation using a monoclonal antibody, that acts as its natural substrate, MCP-1,in blocking the entry of HIV into the cell [217]. This observation is mediated by the heterodimerization of CCR-2 with CCR-5 and CXCR-4.

CCR-5Δ32

The CCR-5Δ32 is a mutant allele containing a 32 base pair deletion in the open reading frame of the CCR-5 gene that induces a frame shift, a premature stop codon, and loss of HIV co-receptor activity [218, 219] (Figure 22). These changes may alter the correct interaction between CCR-5 and its natural ligands, RANTES, MIPs 1-α and β, and then, the chemotaxis might be severely impaired [220]. The frequency of this mutant allele is approximately 1% in the homozygous state and 10 to 20% for the heterozygous state among Caucasians in North America or Europe, but is lower or absent in subjects of African, Asian, and Latin American heritage [221].

“Theoretically this polymorphism should be associated to a protective effect in the development of atherosclerosis

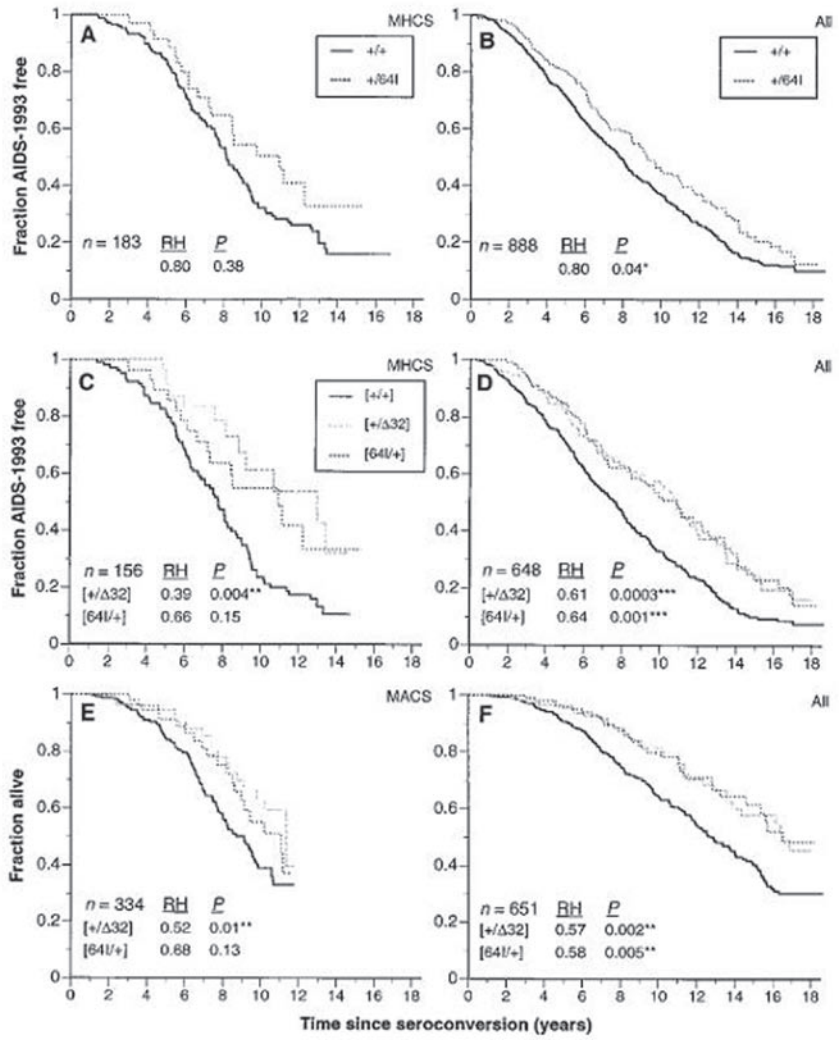
The influence of this mutated allele in HIV infection has been fully studied. Similarly, intense research in animal models prone to develop atherosclerosis, have resulted in relevant insights in atherosclerosis.

Theoretically this polymorphism should be associated to a protective effect in the development of atherosclerosis, but studies did not yield uniform results. Szalai C et al [165], found a lower prevalence of homozygous individuals for CCR-5Δ32 among those cases of coronary artery disease, but it has not been further confirmed [222,223] .

CCR-5 was one of the first HIV co-receptors identified for those M-tropic HIV strains. Thus, research have been focusing on the influence of mutated CCR-5 alleles in HIV infection and disease progression. The highlights of these studies are consistent with the following:

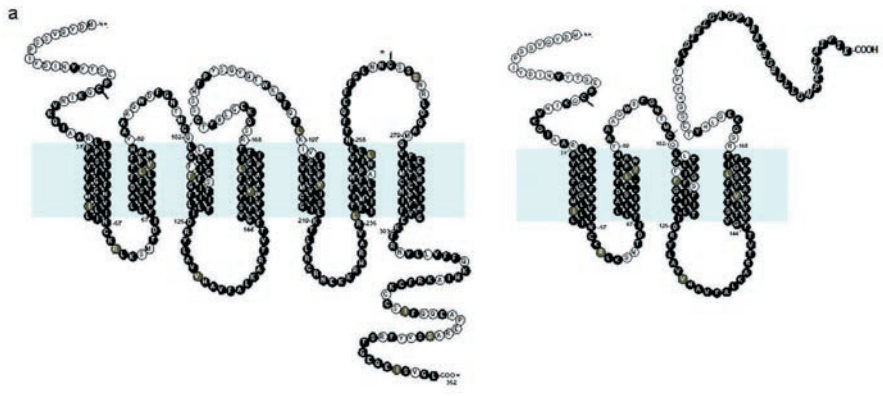
- those individuals homozygous for Δ32 allele in the CCR-5 gene has a natural resistance to HIV-infection although being several times exposed [224].
- Those HIV-infected patients who bear at least one mutated allele for the Δ32 are more prone to delayed HIV-disease progression [225] (Figure 21).

Figure 21. Influence of CCR-5Δ32 and CCR-2 64I polymorphisms in AIDS (From Science 1997;277:959–965).



Footnote: Kaplan Meier survival curves for AIDS-free or alive participants of MHCS cohort (panels A, C and E) and a composite of several cohorts including MHCS (panels B, D and F). ++ indicate CCR-5 wild type; +/64I heterozygote for CCR2-64I; +/Δ32 heterozygote for CCR-5Δ32. RH: hazard ratio.

Figure 22. Schematic representation of CCR-5 receptor.



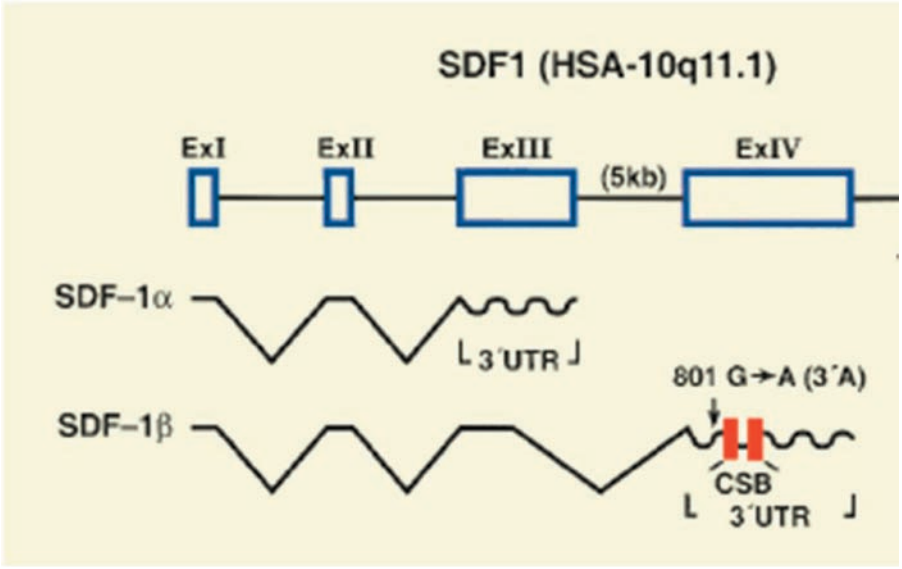
Footnote: Panel a. No-mutated CCR-5 receptor. Panel b. CCR-5 receptor with 32 base pairs deleted.

SDF1-3'A

The screening for polymorphisms of the CXCR4 gene have to date yielded only two nucleotide variants which have little epidemiologic consequences[226]. When the coding region of the only known CXCR4 ligand, SDF, was interrogated, a common SNP variant at position 801 (counting from the AUG codon) in the 3' un-translated region (3'UTR) for SDF-1β was discovered [227] (Figure 23).

studies based on the circulating concentrations of SDF-1 present serious limitations; however, individuals who bear the SDF1-3'A presented a minor SDF-1 expression

Figure 23. Gen map of SDF-1 locus on human chromosome 10q11.1 (Immunological Reviews 2000; 177: 99–111).



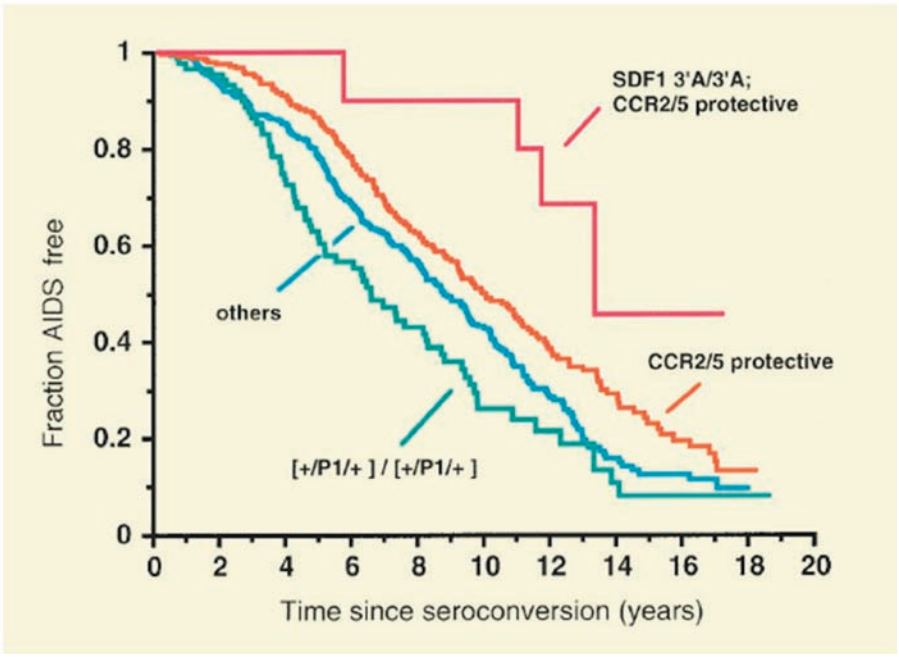
Because SDF expression is limited to stromal cells and other tissues that are not easy to quantify [228], studies based on the circulating concentrations of SDF-1 present serious limitations; however, individuals who bear the SDF1-3'A presented a minor SDF-1 expression and lower circulating levels of SDF-1 [229] . Further, circulating levels of SDF-1α have been found to be lower in an acute coronary heart disease study [230] although no relationship has yet been found between the allelic frequency of the SDF1-3'A and the presence of coronary artery disease [231].

HIV-infected individuals homozygous for the SDF1-3'A/3'A variant show a remarkable level of protection against AIDS

HIV-infected individuals homozygous for the SDF1-3'A/3'A variant show a remarkable level of protection against AIDS in pooled or separated cohorts. Among individuals with both SDF1-3'A/3'A and CCR-5 (or CCR-2) heterozygous mutant alleles, the protective effect is quite strong – several-fold higher than that conferred by CCR-5 or CCR-2 heterozygosity alone [232] (Figure 24), although results from meta-analyses did not support this conclusion [233]. Several hypothesis have arose concerning SDF-1 and HIV,

and in fact, the epidemiologic interaction of CCR-5 or CCR-2 and SDF1-3'A (a genetic phenomenon termed epistasis) suggests that a functional interaction might explain the enhanced protection. One hypothesis is that CCR-2 and CCR-5 variants slow AIDS by limiting the number of CCR-5 co-receptors that mediate the replication and spread of primary, early stage R5 HIV, while the SDF1-3'A variant restricts the emergence of X4 tropic HIV strains and the ensuing AIDS-accelerating process. A possible mechanism would be overproduction of SDF-1 in local compartments, which binds to and blocks the CXCR-4 receptors required for X4 viruses to emerge and predominate.

Figure 24. Kaplan Meyer Curves of the effects of SDF1-3'A on HIV disease progression (Immunological Reviews 2000; 177: 99–111).



Footnote: it is of interest to remark the enhanced protection in those HIV-infected patients who bear the mutated alleles in all (CCR-5 or CCR-2 and SDF-1) to keep AIDS-free. SDF-1 and these chemokine receptors are involved in HIV entry into the cell.

Dipòsit Legal: T.1044-2012

CX3CR-1 V249I/T280M

individuals who bear the mutated allele presented an impaired leukocyte migration

These two polymorphisms in the CX3CR-1 gene are in complete linkage disequilibrium, and those individuals who bear the mutated allele (either 249I or 280 M) presented an impaired leukocyte migration related with a decrease in the CX3CR-1 expression [234]. Therefore, the CX3CR-1/fractalkine axis might be less effective in the chemotaxis of monocytes. These polymorphisms have been largely studied in atherosclerosis. A case control study of coronary artery disease (CAD) affected patients revealed that individuals with the 249I allele were less frequent in the CAD group compared with those without CAD (38% vs. 51%) [235], probably related with an impairment of the monocyte adhesive function [236]. The evaluation of coronary vascular function in 339 subjects according to polymorphisms in CX3CR-1 gene resulted in that both the prevalence and severity of CAD were less relevant in the group that was heterozygous for the 249I allele compared with the group that was homozygous for V249 [237]. Studies using the measurement of carotid IMT revealed a beneficial influence in those participants who bear the 280M [238], although these results are not supported by other studies [239].

In HIV-infected patients the CX3CR-1 gene has also been a focus of intense research and controversial results. HIV-infected patients homozygous for CX3CR1-249I, progressed to AIDS more rapidly than those with other haplotypes. Functional CX3CR-1 analysis showed that fractalkine binding is reduced among patients homozygous for this particular haplotype [240]. However, an study performed in the United States, based in homosexual men did not yield similar results, and the authors did not find any genetic association between the course of HIV infection and the polymorphisms in the CX3CR-1 gene [241]. When these association studies were applied to HIV-infected patients under antiretroviral therapies, the presence of CX3CR-1 280M homozygosity was associated with higher peripheral CD4+ T cell counts than those bearing the wild type allele ($p = 0.003$) [242].

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

9.- Summary of chemokine related polymorphisms in HIV infection and atherosclerosis. (Table 5)

As previously stated, the initial steps of atherosclerosis and the entry of HIV into the cell share similar biological mechanisms. The expression and structural composition of these molecules are influenced by the individual genetic background, resulting in different disease manifestations. For this reason, we have studied molecules implicated in both processes, atherosclerosis and HIV infection. In table 5 we have summarized the main effects of the polymorphisms studied in both diseases.

Gene	SNPs	Effect	Atherosclerosis	HIV infection
MCP-1	-2518A/G	↑ Expression	More common in CAD	Prone to Dementia/AIDS
CCR-2	Val64Ile	Dimerization	Resistant to atherosclerosis	Associated to LTNP
CCR-5	Δ 32	Structural changes	No definitive results	Resistance to HIV infection
SDF-1	3'A	↓ Expression	No definitive results	Associated to LTNP
CX3CR-1	V249I/T280M	↓ Expression	Resistant to atherosclerosis	Rapid progression to AIDS

CAD denotes coronary heart disease. LTNP: long term non-progressors.

Table 5. Influence of several SNPs in genes coding for chemokines and chemokine-receptors in atherosclerosis and HIV infection

The presence of the mutated MCP-1–2518G allele has been described to be protective as far as the HIV infection is concerned, but once infected, patients with the mutated allele would be more prone to HIV associated diseases, and also in the development of atherosclerosis. Conversely, those patients with the CCR2-64I are protected to HIV infection and to the development of atherosclerosis, although human studies are scarce. Those patients with the deletion of 32 base-pairs in the CCR-5 gene are protected to be infected by HIV, progress to AIDS significantly slower, and probably would also be protected in the development of atherosclerosis. The study of SDF1-3'A has yielded contradictory results in HIV disease progression and data concerning atherosclerosis are scarce. Finally, CX3CR-1 V249I/T280M are robustly associated with a lesser expression of atherosclerosis, although its role in HIV is far more controversial.

Hypothesis

HIV-infected patients live longer since the generalization of highly effective antiretroviral therapies. This relevant advance has yielded those patients to the presentation of concomitant diseases. Cardiovascular diseases represent the first cause of morbidity and mortality in non-infected individuals in industrialized countries. However, there is not a uniform presentation concerning atherosclerosis in HIV-infected patients, and consequently, a genetic predisposition could be hypothesized. In the search of these genetic candidates we have explored chemokines and chemokine-receptors, because these systems are involved in both HIV infection and atherosclerosis, and theoretically, HIV infection present a relevant influence in these chemokine networks.

Therefore, the aim of this work is to study the role of selected chemokines, implicated in the entry of HIV into the cell, in the development of atherosclerosis. We have also studied whether this functional polymorphisms influence in the course of HIV infection in patients receiving antiretroviral therapy.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Outline of the thesis

1.- To explore the role of the following chemokine and chemokine-receptor polymorphisms: MCP-1–2518G, CCR-2 64I, SDF1-3'A and CX3CR-1V249I/T280M,
in atherosclerosis (measured by intima-media thickness [IMT]) in HIV-infected patients.

2.- To study the influence of MCP-1–2518G in HIV disease progression, in patients under antiretroviral therapy.

3.- To study the impact of the above-mentioned genetic variants, in the progression/regression of IMT in HIV-infected patients.

4.- To introduce the assessment of IMT in the HIV clinic, in order to determine the presence of atherosclerosis, in addition to the study of classical cardiovascular risk factors.

Original Manuscript #1 published in Circulation 2004;110:2204-2209.

"Atherosclerosis in Patients Infected With HIV is Influenced by a Mutant Monocyte Chemoattractant Protein-1 Allele"

In the first cross-sectional study, we studied 183 HIV-infected subjects (124 men, 59 women, 20 to 66 years old). The aim of this study was to assess atherosclerosis and its related-variables and , therefore, we analyzed data according to a case-control design (presence or absence of atherosclerotic lesions). We performed carotid and femoral ultrasound exams to explore IMT values and the presence of atheromatous plaques. Clinical data and biologic samples (plasma, serum and DNA) were collated.

Participants with atherosclerosis were significantly older than the control group (P<0.001). Most of them were heavy smokers and with higher rates of hypertension and abnormal fasting glucose concentrations. Those HIV-infected patients with atherosclerosis had a significantly higher concentration of plasma MCP-1 than those without atherosclerosis.

The majority of patients (n=139; 76.0%) presented with atherosclerotic lesions in either carotid or femoral arteries. The frequencies of GG and GA genotypes in the MCP-1 gene polymorphism were significantly higher in subjects with atherosclerosis than in those without (47.5% versus 18.2%; P<0.001). When data was analyzed according to age, subjects with at least 1 mutated allele for MCP-1 had higher rates of atherosclerotic lesions in each of the age quartiles, and further, age was significantly associated with atherosclerotic lesions (OR 1.32, 95% CI 1.17 to 1.50, P<0.001) as was the MCP-1–2518G allele (OR 5.72, 95% CI 1.74 to 18.80, P<0.004).

The effect of the MCP-1–mutated allele was evaluated with respect to the clinical course of the atherosclerotic lesions with the stored images available for 40 subjects, and those who bear the mutated MCP-1 allele, presented a significantly higher increase in the area of atheromatous plaques.

Our results attribute a critical role to MCP-1 in the development of atherosclerosis in HIV-infected patients. It is particularly interesting to highlight that both the mutated allele and the plasma concentration of MCP-1 were implicated in atherosclerosis. The HIV infection itself is able to over express MCP-1 gene (boosting the influence in those individuals who bear the mutated allele), and consequently, renders those individuals with both conditions to a higher risk of developing atherosclerosis. The main result is that the study of the inflammatory status is relevant in the assessment of atherosclerosis (studied non-invasively with the IMT) in HIV-infected patients.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Atherosclerosis in Patients Infected With HIV Is Influenced by a Mutant Monocyte Chemoattractant Protein-1 Allele

Carlos Alonso-Villaverde, MD; Blai Coll, MD; Sandra Parra, MD; Manuel Montero, MD; Nahum Calvo, MD; Mònica Tous, PhD; Jorge Joven, MD; Lluis Masana, MD

Background—Patients infected with HIV present with premature atherosclerosis, and the 2 diseases share common pathogenic pathways. We investigated mutations in the monocyte chemoattractant protein-1 (MCP-1) and CCR-2 genes, which are known to control aspects of these pathways, to ascertain whether they are involved in atherogenesis in these patients.

Methods and Results—We performed carotid and femoral artery ultrasonography to detect subclinical atherosclerosis in patients infected with HIV (n=183). MCP-1–2518G and CCR-2 64I polymorphisms were determined in the HIV group and in a population-based control group (n=348). We also determined MCP-1 circulating levels in the HIV group. The presence of MCP-1–2518G in the group of patients with subclinical atherosclerosis was significantly higher than in patients without atherosclerotic lesions (47.5% versus 18.2%, respectively; $P<0.001$). Furthermore, the patients with atherosclerotic lesions had higher MCP-1 plasma concentrations than did patients without lesions (74.15 [4.03] versus 57.81 [3.67] pg/mL, respectively; $P=0.03$). When adjusted for known cardiovascular risk factors, the MCP-1–2518G allele was associated with subclinical atherosclerosis (OR 5.72, 95% CI 1.74 to 18.80, $P=0.004$). Compared with measurements conducted ≈2.5 years earlier in a subset of 40 patients, intima-media thickness (IMT) in the carotid artery progressed at a mean rate of 0.06 mm/y more rapidly in patients bearing the MCP-1–mutated allele ($P=0.08$).

Conclusions—HIV-infected patients with the MCP-1–2518G allele have a 5-fold increased risk for atherosclerosis, as assessed by ultrasonography. **(*Circulation*. 2004;110:2204-2209.)**

Key Words: atherosclerosis ■ HIV ■ inflammation ■ genotype ■ prevention

Patients infected with HIV develop proatherogenic metabolic abnormalities. These abnormalities have been linked to the effects of antiretroviral drugs and to the HIV infection itself. The patients present with premature subclinical atherosclerosis.[1–4] It is conceivable that, as survival of individuals with the infection increases,[5] atherosclerotic vascular disease could become an important complication. Inflammation is of paramount importance in the development of atherosclerosis,[6,7] and HIV, together with other infectious agents, may contribute inflammatory stimuli that could initiate or exacerbate atherogenesis or both.[8] In the development of atherosclerosis, mononuclear phagocytes (monocytes and macrophages) are primarily involved in the inflammatory processes.[9] The monocyte chemoattractant protein-1 (MCP-1) is a potent activator of these mononuclear phagocytes and, once its receptor (CCR-2) is stimulated, the monocytes migrate into the subendothelial space, commence phagocytosis of modified lipoproteins, and become lipid-laden foam cells.[10] Several studies implicate the MCP-1–CCR-2 axis in the clinical course of HIV infection as well as in atherosclerosis.[11–25] The different levels of expression of MCP-1 between the 2518G allele and the 2518A allele may represent a mechanism that in adults provides partial protection from the infection.[11] Furthermore, CCR-2 is a natural co-receptor used by HIV to enter the CD4 lymphocytes.[12] Although controversial, the G allele in this gene has been shown to be related to a different rate of progression for AIDS.[13–15]

In mice, being knockout for either the MCP-1 or CCR-2 genes has been associated with a reduction in atherogenesis, and the atheromatous lesions that are present are far more stable.[16–18] The MCP-1–2518G allele is associated with higher MCP-1 expression,[19] and patients diagnosed as having ischemic heart disease exhibit a higher prevalence of the MCP-1–2518G/G genotype.[20] In patients with acute coronary syndrome, the higher the levels of MCP-1 the higher the likelihood of myocardial infarction or death appears to be.[21] Interestingly, the polymorphism in the CCR-2 gene (A190G or V64I) has been shown to be associated with reduced coronary artery calcification,[22] whereas homozygosity for the CCR-2 64I allele seems to have a protective effect with regard to the development of coronary artery disease.[20]

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Some HIV proteins can induce the overexpression of MCP-1, and the levels of this protein may be altered during the course of HIV progression.[23–25] This disease feature is exacerbated in carriers of the MCP-1 mutant allele. As such, the evidence to date suggests that HIV infection and atherosclerosis share pathways in their pathogenesis. It is a reasonable hypothesis that mutations in genes that control aspects of these pathways could affect the course of both diseases. Hence, we assessed whether known associations between MCP-1 and CCR- 2 mutant alleles and atherosclerosis in the general population also are found in an HIV-infected population.

Methods

Study Design

We performed a case-control study based on the presence or absence of atherosclerosis in 183 subjects infected with HIV. We also evaluated clinical, laboratory, and genotyping results in the cases and controls to assess the risk factors for atherosclerosis in this particular clinical setting. For genotype comparisons we used a general population–based control group of unrelated subjects (n=348), the details of which have been described elsewhere.[26]

Study Participants and Eligibility

From among the patients infected with HIV who attended our clinic (n=305), 183 accepted the invitation to participate in the study and provided fully informed consent. Among the exclusion criteria were being <18 years old, having AIDS-related opportunistic diseases at the commencement of the study, and declining the invitation to participate. The Ethics Committee of the Hospital Universitari de Sant Joan de Reus approved the study.

Outcome Measurements

Clinical and Laboratory Measurements

A detailed clinical record was taken of each subject and a thorough physical examination was performed at interview. The traditional cardiovascular risk factors assessed were smoking status, presence or absence of hypertension, and body mass index (defined as the weight in kilograms divided by the square of the height in meters). A sample of fasting venous blood was taken for the measurement of glucose, total cholesterol, HDL cholesterol, and triglycerides. The analyses were conducted using standard laboratory methods. The LDL cholesterol level was calculated using the Friedewald formula.

Ultrasonography Measurements

Ultrasonography to measure intima-media thickness (IMT) was performed as previously described[27] with a LOGIQ 700 MR system (General Electric). When a plaque was identified at a predefined point, the IMT was determined in adjacent segments. The presence of atherosclerosis was defined as IMT >0.8 mm, the presence of a plaque, or both[27] in either carotid or femoral territories. We used this selection criterion to define the subject as a case or as a control. The concordance between the 2 sonographers responsible for the atherosclerosis evaluations indicated a high correlation coefficient of κ >0.8 for the independently conducted measurements.

Inflammatory Marker Measurements

Venous blood samples were collected into EDTA-containing tubes. The concentration of C-reactive protein (CRP) was measured by a particle-enhanced turbidimetric immunoassay (Quantex hs-CRP kit, Biokit), which had a sensitivity of 0.10 mg/L. The plasma concentration of MCP-1 was measured according to the manufacturer's recommendations with an enzyme-linked immunosorbent assay (Human MCP-1 ELISA Development Kit, PeproTech), which had a measurement range of 8 to 3000 pg/mL.

Genotyping

DNA was extracted by a standard phenol-chloroform procedure. The mutations MCP-1–2518G and CCR-2 64I were identified according to previously published methods.[20]

Risk Factor Analysis

Multivariate logistic regression analyses were performed to adjust for known cardiovascular risk factors. The data on carotid, femoral, or carotid and femoral atherosclerosis were the dependent variables, and the independent variables included age, sex, smoking habit, blood pressure, lipid profile, plasma glucose, mean duration of each antiretroviral treatment (ie, protease inhibitor, non-nucleoside inhibitors, and nucleoside analogues) and the DNA polymorphisms.

Atherosclerosis Progression

In a pilot study conducted ≈3 years previously, 40 patients were examined and clinical and ultrasonography data were documented. The stored images were retrieved and compared with the present measurements to assess atherosclerosis status. Comparisons included the mean IMT change (in millimeters) of predefined carotid arterial segments and the change (in square millimeters) in the area of a previously selected carotid plaque. These data were used to assess changes in the dimensions of the arterial lesions during the specific period.

