The β-Chemokines MIP-1α and RANTES and Lipoprotein Metabolism in HIV-Infected Brazilian Patients

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HIV patients are predisposed to the development of hypertriglyceridemia and hypercholesterolemia as a result of both viral infection and HIV infection therapy, especially the protease inhibitors. Chemokines and cytokines are present at sites of inflammation and can influence the nature of the inflammatory response in atherosclerosis. We investigated the correlation between biochemical variables and β-chemokines (MIP-1α and RANTES) and the apolipoprotein E genotype in HIV-infected individuals. The apolipoproteins were measured by nephelometry. Triglycerides and total cholesterol were determined by standard enzymatic procedures. The β-chemokines were detected by ELISA. The genetic category of CCR5 and apolipoprotein E were determined by PCR amplification and restriction enzymes. Immunological and virological profiles were assessed by TCD_4^+ and TCD_8^+ lymphocyte counts and viral load quantification. Positive correlations were found between apo E and CD_8^+ (p = 0.035), apo E and viral load (p = 0.018), MIP-1α and triglycerides (p = 0.039) and MIP-1α and VLDL (p = 0.040). Negative correlations were found between viral load and CD_4^+ (p = 0.05) and RANTES and CD_4^+ (p = 0.029). The β-chemokine levels may influence lipid metabolism in HIV-infected individuals.

Key Words: β- chemokine; HIV; genotype, lipoproteins; cholesterol, triglyceride.

The global AIDS epidemic has become one of the most pressing public health emergencies of this century. The AIDS epidemic claimed more than 3 million lives in 2002, and an estimated 5 million people acquired the human immunodeficiency virus (HIV) in the same year. The latest statistics provided by the UNAIDS (Joint Commission of the United Nations for HIV/AIDS) indicated 42 million people living with HIV/AIDS worldwide (available at <www.unaids.org>).

In addition to the well-established pathogenesis of the virus, HIV-infected patients are predisposed to a higher risk of having coronary artery disease (CAD) as a result of both the viral infection *per se*, which triggers the development of hypertriglyceridemia and decreased levels of HDL-cholesterol [1-3], and due to standard antiretroviral therapy, including the protease inhibitors (HAART - Highly Active Antiretroviral Therapy), which is accompanied by idiosyncratic effects that affect lipid metabolism, leading to hypertriglyceridemia, hypercholesterolemia, lipodystrophy and lipomegaly [4,5]. Taking these observations into consideration, the HIV-infected patient is afflicted by various factors that can lead to the development of atherosclerosis. Furthermore, knowing that therapy delays disease progression and extends life expectancy, it is worthwhile to assess its implications for lipid metabolism, because antiretroviral treatment is used indefinitely.

Along with these disturbances in lipid metabolism, there is the polygenic and multifactorial inheritance of CAD and atherosclerosis. In this context, one approach

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to understand the etiology of these metabolic alterations is to empirically study candidate genes that could contribute to the pathogenesis of CAD. Human lipid metabolism in humans is strongly regulated by an array of polymorphisms in several genes, and like other common chronic diseases, atherosclerosis depends on an interaction of environmental risk factors and multiple predisposing genes [6,7]. The identification of candidate genes for atherosclerosis has been based on data regarding proteins thought to be implicated in atherogenesis [8]. Of particular interest, apolipoprotein E (apo E) has been implicated in atherogenesis [9,10], due to its cardinal role in the homeostasis of triglycerides and cholesterol [3]. The polymorphic nature of the apo E gene accounts for variations in susceptibility to CAD; the ε4 allele of apo E is associated with an increased risk for CAD, although its impact seems to vary with factors such as gender, ethnic origin and life style [11,12]; this allele is also cardioprotective [9].

Roughly speaking, the atherosclerotic reaction is dependent on various risk factors, such as dyslipemia. These risk factors are essentially injurious agents to the vascular endothelium, which lead to vascular inflammation and culminate in atherosclerotic plaques. As atherosclerosis is an outcome of an inflammatory disease, the recruitment of monocytes to the vessel wall is one of the earliest detectable events in human and experimental atherosclerosis. Monocytes cytokines and growth factors induce the maturation of macrophages and their transformation into lipid-laden foam cells. These events constitute important stages in the initiation and progression of atherosclerotic plaques [13,14]. Multiple cytokines and growth factors are present at sites of inflammation, and each of them can potentially influence the nature of the inflammatory response [15]. Chemokines are a growing superfamily of small (8-10kDa) chemotactic cytokines involved in the recruitment and activation of leukocytes and other types of cells [16]. They have been implicated in a variety of inflammatory conditions, and since 1996 their receptors have been shown to be coreceptors for the infection of host cells by the primate lentiviruses, human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) [17]. A 32bp (Δ32) deletion that interrupts the coding region of the CCR5 chemokine receptor locus is protective against HIV infection and progression to AIDS. There have been several reports of exposed uninfected individuals who had high levels of the endogenous β-chemokines MIP-1α, MIP-1β and RANTES, indicating that β-chemokines can contribute to clinical resistance [18,19].

Atherosclerosis is associated with increased expression of a number of chemokines, including MCP-1, RANTES, MIP-1α/β, eotaxin and IL-8 [20,21], and the apo E gene is implicated in atherogenesis. Thus, since we know that HIV infection provokes dyslipemia, and increasing levels of pro-atherogenic factors and β-chemokines, it is reasonable to affirm that an atherogenic landscape exists during HIV infection. This situation could be a result of a sum of the effects derived from: (i) high levels of lipids and atherogenic lipoproteins, and (ii) the activity of chemokines by which monocytes and other cell types are arrested on the luminal surfaces of vessels to form atheroslerotic lesions. Based upon this observation, we investigated the correlation between biochemical variables and β-chemokines (MIP-1α and RANTES) during HIV infection, and we investigated whether the ε4 allele of apolipoprotein E in HIV-infected patients is associated with a higher level of b-chemokines in comparison to patients carrying the apo E3/3 genotype.

Materials and Methods

Subjects

A heterogeneous group of 24 asymptomatic HIV-infected adults (asymptomatic, based on the common immunological and virological markers, i.e., viral load and CD_4^+ T-lymphocyte count) was selected. The HIV-1 infection was detected by ELISA and confirmed by the western blot technique. The patients were under medical supervision at the local HIV Health Service (SESA, Araraquara SP - Brazil) and they did not have signs of active secondary opportunistic infections at the time of the study. The duration of HIV infection was 4.0 ± 2.9 years. A written informed consent was

obtained from all subjects, and the protocol was approved by the Research Ethics Committee of the Faculty of Pharmaceutical Sciences, São Paulo State University in Araraquara.

There were 9 men and 15 women, with average age of 34 ± 5 years (ranging from 21 to 42 years). The subjects were randomly selected to represent the characteristic race admixture in