Statistical Analyses

Data are presented as means with the standard error of the mean in parentheses. Standard methods (Kolmogorov-Smirnov and Shapiro-Wilk tests) were used to check for normality of the distributions. Analysis of variance was used to compare differences in quantitative variables, and the χ^2 test was used for categorical variables. Allele frequencies were calculated by the gene-counting method. The Hardy-Weinberg equilibrium and the differences in biallelic polymorphisms (genotype distributions and allele frequencies) between groups were tested using the χ^2 test. Analysis of variance was used to compare changes in mean IMT and the area of the plaque over time. The significance of association between the MCP-1 allele and the increase in the variables was assessed using a multiple linear regression model in which adjustment was made for other conventional cardiovascular risk factors. All probability values <0.05 were considered to be statistically significant. All analyses were performed with SPSS statistical software (version 11.0).

Results

We studied 183 HIV-infected subjects (124 men, 59 women, 20 to 66 years old). The subgroup of 40 HIV-infected subjects (34 men, 6 women, 32 to 58 years old) used for assessing atherosclerosis progress did not present any clinical differences when compared with the overall HIV study group. When known cardiovascular risk factors were compared (age, lipid profile, or patients with high blood pressure or abnormal fasting glucose), we did not find any statistically significant differences.

Subclinical Atherosclerosis and Control Groups

The measured variables, including conventional cardiovascular risk factors and segregated with regard to presence of atherosclerosis, are presented in Table 1. Subjects were of the same ethnic (white) background. The participants with atherosclerosis were significantly older than the control group (P<0.001). We evaluated conventional cardiovascular risk factors between groups. Most subjects were heavy smokers. Although we did not find differences in the mean body mass index (BMI) when cases and controls were compared (23.10 [0.27] versus 23.00 [0.52], respectively; P=0.857), we found higher rates of hypertension and abnormal fasting glucose

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

TABLE 1. Characteristics of HIV-Infected Patients Segregated According to Presence or Absence of Atherosclerosis

Characteristic	Atherosclerosis (n=139)	No Atherosclerosis (n=44)	*P*
Age, y	40.71 (0.59)	34.15 (0.89)	<0.001
Sex, %			0.03
Male	71.9	54.5	
Female	28.1	45.5	
Conventional cardiovascular risk factors,* %			
Current smoker	82.6	88.6	0.32
Hypertension	15.6	2.4	0.01
Abnormal fasting glucose	14.4	...	0.01
Dyslipemia	27.0	16.7	0.16
Lipid profile, mmol/L			
Total cholesterol	5.06 (0.12)	4.77 (0.16)	0.22
LDL cholesterol	2.85 (0.09)	2.63 (0.12)	0.22
HDL cholesterol	1.16 (0.04)	1.28 (0.06)	0.15
Triglycerides	2.44 (0.18)	1.82 (0.28)	0.09
Risk factor for HIV infection			0.67
Intravenous drug use, %	59.0	52.3	
Male homosexual contact, %	9.7	13.6	
Heterosexual contact, %	31.3	34.1	
Months since HIV diagnosis	89.25 (4.34)	78.48 (7.83)	0.22
Basal CD4, %			0.99
>500	35.3	34.1	
200–500	44.6	45.5	
<200	20.1	20.5	
Previous antiretroviral therapy, mo			
Nucleoside analogs	100.87 (5.41)	85.25 (8.74)	0.15
Protease inhibitor	29.70 (2.39)	26.06 (3.78)	0.44
Non-nucleoside reverse transcriptase inhibitors	8.35 (0.89)	8.70 (1.69)	0.85
AIDS-related disease, %	32.4	31.8	0.94

*Hypertension defined as >140, >90 mm Hg, or both. Abnormal fasting glucose defined as fasting plasma glucose >6.1 mmol/L. Dyslipemia defined as LDL cholesterol >3.36 mmol/L.

concentrations in the subjects who had atherosclerosis. Only 7 of these subjects were receiving statin therapy (for <1 year), and fibrates had been used in 10 subjects during the previous 6 months. None of the participants included in the study presented with either cardiac or cerebral ischemic events. We did not find any statistically significant differences with regard to HIV-related variables such as baseline CD4 cell count, AIDS-related opportunistic disease, or the time lapse after HIV diagnoses. Seven cases (5.0%) and 5 controls (11.4%) were naïve with regard to antiretroviral therapy ($P=0.163$). Segregation by sex, age, or both did not affect the distribution of genotypes; thus, all subjects were analyzed as a single group. The allelic distribution of MCP-1 and CCR-2 genotypes followed the Hardy-Weinberg equilibrium (χ^2, $P=0.30$ and $P=0.81$, respectively) in patients in the case group as well as in controls. No statistically significant differences between the unrelated subject control group and either of the case groups with regard to genotype distributions or in allelic frequencies were found (Table 2). Also, no differences were found in the distributions of both the mutations.

Analysis of Subclinical Atherosclerotic Lesions

The majority of subjects infected with HIV (n=139; 76.0%) presented with atherosclerotic lesions in one or another of the territories assessed, a percentage that is equivalent to that observed in other similar studies.[1,2] Analysis of the distribution of genotypes according to the presence or absence of subclinical atherosclerosis indicated that the frequencies of GG and GA genotypes in the MCP-1 polymorphism were significantly higher in subjects with atherosclerosis than in those without (47.5% versus 18.2%; $P<0.001$). The results showed that subjects with at least 1 mutated allele were more likely to show evidence of atherosclerosis (89.2%). No differences were observed in the distribution of CCR-2 polymorphism between subject populations (Table 2).

It is worth noting that our study population was relatively young, with >75% of subjects <42 years old. An analysis of

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

TABLE 2. Distribution of Genotypes and Inflammatory Markers Among HIV-Infected Patients With or Without Subclinical Atherosclerosis

	Atherosclerosis (n=139)	No atherosclerosis (n=44)
MCP-1–A2518G genotype distribution, n (%)		
GG + GA	66 (47.5)	8 (18.2)*
AA	73 (52.5)	36 (81.8)
CCR-2–A190G genotype distribution, n (%)		
GG + GA	25 (18.0)	9 (20.5)†
AA	114 (82.0)	35 (79.5)
Inflammatory markers		
CRP, mg/L	3.38 (0.31)	3.46 (0.54)
MCP-1, pg/mL	74.15 (4.03)	57.81 (3.67)‡

*$P<0.001$.
†$P=0.71$.
‡$P=0.03$.

the data segregated with regard to age quartiles indicated that subjects with at least 1 mutated allele for MCP-1 had higher rates of atherosclerotic lesions in each of the quartiles (Figure 1). This finding was especially relevant in subjects <34 years old.

We then analyzed the association between atherosclerosis and inflammatory markers such as CRP and MCP-1. Although no significant differences were found among MCP-1 plasma concentrations according to the MCP-1 polymorphism, a higher concentration was observed when subjects with HIV and atherosclerosis were compared with those without any arterial lesions (74.15 [4.03] versus 57.81 [3.67] pg/mL, respectively; $P=0.03$). We did not find significant differences in CRP concentrations between subjects with atherosclerosis and those without (3.38 [0.31] versus 3.46 [0.54] mg/mL, respectively; $P=0.905$).

Multivariate logistic regression analysis with known cardiovascular risk factors as independent variables revealed that only age and the MCP-1–2518G polymorphism were significantly associated with the presence of subclinical atherosclerosis (Figure 2). Treatments with protease inhibitor or nonnucleoside–based regimens were not associated with the presence of subclinical atherosclerosis ($P=0.64$ and $P=0.56$, respectively). Age was significantly associated with atherosclerotic lesions (OR 1.32, 95% CI 1.17 to 1.50, $P<0.001$) as was the MCP-1–2518G allele (OR 5.72, 95% CI 1.74 to 18.80, $P=0.004$).

The effect of the MCP-1–mutated allele was evaluated with respect to the clinical course of the atherosclerotic lesions with the stored images available for 40 subjects. The time lapse between the 2 ultrasonographic measurements was 2.61 (0.07) years. When subjects were segregated into those with the MCP-1–2518G (n=13) allele and those with the AA genotype (n=27), the subjects with the mutated allele appeared to have a poorer clinical outcome (Figure 3). The data indicated an increase in carotid IMT of 0.06 mm/y in the subject group with the mutated allele (MCP-1–2518G). In the group of subjects with the AA genotype, this increase was 0.03 mm/y; however, the difference did not reach statistical significance ($P=0.08$). When the areas of predefined carotid lesions were analyzed it was apparent that subjects with at least 1 mutated allele experienced a significantly higher increase than did subjects with the wild-type allele (12.9 [4.3] versus 32.3 [6.4] mm^2, respectively; $P=0.04$).

Discussion

Some genetic variants of the chemokines are reputed to influence individuals' susceptibility to HIV infection, the progression of the disease, and even the presence of so-called HIV-associated manifestations.[28] Considerable research has focused on the role of chemokine polymorphic genes implicated in the inflammatory response and, as a consequence, in atherosclerosis.[29]

An important finding of our study is that a mutation in the promoter region of the MCP-1 gene has an atherosclerosis-promoting effect. Infiltration of tissues by monocyte-derived

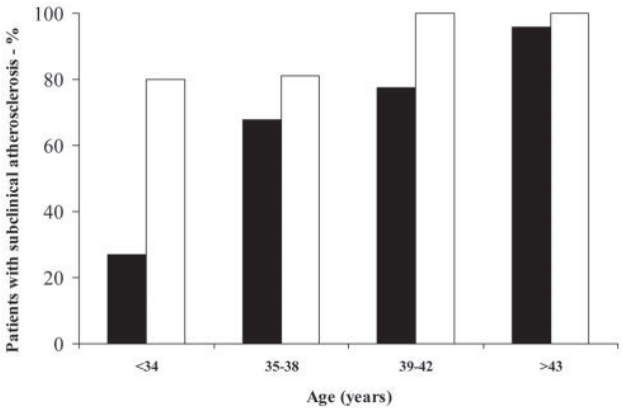

Figure 1. Percentage of subjects infected with HIV with subclinical atherosclerosis according to MCP-1–2518G (□) and –2518A (■) polymorphism and distributed by quartiles of age. Logistic regression model was applied to assess association of carotid and femoral atherosclerosis with presence of MCP-1–mutated allele adjusted for age ($P<0.001$).

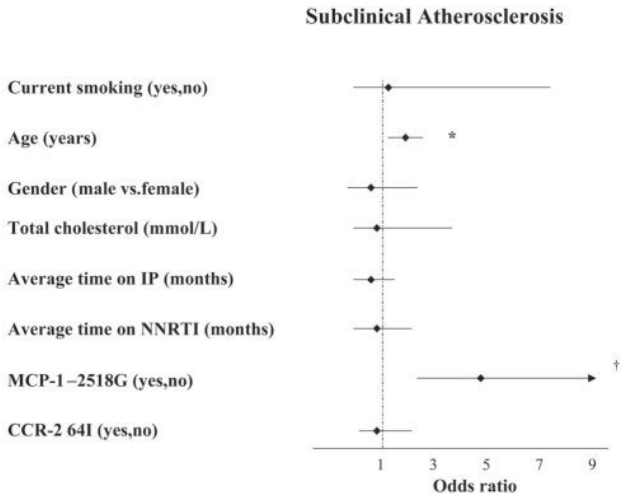

Figure 2. ORs for subclinical atherosclerosis. Horizontal lines represent 95% CI. ◆; indicates that OR is higher than represented. (See Methods for further information.) *$P<0.001$; †$P=0.004$.

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

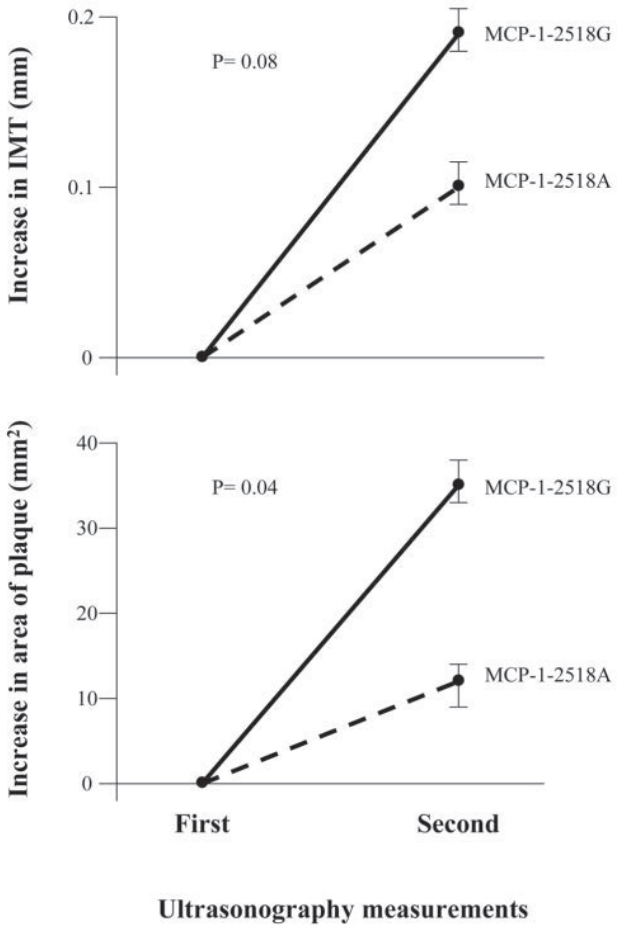

Figure 3. Change in IMT and area of predefined atheromatous plaque of internal carotid artery. Differences were calculated between the 2 ultrasonographs conducted on 40 subjects and segregated with regard to MCP-1 polymorphism.

macrophages is a prominent feature of atherosclerosis. MCP-1 and possibly other chemoattractant proteins are thought to be the molecular signals that direct such infiltration. We measured plasma MCP-1 concentrations and, despite these individuals' being subject to multiple infections and inflammatory insults and receiving antiretroviral therapies that can induce further changes in MCP-1 levels,[23–25] we found an association between higher values of MCP-1 and atherosclerosis. This finding provides support for our hypothesis that MCP-1 may play a crucial role in atherogenesis. In previous studies,[20,22] atherosclerosis was less extensive in patients who had well-established cardiovascular disease and who carried the mutation in the CCR-2 gene. We are unable to confirm such results based on our multivariate analyses. We wish to highlight that with regard to conventional cardiovascular risk factors, subjects with atherosclerosis experienced higher rates of hypertension and abnormal fasting glucose. In multivariate analyses, these variables lost their statistical significance in relation to atherosclerosis.

Ultrasonography, being a noninvasive tool, is widely accepted in the evaluation of IMT, and IMT has been validated as a surrogate marker for atherosclerotic vascular disease.[30] For example, a yearly increase of carotid artery IMT of 0.03 mm is associated with an increase in coronary events in patients with established atherosclerosis.[31] Conversely, the reduction of 0.03 mm/y achieved with high-dose statins appears to have a significant impact on the prevention of coronary artery disease.[32] Although the present statistical analyses are not a case-control study of "atherosclerosis" versus "no-atherosclerosis" comparisons, the yearly increase of carotid IMT in our subjects was clearly >0.03 mm and was more evident in subjects with the MCP-1–2518G allele. Although drug interactions, toxicity, intolerance, and decreased adherence to treatment are common in these subjects, we believe our data suggest that the prescription of statins, fibrates, or both in subjects with HIV could induce favorable outcomes with regard to the development of atherosclerosis in these subjects.

In summary, our results indicate that the MCP-1–CCR-2 gene axis is related to carotid and femoral atherosclerosis in patients infected with HIV. These findings need to be reflected in proposals for new therapies. For example, an increase in the prescription of statins, platelet antiaggregants, or both together with the use of antiretroviral regimes would be appropriate. Conversely, the inducers of metabolic disturbances would need to be reduced to minimize the risk of vascular events in these patients. Knowledge of the activation mechanisms of chemokines in HIV and other inflammatory disorders would provide insight into better management and control of HIV-associated diseases, including atherosclerosis.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
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"Influence of a monocyte chemoattractant protein 1 mutated allele on the response to protease inhibitor-based antiretroviral therapy"

In the second study we addressed the role of MCP-1 and CCR-2 in the course of HIV infection. For this purpose, we selected those patients who had never been treated with Protease Inhibitors (N=164), and their CD4 cell counts and HIV viral load were monitored according to the results of MCP-1 analyses. We did not find significant differences between groups (wild type or mutated alleles for MCP-1-2518G) in genotype distributions of the CCR-2, CCR-5 and SDF-1 mutated alleles. Patients who bear MCP-1-2518G experienced a significantly better CD4 response in months 6 and 21 after initiation of PI therapy. The baseline CD4 cell count and the presence of the MCP-1–2518G mutated allele were significantly ($P<0.01$) related to a better CD4 cell response in a multivariate analyses.
Patients bearing the MCP-1–2518G and CCR-2 64I mutated alleles, i.e. those with a blocked MCP-1–CCR-2 axis (n=22), had a higher likelihood of continuing to have an undetectable HIV-1 viral load than patients (n=142) carrying both wild-type alleles.
These results may be explained by several mechanisms (competition, heterodimerization, etc) but they clearly states an strong relationship between key genetic variables and the course of HIV-infection. The over expression of MCP-1 and /or structural changes in its natural receptor, CCR-2, leads HIV-infected patients to a better response to PI and present longer time with undetectable HIV viral load.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
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ORIGINAL RESEARCH

Influence of a monocyte chemoattractant protein 1 mutated allele on the response to protease inhibitor-based antiretroviral therapy

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Background

Antiretroviral drug efficacy has been widely studied in relation to viral factors. Mutations in the HIV co-receptors and their natural chemokines, however, may be critical in HIV infection and treatment response. We compared the efficacy of protease inhibitor (PI) treatment among PI-naïve patients grouped according to whether they carried the chemokine CC motif receptor 2 (*CCR-2*) 64I and monocyte chemoattractant protein 1 (*MCP-1*)–2518G alleles.

Methods and results

HIV-infected patients who were PI-naive were selected for the study ($n = 164$) but there was no restriction on lymphocyte CD4 count or plasma HIV viral load. Follow-up was for the first 24 months of treatment. Clinical and laboratory data were obtained every 3 months. All the participants were genotyped for the *MCP-1*–2518G, *CCR-2* 64I, *CCR-5* Δ32 and stromal derived factor 1 (*SDF1*) 3′A mutated alleles. The results indicated that patients carrying the mutated allele of *MCP-1* had a higher mean CD4 cell count throughout the follow-up period than those with the common allele ($P = 0.01$). Also, patients with the *MCP-1* and *CCR-2* mutated alleles were more likely to continue to have an undetectable viral load following treatment ($P = 0.05$).

Conclusion

A better response to PI treatment appears to be conferred by mutations in the host *MCP-1* and *CCR-2* genes, and may be related to the cellular axis-of-entry used by the retrovirus.

Keywords: chemokines, genetics, HIV course, monocyte chemoattractant protein 1

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Introduction

Biological differences among HIV isolates and resistance to highly active antiretroviral therapy (HAART) have been explored extensively, but the individual variation in treatment response observed indicates the importance of host genetic determinants, such as the cell chemokine receptor 5 (*CCR-5*) Δ32 and stromal derived factor 1 (*SDF1*) 3′A alleles [1–6].

In the early stages of the infection, the virus adopts a macrophage tropism (M-tropism) using CCR-2 and CCR-5 as CD4 co-receptors [7,8]. Patients who carry a valine-for-isoleucine substitution at position 64 of the *CCR-2* gene (*CCR-2* 64I) have a higher likelihood of being long-term survivors [9–11], with this probably being related to heterodimerization of the mutated CCR-2 with the HIV co-receptor CCR-5 or chemokine, CXC motif receptor 4 (CXCR4) [12]. The natural chemokine of CCR-2 is monocyte chemoattractant protein 1 (MCP-1), which plays a pivotal role in the involvement of monocytes/macrophages in tissue inflammation. However, it also influences the response of cytotoxic T lymphocytes [13], and has effects on both innate and adaptive immunity through the control of T helper cell polarization [14]. The substitution of adenine for guanine at position −2518 in the promoter region has been associated with *MCP-1* over-expression [15] and may have several consequences, for example promotion of the heterodimerization of CCR-2 with HIV co-receptors [16], competitive inhibition of CCR-2, activation of T-cell immunity or lymphokine production [17]. This mutation has been investigated as a genetic marker influencing the risk of HIV transmission and the likelihood of developing HIV-associated dementia [18,19], but here we explored the hypothesis that this mutation in the

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

MCP-1 promoter region improves the response to antiretroviral treatment in HIV-infected patients undergoing protease inhibitor (PI)-based antiretroviral therapy (ART).

Methods

Among those attending our clinic, 232 HIV-infected patients (164 male and 68 female, aged between 20 and 66 years) agreed to participate in the study. We identified, from our computerized database, those patients ($n = 164$) who were naïve to the proposed protease inhibitor (PI)-based HAART regimen. Patients eligible in terms of ART for PI treatment in this study included those who had failed previous ART schemes and naïve patients with CD4 cell counts <350 cells/μL. We did not exclude any patient on the basis of CD4 cell count or viral load. Also, patients who had been treated previously with nucleoside reverse transcriptase inhibitors (NRTIs) were not excluded. Clinical and laboratory data that are routinely obtained every 3 months were collated and analysed during the first 24 months of PI treatment. The Ethics Committee of the Hospital Universitari de Sant Joan approved the study. Fully informed consent to the genetic testing was obtained from all the participants.

Serum HIV-1 RNA viral load was measured with the Amplicor Analyser (Roche Diagnostics, Branchburg, NJ, USA). The lower limit for reliable quantification was considered to be 200 HIV-1 RNA copies/mL, and lower values were regarded as undetectable. CD4, CD8 and lymphocyte cell counts were determined by standard FACscan flow cytometry (Becton-Dickinson, Madrid, Spain).

Venous blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA), and DNA was obtained from leucocytes using the Puregene Kit (Gentra Systems, Minneapolis, MN, USA). The mutations *MCP-1*–2518G, *CCR-2* 64I, *CCR-5* Δ32 and *SDF1* 3′A were identified according to previously published methods [20]. Genotypes were scored independently by two observers who were blind with respect to the clinical data.

Data are presented as the mean and the standard error of the mean (SEM). We used the standard Kolmogorov–Smirnov test to check for normality of distribution. Differences in quantitative variables were assessed using analysis of variance (ANOVA), and the χ^2 test was used for categorical variables. We tested for Hardy–Weinberg equilibrium using the χ^2 test. We considered patients who were heterozygous and homozygous for the mutated allele as a single group so as to compare these patients with those carrying the common allele. Nadir CD4 cell count was the value recorded at the time of diagnosis of HIV infection.

The endpoints analysed were CD4 cell count during the first 24 months of PI therapy and the time for which the patients continued to have undetectable HIV viral load. A general linear regression model was used to test the association between CD4 cell course with genotype. The baseline CD4 cell count and the presence of *CCR-5*, *CCR-2* and *SDF1* mutated alleles were used as covariates in the assessment of the CD4 cell count response. We also performed a single regression analysis in the difference of CD4 T cells from baseline in each time point. The Kaplan–Meier method was used to evaluate the influence of genotypes on the time for which HIV-1 viral load remained undetectable. The log-rank test was used to calculate the overall *P*-value. All analyses were performed with the SPSS 11.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the HIV-infected patients and genotype distributions

Participants were of the same ethnic background (Caucasian). A comparison of variables, including immunological status at initiation of the PI-based ART regimen, between two groups of patients differing in the *MCP-1* polymorphism is presented in Table 1. There were no statistically significant differences between the two groups in any of the characteristics examined. CD4 cell count and viral load did not differ significantly between the groups. Almost half of the population studied had been on NRTI-based ART before commencing the PI regimen. We did not find significant differences between the groups in the genotypic distributions of the *CCR-2*, *CCR-5* and *SDF1* mutated alleles. The allelic distribution of the genotypes studied was consistent with Hardy–Weinberg equilibrium.

Analysis of CD4 cell count

Patients with at least one mutated *MCP-1*–2518G allele did not have different CD4 cell counts from other patients either at baseline or at the commencement of PI therapy. Similarly, we observed no significant difference in CD4 cell count between groups of patients differing in the *CCR-2* polymorphism. However, patients with the mutated allele in the promoter region of *MCP-1* experienced a significantly better CD4 response in months 6 and 21 after initiation of PI therapy (single regression model, $P<0.05$; Fig. 1). Further, in the multivariate model in which the results of the *CCR-5*, *CCR-2* and *SDF1* polymorphisms were included, the baseline CD4 cell count and the presence of the *MCP-1*–2518G mutated allele were significantly ($P<0.01$) related to a better CD4 cell response (Fig. 1).

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Table 1 Characteristics of HIV-infected subjects ($n = 164$) at the start of protease inhibitor (PI)-based treatment grouped according to presence of the monocyte chemoattractant protein 1 (*MCP-1*)–2518G mutated allele

Characteristic	*MCP-1*–2518A ($n = 89$)	*MCP-1*–2518G ($n = 75$)	*P*-value
Age (years)	38.77 (0.58)	39.24 (0.92)	0.65
Gender (male) [*n* (%)]	59 (66.2)	51 (68)	0.78
Time since HIV diagnosis (years)	6.37 (0.33)	5.65 (0.33)	0.13
Risk factor for HIV infection (%)			0.94
Intravenous drug use	54.3	45.7	
Heterosexual contact	51.1	48.9	
Male homosexual contact	52.9	47.1	
Nadir CD4 cell count (cells/μL)	303 (32)	362 (38)	0.23
Nadir HIV viral load ($\log_{10}$ copies/mL)*	4.98 (0.10)	4.68 (0.14)	0.10
CD4 cell count (cells/μL)	252 (26)	311 (32)	0.15
HIV viral load ($\log_{10}$ copies/mL)	4.40 (0.15)	4.17 (0.17)	0.32
Hepatitis C virus coinfection (%)	54.5	45.5	0.93
PI prescribed (%)			0.73
Ritonavir	9.2	12.3	
Indinavir	43.7	39.7	
Saquinavir	28.7	26.0	
Nelfinavir	11.5	9.6	
PI-boosted therapy†	6.9	12.3	
NRTI prescribed (%)			0.11
Zidovudine	53.2	61.4	
Lamivudine	76.4	75.8	
Stavudine	46.1	35.6	
Didanosine	11.4	14.4	
Zalcitabine	9.0	9.7	
Abacavir	5.1	3.0	
Prior NRTI-based therapy (%)	47.4	41.5	0.59
AIDS-related opportunistic disease (%)	32.2	32.9	0.92
CCR-2 64 I (%)	13.8	21.9	0.17
CCR-5 Δ32 (%)	12.6	9.6	0.54
SDF1–3′A (%)	48.8	44.3	0.57

Values are the mean and the standard error of the mean (SEM), unless otherwise stated. Time since HIV diagnosis relates to years of seroprevalence (unknown seroconversion date). The genetic results indicate the percentage of patients carrying the mutated alleles *CCR-2* 64I, *CCR-5* Δ32 and *SDF1*–3′A.
*These data were available for 85 patients.
†PI-boosted regimens were as follows: indinavir + ritonavir, saquinavir + ritonavir, nelfinavir + ritonavir, amprenavir + ritonavir and lopinavir + ritonavir.
CCR, cell chemokine receptor; NRTI, nucleoside reverse transcriptase inhibitor; SDF, stromal derived factor.

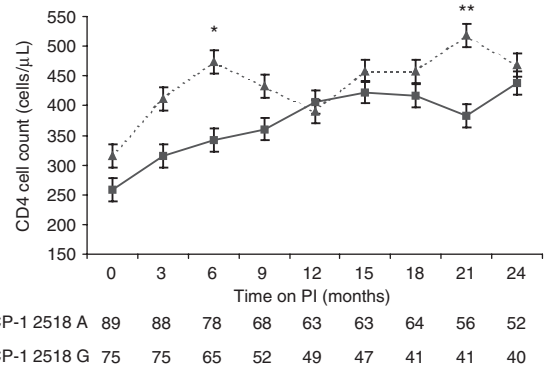

Fig. 1 CD4 cell count (mean and two-tailed standard error of the mean) over the follow-up period for patients on a protease inhibitor (PI)-based highly active antiretroviral therapy regimen grouped according to the monocyte chemoattractant protein 1 (*MCP-1*) polymorphism. The CD4 cell count differed persistently and significantly ($P = 0.01$) in the mixed model analyses of data for all visits, with covariates including cell chemokine receptor 5 (*CCR-5*), *CCR-2* and stromal derived factor 1 (*SDF1*) polymorphisms and baseline CD4 cell count. *$P < 0.01$; **$P < 0.05$ in the single regression model for comparison with the baseline CD4 cell count. Squares (and the solid line) represent individuals ($n = 89$) with the *MCP-1* wild-type allele. Triangles (and the dotted line) indicate individuals ($n = 75$) with the *MCP-1*–2518G allele. Numbers of patients at each time-point are shown below the horizontal axis.

Analysis of HIV-1 viral load

In a comparison of HIV-1 viral load at the commencement of PI therapy among groups of patients differing with respect to the *MCP-1* and *CCR-2* polymorphisms, no statistically significant differences were found. We further analysed the influence of the *MCP-1* mutated allele on the length of time that patients continued to have undetectable HIV viral load. Although those with the *MCP-1*–2518G allele presented a better antiretroviral response, the finding did not reach statistical significance (log rank 0.97; $P = 0.32$). However, the Kaplan–Meier model showed that patients bearing the *MCP-1*–2518G and *CCR-2* 64I mutated alleles, i.e. those with a blocked MCP-1–CCR-2 axis ($n = 22$), had a higher likelihood of continuing to have an undetectable HIV-1 viral load than patients ($n = 142$) carrying both wild-type alleles (log rank 3.65; $P = 0.05$).

Discussion

We assessed the treatment responses of HIV-infected patients initiating PI-based ART in relation to mutated alleles of genes implicated in the entry of HIV into the cell. Our results indicated that an allelic variant of *MCP-1* associated with over-expression of *MCP-1* was related to a better CD4 cell count response in patients undergoing ART. The influence of chemokines and chemokine receptors in HIV infection and disease progression has been widely studied, and several mechanisms have been implicated. It is likely that the better response found in the present study in patients with the mutated *MCP-1* allele can be attributed to substrate competition between the MCP-1 molecule and HIV particles for the CCR-2 receptor, a phenomenon termed 'steric hindrance' [21]. However, the presence of the mutated allele *MCP-1*–2518G may be associated with down-regulation of HIV co-receptors, which can assist the patient's natural mechanism for blocking HIV entry into the cell [6,22].

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

However, as stated by other authors [8], CCR-2 has been recognized as a minor CD4 co-receptor, so the influence of CCR-2 might be exerted indirectly through changes in the activation of the immune system. In fact, it is known that the product of the mutated *CCR-2* 64I allele heterodimerizes in the lipid raft of the cell membrane with the 'major' HIV co-receptors, CCR-5 and CXCR-4, and this effect may be responsible for blocking HIV entry [12]. Interestingly, MCP-1 has the ability to promote CCR-2 heterodimerization with the other receptors, and so over-expression of *MCP-1* as a result of a genetic variant in its promoter region may produce a better immunological response in HIV-infected patients who carry the mutated allele. Similarly, Rodriguez-Frade *et al.* [23] reported the beneficial effects of CCR-2 activation using a monoclonal antibody that acts as its natural substrate, MCP-1, in blocking the entry of HIV into the cell. This effect is mediated by the heterodimerization of CCR-2 with CCR-5 and CXCR4. These data provide new insights into the therapeutic opportunities of HIV-infected patients [23].

Further, MCP-1 plays a pivotal role in the activation of cytotoxic T cells [13], in the production of lymphokines and in turn in lymphocyte proliferation [17]. These effects may contribute to the benefits found to be conferred by the mutated allele in our patients.

The presence of the G allele at position −2518 of the *MCP-1* gene has been related to a lower risk of acquiring HIV-1 infection. Conversely, it has been found that, in patients who are already infected and who carry the *MCP-1* mutated allele, the progression to AIDS is accelerated and the risk of suffering from HIV-associated dementia and *Mycobacterium avium* complex infection is higher than in patients with the common variant [19]. However, the course of CD4 cell count in patients on ART has not been reported. Further, the presence of the mutated *MCP-1* allele has been shown not to be associated with cytomegalovirus infection, *Pneumocystis carinii* pneumonia or Kaposi's sarcoma [19], raising more questions regarding the role of *MCP-1* in HIV infection.

Our study has several limitations, mainly related to the small sample size. Further, nadir CD4 cell count and viral load, time since diagnoses and the genotype distribution of *CCR-2*, were slightly different between groups (mutated and wild type alleles for the MCP-1 genotype), although the differences were not statistically significant, and this could call into question any conclusions drawn from the results. However, these factors were included in the statistical model as covariates in order to eliminate potential bias, and their inclusion did not alter the findings of the study.

Another limitation of the study is that we performed a genotypic assessment of the immunological course of HIV infection in the patients, but we did not obtain phenotypic data. It would be of great value to determine the plasma concentration of MCP-1 and/or *CCR-2* mRNA expression, in order to assess the exact status of the MCP-1–CCR-2 axis.

We did not find a significant influence of the *CCR-5* Δ32 allele on the CD4 cell response, and this was probably related to the low allele frequency (0.05) and the small sample size ($n = 164$). Similarly, we did not find any significant influence of the SDF1–3′A allele in our study population, but the effects of this genetic variant are a matter of debate [11]. However, we took into account these genotypes as covariables in the analysis of the course of CD4 cell count.

Evidence is emerging that immunological pressure on HIV in human hosts is causing population-dependent genetic changes in the virus itself. One of the main concerns discussed in the current literature is the possibility that chemokines might favour the emergence of X4 strains of HIV-1 that use CXCR4 for cell entry [24]. It should be noted that individuals on ART who are carrying the *MCP-1* mutated allele have a better outcome in terms of CD4 response and, according to our data, the emergence of a predominant X4 HIV-1 strain is unlikely.

The beneficial effects of the chemokine receptor gene polymorphisms *CCR-5* Δ32 and *CCR-2* 64I on the course of HIV-1 infection in patients on ART have already been described [25,26]. Moreover, *in vitro* studies have revealed that mutated alleles for these chemokine receptors may modify the interaction between inhibitory fusion agents (T-20) and their activity sites, and hence different therapeutic activity might be expected [27]. Our results indicated that patients carrying a combination of alleles blocking the M-tropism pathway of HIV entry into the cell (i.e. the *MCP-1* and *CCR-2* mutations) had a better response to a PI-based regimen, as measured by the time for which viral load remained undetectable.

In summary, genetic variations affecting host cells need to be taken into account in the management of patients infected with HIV. Mutated alleles of genes affecting the M-tropism of HIV could influence the patient's response to HAART and the clinical evolution of the disease. These findings could be useful in establishing more appropriate and individualized antiretroviral strategies.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
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"The stromal derived factor-1 mutated allele (SDF1-3'A) is associated with a lower incidence of atherosclerosis in HIV-infected patients."

In the third study, we assess the relationship of a functional polymorphism in the SDF-1 gene, with the development of atherosclerosis. Due to the interaction with platelets, tissue factor and with endothelial progenitor cells, SDF-1 might have a significant influence in atherosclerosis development. We found a lower number of individuals carrying the SDF1-3'A allele in the group with carotid atherosclerosis (41.6%) than those without arterial lesions (57.1%; P =0.04). Further, analysis of the distribution of the SDF1-3'A mutated allele indicated that, among those participants with an abnormal IMT (> 0.8 mm), the SDF-1 mutated allele was less likely to be encountered than the wild type (38.4% versus 61.6%; P =0.03). When carotid plaques were measured, we found that those individuals who bore the mutated allele for SDF-1 presented a significantly lower carotid plaque area 20.36 ±5.57 mm2, than those carrying the wild type 37.33 ±4.90 mm2 (P = 0.02).
Age and the presence of dyslipidaemia were significantly (P<0.05) associated with carotid atherosclerosis in the multivariate analysis. Further, the results showed a protective effect of the SDF1-3'A allele on the development of carotid atherosclerosis [odds ratio (OR), 0.45; 95% confidence interval (CI), 0.19–1.02; P <0.05)].
We further explored, in a subset of these participants, which could be the underlying mechanism. We selected patients with the same antiretroviral therapy scheme and follow their lipid profile. We did not find significant differences between groups when total cholesterol, HDL-C and triglycerides were analyzed. However, when LDL-C was considered, those patients without carotid atherosclerotic lesions and who carried the SDF-1 mutated allele (n =19) showed a significantly (P <0.04) lower LDL-C of 2.06 ± 0.34 mmol/l, throughout the follow-up period.
The main results of this study are that SDF1-3'A mutated allele revealed as a protector factor in the development of carotid atherosclerosis in HIV-infected patients, probably related with the lower LDL cholesterol concentration. Mechanisms by which the lower expression of SDF-1 is associated to these phenotype variations are poorly understood, but as occurred with CCR-2 and MCP-1, a complex interaction among cytokines and metabolic variables should be further explored.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

The stromal derived factor-1 mutated allele (SDF1-3′A) is associated with a lower incidence of atherosclerosis in HIV-infected patients

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Background: HIV-infected patients have higher rates of subclinical atherosclerosis. The chemokine stromal derived factor 1 (SDF-1) is the natural ligand for the CXCR4 HIV co-receptor, is highly expressed in atherosclerotic plaques, and the plasma concentration is lower in individuals homozygous for the mutant allele (SDF1-3′A). We tested the influence of SDF1-3′A on atherosclerosis in HIV-infected patients.

Methods: We performed carotid ultrasonography and determined the SDF1-3′A DNA polymorphism in 183 HIV-infected patients. Classical cardiovascular risk factors and antiretroviral therapy were also recorded. From these patients, we selected a group of 134 patients taking protease inhibitor-based antiretroviral therapy and in whom the lipid profile over an 18-month follow-up was collated.

Results: We found atherosclerosis in 113 (61.7%) and a lower number of patients with the SDF-1 mutated allele in the group with carotid atherosclerosis compared to those without (41.6% versus 57.1%; $P = 0.04$). Using a logistic regression analysis, age and dyslipidaemia were significantly associated with atherosclerosis but the SDF1-3′A allele exerted a protective effect on the development of atherosclerosis (odds ratio, 0.45; 95% confidence interval, 0.14–1.02; $P = 0.05$). Further, we observed that, in the selected group of patients there were lower plasma low-density lipoprotein cholesterol concentrations [mean ± SEM, 2.06 ± 0.34 mmol/l] throughout follow up in those patients without carotid lesions and who also carried the mutated SDF1-3′A allele ($P = 0.04$).

Conclusion: The SDF1-3′A allele is associated with a lower presence of subclinical carotid atherosclerosis in an HIV-infected population.

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Introduction

HIV-infected patients present with higher rates of atherosclerosis [1,2] and the incidence of myocardial infarction has been rising in this group of patients [3], especially in those under a protease inhibitor (PI)-based regimen. This may be explained by the metabolic abnormalities related to the highly active antiretroviral therapies (HAART) coexisting with the classic cardiovascular risk factors in these patients [4,5]. However, several polymorphisms of genes related to inflammation have also been implicated in the development of atherosclerosis [6,7]. Of these stromal derived factor-1 (SDF-1) is an additional candidate because: it is a powerful chemoattractant cytokine for lymphocytes and monocytes [8]; it has been associated with aggregation-activation of platelets [9,10]; and it is highly expressed in atherosclerotic plaques [9,11].

SDF-1 also plays a significant role in HIV infection because it is the natural ligand for a well-known CD4 co-receptor, CXCR4, which is used by HIV T-tropic strains

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Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

to enter into the cells in advanced stages of the disease [12]. A polymorphism in the un-translated region of the SDF-1 β gene, SDF1-3′UTR-801 G→A (abbreviated to SDF1-3′A) influences the course of HIV infection [13–18], and individuals who bear the mutated allele present with a minor SDF-1 expression and lower circulating levels of SDF-1α [16]. Although a clear association between SDF-1 and atherosclerosis has been established in *in vitro* studies [9–11], these findings have not been confirmed clinically. The circulating levels of SDF-1α have been found to be lower in an acute coronary heart disease study [19] and no relationship has yet been found between the allelic frequency of the SDF1-3′A and the presence of coronary artery disease [20].

Hence, the aims of our study were to test the association between SDF1-3′A and the presence of atherosclerosis in a group of HIV-infected patients and to analyse, in a group of these patients on a PI-based regimen, the known metabolic risk factors of cardiovascular disease, such as circulating lipid concentrations.

Methods

Study design: participants and eligibility

The study was case-controlled based on the presence or absence of carotid atherosclerosis in 183 HIV-infected subjects. We evaluated clinical, laboratory and genotyping data in the cases and controls as a measure of the atherosclerosis risk factors in this specific clinical setting.

Among the exclusion criteria were age < 18 years and an AIDS-related opportunistic disease at the commencement of the study. The Ethics Committee of the Hospital Universitari de Sant Joan de Reus approved the study.

Clinical and laboratory measurements

A detailed clinical record was taken and a thorough physical examination performed at interview. The cardiovascular risk factors assessed were smoking habit, presence or absence of hypertension, and body mass index. Blood pressure was measured following standardized recommendations of the Spanish Society of Cardiology [21]. The measurements of glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were performed using standard laboratory methods. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. Dyslipidaemia was considered when total cholesterol was > 6.2 mmol/l or LDL-C > 4.1 mmol/l or HDL-C < 0.9 mmol/l. The concentration of C-reactive protein (CRP) was measured by a particle-enhanced turbidimetric immunoassay (Quantex hs-CRP kit, Biokit, SA, Barcelona, Spain) which has a sensitivity of 0.10 mg/l. CD4, CD8 and lymphocyte cell counts were determined by standard FAC scan flow cytometry (Becton-Dickinson, Madrid, Spain).

Lipid course analysis

We evaluated the course of lipid profile changes in those patients who had commenced treatment with a PI-based HAART regimen (134 of the 183 patients) since these formed the largest treatment group in the study. This is a retrospective substudy in which the eligibility criteria were that patients had been naive for PI therapies and had been identified from our computerized database. Based on the effects that had been demonstrated by different PI on lipid profiles [22], we analysed the course of lipid-profile changes segregated into two patient groups: those patients on a PI-boosted regimen with ritonavir or ritonavir as a single PI, and the rest of the patients who had been treated with indinavir, saquinavir or nelfinavir. Analyses included total cholesterol, HDL-C, LDL-C, and triglyceride concentrations measured over the first 18 months of PI treatment. Patients with other treatment regimens represent a small proportion of our patient population and were not considered for this part of the study.

Ultrasonography

Ultrasonography was used to measure intima-media thickness (IMT) in the common carotid artery 1 cm proximal to the carotid bifurcation, as previously described [7,23] using a GE Logiq 700MR system (Milwaukee, USA). The presence of atherosclerosis was defined as IMT > 0.8 mm and/or the presence of a plaque [23]. We used this selection criterion to define the patient as a case or as a control.

Genotyping

Venous blood samples were collected into tubes containing EDTA, and the DNA was obtained using the Pure Gene Kit (Gentra, Gentra Systems Inc., Minneapolis, USA). The SDF1-3′A mutation was identified according to previously published methods [13]. For genotype comparisons we used a general population-based control group of unrelated subjects ($n = 348$) the details of which have been described elsewhere [24]. Briefly, they were healthy people (167 female, 181 male) with an overall mean age 42 years (range, 19–75 years). All the participants were of Caucasian–Mediterranean ethnic origin. Exclusion criteria were the use of medication and vitamin supplements, having dementia or pregnancy.

Statistical analyses

Data are presented as means with SEM in parentheses. The Shapiro–Wilk test was used to check for normality of distributions. ANOVA was used to compare differences in quantitative variables and the χ^2 test for categorical variables. The Hardy–Weinberg equilibrium together with the differences in genotype distributions and allele frequencies between groups were tested using the χ^2 test. The SDF-1 genotype results indicated 96 patients

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

homozygous for the wild type, 78 patients heterozygous and nine patients homozygous for the mutated allele (SDF1-3′A). Because of this allelic distribution and previously published precedent [25] the latter two groups were considered as a single group for statistical purposes. To test for significance in the changes in the lipid profile, we combined the results of the carotid assessment and the SDF1-3′A genotype. As such, there were four possible combinations taken into account: patients with no atherosclerotic lesions and with the common SDF-1 allele; patients with no atherosclerotic lesions and SDF1-3′A allele; patients with carotid lesions and the common SDF-1 allele; patients with atherosclerotic lesions and the SDF1-3′A mutated allele. A general linear regression model was used to test the differences in the lipid profile segregated on the basis of these four groups. The data were adjusted for ritonavir-boosted PI regimens or ritonavir-based therapy as a single PI and with Bonferroni adjustment for multiple comparisons. All P values < 0.05 were considered statistically significant. All analyses were performed with the SPSS statistical package (version 11.0; SPSS Inc, Chicago, Illinois, USA).

Risk factor analysis

Multivariate logistic regression analyses were performed to evaluate the risk factors in the development of carotid atherosclerosis. Variables included in the multivariate analyses were those factors that represent known cardiovascular risk, e.g., age, body mass index, smoking habit, CRP, dyslipidaemia, diabetes, hypertension, PI regimens, as well as those that showed significant associations in the univariate analyses. The data on carotid atherosclerosis were the dependent variable and the independent variables included age, hypertension, smoking habit, dyslipidaemia, diabetes, CRP, PI regimens and the SDF-1 polymorphism.

Results

From among the HIV-infected patients attending our clinic ($n = 305$), 183 accepted the invitation to participate in the present study, and provided fully informed consent. Selected characteristics of the participants, segregated according to the presence or absence of carotid atherosclerosis, are presented in Table 1. There was no case of any vascular event either in the coronary or in the cerebral areas. Only seven of the patients had been receiving a statin therapy (but over a period of less than 1 year) and fibrates had been prescribed in 10 patients over the previous 6 months. Segregation with respect to sex and/or age did not affect the distribution of the genotype and, hence, all patients were analysed as a single group. The allelic distribution of SDF1-3′A genotype followed Hardy–Weinberg equilibrium (χ^2; $P = 0.39$) in HIV-infected patients as well as in the general population-based control group. The genotype distribution and the allelic frequency in the HIV-infected group did not differ significantly from that of the population-based control group. We did not find significant differences when comparing patients with atherosclerosis and those without, when considering different types of PI-based therapies (Table 2). Further, there were no significant differences in PI treatment schemes when comparing those patients with the SDF-1 mutated allele with those with the wild-type allele (data not shown).

Table 1. Selected characteristics of HIV-infected patients according to the presence or absence of carotid atherosclerosis.

	All patients ($n = 183$)	With atherosclerosis ($n = 113$)	Without atherosclerosis ($n = 70$)	P
Age (years) [mean (± SEM)]	39.13 (0.54)	41.54 (0.67)	35.24 (0.68)	< 0.001
Male (%)	67.8	72.6	60.0	0.07
Current smoker (%)	84.1	80.4	90.0	0.07
Hypertension (%)	12.3	16.0	6.3	0.05
BP_S (mmHg)[a] [mean (± SEM)]	119.12 (1.37)	122.27 (1.86)	113.89 (1.82)	0.03
BP_D (mmHg)[a] [mean (± SEM)]	76.98 (0.92)	78.60 (1.22)	74.26 (1.38)	0.02
Body mass index (kg/m^2) [mean (± SEM)]	19.47 (0.19)	19.18 (0.26)	19.68 (0.31)	0.23
Abnormal fasting glucose[a] (%)	10.9	15.0	4.3	0.01
Dyslipidaemia[a] (%)	35.2	50.4	28.6	0.003
C-reactive protein (mg/l) [mean (± SEM)]	4.09 (0.38)	4.08 (0.48)	4.61 (0.88)	0.56
Months since HIV diagnosis [mean (± SEM)]	86.66 (3.80)	90.60 (4.79)	80.31 (6.21)	0.18
CD4 cell count; cells/ mm^3 (%)				
< 350	42.1	41.6	42.9	0.98
350–500	23.0	23.0	22.9	
> 500	35.0	35.4	34.3	
AIDS defining condition[b] (%)	32.2	31.9	32.9	0.88
Nadir CD4 cell count; cells/mm^3 [mean (± SEM)]	344 (21)	322 (24)	380 (41)	0.19
SDF1-3′A (%)	47.5	41.6	57.1	0.04

[a]Hypertension was considered as systolic blood pressure (BP_S) > 140 mmHg or diastolic blood pressure (BP_D) > 90 mmHg; abnormal fasting glucose was defined as fasting plasma glucose > 6.1 mmol/l; dyslipidaemia was considered as total cholesterol > 6.2 or low-density lipoprotein cholesterol > 3.36 mmol/l or high-density lipoprotein cholesterol < 0.9 mmol/l.
[b]AIDS-defining condition was considered according to Clinical Report of the Guidelines for National Human Immunodeficiency Virus Case Surveillance [30].

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Table 2. Antiretroviral agents used in the 183 participants according to the presence or absence of carotid atherosclerosis.

	Atherosclerosis		No atherosclerosis		
	Time (months) [mean (± SEM)]	*n* (%)	Time (months) [mean (± SEM)]	*n* (%)	*P*
Not ritonavir boosted[a]	22.85(1.97)	77 (61.6)	22.12 (2.68)	48 (38.4)	0.95
Ritonavir boosted	6.46 (1.19)	38 (65.5)	5.40 (1.50)	20 (34.5)	0.47
Efavirenz	8.09 (1.29)	61 (62.2)	7.79 (0.96)	37 (37.8)	0.88

[a]No ritonavir boosted agents include indinavir, nelfinavir and saquinavir.

Analyses of subclinical carotid atherosclerosis

We found that 113 participants (61.7%) presented with subclinical carotid atherosclerosis despite being relatively young (mean age, 39.13 ± 0.54 years). Most of the participants were heavy smokers. In the atherosclerosis group, we found higher rates of hypertension, abnormal fasting glucose and dyslipidaemia. Conversely, we did not find differences in body mass index, or HIV-related variables such as time since diagnosis (indicating time of seroprevalence), duration of antiretroviral therapy, CD4 cell count, and the prevalence of AIDS-defining conditions. When the distribution of the genotype results were analysed, we found a lower number of individuals carrying the SDF1-3′A allele in the group with carotid atherosclerosis (41.6%) than those without arterial lesions (57.1%; $P = 0.04$). Further, analysis of the distribution of the SDF1-3′A mutated allele according to the results of IMT indicated that, among those participants with an abnormal IMT (> 0.8 mm), the SDF-1 mutated allele was less likely to be encountered than the wild type (38.4% versus 61.6%; $P = 0.03$). When carotid plaques were measured, we found that those individuals who bore the mutated allele for SDF-1 presented a significantly lower carotid plaque area 20.36 ± 5.57 mm^2, than those carrying the wild type 37.33 ± 4.90 mm^2 ($P = 0.02$).

To ascertain whether the SDF1-3′A allele was exerting any protective effect on the development of carotid atherosclerosis, we used a multivariate regression analyses to assess the influence of several known cardiovascular risk factors on the findings of SDF-1 genotype. Age and the presence of dyslipidaemia were significantly ($P < 0.05$) associated with carotid atherosclerosis in the multivariate analysis (Table 3). Further, the results showed a protective effect of the SDF1-3′A allele on the development of carotid atherosclerosis [odds ratio (OR), 0.45; 95% confidence interval (CI), 0.19–1.02; $P = 0.05$)]. We did not find any significant effect of the different antiretroviral regimens used on carotid atherosclerosis.

Table 3. Odds ratios (OR) for carotid atherosclerosis in the multivariate analysis.

	OR	95% CI	*P*
Age (years)	1.21	1.11–1.31	< 0.001
Male	1.49	0.61–3.59	0.37
Body mass index (kg/m^2)	0.91	0.80–1.03	0.15
Current smoking	1.22	0.34–4.31	0.75
Hypertension	2.67	0.57–12.54	0.21
Dyslipidaemia	2.88	1.21–6.87	0.01
Diabetes mellitus	3.28	0.53–20.15	0.19
C-reactive protein (mg/l)	0.97	0.91–1.03	0.39
Non-boosted PI regimens	1.00	0.98–1.01	0.99
Boosted PI regimens	1.00	0.97–1.03	0.95
Efavirenz-based regimen	1.03	0.99–1.07	0.12
SDF1-3′A	0.45	0.19–1.02	0.05

CI, Confidence interval.

We analysed the lipid profile changes in these individuals ($n = 134$) during a period of 18 months from the start of a PI-based therapy. The relevant characteristics of these patients are given in Table 4. Of note was that higher rates of systolic and diastolic blood pressure were found in the

Table 4. Selected characteristics of patients under a protease inhibitor (PI)-based therapy.

	PI-naive group ($n = 134$)	Atherosclerosis ($n = 84$)	No atherosclerosis ($n = 50$)	*P*
Age (years) [mean (± SEM)]	38.99 (7.29)	41.23 (0.75)	35.22 (0.88)	< 0.001
Male (%)	67.2	70.2	62.0	0.32
Current smoker (%)	85.7	82.1	92.0	0.10
BP_S (mmHg) [mean (± SEM)]	120.34 (1.64)	123.62 (2.18)	114.71 (2.19)	< 0.05
BP_D (mmHg) [mean (± SEM)]	77.40 (1.11)	79.20 (1.46)	74.32 (1.61)	< 0.05
Body mass index (kg/m^2)	19.52 (0.23)	19.41 (0.31)	19.70 (0.36)	0.57
Abnormal fasting glucose (%)	11.9	16.7	4.0	< 0.05
Dyslipidaemia (%)	41.8	48.8	30.0	< 0.05
C-reactive protein (mg/l) [mean (± SEM)]	4.79 (0.57)	4.69 (0.60)	4.95 (1.17)	0.82
Time since HIV diagnoses (months) [mean (± SEM)]	94.71 (4.07)	96.41 (5.37)	91.86 (6.19)	0.59
Prior NRTI therapy	63.5 %	67.4%	59.6%	0.36

BP_S, Systolic blood pressure; BP_D, diastolic blood pressure; NRTI, nucleoside reverse transcriptase inhibitor.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

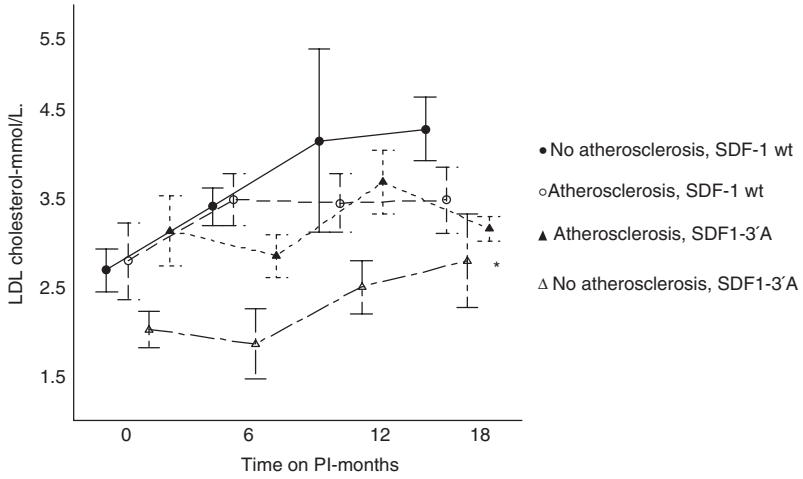

Fig. 1. LDL-C values (means ± SEM) in HIV-infected patients on PI-based therapy ($n = 134$) segregated with respect to SDF-1 genotype and the results of the carotid ultrasonography. A general linear regression model adjusted for ritonavir-boosted or ritonavir as a single PI-based antiretroviral therapy was applied. $^{*}P = 0.04$.

atherosclerosis group, and that the patients with atherosclerosis were significantly older. We did not find significant differences between the groups when total cholesterol, HDL-C and triglycerides were analysed (data not shown). However, when LDL-C was considered, those patients without carotid atherosclerotic lesions and who carried the SDF-1 mutated allele ($n = 19$) showed a significantly ($P = 0.04$) lower LDL-C of 2.06 ± 0.34 mmol/l, throughout the follow-up period (Fig. 1). Multiple comparisons (with Bonferroni adjustments) resulted in significant ($P < 0.05$) differences when those patients without atherosclerosis and SDF1-3′A allele were compared with those patients with wild type SDF-1.

Discussion

The identification of host genetic factors that can exert influence in the development of cardiovascular diseases in HIV-infected patients becomes relevant due to the incidence of atherosclerosis observed in these patients. Knowledge of these host variables should lead to an individualized, and better fitted, antiretroviral approach which should minimize the advent of cardiovascular events in the long-term. Our main finding is that an allelic variant of the SDF-1 gene, SDF1-3′A, was significantly less prevalent in HIV-infected patients with carotid atherosclerosis and, further, these patients presented with a better LDL-C profile in the 18 months of follow-up.

Classic cardiovascular risk factors such as advanced age, hypertension, abnormal fasting glucose and dyslipidaemia were far more prevalent in the group of patients with carotid atherosclerosis. However, one of the most commonly attributed cardiovascular risk factors in these patients has been the treatment with PI [3] but, according to our data, multivariate analyses did not reveal any significant effect of these drugs on the development of subclinical atherosclerosis. Conversely, a lower number of patients bearing the mutated allele for SDF-1 were found in this group, and the results in the multivariate analysis confirmed the protective effect of the SDF1-3′A mutated allele in the development of carotid atherosclerosis.

Several chemokines have been identified as being involved in the development of the atherosclerotic lesion, and polymorphisms in these genes could exert a critical influence on the process. SDF-1 has been shown to be involved in different atherosclerosis developmental processes such as platelet activation and aggregation as well as in the activation of arterial smooth muscle cells [9–11]. It is feasible, then, that the influence of SDF-1 on atherosclerosis might be exerted in a dose-dependent manner, and it is likely to be modulated by genotype. Although this hypothesis was not confirmed when explored in a group of patients with unstable angina [19], the variations in the plasma concentrations of SDF-1 are subject to various interpretations. SDF-1 is a chemokine of stromal origin [8] so it could be that low circulating levels of SDF-1 do not correspond to the levels in tissues. Further, it has been demonstrated in HIV-infected patients that circulating SDF-1 is converted into an inactive form which appears to be smaller in size that the original [26]. These possibilities make the interpretation of plasma concentration of SDF-1 difficult. However, in a non-HIV-infected population, SDF-1 expression was decreased in those bearing the SDF1-3′A allele [16] and, therefore, it would seem reasonable to postulate a protective effect against atherosclerosis. We did not measure SDF-1 plasma concentration because, in HIV-infected patients, several factors can influence its

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

measurement [26,27] and, as such, a single determination is unlikely to be of much statistical value. We have not typed the viruses for CXCR4, and this may represent a limitation in our study. Most of the patients studied were asymptomatic and it may be assumed that R5 HIV strains are predominant [28]. However, we did not aim to study the influence of SDF1-3′A mutated allele on HIV progression but, more importantly, the influence of these polymorphisms on the development of atherosclerosis in a cohort of HIV-infected patients. As such, knowledge of the virus type, whether R5 or X4, would not add much additional insight into the development of atherosclerosis in response to the inflammatory state induced by the HIV infection.

We found a striking association between the lipid profile and the SDF-1 genotype. LDL-C values were greatly decreased in those individuals carrying the mutated allele. This could explain, in part, the protective influence of the mutated allele on the development of atherosclerosis. Although it might represent merely an epigenetic phenomenon, it is of note that patients without atherosclerotic lesions and who bear the mutated allele had LDL-C values < 2.5 mmol/l throughout the course of the follow-up period. This would need to be confirmed in large prospective studies since it is of considerable interest to evaluate the influence of SDF-1 on the development of carotid atherosclerosis and on the course of lipid changes and, as such, to assess whether the host genetic background has any influence in the progression/regression of carotid IMT. One further limitation of our study is that we did not evaluate patients with antiretroviral therapies other than the PI–HAART scheme. As such, for example, we have not analysed patients receiving non-nucleoside reverse transcriptase inhibitor-based regimens as the numbers in this subsample are small. However, it has been documented that this antiretroviral therapy can exert a beneficial effect on the lipoprotein profile by inducing increases in HDL-C concentrations and, as such, would have represented a confounding variable [29] in the present study.

In summary, the allelic variant SDF1-3′A of SDF-1 appears to be protective against the development of carotid atherosclerosis in a group of HIV infected patients and is associated with a less-atherogenic lipid profile, including lower concentrations of LDL-C. The identification of this host polymorphism could be of major interest in individualizing antiretroviral therapy and in minimizing the risk of cardiovascular disease in these patients.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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UNIVERSITAT ROVIRA I VIRGILI
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"The role of immunity and inflammation in the progression of atherosclerosis in HIV-infected patients."

The fourth study is the result of the analyses of atherosclerosis progression/regression of participants in the study. We applied the same protocol (carotid and femoral IMT, clinical assessment and biologic samples) after a median of 2.5 years of follow-up. The main result is that IMT values, in both carotid and femoral arteries, progressed at a rate of 0.045 mm/year, indicating a significant atherosclerosis progression in HIV-infected patients (normal rate of progression is set at 0.01 mm/year). Classical cardiovascular risk factors, such as age or total cholesterol, were influencing significantly the rate of IMT increase, but interestingly, the number of CD4 cell count and certain chemokine-related polymorphisms were also determinant in the course of atherosclerosis. The lower the nadir CD4 cell count the higher was the rate of IMT increase, which might indicate that the immune reconstitution experienced by HIV-infected patients severely immunodepressed before starting any HAART, may be deleterious in terms of atherosclerosis. Further, those patients who bear the SDF1-3'A or CX3CR-1 249I presented a significantly reduced rate of IMT increase. These data suggest a relevant influence of inflammatory and immunologic-related variables in the course of atherosclerosis in HIV-infected patients.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
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The role of immunity and inflammation in the progression of atherosclerosis in HIV-infected patients

Blai Coll, MD; Sandra Parra, MD; Carlos Alonso-Villaverde, MD; Gerard Aragonés, Manuel Montero, MD; Jordi Camps, PhD; Jorge Joven, MD; Lluis Masana, MD.

Background and Purpose- The initial steps of atherosclerosis and the entry of HIV into the cell share similar biological mechanisms. Therefore, our hypothesis is that the progression of atherosclerosis in HIV-infected patients can be influenced by variations in genes implicated in both processes.

Methods and Results- The progression of atherosclerosis over a 2-year follow-up period was measured as the combined carotid and femoral intima-media thickness (IMT) in 141 HIV-infected patients. The ΔIMT ($IMT_{follow-up}$- $IMT_{baseline}$) values were used to segregate patients as minimal progressors or regressors (lowest ΔIMT tertile), slow progressors (mid ΔIMT tertile) and rapid progressors (highest ΔIMT tertile). Mutations CCR-5Δ32, CCR-2 64I, MCP-1–2518G, SDF1-3'A and CX3CR-1 (T280M and V249I) in the host DNA were determined. Mean age of the patients was 38.96 (SEM: 0.61) and 68.8% were male. The mean ΔIMT was 0.045 mm (0.01) per year which represented a significant progression ($p<0.001$) with respect to baseline values. Patients with minimal progression or regression had a significantly ($p=0.01$) higher CD4 cell count than slow progressors and rapid progressors. Multivariate analyses indicated that age and total cholesterol were positively associated with IMT progression. In contrast, the CD4 cell count, the SDF1-3'A and the CX3CR-1 249 I mutated alleles were associated with a lesser IMT progression.

Conclusion- The course of atherosclerosis in HIV-infected patients is influenced by polymorphisms in the SDF1 and CX3CR1 genes, by metabolic variables and by the CD4 cell count. These data would be of help in assessing therapeutic needs of these patients. (**Stroke 2007; in press).**

Key words: atherosclerosis • intima-media thickness • HIV • chemokine polymorphisms • non-conventional cardiovascular disease risk factors

HIV-infected patients have higher rates of atherosclerosis [1, 2] and the progression is faster than in non-infected individuals [3]. Chemokines play a significant role in atherosclerosis and HIV infection [4, 5] and chemokine-related genetic variants are implicated in the development of atherosclerosis as well as in the natural course of HIV-infection [5-8].

Circulating monocytes are attracted to the sub-endothelial space, mainly mediated by monocyte chemoattractant protein-1 (MCP-1), where they become foam cells [7, 9]. A polymorphism in the promoter region of the MCP-1 gene (MCP-1–2518G) is associated with a higher MCP-1 expression [10]. The bearers of this mutation are more prone to the development of AIDS-associated dementia [11], or sub-clinical atherosclerosis [12]. Its natural receptor, CCR-2, a minor co-receptor for the entry of HIV into the cell [13], has been implicated in the development of atherosclerosis [14] as well as in disease progression in HIV-infected individuals [15]. Similarly, the stromal derived factor-1 (SDF-1) [16], activates platelets within the atheromatous plaque [17] and promotes the migration of smooth muscle cells to the sub-endothelial space [18]. A polymorphism in the untranslated region, SDF1-3'A, which is associated with a lower SDF-1 expression [19], influences the disease progression as well as the presence of sub-clinical atherosclerosis in HIV-infected patients [20, 21].

Another chemokine, fractalkine, is up-regulated in inflamed tissues and it functions as a chemo-attractant as well as an adhesion molecule. DNA polymorphisms in the gene for its specific receptor CX3CR1 (V249I and T280M) lead to a reduced number of fractalkine binding sites, to a reduced risk of coronary artery disease, and to a more rapid progression to AIDS [22-23].

As a consequence, patients with HIV infection are prone to continuous inflammatory stimuli which may trigger a cytokine imbalance that can influence the development of atherosclerosis [24]. However, these patients also present with well-known, pre-existing cardiovascular risk factors [25] and our aim was to evaluate these variables and to evaluate their relative contribution to the progression of atherosclerosis.

Design and Methods

Participants and design

We performed a prospective study aimed at evaluating atherosclerosis and its related factors in a cohort of HIV-infected patients (n=305) who were receiving regular attention in our out-patient HIV clinic. The results of the initial assessments have been published previously [12, 21] and the current manuscript summarizes the follow-up data. In the first clinical consultation, a complete physical examination was performed including the assessment of body weight, height and blood pressure according to standard guidelines [26] and a venous blood sample was drawn for blood chemistry and DNA analyses. At the same visit the first ultrasound baseline scans of the carotid and femoral arteries were performed. The identical protocol was applied by the same investigators after two years of follow-up. During this period, data on smoking habit, alcohol consumption, time since HIV diagnoses, nadir of the CD4 cell count (CD4 cell count before starting antiretroviral therapy), time on highly active antiretroviral therapy (HAART) and opportunistic infections were collated. Lipodystrophy and metabolic syndrome were diagnosed using accepted criteria [27, 28]. The laboratory measurements included plasma total cholesterol, HDL cholesterol, triglycerides, and glucose as well as serum HIV-1 RNA and blood lymphocyte T

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INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

CD4+ cell count. LDL cholesterol was calculated from the Friedewald formula. The study was approved by the Ethics Committee of Hospital Universitari Sant Joan.

Genotyping

DNA was obtained using the Pure Gene Kit (Gentra Systems, Inc.). We determined MCP-1–2518G, CCR-2 V64I, SDF1-3'A, CCR-5 Δ32 and CX3CR-1 (V249I and T280M) variants using previously-published methods [11, 16, 20, 24]. For genotype comparisons we used a general population-based control group of unrelated subjects (n=348) the details of which have been described previously [29]. Briefly, they were healthy people (167 female, 181 male), mean age 42 years (range 19-75) and who were considered representative of the general population in our area.

Ultrasound standardized protocol

The carotid and femoral ultrasound measurements were performed under the identical protocol by the same investigators (MM, BC) who were blinded with respect to the results of the other measured variables. We used a GE Logiq 700 with an ultrasound probe of 7-10 MHz. We identified and digitally recorded the far wall of the common carotid artery (1cm proximal to the bifurcation), the carotid bulb (in the bifurcation), the internal carotid artery (1cm distal to the bifurcation) and the common femoral artery. All the IMT measurements were performed at the pre-defined points using the image processing software AnaliSYS ™ (Soft Imaging System, Münster, Germany). IMT measurements on each arterial segment were averaged and used in the statistical analyses as combined IMT, as previously described [13]. The intraclass correlation coefficient between the 2 examiners in evaluating the images from 20 IMT measurements was 0.89 ($p<0.001$), and the absolute IMT difference was 0.01 mm (0.025). To assess reproducibility of measurements, the images of 20 randomly-selected patients were re-measured applying the same protocol. The intraclass correlation coefficient between the 2 sets of measurements was 0.91, and the absolute difference in IMT was 0.007 mm (0.018). To assess the reproducibility of re-scanning and re-measurement, 10 patients underwent a re-scan within 1 month. The intraclass correlation coefficient for the measurement was 0.94.

Statistical analyses

Results are expressed as the mean with standard error of the mean (SEM) in parentheses, or in percentages. Kolmogorov-Smirnov test was applied to test the distribution of variables. Paired Student t-test was applied to evaluate differences in measurements of continuous variables at baseline and at follow-up and the Wilcoxon test when the data were not normally distributed. The $\chi 2$ test was used to compare categorical variables. The difference in the combined IMT value between the 2 examinations was calculated as ΔIMT = IMT follow-up – IMT baseline. Univariate analyses were used to identify the variables having an impact on ΔIMT. We defined as "rapid progressors" those individuals with a ΔIMT value in the highest tertile (ΔIMT ≥0.18 mm), "slow progressors" as being those patients with ΔIMT between 0.02 and 0.179 mm, and "minor progressors" or "regressors" as those in the lowest tertile (ΔIMT ≤0.019 mm). Differences among these groups were tested with ANOVA and a post-hoc analysis (Bonferroni test) was applied.

Stepwise regression analyses were performed to determine prognostic factors for baseline sub-clinical atherosclerosis (defined as the presence of an atherosclerotic plaque or as a combined IMT ≥0.8mm) and for the change in the combined IMT at 2 years of follow-up. The stepwise analysis method added variables one-by-one into the model, with the variable with the smallest probability value significant at the 0.05 level.

For the identification of variables related to baseline sub-clinical atherosclerosis we applied a logistic regression analyses and, for the ΔIMT, a linear regression analyses in which the following were the independent variables: age, gender, BMI, systolic and diastolic blood pressure, fasting plasma lipid concentrations, lipodystrophy, metabolic syndrome and CD4 cell count. Also included were the duration of the patient's antiretroviral treatment and the results of the genotype analyses. The multivariate linear regression analysis was adjusted for the baseline IMT values.

Results

General characteristics of HIV-infected participants

From the 183 participants who accepted to participate, 8 died from HIV-associated diseases, 27 were lost to follow-up or declined to participate in the second evaluation, and in 7 patients the recorded images were not of sufficient quality to perform the analyses.

The clinically-relevant characteristics of the 141 patients included are summarized in Table 1. The mean time-lapse to HIV sero-prevalence (time from diagnoses) was 7.24 (0.36) years and 79 (56%) patients were co-infected with the hepatitis C virus. Most patients (56%) were current or past intravenous drug abusers and sexual intercourse-related factors were identified as the cause of the infection in the remaining patients. In the baseline examination, most patients were heavy smokers, relatively young, without significant obesity and with normal blood pressure values. Mean plasma lipid and glucose concentrations were within the laboratory reference ranges. Mean CD4 cell count was significantly higher in the second evaluation and there was a trend towards more patients having undetectable HIV viral load (Table 1). Most patients (92.2%) were receiving HAART treatment schemes and, during the follow-up, there were no major changes in the prescriptions (Table 1).

We found higher total plasma and LDL cholesterol concentrations in the baseline examination but higher mean systolic blood pressure was observed in the second evaluation (Table 1). Similarly, we found more patients who fulfilled the criteria of lipodystrophy and metabolic syndromes in the second examination, although this difference did not reach statistical significance. During the follow-up, 3 patients had cardiovascular disease events (2 patients with stroke and 1 patient with an acute coronary syndrome) and statins were prescribed to 10 patients.

Table 1. Characteristics of participants (N=141) at baseline and at follow-up.

Characteristic	Baseline	Follow-up	P value
Gender; male	97 (68.8)	NA	NA
Age; years	38.9 (0.6)	40.7 (0.6)	NA
Body mass index; kg/m^2	23.31 (0.27)	23.52 (0.29)	0.02
Systolic blood pressure; mmHg	118.04 (1.44)	120.46 (1.54)	0.07
Diastolic blood pressure; mmHg	76.96 (1.04)	78.35 (0.97)	0.16
Smoking; yes	94 (66.6)	88 (62.4)	0.09
Lipodystrophy; yes	42 (29.8)	46 (32.6)	0.09
CD4 cell count; cells/mm^3	462.97 (27.02)	523.63(27.81)	<0.001
Undetectable HIV-1 viral load*	89 (63.1)	98 (69.5)	0.08
Total cholesterol; mmol/L	5.06 (0.11)	4.90 (0.12)	0.004
HDL cholesterol; mmol/L	1.18 (0.04)	1.19 (0.03)	0.56
LDL cholesterol; mmol/L	2.82 (0.09)	2.68 (0.09)	0.01
Triglycerides; mmol/L	2.50 (0.19)	2.53 (0.21)	0.38
Glucose; mmol/L	5.42 (0.08)	5.40 (0.09)	0.48
Metabolic syndrome; yes	26 (18.4)	35 (24.8)	0.64
Antiretroviral therapy‡			
NRTIs	129 (92.2)	127 (90.2)	0.43
PIs	104 (73.8)	90 (64.2)	
NNRTIs	77 (55.3)	82 (58.2)	

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Genotype distribution

As shown in Table 2, there were no significant differences either in allele frequencies or genotype distributions between the group of HIV-infected patients (N=141) and the general population (N=348).

For statistical purposes, those patients with at least one mutated allele for MCP-1, SDF-1, CCR-5 or CCR-2, were analyzed as a single group. From among the 9 possible CX3CR-1 haplotypes, we did not find any study participant with the VV/TM, VV/MM and VI/MM genotypes. This indicated that the CX3CR-1 polymorphisms were in complete linkage disequilibrium. Since the 249I allele has been shown to be associated with less-extensive development of atherosclerosis (24), we compared the V249V genotype against the other genotypes as a combined group (V249I or I249I).

There were no significant differences in the CD4 cell count and HIV viral load between those patients carrying the mutated alleles compared to those carrying the wild type and, as such, the HIV-related variables did not segregate with genotypes.

Table 2. Genotype distribution of the polymorphisms in the HIV-infected patients and the general population

Alleles	HIV-infected (N=141)	General population (N=348)	P Value
MCP-1–A2518G			0.20
GG	3 (2.1)	19 (5.5)	
GA	51 (36.2)	128 (36.8)	
AA	87 (61.7)	201(57.8)	
CCR-5? 32			0.69
? 32/? 32	-	1 (0.3)	
wt/? 32	18 (12.8)	47 (13.5)	
wt/wt	123 (87.2)	300 (86.2)	
CCR-2 V64I			0.24
II	-	4 (1.1)	
IV	26 (18.4)	67 (19.3)	
VV	115 (81.6)	277 (79.6)	
SDF1-3'A			0.07
3'A/3'A	7 (5.0)	22 (6.3)	
wt/3'A	66 (46.8)	120 (34.5)	
wt/wt	68 (48.2)	206 (57.5)	
CX3CR-1 V249I			0.11
II	7 (5.0)	32 (9.2)	
VI	58 (41.1)	116 (33.3)	
VV	76 (53.9)	200 (57.5)	
CX3CR-1 T280M			0.10
MM	-	6 (1.7)	
TM	30 (21.3)	84 (24.1)	
TT	111 (78.7)	258 (74.1)	

Factors influencing baseline and follow-up values of combined IMT

There was a significant (p<0.001) mean annual increase (0.045 mm) in the combined IMT between the baseline measurements [0.75 (0.01) mm] and the follow-up values [0.84 (0.01)] (Figure 1), and this tendency did not vary when carotid and femoral arteries were analyzed separately (data not shown). Males showed higher baseline IMT values than females, although patients of both genders had similar rates of increase (Figure 1). Baseline values were not significantly influenced by the lipid profile, the presence of metabolic syndrome or the treatment with protease inhibitors (PI).

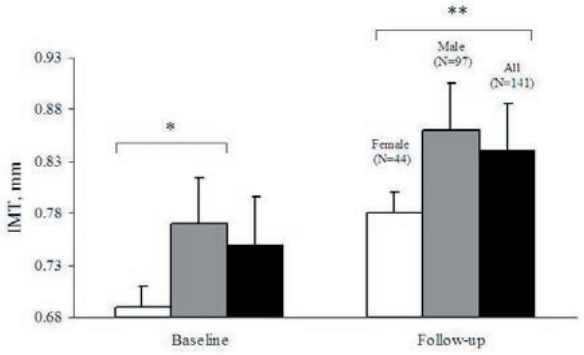

Figure 1. Combined IMT in baseline and follow-up examination in females (open bars), males (hatched bars) and overall study participants (closed bars). * p<0.05 comparing baseline IMT values between females and males (ANOVA) ** P<0.05 follow-up IMT compared to baseline in the 3 groups (*t*-test of repeated measures)

We used the ΔIMT values to segregate the patients into regressors (N=45), slow progressors (N=48) and rapid progressors (N=48) so as to identify variables that may influence the course of atherosclerosis (Table 3). None of the classical cardiovascular disease risk factors were significantly associated with the course of IMT. The proportion of patients with metabolic syndrome was lower among the regressors but the difference did not reach statistical significance (p=0.09). There were significant differences in the CD4 cell counts among groups. Regressors had a higher nadir and baseline CD4 cell counts than slow progressors and rapid progressors (Table 3 and Figure 2), indicating that the better the immune status the lower the likelihood of atherosclerosis progression.

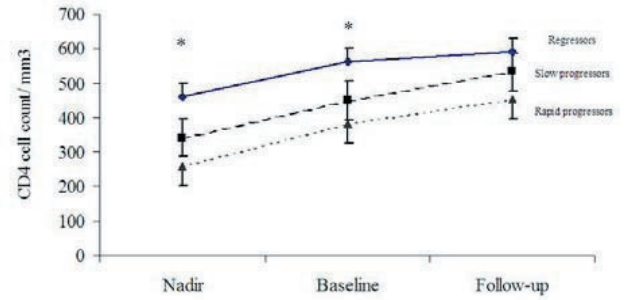

Figure 2. Rate of IMT progression segregated with respect to selected genotypes. Open bars represent patients bearing the wild type allele and closed bars those with the mutated allele. * P=0.05 and ‡ P=0.04 comparing the different rates of IMT progression segregated with respect to SDF-1 and CX3CR-1, respectively (ANOVA).

Genetic variables were distributed in a similar manner among groups, although the variant CX3CR-1 249I was more frequent among the regressors (p=0.08). Moreover, the simultaneous presence of the putatively atherosclerosis-protective alleles (i.e. SDF1-3'A + CX3CR-1 249I + MCP-1–A2518), was significantly (p=0.008) higher in the group of regressors.

The differences in the rates of progression were not statistically significant with respect to the genetic variants in CCR-5, CCR-2 and MCP-1. However, those patients with

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
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either the SDF1-3'A or the CX3CR-1 249I alleles showed a significantly lower IMT increase than those with the corresponding wild-type alleles (Figure 3).

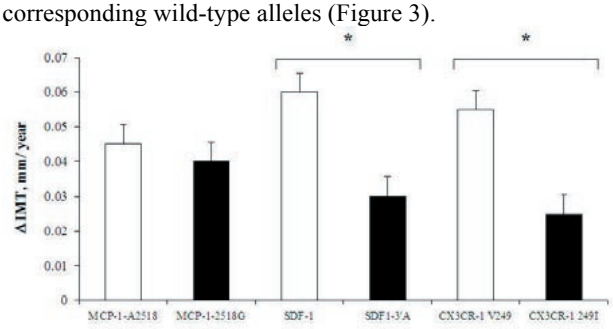

Figure 3. Progression of CD4 cell counts in relation to the course of atherosclerosis. Nadir (at the moment of initiating antiretroviral therapy), Baseline (first clinical evaluation) and Follow-up (second clinical evaluation). CD4 cell counts are presented as the mean (SEM). There were no significant differences between the groups with respect to time-lapse to HIV sero-prevalence; * P=0.01 comparing the 3 groups of atherosclerosis progression rates (ANOVA).

Variables related with atherosclerosis progression

We applied multivariate regression analyses to identify those variables that could influence baseline IMT values and atherosclerosis progression. The results of the analyses revealed that age, diastolic blood pressure and the MCP-1–2518G mutated allele were positively, and significantly, associated with a higher likelihood of atherosclerosis in the baseline measurements (Table 4). The combined IMT in the baseline measurement was the strongest predictor of the course of IMT i.e. the higher the baseline values the slower the rate of IMT increase. Age and total cholesterol were positively associated with a higher IMT increase (Table 4). The CD4 cell count was also an important determinant i.e. higher CD4 cell counts predicting slower IMT increase. Finally, the mutated SDF1-3'A allele and the presence of the I allele in position 249 of the CX3CR1 gene were also identified as protective factors in the increase of IMT (Table 4).

Table 4. Multivariate stepwise analyses of atherosclerosis. R^2 of the linear regression analyses for the ΔIMT = 0.56.

Baseline atherosclerosis	**Odds ratio**	**95%CI**	**P value**
Age ; years	1.23	1.11- 1.36	<0.001
Diastolic blood pressure, mmHg	1.06	1.01- 1.10	0.006
MCP-1–2518G	7.78	2.31- 26.16	0.001
4b. Follow-up examination			
Δ IMT; mm	**β**	**95%CI**	**P value**
Baseline combined IMT ; mm	–0.69	–0.84- –0.59	<0.001
Age ; years	0.21	0.002- 0.009	0.001
Total cholesterol ; mmol/L	0.17	0.008- 0.04	0.003
CD4 cell count ; cells/mm^3	–0.13	–0.03- –0.01	0.02
CX3CR1-249I	–0.14	–0.09- –0.01	0.01
SDF1-3'A	–0.12	–0.09- –0.002	0.04

The formula resulting from the application of the multivariate model predicts 60% of the variance of ΔIMT, and the predicted values correlated closely (Pearson coefficient=0.74, p<0.001) with those observed (Figure 4).

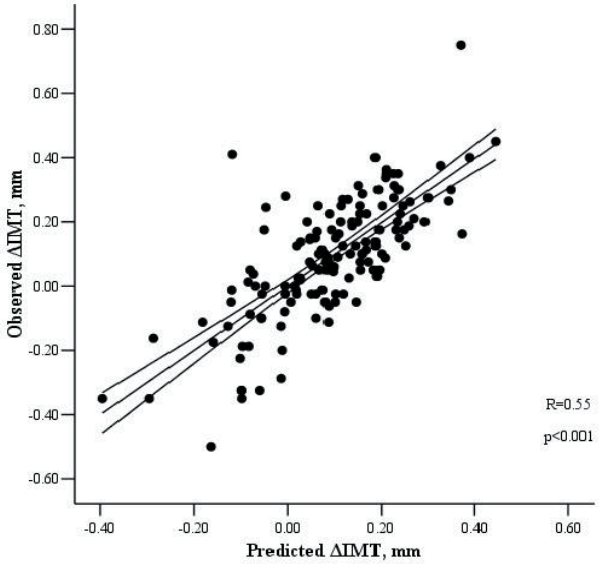

Figure 4. Correlation between observed and predicted ΔIMT
The predicted ΔIMT was obtained using the following formula: ΔIMT= 0.43 + [–0.72* baseline IMT(mm)] + [0.006* age(years)] + [0.025* Total cholesterol (mmol/L)] + [–0.00008* CD4 cell count(cells/mm^3)] + [–0.05* CX3CR-1(0 wild type, 1 mutation)] + [–0.04* SDF-1 (0 wild type, 1 mutation)] Data presented as mean and 95 % confidence interval.

Discussion

Our data indicate that the IMT in HIV-infected patients increases at a faster rate (0.045 mm/year) than that considered as the threshold value (0.03 mm/year) beyond which the risk of suffering from cardiovascular disease events is significantly higher [30]. Among the causes of such progression, only age and total cholesterol are among the classical cardiovascular risk factors that we observed to be significantly associated with a higher rate of IMT increase. Our data are similar to that of a previous study by Hsue et al [3] which had shown an IMT increase of 0.07 mm/year. The mean age of participants and different genetic background may explain the differences in the rates of atherosclerosis progression between studies. Further, one relevant source of variability among studies, is that we used different protocols to acquire and read the IMT images. However, their data reached similar conclusions and, as such, are highly informative.

Another variable that influences the rate of IMT increase is the baseline IMT. The use of a structural measure to assess atherosclerosis reflects the retrospective lifetime exposure to risk factors. Indeed, it may indicate that those patients with higher baseline IMT had been also exposed to a larger number of classical risk factors, and which have been prospectively controlled. This may explain the negative association between baseline IMT and progression. However, a specifically designed controlled clinical trial would need to be performed to answer to this question.

We observed, as well, that the CD4 cell count may play a relevant role in atherosclerosis progression; the higher the CD4 cell count the lower the rate of atherosclerosis progression. It is plausible that patients with a better CD4 recovery were not under the deleterious influence of CD4 activation [31-33]. Atherosclerotic plaques are constantly being

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

remodeled, and this determines the rate of progression/regression of the disease. CD4+ cells can influence this dynamic process, as has been described for monocytes in animal models of atherosclerosis regression [34]. The detrimental influence of low CD4 cell counts in the IMT increase may be explained by a higher rate of antiretroviral prescriptions. However, in the multivariate analyses none of the treatments analyzed were positively associated with atherosclerosis progression.

The progression of atherosclerosis in HIV-infected patients can be controlled through a reduction in cardiovascular risk factors, a reduction in the prescription of PIs and an increase in concomitant treatments with lipid-lowering agents [35]. These results warrant confirmatory large-scale multi-centered trials ought to incorporate an assessment of the IMT within a standardized protocol. This is being recognized in clinical trials assessing the efficacy of lipid-lowering drugs [36].

Chemokines and their natural receptors have received increasing attention in atherosclerosis research [5]. This is especially relevant in HIV-infected patients because the chemokines have been implicated in both processes: the entry of HIV-into the cell, and the ability of the monocyte to enter the sub-endothelial space. MCP-1 is the more active molecule implicated in chemotaxis [7], a phenomenon in which other molecules such as fractalkine and its receptor CX3CR-1 participate. The fractalkine/CX3CR-1 axis is of particular interest because it can function as a chemo-attractant and as a direct adhesion molecule [23]. The presence of the 249I mutated allele has been associated with a lower CX3CR-1 expression [24] and, as such, the interaction between fractalkine and CX3CR-1 is less likely. Our results indicate that HIV-infected patients who bear the 249I allele have significant protection against atherosclerosis progression. Since heterozygous patients may have only a partial deficiency in CX3CR-1 function, and since alternative monocyte recruitment pathways can exist, our study addressing the role of MCP-1 and SDF-1 adds a different perspective to the atherosclerosis process. The SDF1-3'A mutation is associated with a lower SDF-1 expression. It appears not to have relevance in determining baseline sub-clinical atherosclerosis but seems, however, to have a beneficial influence on the progression of the disease. Moreover, the putative "atherosclerosis-protective" combination, such as CX3CR-1 249I+ SDF1-3'A+MCP-1–2518A, is associated with a lower rate of IMT increase.

Limitations of the study

Previous reports in non-HIV-infected patients, assessed the influence of genetics in the values of IMT, It was estimated that 35–45% of the variability in multivariable-adjusted carotid IMT is explained by genetic factors [37]. One of the aims of our study was to investigate the relationship between selected genetic polymorphisms and the IMT course. Although the associations observed in univariate were not very robust, we included these variables in the multivariate model in order to determine, specifically, the influence of certain genetic variations in this clinical setting. However, to confirm such genetic associations, a larger patient population sample would be necessary. Further, to preclude the effect of confounding variables associated with HAART therapies, the study would need to be performed in treatment-naïve patients. There are lines of evidence suggesting a deleterious effect of PIs on the atherosclerosis process [38-40] but, to-date, no studies have been specifically designed to prove such hypotheses and, as such, they have yet to be definitively demonstrated.

We expressed our data from carotid and femoral IMT as combined IMT mainly because the course of IMT in both territories was observed to be similar (significant progression was found in both carotid and femoral arteries). Also, atherosclerosis is a systemic disease in which disturbances in carotid and femoral arteries have been associated with a higher incidence of cardiovascular and peripheral vascular disease, respectively [41].

Implications

Our multivariate analyses resulted in the configuration of a formula that predicts 60% of the variance in ΔIMT. The only significant modifiable factors were total cholesterol and the CD4 cell counts. For example, in a 40 year-old patient with a baseline IMT of 0.8 mm and wild type for the 2 critical genotypes (SDF1-3'A and CX3CR-1 249I), the reduction of 1 mmol/l of total cholesterol and the increase of 100 CD4 cells/mm^3 would result in a 20% reduction in the predicted ΔIMT. Our data also indicate that the putative deleterious effect of antiretroviral therapy is unlikely to occur if the patient's plasma cholesterol concentration is properly managed.

In measuring IMT, we support the exploration and measurement of the degree of atherosclerosis and not just the assessment of risk-factor status in the development of the disease. We propose that lipid-lowering agents should be used not only for the patient with high values of total plasma cholesterol and LDL cholesterol but also for those patients with pathological baseline IMT as well as those with a higher rate of IMT increase.

Summary

The progression of atherosclerosis in HIV-infected patients is influenced by metabolic, inflammatory and immunological variables. Our results indicate that, in the assessment of a patient population with HIV infection, the classical cardiovascular disease risk factors should be complemented with the study of the inflammatory response (especially those genetic factors that have an influence in HIV and in atherosclerosis. These include MCP-1, SDF-1 and CX3CR-1, and immune status (CD4 cell count at the moment of HIV diagnoses). This conclusion has been reached by another study in non-HIV-infected patients in which a combination of genetic polymorphisms and a pro-inflammatory score was strongly related with carotid and femoral IMT [42]. Standardized protocols to measure IMT and the identification of the genetic susceptibility should be implemented for a better, and more individualized, cardiovascular disease risk assessment of the patient.

Acknowledgements

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Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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UNIVERSITAT ROVIRA I VIRGILI
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Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

SUPPLEMENTARY ON-LINE MATHERIAL

Table 3. Comparison of relevant variables in groups segregated by tertiles of the course of intima-media thickness

Variable	Regressors N = 45	Slow Progressors N = 48	Progressors N = 48
Gender; male	34 (75.6)	32 (66.7)	**31 (64.6)**
Age; years	40.1 (1.1)	37.9 (0.9)	**38.9 (1.1)**
Body mass index; kg/m2	22.90 (0.43)	23.98 (0.50)	**23.02 (0.45)**
Smoking; yes	41 (91.1)	38 (79.2)	**36 (75.0)***
Systolic Blood Pressure; mmHg	119.97 (2.79)	118.39 (2.39)	**115.92 (2.35)**
Diastolic Blood Pressure; mmHg	77.72 (2.03)	78.84(1.79)	**74.42(1.60)**
Lipodystrophy; yes	15 (33.3)	17 (35.4)	**10 (20.8)**
Metabolic syndrome; yes	4 (8.9)	12 (25.0)	**10 (20.8)**
Total cholesterol; mmol/L	4.98 (0.20)	4.83 (0.16)	**5.37 (0.22)**
HDL cholesterol; mmol/L	1.25 (0.10)	1.12 (0.06)	**1.19 (0.07)**
LDL cholesterol; mmol/L	2.76 (0.16)	2.79 (0.12)	**2.95 (0.17)**
Triglycerides; mmol/L	2.52 (0.33)	2.03 (0.17)	**2.88 (0.44)**
Glucose; mmol/L	5.43 (0.19)	5.46 (0.14)	**5.33 (0.11)**
Nadir CD4 cell count, cells/mm3	459.66 (62.21)	338.60 (33.40)	**268.50 (37.49)* ‡**
CD4 cell count, cells/mm3	562.66 (61.46)	444.93 (40.90)	**380.41 (29.99)* ‡**
CRP, mg/L	4.65 (0.96)	4.51 (0.70)	**3.18 (0.49)**
ΔIMT, mm	-0.11 (0.01)	0.09 (0.006)	**0.27 (0.01)* ‡**
Δ Carotid IMT, mm	-0.05 (0.02)	0.06 (0.02)	**0.22 (0.01)* ‡**
Δ Femoral IMT, mm	-0.19 (0.03)	0.13 (0.02)	**0.28 (0.02)* ‡**
Genetic variables			
CCR-5Δ32	7 (15.6)	4 (8.3)	**7 (14.6)**
CCR-2 64I	5 (11.1)	9 (18.8)	**12 (25.0)**
MCP-1–2518G	22 (48.9)	14 (29.2)	**18 (37.5)**
SDF1-3'A	27 (60.0)	24 (50.0)	**24 (50.0)**
CX3CR1- 249I	25 (55.6)	23 (47.9)	**18 (37.5)**

Notes to Table 3: For definition of "regressors", "standard progressors" and "rapid progressors" see text for details; * P <0.05 of the differences between rapid progressors and regressors (post hoc analysis, Bonferroni test); ‡Indicate overall p value <0.01 (ANOVA). Definition of the Metabolic syndrome (see text). All values are expressed as mean (Standard Deviation)

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General Discussion

This thesis analyses the influence of genetics on different fields regarding HIV infection, such as atherosclerosis and the course of immune and virologic variables. The study was designed prospectively in 2002 to assess the incidence, causes and mechanisms of atherosclerosis in a population under the influence of a chronic infection, and to further explore those genetic variables that may impact in both processes, atherosclerosis and HIV-infection (ATEROVIR study, which was partially financed by the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, 2004-2007). This approximation lead us to collate clinical data, not only related to infectious diseases but also, metabolic and cardiovascular risk factors. We have performed a collection of images of carotid and femoral arteries of each participant for the measurement of IMT. The design of the study includes also the storage of biological samples, such as DNA, plasma, serum and lymphocytes.

I present in this thesis the first four original manuscripts although the study is under a constant actualization and revision. As such, it is still open and active for further studies and modifications.

Genetics, atherosclerosis and HIV

The identification of markers for the early detection of atherosclerosis is highly relevant. This is especially interesting in young populations, because primary preventive strategies are available, and therefore, the incidence and consequences of cardiovascular diseases should be positively diminished. In that sense, the use of IMT has revealed a very effective tool in assessing the risk of suffering from cardiovascular diseases, and as such, it is a very effective tool to study atherosclerosis in different populations [243]. We have studied HIV-infected patients for many reasons. First, they are under the influence of a chronic infection, that might provoke a chronically inflamed status in most of these patients [244]. It is known that a chronic inflammatory state has been strongly associated to a higher incidence of atherosclerosis related events [245], and in fact, several studies addressing the influence of chronic infections such as, *Chlamydia or Helicobacter,* on the development of atherosclerosis have already been published [246]. Second, the generalization of HAART schemes are closely linked with a longer life expectancy, and then the appearance of age-related conditions, such as atherosclerosis, are more likely. Third, as previously stated, the mechanisms

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by which HIV entry into the cell and monocytes reach the subendothelial space, are similar, i.e.: a complex interactions of chemokines and their natural receptors. Interestingly, HIV itself is able to influence the expression of these chemokines, the inflammatory response (for instance, Tat and MCP-1) and also the metabolism of the infected cells (blocking the properties of macrophage ABCA1) [247].

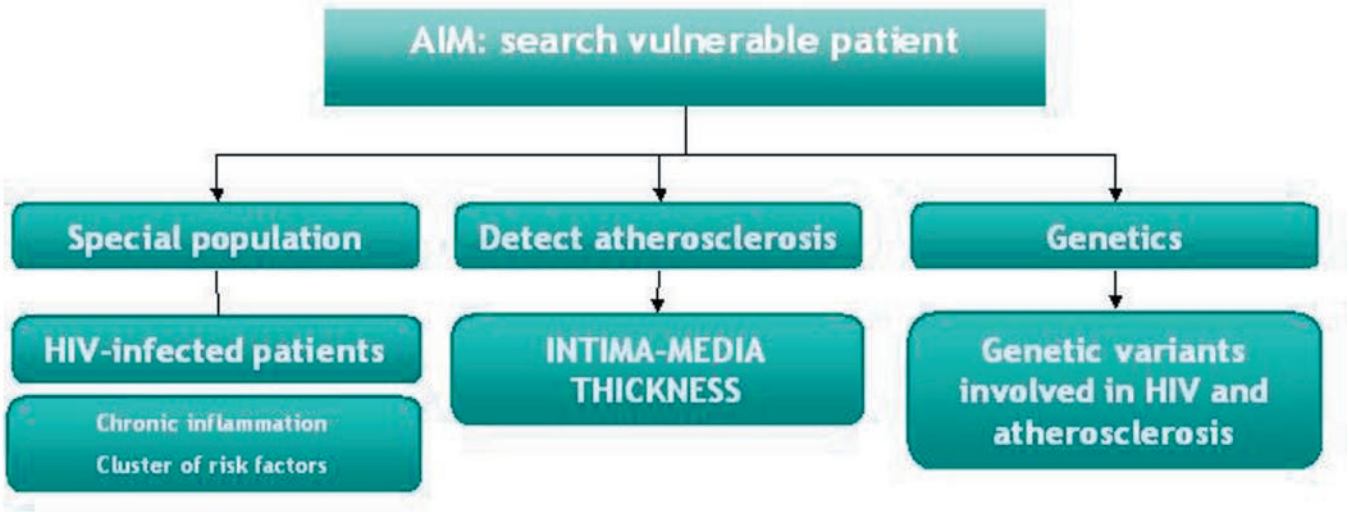
For all these reasons, and taken into account that the identification of these patients more vulnerable to the development of atherosclerosis might be critical, we have developed a prevention strategy, summarized in the Figure 25, which is based in:

1.- to detect atherosclerosis non-invasively: using in a standardized fashion the measurement of the IMT.

2.- to collate classical cardiovascular risk factors.

3.- to analyze genetic polymorphisms influencing both HIV and atherosclerosis.

Figure 25. General approach to the study of atherosclerosis in HIV-infected patients.



The first study revealed the impact of MCP-1 in the development of atherosclerosis in HIV-infected patients; either the mutated MCP-1–2518G allele or the plasma concentration of MCP-1 are closely related with a higher likelihood of having atherosclerosis. We also studied [248] which variables were related to MCP-1 concentration in HIV-infected and un-infected population (Annex #1). Plasma MCP-1 concentration was significantly higher in HIV-infected patients than in healthy participants, in whom, MCP-1 were significantly correlated with age, smoking status, triglyceride concentrations and with the presence of MCP-1-2518G mutated allele. In HIV-infected patients, MCP-1 was significantly and positively correlated with HIV viral load and inversely correlated with the number of CD4+T cells, indicating that a close interaction between HIV and MCP-1 may exist. However, in a multiple regression model, only age, the MCP-1 genotype and smoking status showed significant associations with plasma MCP-1 concentrations.

loss of association between C-reactive protein and circulating MCP-1

lipodystophic HIV-infected patients presented higher plasma concentration of MCP-1 closely related with a higher carotid IMT

MCP-1 and CRP might represent different ways of inflammation and thereby studies based on both molecules should not be taken together

metformin or rosiglitazone decreased plasma concentrations of MCP-1

An interesting conclusion of these studies is the loss of association between C-reactive protein and circulating MCP-1 [157, 248]. This feature is of particular interest because they are usually studied together, in order to better assess the inflammatory status [249]. According to our results, MCP-1 and CRP might represent different ways of inflammation and thereby studies based on both molecules should not be taken together.

One of the conclusions of our first study is that the higher the MCP-1 concentration, the higher the IMT values, and it is specially relevant in those HIV-infected patients who bear the mutated allele. This is also applicable to HIV-infected patients who have developed lipodystrophy, in whom a different expression of cytokines and adypokines have been observed [250]. We have studied in a sub-set of HIV-infected patients the relationship between carotid IMT and MCP-1 [251] (Annex #2), and we found that lipodystophic HIV-infected patients presented higher plasma concentration of MCP-1, that in turn were closely related with a higher carotid IMT (Figure 26). This observation lead us to hypothesize that a possible mechanism explaining the higher IMT values in these patients was mediated by MCP-1.

Further, we have studied which would be an effective way to control the expression of MCP-1. In non-infected population, the use of statins [252] or insulin sensitizers [253] have been associated to a decrease in the plasma or serum concentrations of MCP-1. In a collaboration study with the group of the Erasmus Medical Center in Rotterdam, we studied the impact of several cytokines and anti-oxidant variables in lipodystrophic HIV-infected patients who had received metformin or rosiglitazone [254] (Annex #3). Both treatments decreased plasma concentrations of MCP-1 significantly after 26 weeks of therapy. Whether this action has a direct impact in the development of atherosclerosis remains to be shown, and further

Figure 26. Relationship between carotid IMT and plasma MCP-1 in HIV-infected patients with lipodystrophy (A) and without lipodystrophy (B).

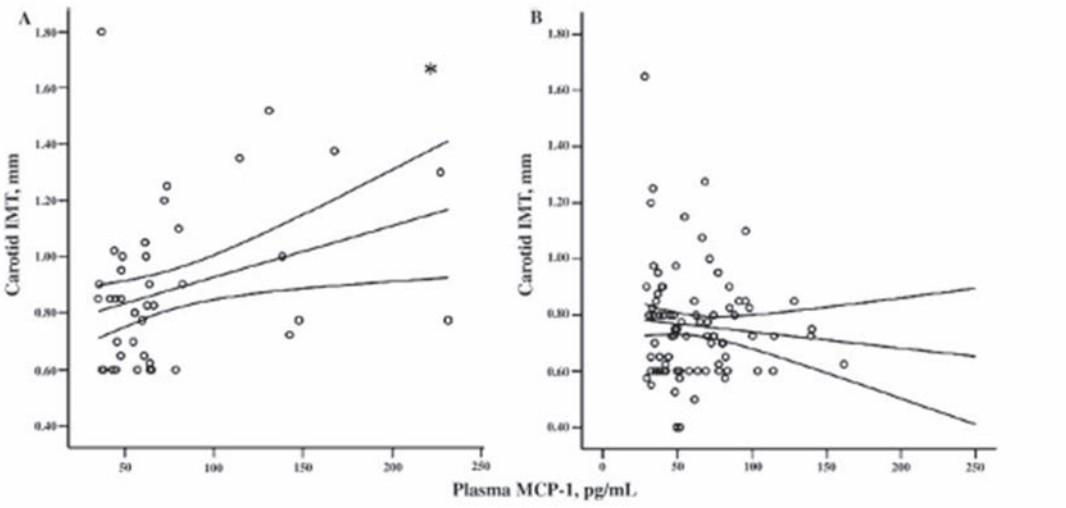
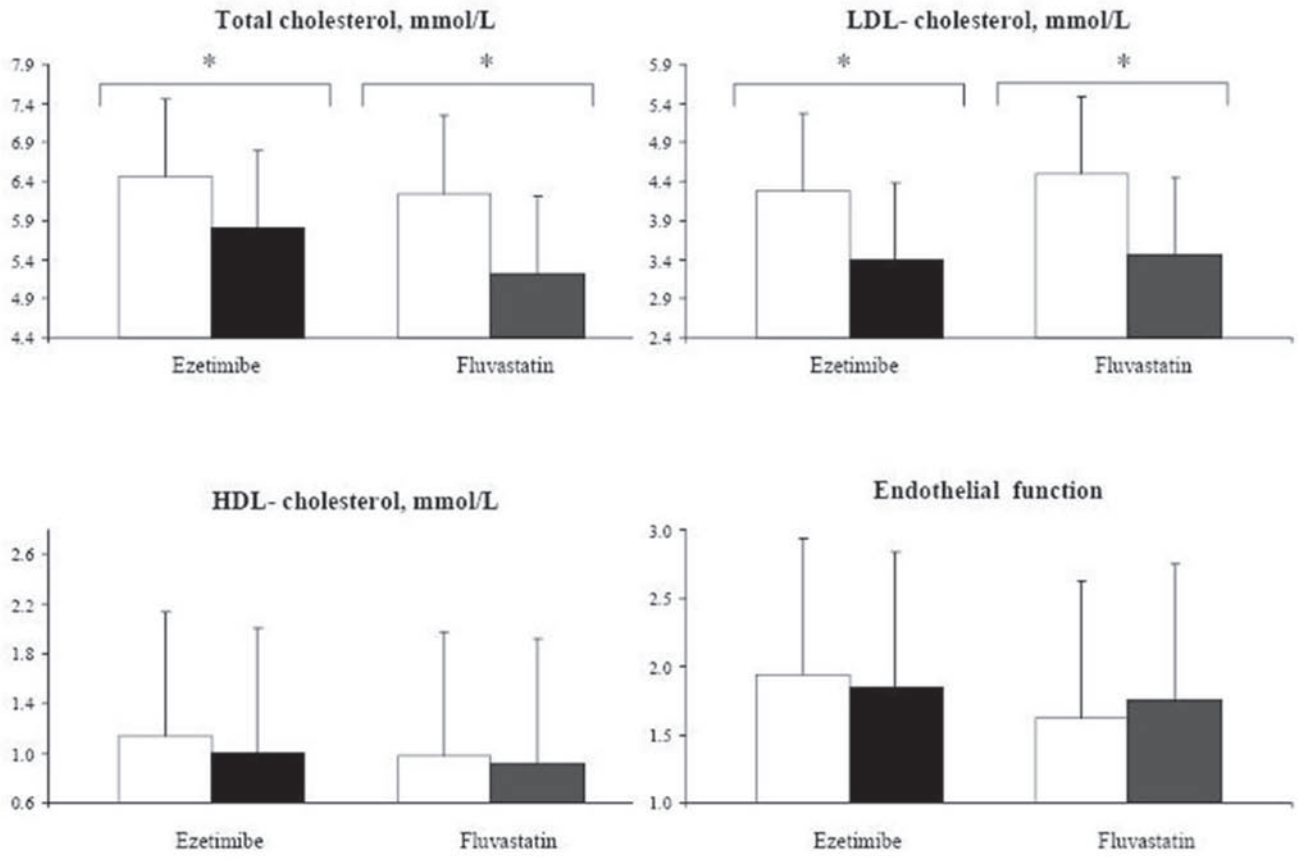


Fig. 2. Correlation plots between carotid IMT and MCP-1 plasma concentration in LD-HIV-1-infected patients (A, solid circles) and non-LD-HIV-1-infected patients (B, open circles). Mean and 95% CI values are depicted. *MCP-1 plasma concentration and carotid IMT values have been log-transformed for statistical purposes Pearson coefficient 0.31, $p = 0.03$.

and larger clinical trials should be initiated. van Wijk et al. [255] reported in a clinical trial of lipodystrophic HIV-infected patients that rosiglitazone increased subcutaneous and visceral abdominal fat. They also assessed cardiovascular status using endothelial function parameters, such as flow-mediated vasodilation, that was significantly increased with metformin. They also performed carotid IMT studies of these patients and those values were compared with diabetic patients and with healthy participants [256]. Carotid IMT was similarly increased in the group of HIV-infected patients with metabolic syndrome (comparable to those diabetic patients), and significantly higher than healthy participants.

In a subset of HIV-infected patients with hypercholesterolemia we have used lipid lowering agents to ascertain its impact on an early atherosclerosis marker, the assessment of endothelial function [257] (Annex #4). Although we have not data concerning the course of cytokines in these treated patients, either fluvastatin or ezetimibe effectively reduced LDL cholesterol concentration. They improve endothelial function after the treatment period, although differences did not reach statistical significance; however, these differences may warrant the utilization of these agents not only as lipid lowering drugs, but also to their pleiotropic effects in HIV-infected patients.

Figure 27. Overview of the main results of treating HIV-infected patients either with fluvastatin or ezetimibe.



Footnote: white bars represent the results of the exams performed before treatment and filled bars after treatment.

A part from these results, chemokine-derived research has revealed that chemokines are key mediators in directing the deleterious influence of classical cardiovascular risk factors in the artery wall, and as such, focusing therapies beyond lowering cholesterol concentration is, theoretically, highly desirable. In Phase I-II clinical trials, several compounds have directed its action to block the interaction between MCP-1 and CCR-2 [258]. Blocking some chemokine receptors may lead to a down or up regulation of other receptors. A proof of this is the ability of Tak-779 to block CCR-5 and CxCR-3 making this agent a good candidate for the HIV treatment, but also, it has been demonstrated in a pro-atherogenic animal model, to be highly effective in reducing the development of atherosclerosis [171]. This study deserved an editorial in *Arteriosclerosis Thrombosis and Vascular Biology* entitled "*Killing two birds with one stone*", indicating that chemokine research focused at both conditions, HIV-infection and atherosclerosis, deserve further consideration [259].

"it would be highly desirable to design therapeutic strategies directed to MCP-1/CCR-2 axis, in order to treat HIV-infection and atherosclerosis

Summarizing the first two studies of this thesis, we identified a functional polymorphism in the promoter region of MCP-1 that, in HIV-infected patients, although it is associated with a better course of the HIV-infection (in terms of CD4 cell course and undetectable HIV-viral load) yields patients with the mutated allele to a higher likelihood of developing atherosclerosis (a five-fold increased risk). Taking both results into account, it would be highly desirable to design therapeutic strategies directed to MCP-1/CCR-2 axis, in order to treat HIV-infection and atherosclerosis, as it has been similarly proposed in other conditions such as multiple sclerosis[260], cancer[261] or graft versus host disease [262].

In the search of susceptible genes for the development of atherosclerosis, we studied the role of SDF-1. SDF-1 is the natural ligand of CXCR-4, a known HIV co-receptor, specially involved in the late stages of the HIV infection. The lower gene expression related to the presence of the mutated SDF1-3'A allele, confers protection for the development of carotid atherosclerosis in HIV-infected patients. The relationship between SDF-1 and cardiovascular events is scarce, and the case-control studies published in the literature [165] showed no association between coronary artery disease and the presence of the SDF1-3'A allele. However, we found an striking association between the mutated allele and the lower concentrations of LDL cholesterol, that might be indicating an indirect effect in the appearance of carotid lesions. This analyses was performed taking into account the results of previous studies, in which the MCP-1 mutated allele was obtained. Again, a functional polymorphism in a cytokine-related gene has revealed as protector in the development of atherosclerosis in a chronically inflamed population. These results were further confirmed in the study of atherosclerosis progression. Those patients who bear the mutated alleles either SDF1-3'A or CX3CR-1 249 I were resistant to a higher rate of IMT increase.

Any of the polymorphisms presented in this study, met the set criteria to be considered as clinically relevant [263]:

1. The change in the gene causes a relevant alteration in the function or level of the gene product.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

2. The number of cases associating an allele with a particular phenotype must be large enough to be convincing.
3. The beneficial and harmful phenotypes being studied must have clear-cut clinical differences.
4. The plausibility of the hypothesis must be convincing.

According to our results, the study and application of future therapeutic molecules targeting chemokine and chemokine receptors studied in our work, should be specially warranted.

We observed, as well, that CD4 cell count may play a relevant role in atherosclerosis progression; the higher the CD4 cell count the lower the atherosclerosis progression. It is plausible that patients with a better CD4 recovery, were not under the deleterious influence of CD4 activation[264,265], and this may explain a lesser IMT increase. These results are in accordance with those presented by Hsue P. et al [46], and it should have clinical implications. HIV-infected patients did not receive antiretroviral therapy since their CD4 cell counts get down below 350 cells/mm^3 [266]. If the deleterious impact of the antiretroviral therapies were minimized, we probably should reconsider these recommendations, and start therapy earlier in life with higher CD4 cell counts. However, some authors agreed that the progression of atherosclerosis in HIV-infected patients can be controlled through a reduction in cardiovascular risk factors, a reduction in the prescription of PIs and an increase in concomitant treatments with lipid-lowering agents [267]. These results warrant confirmatory large-scale multi-centered trials, as those employed in the validity of lipid-lowering therapies, which should incorporate an assessment of the IMT within a standardized protocol [268].

These results warrant confirmatory large-scale multi-centered trials, as those employed in the validity of lipid-lowering therapies, which should incorporate an assessment of the IMT within a standardized protocol

Limitations and Perspectives

We have not performed a case-control study (infected and non-infected) by several reasons. Firstly, our aim was not to compare the incidence of atherosclerosis in HIV-infected patients (other research groups have been involved in that issue), but the influence and genetic associations determining the appearance of these lesions. Secondly, it is very difficult to obtain suitable controls to HIV-infected patients, because, they usually represent a highly specific population (cultural and socio-economic background, addicted to a great number of substances) that make the comparison very difficult to be standardized.

Atherosclerosis has been considered as a paradigm of a complex disease, and as such, a very intriguing interaction between environmental and genetic variables take place in its origin and development. This is particularly important when considering patients with a chronic infection caused by HIV, that in turn interacts with molecular pathways of the inflammatory lesions of the artery wall. We have approached atherosclerosis focusing in selected chemokines, genetic polymorphisms, clinical data and the information coming from the image (carotid and femoral IMT), but the next step should be to direct our efforts in assessing atherosclerosis with the information from the gene expression. We are conducting such an study considering not only the above mentioned genes, but also a wide range of metabolic and inflammatory related genes. The rationale for doing so is that we should be able to know the influences on the expression of key genes, in patients who bear a mutated allele. This strategy should lead us to a better comprehension of the molecular processes implicated, and to a better design of therapeutic targets. These results are planned to be analyzed in the present year.

Atherosclerosis is a highly devastating disease [269], in which hundreds of risk factors have been identified [270]. Preventive strategies are based on the calculation of risk according to several scores, Framingham, Score or Regicor, that basically collect clinical and laboratory information. These preventive strategies have revealed useful in identifying patients at very high risk, but their role as an effective and sensitive preventive strategy is far to be accomplished [271]. All these preventive strategies are focused on the individual assessment of cardiovascular risk, based on factors that are highly prevalent in the general population. Therefore, they provide limited prognostic value in an individual setting [272]. Probably, this might be the reason why 6 over 10 patients with a myocardial infarction are considered to have a low/intermediate risk [273], in whom the current guidelines do not indicate the use of drug therapies to control risk factors [274]. In this context, the use of the image (the measurement of carotid IMT, for example) and the genetic information should strengthen the assessment of individuals, in order to better identify those vulnerable patients, and not only those at risk.

The study developed by our group is the result of this approach in HIV-infected patients. It has several limitations, and the results have to be validated in larger population studies, but the main conclusion is that in the study of atherosclerosis, the assessment of risk factors are clearly insufficient to accomplish with the goal of reducing atherosclerosis-related events. A wider assessment, which should include genetic and image information, should guide future cardiovascular medicine, at least in special populations, such as those with the HIV-infection.

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Annex #1:

The influence of HIV infection on the correlation between plasma concentrations of monocyte chemoattractant protein-1 and carotid atherosclerosis.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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The influence of HIV infection on the correlation between plasma concentrations of monocyte chemoattractant protein-1 and carotid atherosclerosis

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Abstract

Background: Monocyte chemoattractant protein-1 (MCP-1) plays a crucial role in atherosclerosis and it has been recently proposed as a surrogate biomarker of long-term clinical outcomes in patients with acute myocardial infarction. Little is known of the factors that may influence plasma MCP-1 concentrations.
Methods: We studied 384 healthy volunteers and 226 HIV-infected patients as a model of chronic inflammatory condition that predisposes to sub-clinical atherosclerosis.
Results: In healthy participants there were significant associations between plasma MCP-1 concentration and age, smoking status, and serum triglyceride concentrations that were not observed in the HIV-infected patients. The plasma concentration of MCP-1 was significantly associated with the polymorphism at position −2518 of the MCP-1 gene and, in patients, with the carotid artery intima–media thickness. There were also significant correlations indicating a close association between MCP-1 and HIV disease activity. However, in a multiple regression model, only age, the MCP-1 genotype and smoking status showed significant, and independent, associations with plasma MCP-1 concentrations.
Conclusion: Plasma MCP-1 concentration is genetically determined and associated with age and smoking habit and it also correlates with subclinical atherosclerosis in HIV-infected patients.
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Keywords: Cardiovascular risk factors; Human immunodeficiency virus; Infection; Inflammation

1. Introduction

Inflammation plays an important role in the development of atherosclerosis, and may be a predictive factor for the further progression of acute coronary syndromes [1,2]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine responsible for the recruitment of monocytes to sites of inflammation where they promote atherosclerotic lesions and plaque vulnerability [3–5]. The data obtained from clinical and experimental studies provide support for a role of MCP-1 on the initiation, and progression, of atherosclerosis [6,7]. It is of note that plasma MCP-1 concentration seems to be genetically determined [8], and appears to be influenced by certain therapeutic agents and conditions such as myocardial infarction, hypertension, hypercholesterolemia and stroke [9–14].

To further explore the relationship between plasma MCP-1 concentration and atherosclerosis, we sought associations with cardiovascular disease risk factors in a population-based

Abbreviations: BMI, body mass index; CRP, C-reactive protein; PI, protease inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

sample of ostensibly healthy participants. We studied, as well, a group of HIV-infected patients since they are constantly challenged by various inflammatory stimuli which result in an imbalance in circulating cytokines [15]. In these patients, premature sub-clinical atherosclerosis is frequent and influenced by a mutant MCP-1 allele [16,17]. As such, we hypothesize that there would be significant alterations in circulating markers of vascular inflammation such as C-reactive protein (CRP) and, as we currently propose, MCP-1. We also sought associations between these markers and the carotid intima–media thickness (CIMT), a well-validated surrogate marker for atherosclerotic vascular disease [18].

2. Materials and methods

2.1. Study population

A population-based sample of 384 subjects (187 women, 197 men; mean age 42.1 years; range 19 to 75 years) was randomly selected from the local town hall's population register. The detailed characteristics have been presented elsewhere [19]. All the participants were of Mediterranean (Caucasian) ethnic origin, and were ostensibly healthy with no evidence of renal insufficiency, hepatic damage, cancer, or psychiatric disease that may alter their lifestyle habits. Also, there was no family history of premature cardiovascular disease and none had noted chest pain or claudication on usual or recreational physical efforts. All the selected subjects were invited to attend a clinical examination at which their socio-demographic details were solicited and a venous blood sample taken for analyses. Current medication use was an exclusion criterion. From among the patients attending our Outpatient Clinic, 226 HIV-infected subjects (68 women, 158 men; mean age 39.7 years, range 22 to 66 years) agreed to participate in the present study. The variables recorded at interview were age, gender, smoking habit, time since HIV diagnosis, main risk factor for HIV-infection, CD4 cell count at diagnosis, antiretroviral therapy history, height, weight, and body mass index (kg/m^2). No patient had evidence of atherosclerosis-related events. The study was approved by the Ethics Committee of the Hospital Universitari de Sant Joan de Reus and all patients provided full informed consent to participation.

2.2. Laboratory measurements

Blood samples were obtained during the clinical examination and collected into potassium EDTA-containing tubes for MCP-1 and lipoprotein(a) [Lp(a)] determinations, or into tubes with no anticoagulants added for the other biochemical analyses. Assays for plasma MCP-1 were performed by ELISA (Peprotech, London, UK) according to the instructions of the manufacturer. Recombinant human MCP-1 antigen was used as the calibrator for assay standardization. We used plasma EDTA because we had previously observed an inconsistent increase of between 40% and 80% in concentrations in serum, relative to those in plasma. The measurable range was between 10 and 1000 ng/L. Coefficients of variation at 60 and 120 ng/L were <2% and 4.1%, respectively (intra-assay); and <4% and 8.2%, respectively (inter-assay). Plasma Lp(a) was measured as previously described [20]. The serum concentration of C-reactive protein was measured using a high sensitivity method with a lower limit of detection of 0.10 mg/L [21]. HDL-cholesterol concentration was analyzed using a homogeneous method [22]. Serum cholesterol and triglycerides were determined by enzymatic techniques (ITC Diagnostics, Barcelona, Spain). Serum viral load was measured with the Amplicor HIV-1 monitor assay (Roche, Basel, Switzerland) and CD4+ T-cell counts by standard FACscan flow cytometry (Becton-Dickinson, Madrid, Spain).

2.3. Genotyping

Reactions were carried out in a 10 μL total volume containing 10 ng of genomic DNA, 0.25 U of Taq DNA polymerase and its buffer (1×), $MgCl_2$ 1.5 mM, DNTP mix 0.2 mM, and 0.2 μM each of sense and antisense primers. The primers used were: 5′-TCT CTC ACG CCA GCA CTG ACC-3′ and 5′-GAG TGT TCA CAT AGG CTT CTG-3′ for MCP-1 A−2518G and 5′-TTG TGG GCA ACA TGA TGG-3′ and 5′-GAG CCC ACA ATG GGA GAG TA-3′ for CCR-2 V64I. To type the MCP-1 allele, the amplicon (234 bp) was digested with 1 U of *Pvu*II. This results in fragments of 159 and 75 bp when a G is present at nucleotide position-2518. For the CCR-2 allele, the amplicon (128 bp) was digested with *BsaB*I which produces fragments of 110 and 18 bp when A is present at nucleotide position 160.

2.4. Ultrasonography

Ultrasonography was performed with a GE Logiq 700MR system (Milwaukee, USA) equipped with a 7–9 MHz linear array transducer, in high-resolution B-mode. The specialist physician performing this procedure was blinded with respect to specific clinical information of the patients. The evaluation included bilateral measurements of the common carotid artery, the carotid bifurcation and the proximal portion of the internal carotid artery as described previously [17].

2.5. Statistical analyses

The Kolmogorov–Smirnov test was used to check for normality of distributions. Because the distribution of plasma MCP-1 concentration is highly skewed, the analyses were conducted on the concentration quartiles. The log transformation of these values did not completely normalize the distribution and we preferred to use non-parametric tests. The probability values (P values) for trends were obtained by linear regression. To test the association of

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

MCP-1 with other variables we used the Spearman correlation test. To evaluate differences between MCP-1 quartiles with respect to the variables studied, ANOVA was used, or the Kruskal–Wallis method followed by the Mann–Whitney *U*-test corrected for multiple comparisons, when appropriate. To preclude undue outlier value influence in the regression analyses, we excluded values of MCP-1 >200 ng/L. Multiple linear regression models were used to verify associations between MCP-1 and the other variables. The stepwise backward procedure includes only the most influential of the variables. All statistical procedures were performed with the SPSS 11.0 statistical package.

3. Results

3.1. Distribution of plasma MCP-1 concentrations

There were no statistically significant differences in plasma MCP-1 concentrations between males and females, nor in the healthy volunteers [median (inter-quartile range) 49.0 (25.7) ng/L and 48.3 (24.6) ng/L, respectively; $P=0.55$] neither in the HIV-infected patients [median (inter-quartile range) 61.2 (38.5) ng/L and 56.4 (30.2) ng/L, respectively; $P=0.10$]. Consequently, in all subsequent analyses data are presented without considering sex. The median MCP-1 concentration was 49.3 ng/L (inter-quartile range 28.3 ng/L) for healthy participants and 59.7 (inter-quartile range, 37.4) ng/L for HIV-infected patients. The values for the 2.5th, 25th, 50th, 75th and 97.5th percentiles were 31.0, 39.6, 49.3, 67.7 and 136 ng/L, respectively, for healthy participants and 32.1, 44.7, 59.7, 82.1 and 195.7 ng/L, respectively, for the patients. The distributions are shown in Fig. 1. Plasma MCP-1 concentration was significantly higher in HIV-infected patients than in healthy participants ($P<0.001$)). The same trend was observed in the values of serum hs-CRP (4.1±5.6 vs. 2.6±4.2 mg/L; $P<0.001$) indicating the presence of chronic inflammatory stimuli. However, the correlation between MCP-1 and hs-CRP values was not statistically significant ($n=610$; $\rho=-0.02$; $P=0.85$).

3.2. Relationship between CIMT and risk factors for atherosclerosis

In HIV-infected patients, univariate analysis showed that the average CIMT values correlated with age ($r=0.360$; $P<0.001$) and plasma MCP-1 concentration ($\rho=0.214$; $P=0.002$), but not with other parameters. Interestingly, there was no significant correlation with hs-CRP values ($r=0.012$, $P=0.89$), indicating that the association with MCP-1 was specific and not a consequence of a global inflammatory response.

3.3. Associations between plasma MCP-1 concentrations and other variables

The associations are summarized in Tables 1 and 2. In healthy participants, plasma MCP-1 concentration was positively associated with age, smoking status and plasma triglycerides, and negatively associated with serum HDL-cholesterol. The non-smokers ($n=257$) showed significantly lower plasma MCP-1 concentrations than those measured in smokers [median (inter-quartile range) 46.2 (25.7) ng/L and 56.4 (39.7) ng/L, respectively; $P=0.006$]. In contrast, there were no significant associations with gender, BMI and the concentrations of serum cholesterol, serum CRP and plasma Lp(a). In HIV-infected patients, no significant associations between these variables were observed. The data were further analyzed with respect to variables defining infection

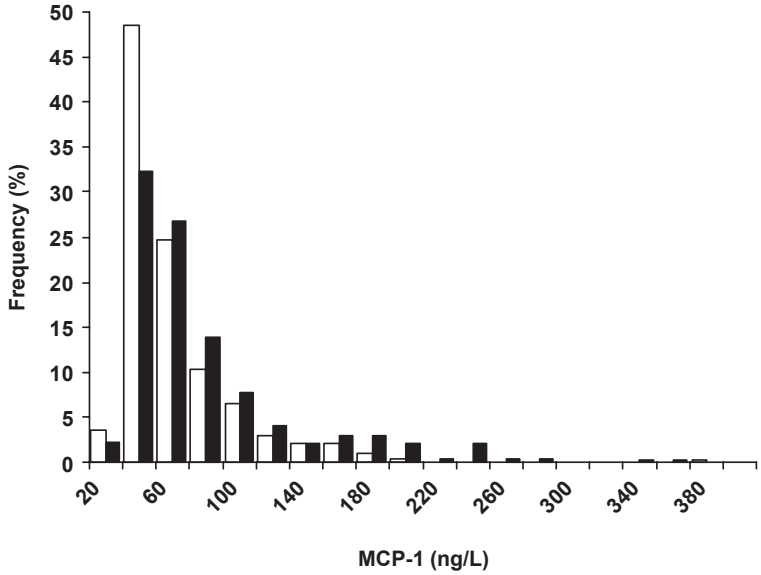

Fig. 1. Distribution of plasma MCP-1 concentrations in healthy volunteers (white bars) and HIV-infected patients (black bars).

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Table 1
Baseline characteristics of healthy participants segregated on quartile distributions of MCP-1

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Trend; *P*
Range, ng/L	26.9–39.6	39.7–49.3	49.3–67.7	68.1–425.7	–
Age, years	38.4 (14.8)	43.3 (15.6)	43.6 (15.0)	44.1 (14.9)	0.043
Males, %	55	52	49	53	0.553
Body mass index, kg/m^2	26.3 (4.9)	27.0 (5.2)	26.9 (5.0)	27.1 (4.9)	0.314
Alcohol intake, g/day	9.5 (16.2)	9.3 (15.6)	9.2 (16.3)	12.3 (22.6)	0.310
Smoking status					
Current smoker, %	27.1	25.0	37.5	42.7	0.009
Cigarettes per day	4.5 (9.7)	3.6 (7.8)	5.4 (8.3)	8.7 (12.5)	0.002
Duration, years	8.1 (10.1)	8.7 (13.2)	11.3 (13.4)	10.2 (11.5)	0.104
Biochemical variables					
Cholesterol, mmol/L	5.13 (0.86)	5.39 (1.01)	5.21 (1.09)	5.35 (1.11)	0.319
Triglycerides, mmol/L	1.11 (0.63)	1.26 (0.74)	1.32 (0.79)	1.64 (1.48)	<0.0001
HDL-cholesterol, mmol/L	1.57 (0.34)	1.56 (0.36)	1.52 (0.39)	1.45 (0.40)	0.018
Lipoprotein(a), mg/L	238 (253)	240 (305)	201 (268)	247 (299)	0.939
C-reactive protein, mg/L	2.23 (3.12)	3.07 (4.95)	2.96 (5.56)	2.37(2.51)	0.875

Values are presented as means (SD).

activity i.e. the number of CD4+ lymphocytes and serum HIV-RNA. These variables were related to plasma MCP-1 concentrations; the lower the MCP-1 levels the lower the HIV RNA, and the higher the CD4+ cells (Table 2). Also, in HIV-infected patients, there was a positive and significant correlation between plasma MCP-1 concentration and the serum HIV RNA ($\rho = 0.380$; $P < 0.001$).

3.4. The influence of CCR-2 and MCP-1 polymorphisms

The genotype frequencies were similar to those reported for other Caucasian populations, and there were no significant differences between the control and patient groups in our study (Table 3). The inheritance of the G allele predisposes to higher MCP-1 concentrations in both groups, but this was only significant in the healthy participants. In patients with serum HIV RNA <200 copies/mL, the absence of the G allele was significantly more frequent than its presence (61.3% vs. 38.7%, respectively; $P < 0.05$). We found no differences in the other variables measured with respect to carriers and non-carriers of the G allele. The presence of the CCR-2 mutation (64I) did not appear to affect the concentrations of plasma MCP-1. None of these genetic variants influenced significantly the CIMT values.

3.5. Stepwise backward multivariate linear regression analyses

Variables shown to be significant in the univariate analyses were retained in the multivariate analysis both in the healthy participants and in HIV-infected patients. An additional model which included the whole population study ($n = 610$) indicated that the significant predictors were age,

Table 2
Baseline characteristics of HIV-infected patients segregated by quartile distributions of MCP-1

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Trend; *P*
Range, ng/L	28.1–44.4	44.8–59.7	59.8–81.9	82.4–344	–
Age, years	38.8 (8.8)	37.7 (6.7)	39.1 (6.1)	39.2 (5.6)	0.517
Males, %	70	60	72	79	0.175
Body mass index, kg/m^2	19.6 (2.7)	18.9 (2.5)	19.7 (3.1)	19.7 (2.8)	0.448
Current smoker, %	85.7	80.7	82.5	87.5	0.774
Biochemical and other clinical variables					
Cholesterol, mmol/L	4.91 (1.23)	4.89 (1.30)	5.29 (1.49)	4.57 (1.33)	0.488
Triglycerides, mmol/L	2.07 (1.75)	2.44 (2.74)	3.64 (4.86)	2.09 (1.98)	0.477
HDL-cholesterol, mmol/L	1.24 (0.46)	1.18 (0.41)	1.09 (0.41)	1.14 (0.43)	0.148
C-reactive protein, mg/L	3.21 (4.51)	5.96 (8.23)	3.12 (3.23)	3.94 (4.85)	0.840
Log HIV RNA	2.5 (0.9)	3.1 (1.2)	2.7 (1.1)	3.1 (1.3)	<0.05
CD4+ lymphocytes, cells/mL	533 (338)	393 (240)	477 (339)	369 (272)	<0.05
PI treated, %	26.8	33.3	47.4	32.1	0.123
NNRTI treated, %	48.2	35.1	26.3	25.0	<0.05
Untreated, %	25.0	31.6	26.3	42.9	0.598

Values are presented as means (SD).

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Table 3
Genotype distributions of the MCP-1 A-2518G and the CCR2 V64I (A190G) polymorphisms in healthy participants and HIV-infected patients

	Healthy participants			HIV-infected patients		
	N (%)	CRP, mg/L	MCP-1, ng/L	*N* (%)	CRP, mg/L	MCP-1, ng/L
MCP1 A-2518G						
AA	230 (59.9)	2.8 (4.9)	56.7 (25.1)	132 (58.4)	4.4 (6.2)	69.8 (40.7)
AG	132 (34.4)	2.4 (2.9)	58.2 (25.5)	89 (39.4)	3.7 (4.8)	72.5 (46.8)
GG	22 (5.7)	2.1 (2.9)	70.1 (40.2)	5 (2.2)	2.4 (1.7)	68.5 (41.9)
ANOVA	–	$P=0.550$	$P<0.05$		$P=0.552$	$P=0.903$
CCR2 A190G						
AA	302 (78.7)	2.9 (4.6)	58.9 (35.1)	186 (82.3)	4.3 (6.1)	71.4 (45.7)
AG	77 (20.1)	2.5 (3.9)	60.6 (26.2)	40 (17.7)	2.9 (3.3)	69.1 (31.6)
GG	5 (1.2)	1.6 (1.4)	51.4 (16.8)	0	–	–
ANOVA	–	$P=0.758$	$P=0.837$		$P=0.163$	$P=0.757$

Quantitative values are presented as the means (SD).

smoking habit, and the presence of the −2518 G mutation. Interestingly, the association with serum triglycerides did not reach statistical significance.

4. Discussion

We have studied the association of plasma MCP-1 values with other relevant variables in a healthy population, and compared to a group of HIV-infected patients. Plasma MCP-1 concentration is regulated by genetic factors [8] and different inflammatory and/or oxidative conditions [10–14,23]. Particularly, the presence of the G allele in the functional A-2518G MCP-1 promoter polymorphism influences the expression of MCP-1 and it has been shown to be associated with inflammatory diseases [24–26] and with coronary artery disease [27]. We observed that plasma MCP-1 concentration is higher in HIV-infected subjects and the increase in circulating levels of MCP-1 in healthy participants is related to aging, smoking and serum lipid alterations, which are major cardiovascular disease risk factors. This may represent further evidence that the plasma MCP-1 concentration could be considered a candidate marker for atherosclerosis [23,28]. The associations found, however, are lost in the group of HIV-infected subjects indicating the influence of inflammation and/or a cytokine imbalance.

Unexpectedly [29–31], however, we did not find any relationship between circulating levels of C-reactive protein and MCP-1 concentrations in the group of HIV-infected patients or in the group of non-infected participants. This lack of correlation should be considered within the overall assessment of cardiovascular risk, and warrants further investigation. More important is the finding that in our patients CIMT values are correlated with plasma MCP-1 concentrations but not with CRP levels confirming data obtained in healthy Japanese population [23] and in non-diabetic patients under chronic haemodialysis [32].

We also found that the MCP-1 A-2518 G polymorphism is a genotypic, heritable, predictor of plasma MCP-1 concentration that may help explain inter-individual and inter-population differences in MCP-1 dependent immune responses. It is also of note that the presence of the CCR-2 mutation (64I) has no effect on circulating plasma MCP-1 concentrations and of other biochemical variables in our study population. The presence of the MCP-1 mutation is frequent in our population (>40%), indicating that a higher MCP-1 expression represents a possible evolutionary advantage likely to be related to the inflammatory response [33]. However, there is growing evidence that the overproduction of pro-inflammatory cytokines, and MCP-1 in particular, could be an adverse factor in the clinical evolution of some diseases [10–14,24–27] as well as a predisposition to AIDS-related complications [34,35]. This is further reinforced by our finding in HIV-infected patients of a direct relationship between the plasma MCP-1 concentration and the serum viral load, and the corresponding inverse association with CD4+ T-cells. Therefore, we conclude that increased plasma MCP-1 concentration may exert detrimental effects in the clinical progression of the disease and its complications. The relationship of MCP-1 with clinical atherosclerosis [36] and the correlation we found between cardiovascular risk factors and plasma MCP-1 concentrations suggest that this variable may represent a useful marker in the evaluation of inflammatory diseases.

Acknowledgements

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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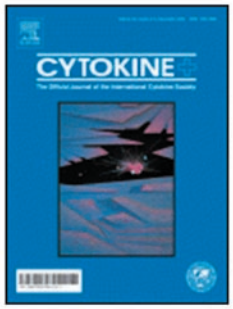
UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Annex #2:



HIV-infected patients with lipodystrophy have higher rates of carotid atherosclerosis: the role of monocyte chemoattractant protein-1.

Coll B, Parra S, Alonso-Villaverde C, de Groot E, Aragones G, Montero M, Tous M, Camps J, Joven J, Masana L.
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UNIVERSITAT ROVIRA I VIRGILI
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HIV-infected patients with lipodystrophy have higher rates of carotid atherosclerosis: The role of monocyte chemoattractant protein-

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Abstract

Individuals with HIV-1 infection are at increased risk for cardiovascular events, and lipodystrophy is generally associated with pr atherogenic metabolic disturbances. We conducted a case–control study to assess the presence of sub-clinical atherosclerosis in HIV- infected patients with or without lipodystrophy (LD) and to evaluate the influence of monocyte chemoattractant protein-1 (MCP-1) c the development of both carotid atherosclerosis and LD. The study population consisted of 43 patients with LD and 86 patients witho LD. We determined carotid intima-media thickness (IMT), MCP-1 concentrations in plasma, and MCP-1 genotype (presence or absen of the −2518G allele). HIV-1-infected patients with LD showed increased risk (OR = 3.71, 95% CI = 1.10–12.47, $p = 0.03$) for sub-cli ical atherosclerosis, and MCP-1 plasma concentration was significantly correlated with IMT in these patients (Pearson = 0.31, $p = 0.03$ Furthermore, presence of LD was a determinant for MCP-1 plasma concentration ($\beta = 0.18$, $p = 0.05$). In summary, HIV-1-infecte patients with clinically manifest LD are at higher risk for atherosclerosis and our observations support the relationship between inflan mation and atherosclerotic disease.
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Keywords: MCP-1; Sub-clinical atherosclerosis; Lipodystrophy; HIV; Inflammation

1. Introduction

Highly active antiretroviral therapy of HIV rapidly decreases morbidity and mortality in HIV-1-infected patients [1]. However, due to the long-term survival, other deleterious effects due to treatment and the chronic inflammatory state emerge. In particular, the effects of lipodystrophy and the increased cardiovascular disease risk are of great clinical impact [2]. In an earlier report we considered the risk of atherosclerosis in HIV-1-infected patients from the perspective of inflammatory disease, by examining the role of the pro-inflammatory β chemokine monocyte ch moattractant protein 1 (MCP-1) [3] in sub-clinical ather sclerosis, assessed by carotid intima-media thickne (IMT), a validated surrogate marker of atherosclero [4]. MCP-1 is mainly produced by monocytes, endothelia cells, and adipose tissue, and has been shown to activat and recruit monocytes/macrophages in the arterial wa [5]. Changes in adipose tissue have been correlated wit changes in MCP-1 gene expression [6], and MCP-1 releas is higher in visceral than subcutaneous human adipo tissue [7].

We hypothesized that the changes in distribution fat tissue observed in patients with LD may influence pla ma MCP-1 concentrations, which in turn may exert

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Blai Coll Crespo
Dipòsit Legal: T.1044-2012

detrimental effect in the development of atherosclerosis. We also investigated whether HIV-1-infected patients with LD present higher rates of sub-clinical atherosclerosis than do those without LD.

2. Materials and methods

We designed a case–control study to examine sub-clinical atherosclerosis in HIV-1-infected individuals with or without LD. This study is a part of a longitudinal follow-up design in which the investigators collate clinical and laboratory data besides the analyses of surrogate markers of atherosclerosis in HIV-infected patients. LD was defined as the presence of body-fat changes that could be clearly recognised by the patient and confirmed by the doctor. Body-fat changes included subcutaneous lipoatrophy (hollow cheeks, prominent superficial veins in the limbs or flattening of the buttocks) and central obesity (increased abdominal girth, breast enlargement or dorsocervical fat pad) [8].

The study population [3] consisted of 305 HIV-1-infected patients aged 18 years or older who regularly attend our Hospital. They were eligible to participate if they had no AIDS-related opportunistic diseases at study entry. We identified a total of 43 patients with LD who met the case criteria, and we then selected 2 controls for each case among the HIV-1-infected patients without LD who were matched for age (±5 years), sex and length of clinic follow-up from the initial visit. All participants gave informed consent and the Ethics Committee of Hospital Universitari de Sant Joan approved the study.

The clinical data collected were time from seroprevalence, history of antiretroviral therapy, CD4 cell counts, and HIV-1 viral load. High blood pressure was defined as systolic blood pressure >140 or diastolic >90 mmHg. A diagnosis of dyslipaemia was made when one of the fo lowing conditions was met: (i) total cholesterol >5.3 mmo L; (ii) HDL cholesterol <0.9 mmol/L; or (iii) LDL chole terol >3.3 mmol/L. Hyperglycaemia was defined as fastin glucose >6.1 mmol/L. Metabolic syndrome was define according to the ATPIII report [9]. Patients were geno typed for MCP-1-2518G polymorphism, and plasm MCP-1 concentrations were determined as previousl described [3]. All study participants underwent an ultraso nography examination of the carotid arteries. The IM was measured in the common carotid artery 1 centimeti proximally to the carotid bulb. IMT values over 0.8 mi were considered to represent an early sign of atherosclero sis [10].

Results are expressed as mean ±SEM. Differenc between categorical variables were analysed with the χ^2 te and continuous variables with the *t*-test. Bivariate correla tions were tested using Pearson coefficient and interactio tests using the univariate general linear model were pe formed. When appropriate we analysed data using non parametric tests. For analyses of the MCP-1 genotyp patients homozygous for the −2518G allele were groupe with patients heterozygous for the −2518G allele. A logi tic regression analysis was performed to determine whic variables were associated with IMT values. Multiple linea regression analysis was performed to evaluate the influenc of several variables on the plasma concentration of MCP- The significance level was set at a p-value < 0.05.

3. Results

Selected characteristics of the 129 study participants ar depicted in Table 1. LD patients, representing 14.1% of th infected patients, had a higher prevalence of dyslipaemia abnormal fasting glucose and metabolic syndrome tha

Table 1
Characteristics of participants according to the presence or absence of lipodystrophy (LD)[a]

	LD–HIV ($N = 43$)	Non-LD–HIV ($N = 86$)	p valu
Age, years	42.48 (1.09)	40.59 (0.75)	0.15
Sex, male	26 (60.5)	54 (62.8)	0.79
Current smoker	31 (72.1)	71 (82.6)	0.17
Body mass index, kg/m^2	23.43 (0.46)	22.65 (0.33)	0.17
High blood pressure	7 (17.5)	9 (11.1)	0.33
Waist-to-hip ratio	0.94 (0.01)	0.89 (0.01)	0.05
Dyslipaemia	37 (86.0)	56 (65.1)	0.00
Abnormal fasting glucose	10 (23.3)	9 (10.5)	0.06
Metabolic syndrome	7 (16.3)	5 (5.8)	0.06
Time on Estavudine, months	25.56 (2.31)	11.57 (1.83)	<0.00
Time on PIs, months	34.76 (3.98)	25.83 (2.81)	0.07
Time since diagnoses, years	7.56 (0.59)	7.22 (0.48)	0.67
CRP, mg/L	4.19 (0.65)	4.63 (0.82)	0.72
MCP-1, pg/mL	75.47 (7.21)	62.10 (3.03)	0.04
MCP-1-2518G	14 (32.6)	32 (37.2)	0.26
Carotid IMT, mm	0.88 (0.04)	0.76 (0.02)	0.00
Atherosclerotic lesion	36 (83.7)	48 (55.8)	0.01

[a] Results are expressed as mean (SEM) or in percentages. PIs indicate protease inhibitors. CRP refers to C-reactive protein and MCP-1 to monocy chemoattractant protein-1. IMT is intima-media thickness and atherosclerotic lesion refers to a carotid IMT >0.8 mm or the presence of an atherosclerot plaque [10].

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira i Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

control patients (Table 1). Patients with LD had been treated for a significantly ($p < 0.001$) longer period of time with stavudine than had patients without LD (25.56 ± 2.31 months vs. 11.57 ± 1.83 months, respectively). The two groups showed no significant differences in treatment history with respect to any other antiretroviral agents used.

Although the differences between the two groups in mean CRP values did not reach statistical significance, plasma MCP-1 concentrations were significantly higher in HIV-1-infected patients with LD compared to those without LD (75.47 ± 7.21 pg/mL vs. 62.10 ± 3.03 pg/mL, respectively; $p = 0.04$). Moreover, carotid IMT was significantly higher in patients with LD than in those without LD (0.88 ± 0.04 mm vs. 0.76 ± 0.02 mm, respectively; $p = 0.007$) and those LD + HIV-infected patients presented higher rates of carotid atherosclerotic lesions (Table 1). When patients were segregated according to the diagnosis of lipodystrophy and carotid atherosclerotic lesion, we observed four groups of patients in which the distribution of plasma MCP-1 concentration was significantly different (Kruskal–Wallis test, $p = 0.04$). LD + patients with carotid atherosclerotic lesions had the highest MCP-1 plasma concentration (Fig. 1).The carotid IMT was positively and significantly ($p = 0.04$) correlated with MCP-1 concentration in plasma in those patients with LD (Fig. 2A), and the results of the interaction test revealed that the presence of lipodystrophy and the plasma concentration of MCP- may exert a significant influence in the variability of th carotid IMT (F coefficient = 4.53, $p = 0.03$). We did no find the same trend in patients without LD (Fig. 2B). Sinc MCP-1 and carotid IMT were correlated in HIV-1-infecte patients with LD, we next applied a multivariate model t assess the role of variables known to influence MCP- expression, such as age, weight, MCP-1 genotype, duratic of treatment with PIs, HIV-1 viral load plus the presence o absence of LD (Table 2a). The presence of LD was the on variable significantly associated with MCP-1 concentratic in plasma, with a β coefficient of 0.18 ($p = 0.05$).

Using a multivariate model, we next analysed those var ables related to the development of carotid atherosclerosi including age, sex, body mass index, and metabolic syn drome, as well as presence or absence of LD, time on pro tease inhibitors (PIs), MCP-1 genotype and MCP- concentration in plasma (Table 2b). In confirmation of pr vious results in a general population of HIV-1-infecte patients [3], MCP-1-2518 genotype [OR = 5.29; 95% C (1.67–16.76), $p = 0.005$] was associated with carotid athero sclerosis. Notably, presence of LD was also significant associated with the development of carotid atherosclerosi [OR = 3.71, 95% CI (1.10–12.47), $p = 0.03$].

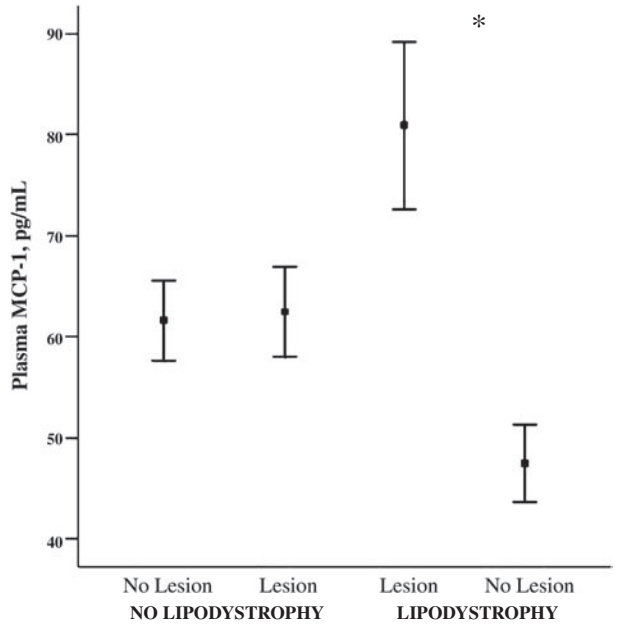

Fig. 1. Distribution of plasma concentration of MCP-1 (mean ± SEM) according to the presence or absence of LD ± carotid atherosclerotic lesion. *Overall p value using the Kruskal–Wallis test = 0.04.

	N	Mean	SEM
LD−, No lesion	38	61.61	3.97
LD−, Lesion	48	62.49	4.47
LD+, Lesion	36	80.90	8.29
LD+, No lesion	7	47.53	3.80

4. Discussion

The higher rates of sub-clinical atherosclerosis seen i HIV-1-infected individuals, although controversial, ar usually attributed to classic cardiovascular risk facto and to side effects of antiretroviral therapies [11–13]. How ever, the chronic infection status suffered by these indivi uals might also lead to a higher risk for atherosclerosis b triggering the inflammatory response. As a disease tha causes an aberration in several metabolic pathways, suc as lipid and glucose metabolism, LD might also increas cardiovascular risk in individuals with HIV-1 infectio Taken together, these factors suggest that HIV-1-infecte patients with LD might be at an even higher risk o atherosclerosis.

Our results indicate that the presence of LD increases b 3-fold the risk of sub-clinical carotid atherosclerosis i patients with HIV-1 infection. Mercie et al. [14] showed significant association between carotid IMT and LD in univariate analysis of 161 HIV-1-infected patients wit LD, but the effect disappeared in the multivariate mode A likely explanation for the discordance between ou results and those obtained by Mercie et al. is that the latt group evaluated a healthier population since the mea carotid IMT was 0.54 mm (range 0.50–0.60 mm), while i our patients the mean carotid IMT was 0.80 mm (rang 0.40–1.80 mm).

MCP-1 has been shown to influence the development o atherosclerosis both in individuals with HIV infection [and in those without [15]. Several inflammatory state as well as MCP-1 genotype, may increase MCP-1 ger expression [16], which in turn leads to higher MCP-1-d

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

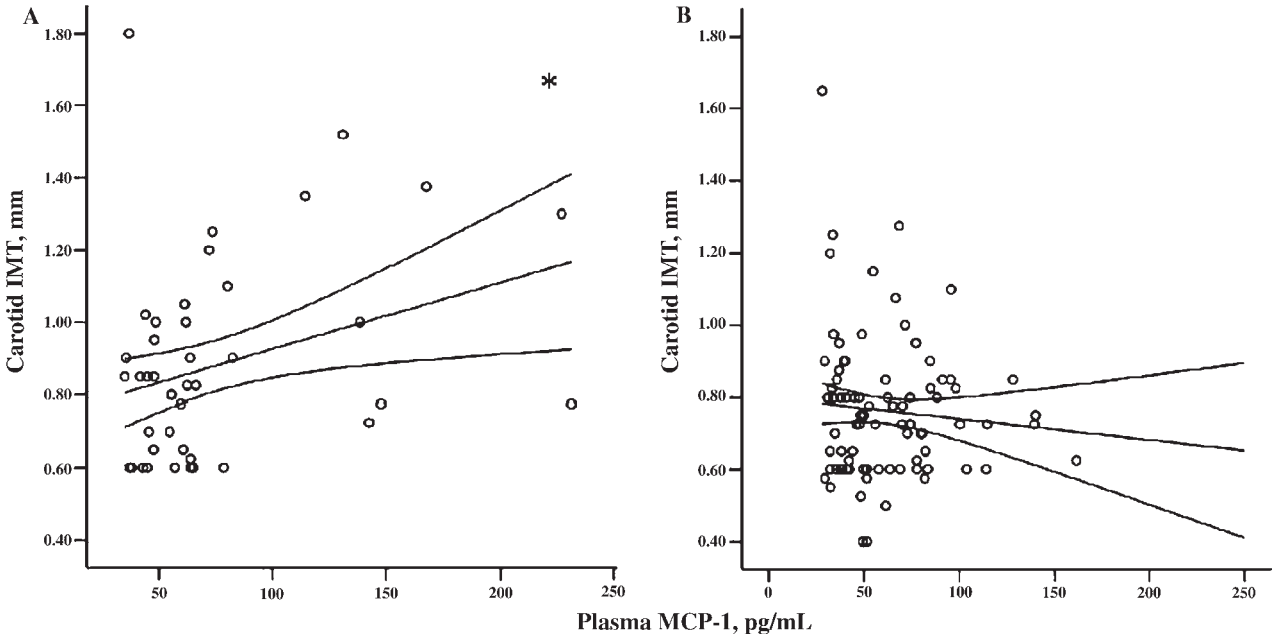

Fig. 2. Correlation plots between carotid IMT and MCP-1 plasma concentration in LD–HIV-1-infected patients (A, solid circles) and non-LD–HIV 1-infected patients (B, open circles). Mean and 95% CI values are depicted. *MCP-1 plasma concentration and carotid IMT values have bee log-transformed for statistical purposes Pearson coefficient 0.31, $p = 0.03$.

Table 2a
Multivariate analyses of the determinants of MCP-1 (2a) and the presence of carotid atherosclerosis (2b). Determinants of MCP-1 plasma concentration using a linear regression analyses

	B Coefficient	*p* value
Age, years	0.03	0.72
Body mass index, kg/m^2	0.14	0.12
Saquinavir, months	0.01	0.91
Indinavir, months	0.05	0.60
Nelfinavir, months	−0.05	0.54
Ritonavir, months	−0.03	0.69
Efavirenz, months	−0.03	0.73
HIV-1-viral load	0.08	0.39
Lipodystrophy, yes	0.18	0.05
MCP-1-2518G, yes	−0.08	0.38

Dependent variable: plasma concentration of MCP-1, pg/mL. The criteria defining lipodystrophy are further detailed in the text.

Table 2b
Multivariate analyses of the determinants of MCP-1 (2a) and the presenc of carotid atherosclerosis (2b). Determinants of sub-clinical caroti atherosclerosis using logistic regression analyses

	Odds ratio	Confidence interval	*p* valu
Age, years	1.21	1.06–1.37	0.03
Sex, male	2.11	0.80–5.56	0.12
Body mass index, kg/m^2	0.88	0.76–1.03	0.13
High blood pressure, yes	7.07	0.73–68.14	0.09
Dyslipaemia, yes	1.62	0.41–6.30	0.48
Fasting glucose, mmol/L	1.06	0.52–2.15	0.85
Time on PI, months	0.99	0.97–1.01	0.32
Lipodystrophy, yes	3.71	1.10–12.47	0.03
MCP-1, pg/mL	1.01	0.99–1.03	0.06
MCP-1-2518G, yes	5.29	1.67–16.76	0.005

We used carotid atherosclerosis as a dependent variable, that is a con posite variable of the presence of an atherosclerotic plaque or a caroti IMT >0.8 mm [10]. High blood pressure was defined as systolic pressu >140 mmHg or diastolic pressure >85 mmHg. Dyslipaemia was defined a total cholesterol >5.3 mmol/L, HDL cholesterol <0.9 mmol/L, or LD cholesterol >3.3 mmol/L (further details in text).

rived inflammatory responses. Notably, adipose tissue is one source of MCP-1 production. Obese subjects have higher plasma MCP-1 levels before weight reduction [6], and MCP-1 release is higher, in vitro, in visceral human adipose tissue than in subcutaneous human adipose tissue [7]. These observations suggest that a similar scenario might occur in the LD syndrome. In our study, multivariate analysis showed that, although statistically weak, LD may be a determinant of MCP-1 in plasma, and IMT values were positively correlated with MCP-1 in LD patients. Due to the fat redistribution syndrome, adipose tissue in HIV-1-infected patients with LD might be overproducing MCP-1, and higher levels of MCP-1 may in turn have a detrimental effect on the development of atherosclerosis in these patients. This association should be further explored in larger studies in order to ascertain the detr mental effects of lipodystrophy on MCP-1 concentratio and in turn in the development of atherosclerosis. This i especially warranted because we found a minimum numbe of patients who fulfilled the criteria of LD without atherc sclerotic lesions ($N = 7$), and this fact may be a bias i interpreting the conclusions.

One of the limitations of the study is that we diagnose LD using clinical criteria without objective measurement of fat redistribution, but such measurements are not star dardized. However, those patients assigned to the LI group based on the clinical criteria are severely affecte

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

by the fat redistribution syndrome, and therefore easily identifiable, and further LD + HIV-infected patients presented with higher rates of dyslipaemia and a significantly higher waist-to-hip ratio than non-lipodystrophic patients, indicating that they probably are influenced by the metabolic abnormalities usually diagnosed in these patients [17].

Another limitation is that the over expression of MCP-1 might be associated with down-regulation of its receptor CCR-2. We did not analyze CCR-2 expression in circulating monocytes, however, CCR-2 is a known HIV-co-receptor [18], and its expression might also be altered according to HIV status (R5 vs. X4). Moreover, an alternative receptor for MCP-1 has been identified in smooth muscle cells [19], indicating a non-unique receptor for MCP-1, and another pathway to exert its pro-inflammatory influence. In order to reduce the inflammatory stimulus, we should investigate the effects of switching antiretroviral therapies or offering statins to HIV-infected patients with LD and sub-clinical atherosclerosis.Statins are known to have beneficial effects on the arterial wall [20] and in the expression of the MCP-1/CCR-2 axis [21].

In summary, the study of the inflammatory status and the arterial wall thickness in LD–HIV-infected patients supports the relationship between inflammation and atherosclerosis development.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Annex #3:

Effects of rosiglitazone and metformin on postprandial paraoxonase-1 and monocyte chemoattractant protein-1 in human immunodeficiency virus-infected patients with lipodystrophy.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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Effects of rosiglitazone and metformin on postprandial paraoxonase-1 and monocyte chemoattractant protein-1 in human immunodeficiency virus-infected patients with lipodystrophy

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Abstract

Highly active antiretroviral therapy in Human Immunodeficiency Virus (HIV) has been associated with lipodystrophy, insulin resistance and atherosclerosis. We investigated the effects of rosiglitazone or metformin on fasting and postprandial inflammatory and antioxidant variables in HIV-infected males with lipodystrophy.

Thirty-one patients were randomly assigned to receive either rosiglitazone (4 mg twice daily) or metformin (1 g twice daily) for 26 weeks. At baseline and after treatment, standardized 10-h oral fat loading tests were performed. Before treatment, inflammatory variables remained unchanged but there was a postprandial decrease in high density lipoprotein (HDL)-cholesterol and paraoxonase (PON1) activity. Rosiglitazone and metformin reduced homeostasis model assessment index (HOMA) similarly (−34% and −37%, respectively, $P<0.05$ for each). Both treatments increased fasting and postprandial PON1 activity and decreased postprandial monocyte chemoattractant protein 1 (MCP-1) concentrations. However, plasma C-reactive protein (CRP) and Interleukin-6 (IL-6) concentration did not change throughout the study.

To decrease insulin resistance results in a higher anti-oxidant and consequent lower pro-inflammatory action of HDL. This may confer protection against accelerated atherosclerosis in these patients.
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Index words: HIV; Paraoxonase; Monocyte chemoattractant protein-1; Postprandial; Metformin; Rosiglitazone

1. Introduction

The use of highly active antiretroviral therapy (HAART) in Human Immunodeficiency Virus (HIV) has greatly reduced morbidity and mortality due to acquired immunodeficiency syndrome (AIDS) (Palella et al., 1998), but it is strongly associated with changes in fat distribution (lipodystrophy), dyslipidemia and insulin resistance, which increase the risk of atherosclerosis (Carr et al., 1998; Balasubramanyam et al., 2004; Hadigan et al., 2001; Friis-Moller et al., 2003). Atherosclerosis is generally accepted to be a low-grade inflammatory disease, initiated by the oxidation of lipoproteins in the subendothelial space, which induces chemotaxis and adhesion and transmigration of circulating monocytes into the arterial wall (Ross, 1999). Inflammation is mediated by cytokines. Particularly, the recruitment of monocytes to the subendothelial space is stimulated by monocyte chemoattractant protein 1 (MCP-1) (Gu et al., 1997). Other biomarkers of inflammation, C-reactive protein (CRP), has shown to be a direct toxic of endothelial cells (Libby and Ridker, 2004), and Interleukin-6 (IL-6) has been demonstrated to be higher in HIV-infected

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UNIVERSITAT ROVIRA I VIRGILI
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Blai Coll Crespo
Dipòsit Legal: T.1044-2012

patients (Dolan et al., 2005), that represent predictors of cardiovascular events.

The enzyme paraoxonase 1 (PON1), located on High Density Lipoprotein (HDL), has potent antioxidant properties by hydrolyzing oxidized lipids formed on Low Density Lipoprotein (LDL) and HDL (Mackness et al., 1993). As such, it inhibits the oxidation of lipoproteins in the subendothelial space that, otherwise, could lead to the formation of foam cells. Supporting this concept, serum PON1 activity is low in patients with insulin resistance and atherosclerosis (Durrington et al., 2001; Mackness et al., 2000) and mice lacking serum PON1 activity are more susceptible to atherosclerosis (Shih et al., 1998). Furthermore, it has been demonstrated *in vitro* that PON1 attenuates the endothelial production of MCP-1 mediated by oxidized-LDL (Mackness et al., 2004). Therefore, PON1 has anti-inflammatory and anti-atherogenic properties and it is related to the action of MCP-1.

Since humans are non-fasting most part of the day, this period may be of particular importance in the pathogenesis of atherosclerosis. It is known that HIV-infected patients have delayed clearance of postprandial triglyceride-rich lipoproteins compared with healthy controls (Stein et al., 2005) and increased postprandial lipemia has been linked to accelerated atherosclerosis (Groot et al., 1991; Weintraub et al., 1996). The underlying mechanisms may involve increased generation of oxidative stress leading to endothelial dysfunction (van Oostrom et al., 2003; Ceriello et al., 2004). Metformin and rosiglitazone are used in clinical medicine to improve insulin resistance and glycemic control in patients with type 2 diabetes (Yki-Jarvinen, 2004; Hundal and Inzucchi, 2003). However, these agents may also have a role in treating patients with nondiabetic insulin-resistant conditions, such as HIV-lipodystrophy (Hadigan et al., 2000, 2002, 2004; Saint-Marc and Touraine, 1999; Carr et al., 2004; Sutinen et al., 2003). Presumptively, the treatment of the metabolic and inflammatory derangements may exert a protective effect from atherosclerosis in HIV-infected patients. We have studied the effects of a high-fat meal on inflammatory (MCP-1, CRP and IL-6) and antioxidant (PON1 activity and HDL-cholesterol) variables in HAART-treated HIV-infected patients with lipodystrophy, and investigated the effects of the insulin-sensitizing agents rosiglitazone and metformin on these variables.

2. Materials and methods

2.1. Study population

We included thirty-one males aged between 30 and 65 with a documented HIV infection and HIV-RNA values < 10,000 copies/ml, who were on HAART for at least 18 months with no changes in the treatment regimen during the 6 months prior to inclusion. We did not consider those with HIV-related symptoms (opportunistic infectious disease, malignancies or unexplained weight loss), renal, thyroid and/or liver diseases, diabetes mellitus and an alcohol intake > 3 U a day. The presence of HIV-lipodystrophy was defined as signs and symptoms of loss of subcutaneous fat (face, arms, legs and buttocks) with or without increased abdominal girth or development of a buffalo hump. The research project was primarily designed to study the effects of treatment on lipodystrophy course as well as endothelial function studies, the results of which have been already published (van Wijk et al., 2005).

2.2. Study design

At inclusion, a fasting blood sample was obtained and height, weight, blood pressure and waist and hip circumference were measured. A complete medical record was obtained and a thorough physical examination was performed. Participants underwent a standardized 10-h oral fat loading test, and were randomly assigned to receive either rosiglitazone (4 mg twice daily) or metformin (1000 mg twice daily) for 26 weeks. Patients visited the hospital after 2 and 4 months of treatment for safety evaluation. At the end of the period the second oral fat loading test was performed. The study protocol was approved by the local research ethics committees of the University Medical Center (Utrecht, the Netherlands) and the Hospital Universitari de Sant Joan (Reus, Spain).

2.3. Oral fat loading test

The subjects fasted overnight for at least 12 h and did not drink alcohol on the day before the test. After placing a cannula for venous blood sampling, subjects rested for 30 min before the fat load was administered. Fresh cream was used as the fat source. It was a 40% (weight/volume) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates. The total energy content was 3700 kcal/l. Cream was ingested within 5 min at a dose of 50 g fat and 3.75 g glucose per m^2 body surface. The participants remained supine during each test and were only allowed to drink mineral water. Blood samples were collected into sodium EDTA-containing tubes for MCP-1, IL-6 and lipoprotein measurements, and most of the other biochemical analyses. Samples were collected into tubes with no anticoagulants added to measure PON1 activity. Samples were obtained before the fat load meal and at 2-h intervals up to 10-h postprandially.

Table 1
Selected baseline characteristics for each treatment group

	Metformin (N=16)	Rosiglitazone (N=15)	P value
Age, years	48.3 (1.9)	48.4 (1.8)	0.95
HIV diagnoses, years	8.4 (0.8)	12.2 (1.1)	0.01
Time under HAART*, years	7.6 (0.8)	9.06 (0.9)	0.23
ART prescribed, %			
Nucleoside analogues	100	100	
Protease inhibitors	61	68	0.59
Non nucleoside analogues	39	32	0.16
Cardiovascular risk factors (see also Table 2)			
Current smoker, %	22	26	0.71
Body Mass Index, kg/m^2	24.9 (0.4)	22.5 (0.6)	0.002
Systolic blood pressure, mm Hg	132 (3.45)	133 (3.73)	0.88
Diastolic blood pressure, mm Hg	81 (1.83)	82.2 (2.71)	0.77
HIV viral load, $\log_{10}$/ml	1.96 (0.14)	2.20 (0.22)	0.37
CD4 cell count, cells/mm^3	573 (58)	868 (87)	0.008

Unless otherwise indicated, values are mean (S.E.M.).
*HAART = highly-active antiretroviral therapy.

Blai Coll Crespo. Hospital Universitari Sant Joan- Universitat Rovira I Virgili, Reus. Tarragona

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

Table 2
Effects of rosiglitazone and metformin on fasting clinical and laboratory variables

	Overall (N=31)			Metformin (N=16)			Rosiglitazone (N=15)		
	Pre treatment	Post treatment	P value	Pre treatment	Post treatment	P value	Pre treatment	Post treatment	P value
Body Mass Index, kg/m^2	23.8 (0.4)	24.0 (0.3)	0.43	24.9 (0.4)	24.5 (0.4)	0.05	22.5 (0.6)	23.2 (0.5)	0.02
Waist to Hip Ratio, m	0.98 (0.01)	0.98 (0.01)	0.31	0.99 (0.01)	0.97 (0.01)	0.02	0.98 (0.01)	0.98 (0.01)	0.43
Glucose, mmol/l	5.51 (0.13)	5.15 (0.18)	0.003	5.64 (0.24)	5.30 (0.35)	0.10	5.37 (0.09)	5.0 (0.11)	0.004
Insulin, mU/l	7.90 (1.18)	5.64 (0.74)	0.005	8.06 (1.13)	5.51 (0.67)	0.03	7.72 (2.17)	5.78 (1.38)	0.08
HOMA index*	2.03 (0.34)	1.31 (0.19)	0.002	2.16 (0.43)	1.36 (0.26)	0.01	1.88 (0.54)	1.26 (0.28)	0.06
Total cholesterol, mmol/l	5.70 (0.16)	5.70 (0.23)	0.98	5.68 (0.24)	5.33 (0.28)	0.08	5.72 (0.24)	6.11 (0.35)	0.12
HDL cholesterol, mmol/l	1.12 (0.05)	1.10 (0.04)	0.67	1.03 (0.04)	1.10 (0.06)	0.17	1.22 (0.09)	1.11 (0.08)	0.06
LDL cholesterol, mmol/l	3.42 (0.15)	3.27 (0.18)	0.34	3.55 (0.22)	3.15 (0.25)	0.05	3.26 (0.20)	3.41 (0.28)	0.53
Triglycerides, mmol/l**	3.02 (0.53)	3.81 (0.47)	0.09	3.61 (0.94)	4.05 (0.77)	0.59	2.39 (0.43)	3.56 (0.56)	0.01
MCP-1, pg/ml	85.99 (5.33)	76.96 (3.20)	0.10	83.50 (6.71)	70.14 (2.71)	0.05	88.64 (8.56)	84.25 (5.45)	0.61
CRP, mg/l**	4.63 (0.93)	3.41 (0.71)	0.15	4.75 (1.58)	2.78 (1.20)	0.15	3.99 (1.20)	3.78 (0.99)	0.52
IL-6, pg/ml**	284.32 (56)	246.20 (51)	0.36	147.90 (23)	165.88 (66)	0.91	390.42 (105)	321.01 (84)	0.28
PON1, U/l	340.01 (30)	392.64 (33)	0.002	364.70 (48)	426.45 (52)	0.04	313.66 (35)	356.58 (42)	0.003

Values are expressed as mean (S.E.M.). *HOMA = Homeostasis Model Assessment. **These were log transformed for statistical purposes.

2.4. Laboratory measurements

Plasma glucose, total cholesterol and triglycerides were measured by standard methods. LDL was isolated by ultracentrifugation. HDL-cholesterol was analyzed using a homogeneous method (ITC Diagnostics, Barcelona, Spain). Insulin, plasma MCP-1 and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden and Peprotech, London, UK). The Homeostasis model assessment index (HOMA) was calculated as [fasting insulin (mU/l) × fasting glucose (mmol/l)]/22.5. The serum concentration of CRP was measured using a high sensitivity method (hs-CRP) (Quantex hs-CRP kit, Biokit, S.A., Barcelona, Spain) with a lower limit of detection of 0.10 mg/l. PON1 activity towards paraoxon was measured after the reaction of paraoxon hydrolysis into *p*-nitrophenol and diethylposphate, as described previously, Ferré et al. (2003).

2.5. Statistical analysis

Results are expressed as means (S.E.M.). Continuous variables were compared using analyses of variance (ANOVA) and the categorical variables using the Chi square test. During serial measurements, time effects were tested by repeated measures ANOVA. The effects of treatment were analyzed with paired *t*-tests, and in non-normal distributed variables log transformation was applied. To compare the differences between the effects of treatment an ANOVA of the mean change was applied. To evaluate the effect of treatment and during serial measurements, we took into account these variables that presented significant differences between groups in the baseline situation. Calculations were performed using SPSS 12.0 (SPSS Inc. Chicago, IL, USA). Statistical significance was taken at the 5% level. The size of the sample was calculated with β=0.10 and α=0.05 and based on a reduction of 20% in inflammatory variables. A multistep regression analyses was performed to study the variables that might have an influence on the response of MCP-1, PON1 activity, CRP and HDL cholesterol. We considered dependent variables ΔAUC MCP-1, ΔAUC PON1, ΔAUC CRP, ΔAUC IL-6 and ΔAUC HDL cholesterol, and independent variables those which had been correlated in a significant manner in a univariate analyses.

3. Results

3.1. General characteristics

Thirty-one HIV-infected patients were included in the study. The baseline characteristics of the study group are depicted in Table 1. Patients assigned to rosiglitazone were diagnosed of HIV infection significantly earlier and their CD4 cell count was higher than those patients in the group of metformin (Table 1).

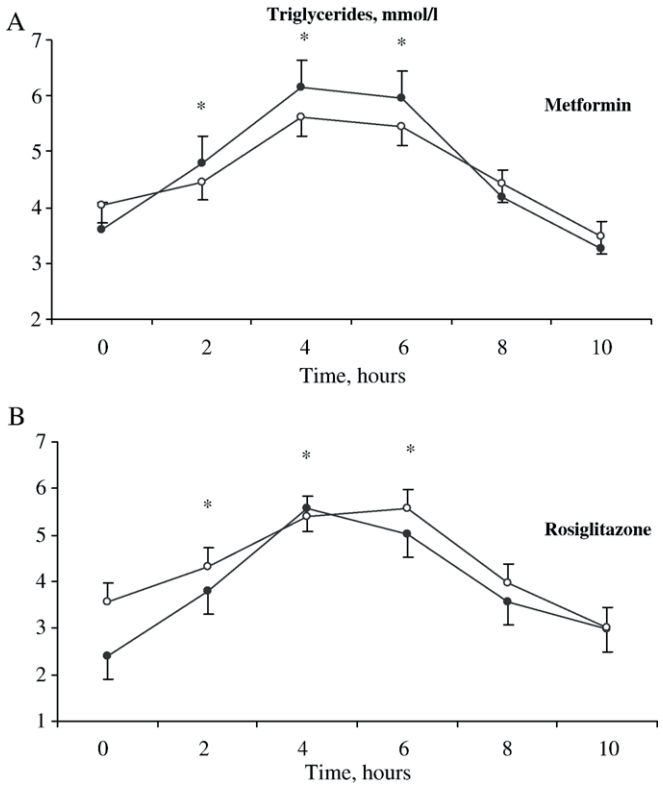

Fig. 1. Mean (S.E.M.) plasma triglyceride concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (A) and rosiglitazone (B)-treated groups. There were no significant differences before and after treatment in the triglyceride response to fat overload. * Indicates P<0.01 comparing with t=0 when a paired t-test was applied.

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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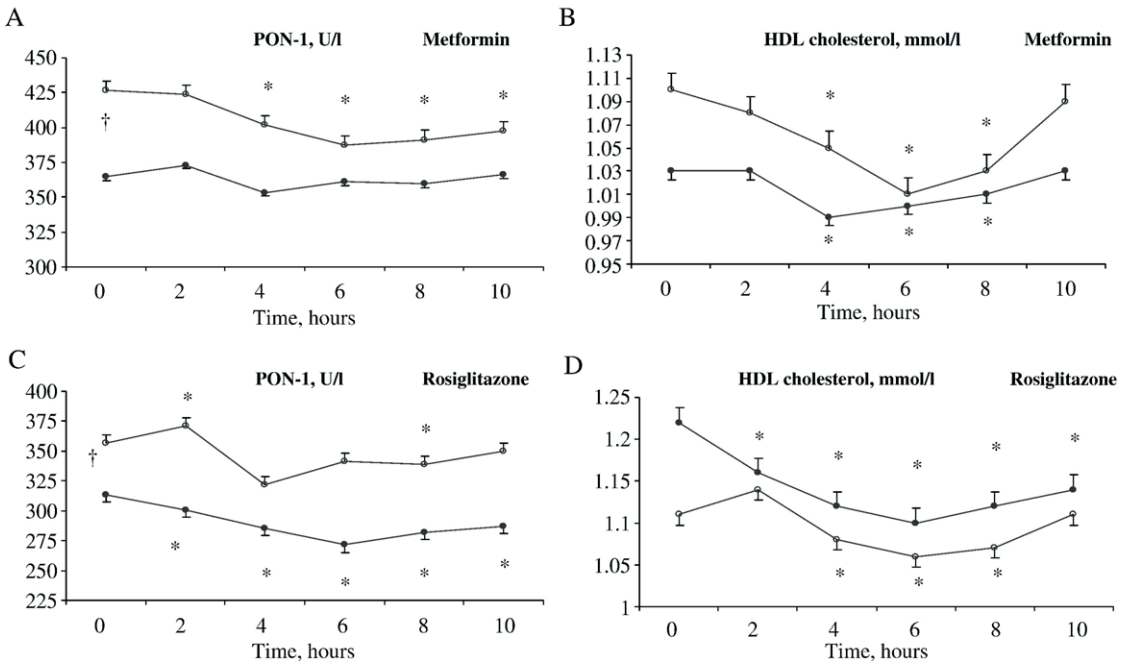

Fig. 2. Mean (S.E.M.) serum PON1 activity and HDL cholesterol in HIV-infected patients before (●) and after treatment (○) in metformin (panels A and B) and rosiglitazone-treated patients (Panels C and D). Although a significant ($P<0.05$) increase in fasting PON1 activity was observed in both groups (†), postprandial decreases were found significant (*$P<0.03$) when compared to $t=0$.

Further, patients in the group of metformin presented with a significant higher body mass index. All participants were on a nucleoside analogue agent plus a protease inhibitor and/or a non-nucleoside reverse transcriptase inhibitor. We did not find significant differences either in the baseline lipid profile or in the inflammatory (MCP-1, CRP) and anti-oxidant (PON1 activity and mass) variables, among patients assigned to receive rosiglitazone or metformin.

Both, rosiglitazone and metformin significantly decreased fasting insulin and HOMA index compared with baseline. Patients assigned to rosiglitazone, experienced a significant increase in the fasting triglyceride concentration (Table 2). Patients

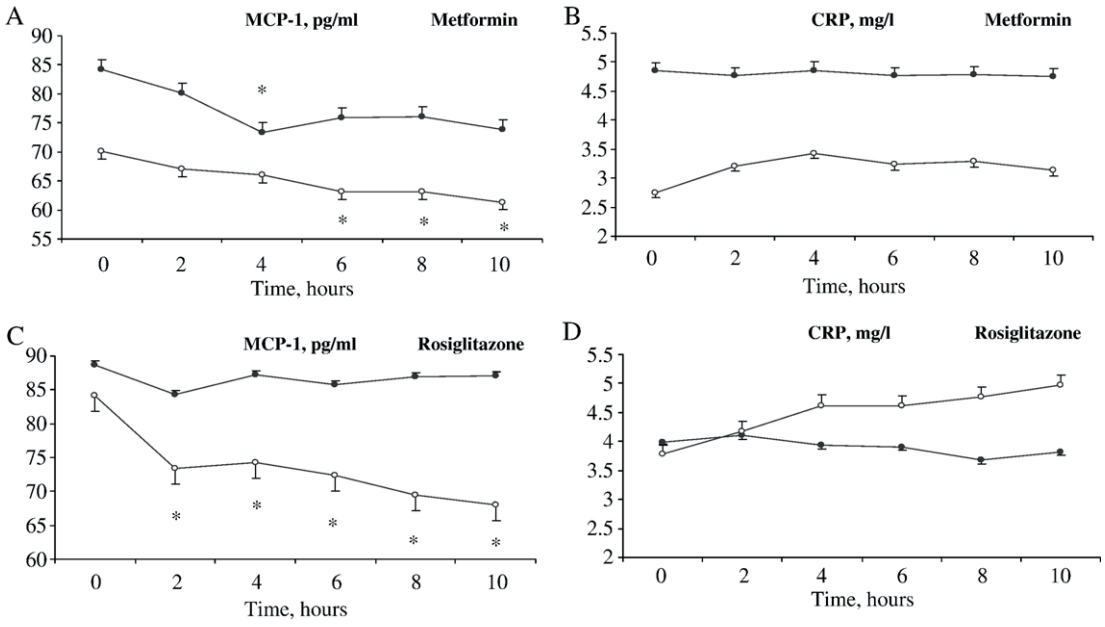

Fig. 3. Mean (S.E.M.) plasma MCP-1 and CRP concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (panels A and B) and rosiglitazone-treated patients (panels C and D). We did not find significant differences among groups but there was a significant decrease in postprandial MCP-1 in the oral fat loading test performed after treatment with metformin and rosiglitazone *$P<0.01$, when compared to $t=0$.

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
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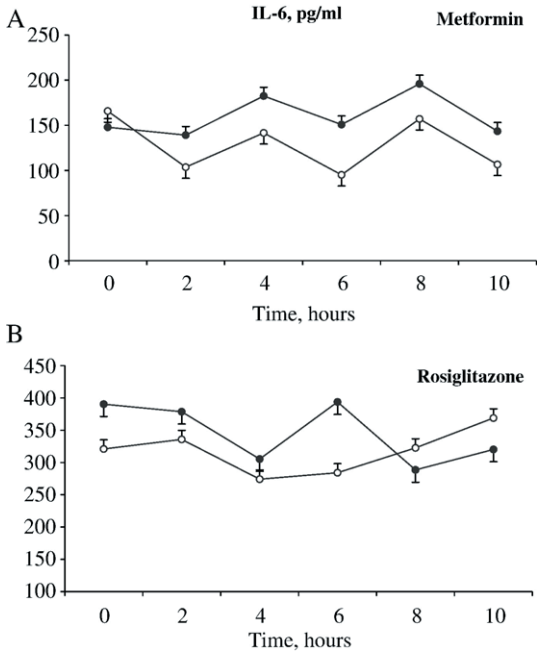

Fig. 4. Mean (S.E.M.) plasma IL-6 concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (A) and rosiglitazone (B)-treated groups. There were no significant differences before and after treatment in the IL-6 response to fat overload.

assigned to metformin experienced a significant increase in fasting HDL cholesterol (0.06 mmol/l) when compared to those assigned to rosiglitazone (−0.10 mmol/l, $P=0.02$). Furthermore, in the patients on metformin, fasting total cholesterol showed a decrease (−0.35 mmol/l) that was significantly different when compared to those with rosiglitazone (+0.38 mmol/l, $P=0.02$ for treatment effect). We did not find significant differences between groups, before the randomization, in MCP-1, CRP, PON1 and IL-6.

3.2. Postprandial study

Plasma triglycerides increased significantly after fat ingestion, reaching maximum concentrations 4-h postprandially, and returning to baseline at the end of the test. Both, rosiglitazone and metformin did not change the AUC for triglycerides compared with the pre-treatment situation (Fig. 1). PON1 activity experienced a significant postprandial decrease throughout the postprandial test (Fig. 2). Both, metformin and rosiglitazone increased fasting PON1 activity (Table 2), but the postprandial course of PON1 showed a progressive and significant decrease in the postprandial period after both treatments. The course of HDL-cholesterol after the oral fat load was similar to that observed for PON1 activity, reaching a nadir 6 h postprandially (Fig. 2). Treatment with metformin or rosiglitazone did not significantly affect the response of HDL cholesterol to the oral fat load.

Before treatment, plasma MCP-1 decreased significantly in the group treated with metformin reaching a nadir at 4 h; however MCP-1 did not change after the oral fat load in rosiglitazone-treated participants (Fig. 3). After 26 weeks of treatment, however, fasting MCP-1 decreased with metformin, but not with rosiglitazone (Table 2). There was a progressive and significant decrease in plasma MCP-1 concentration in the postprandial period that was observed with both treatments. Metformin, but not rosiglitazone, tended to reduce fasting CRP levels, but there were no postprandial changes as a effect of treatment. Although patients assigned to receive rosiglitazone presented with higher concentration of plasma IL-6 before treatment, the difference was not statistically significant ($P=0.10$). Similarly, the postprandial response of IL-6 did not present significant variations when compared the effect of treatment and throughout the postprandial period (Fig. 4).

In a univariate analyses, we found a significant and positive correlation between the ΔAUC of MCP-1 and the increase in the total cholesterol and LDL cholesterol concentrations ($P=0.01$), but changes in insulin concentration or body mass index throughout the study did not correlate with changes in inflammatory variables, such as MCP-1 and CRP concentrations. Further, the ΔAUC of PON1 activity was correlated with HDL cholesterol ($P=0.02$). However, when a multistep, multivariate analyses was applied we did not find any significant association with any of the dependent variables in any of the treatment groups.

4. Discussion

As the survival of subjects with HIV increases, metabolic and vascular complications will become an increasingly important aspect in the management of these patients, specially those derived from accelerated atherosclerosis (Carr et al., 1998; Balasubramanyam et al., 2004; Hadigan et al., 2001; Friis-Moller et al., 2003). Since humans in Western societies are in a postprandial state most of the day, it is important to explore the postprandial period, which is thought to play an important role in the pathogenesis of atherosclerosis (Groot et al., 1991; Weintraub et al., 1996; van Oostrom et al., 2003; Ceriello et al., 2004). This effect will be presumably more intense in HIV-infected patients with lipodystrophy who show insulin resistance and increased postprandial lipaemia (Stein et al., 2005). These metabolic disturbances and the infection itself, represent a considerable degree of inflammation and oxidative stress to these patients. We determined the effects of two different insulin-sensitizing agents on fasting and postprandial inflammatory and antioxidant variables. We found that rosiglitazone and metformin both increased fasting PON1 activity, suggesting increased protection from oxidation. In addition, both treatments decreased the postprandial response of MCP-1, suggesting a reduction of the postprandial inflammatory response. These potentially beneficial effects may be the result of improved insulin sensitivity.

Both treatments significantly increased fasting PON1 activity, despite their different modes of action. Metformin mainly acts by decreasing hepatic glucose output, while the molecular mechanisms underlying the improved insulin sensitivity remain controversial (Hundal and Inzucchi, 2003). Rosiglitazone improves peripheral insulin sensitivity through transcriptional mechanisms (Yki-Jarvinen, 2004). It is likely that increased insulin sensitivity, which occurred similarly in both groups, may partially explain the observed effects on PON1 activity (Yamada et al., 2001; Mackness et al., 1998; Senti et al., 2003), however, these variables

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INFLUENCE OF CHEMOKINE RELATED POLYMORPHISMS IN ATHEROSCLEROSIS AND HIV-INFECTIONS
Blai Coll Crespo
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were not significantly correlated in a linear regression model. Moreover, insulin supplementation in apolipoprotein E-deficient mice reduced the atherosclerotic lesion size by 22–37%, and showed a concomitant 30% increase in PON1 activity and an 18% reduction in lipid peroxide levels (Shamir et al., 2003). The postprandial response of PON1 was consistent with this concept and PON1 activity was higher at all time points which is probably due to a significant increase in fasting serum PON1 activity. Whether the increment in PON1 activity produced by rosiglitazone or metformin in our patients translates into clinical benefit with a reduced cardiovascular risk remains to be shown. We also expected changes in inflammatory variables. Serum CRP is a reliable and easily accessible marker of inflammation and is a strong predictor of cardiovascular events (Libby and Ridker, 2004). We found that the postprandial serum CRP concentrations did not change. Previous studies in type 2 diabetic patients treated with rosiglitazone have shown marked reductions in serum CRP concentrations (Haffner et al., 2002; Mohanty et al., 2004) and the postprandial concentration of IL-6 experimented a significant increase in type 2 diabetic patients (Ceriello et al., 2005). However, we should take into account that our HIV-infected patients are under an inflammatory stimulus which is probably unaffected by insulin-sensitizing agents. Also, the population studied was receiving HAART, which may have direct effects on inflammation. Metformin tended to reduce serum CRP concentration but did not reach statistical significance. However, we should take into account that larger trials assessing these variables should be of great value, since we are aware of the limitation of our study due to, at least in part, to sample size. The lack of a placebo control group is a limitation of our study; it should be of great value to perform the same design in non-HIV-infected participants with the concomitant presence of classic cardiovascular risk factors, such as patients with high blood pressure, diabetes or overweight. This comparison could give us interesting data in recognizing HIV infection as a pro-atherogenic status.

MCP-1 plays a crucial role in initiating atherosclerosis by recruiting inflammatory cells to the subendothelial space (Gu et al., 1997). Although the response of MCP-1 to a high-fat challenge has not been studied before, *in vitro* studies have suggested that chylomicrons induce a higher expression of MCP-1 (Domoto et al., 2003). At baseline, MCP-1 levels did not change postprandially. However, both metformin and rosiglitazone modified the postprandial response of MCP-1 by reducing the postprandial plasma concentrations of MCP-1. This may be a beneficial effect, because MCP-1 has been identified as a crucial mediator of atherosclerosis, and its plasma concentration has been positively correlated with subclinical atherosclerosis (Deo et al., 2004). We have also shown that HIV-infected patients with the MCP-1−2518G allele, which increases the expression of the MCP-1 gene, have a 5-fold increased risk of atherosclerosis (Alonso-Villaverde et al., 2004). Such a postprandial decrease in MCP-1 observed after treatment might be the direct result of improved insulin sensitivity, but the changes in body weight may be an additional factor to be considered (Christiansen et al., 2005).

In summary, a high-fat meal has only minor effects on inflammatory and antioxidant variables in HIV-infected patients with lipodystrophy, but treatment with metformin or rosiglitazone increased fasting and postprandial protection from oxidation and reduced the postprandial pro-inflammatory response by reducing plasma MCP-1 concentrations.

Acknowledgements

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UNIVERSITAT ROVIRA I VIRGILI
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Annex #4:

Ezetimibe effectively decreases LDL-cholesterol in HIV-infected patients.

Coll B, Aragones G, Parra S, Alonso-Villaverde C, Masana L.

AIDS. 2006;20:1675-7.

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Research Letter

AIDS 2006, **20**:000–000

Ezetimibe effectively decreases LDL-cholesterol in HIV-infected patients

Blai Coll[a,b], Gerard Aragonés[a], Sandra Parra[b], Carlos Alonso-Villaverde[b] and Lluis Masana[b]

We tested the security and efficacy of ezetimibe in the treatment of HIV-associated dyslipemia. Twenty HIV-infected patients were randomly assigned to receive ezetimibe 10 mg/day or fluvastatin 80 mg/day. Patients receiving ezetimibe experienced a statistically significant ($P = 0.003$) 20% reduction in the concentration of LDL-cholesterol, similar to that observed with fluvastatin (24%, P between groups 0.70). We concluded that ezetimibe monotherapy effectively decreases LDL-cholesterol in HIV-infected patients.

The control of cardiovascular risk factors in HIV-infected patients is relevant because the incidence of myocardial infarction [1] and other atherosclerosis-related events [2] are increasing. Lipid abnormalities are commonly present in this clinical setting [3], but the majority of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, with the exception of pravastatin and fluvastatin, are metabolized by the cytochrome P450 3A4, which in turn is also the metabolic pathway of many of the antiretroviral agents, especially the protease inhibitors (PI) [4]. Recent evidence supports the use of ezetimibe in combination with statins to add a powerful lipid-lowering effect [5]. However, the metabolism of ezetimibe, which is P450 independent, and the low incidence of drug interactions and side-effects, make this drug suitable to be tested in monotherapy in HIV-infected patients.

Patients were enrolled during the 3 months of the inclusion period if they fulfilled the eligibility criteria: more than 6 months on stable HAART, more than 18 years of age, and a fasting LDL-cholesterol concentration of 3.30 mmol/l or greater. Classic cardiovascular risk factors were recorded, and fasting total cholesterol, HDL-cholesterol, triglycerides, and glucose were determined. LDL-cholesterol values were obtained using the Friedewald formula. CD4 and CD8 lymphocyte counts and HIV-1 viral load were determined using standard techniques.

Endothelial function was analysed using peripheral arterial tonometry [6]. Briefly, this system (Itamar Medical Ltd., Caesarea, Israel) utilizes a finger probe to assess digital volume changes accompanying pulse waves. The peripheral arterial tonometry data were analysed by a computer in an operator-independent manner. A ratio of less than 1.6 was considered to be a marker for endothelial dysfunction [6].

Patients were then randomly assigned (according to the HAART regime: boosted PI or non-nucleoside analogues) to receive ezetimibe 10 mg/day or fluvastatin extended release 80 mg/day in a 1 : 1 ratio. Patients were evaluated 2–3 weeks after the initiation of lipid-lowering agents to assess tolerability and adherence, and at 6 weeks, the baseline protocol was re-applied.

Results are expressed as mean (SEM) or in percentages. Univariate analyses, using non-parametric tests, were used to compare differences between groups (Mann–Whitney) and between pre and posttreatment (Wilcoxon and McNemar tests). The independent Ethics Committee of the Hospital Universitari Sant Joan approved the study.

There were no significant differences in lipid values, age, and baseline endothelial function between patients taking non-nucleoside analogues ($N = 10$) and those with boosted PI ($N = 10$). Patients randomly assigned to receive ezetimibe exhibited higher systolic [137 (5.6) versus 117 (2.7) mmHg, $P = 0.01$] and diastolic blood pressure [90 (3.3) versus 76 (4.5) mmHg, $P = 0.02$] than those on fluvastatin. There were no significant differences either in the other cardiovascular risk factors or in the HIV-related variables between groups, although most of the patients assigned to be given ezetimibe were receiving lopinavir/ritonavir. None of the participants experienced related side-effects and none of them interrupted the lipid-lowering therapies.

In both groups, total cholesterol and LDL-cholesterol were significantly decreased after therapy (Fig. 1). Those patients receiving ezetimibe 10 mg presented a statistically significant ($P = 0.03$) 10% reduction in total cholesterol and a 20% reduction in LDL-cholesterol concentrations ($P = 0.02$, Fig. 1). The results in the fluvastatin group were similar, showing a 17% reduction in total cholesterol ($P = 0.06$) and a 24% decrease in the concentrations of LDL-cholesterol ($P = 0.02$). We did not find significant differences in either triglycerides or HDL-cholesterol (Fig. 1). There were no significant differences in the reduction of lipid concentrations between groups, showing a similar effect of both lipid-lowering agents (Wilcoxon test, $P = 0.2$ for total cholesterol and $P = 0.4$ for LDL-cholesterol).

Furthermore, those patients receiving ezetimibe did not exhibit significant changes in endothelial function after

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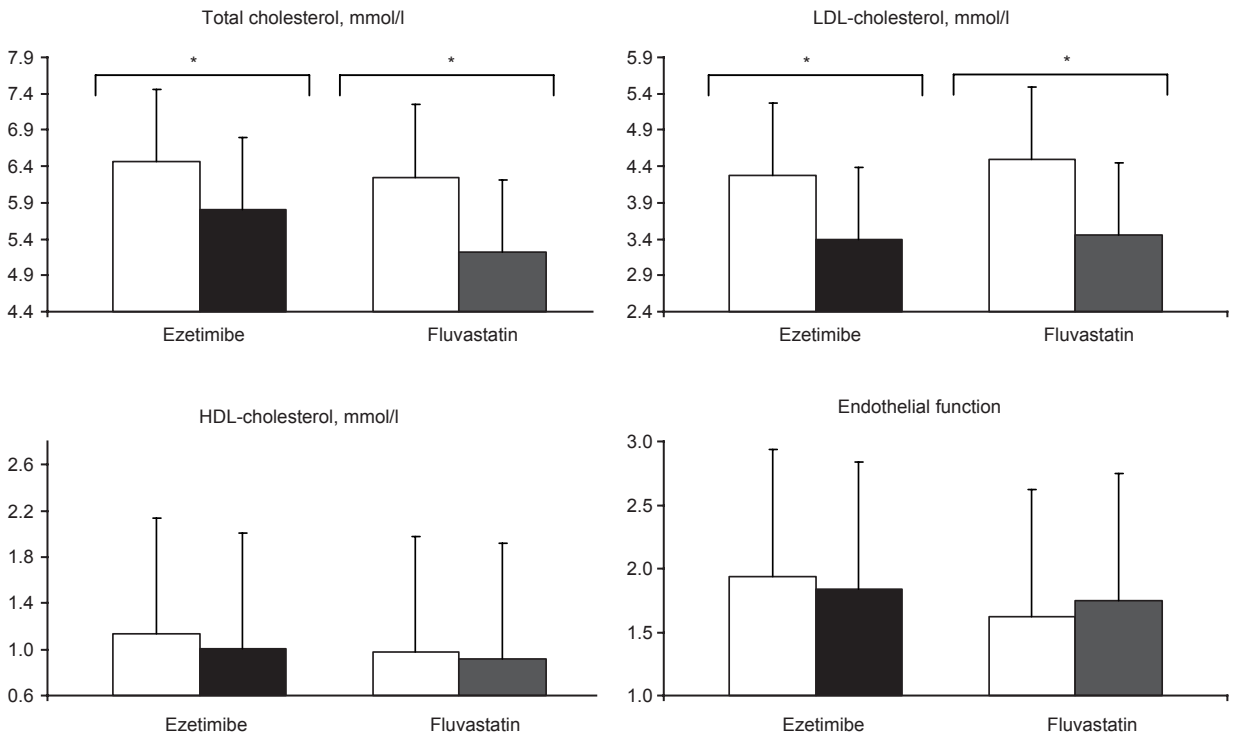

Fig. 1. Results of lipid parameters and endothelial function in HIV-infected patients before (white bars) and after (filled bars) 6 weeks of ezetimibe ($N = 10$) or fluvastatin ($N = 10$). $*P < 0.05$ comparing pre and post-treatment assessment (Wilcoxon test).

6 weeks of therapy (Fig. 1). However, those in the fluvastatin group experienced a non-significant ($P = 0.5$) increase in the rate of endothelial function (11% better result in the second evaluation, Fig. 1).

The treatment of HIV-associated dyslipidemia should avoid drug–drug interactions and potentially detrimental concentrations of statins [7–10]; therefore, the use of lipid-lowering agents without significant interactions with HAART is highly desirable.

Pravastatin is not preferentially metabolized by cytochromes [10], and fluvastatin is metabolized through the CYP2C9 enzyme. Both reduce the concentrations of total cholesterol without significant toxicities [10,11], but other lipid-lowering drugs should be evaluated in this particular group of patients in order to control potential toxicities. Ezetimibe is the first available selective cholesterol absorption inhibitor, blocking cholesterol absorption at the intestinal brush border to reduce LDL-cholesterol [12]. The dual inhibition of cholesterol synthesis and absorption, through the co-administration of a statin and ezetimibe, has been shown to provide significantly greater reductions in LDL-cholesterol than statin monotherapy alone [5]. However, if confirmed, we also support the use of ezetimibe in monotherapy in HIV-infected patients, because it yields similar results to those obtained with fluvastatin, and none of the participants experienced any ezetimibe-related side-effects.

The small sample size and the different distribution of the boosted PI between groups are severe limitations to be considered. Even then, the results favour the use of ezetimibe to control lipid-related cardiovascular risk factors.

An important issue addressed in our study was the influence of these agents on endothelial function; HIV-infected patients exhibit higher rates of endothelial dysfunction than the non-infected population [13], and those on PI have even worse values [14]. Agents that ameliorate endothelial function contribute to a reduction in cardiovascular risk, because endothelial dysfunction is the initial disturbance in the development of atherosclerosis [15]. Those patients on ezetimibe, although reducing significantly the values of LDL-cholesterol, did not experience any change in endothelial function. Conversely, patients receiving fluvastatin ameliorated, although not significantly, their endothelial function.

In summary, ezetimibe monotherapy effectively decreases LDL-cholesterol in HIV-infected patients. Because of the small sample size, these results should be further addressed in larger trials, but the use of ezetimibe in these patients can be advised.

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UNIVERSITAT ROVIRA I VIRGILI
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Blai Coll Crespo
Dipòsit Legal: T.1044-2012

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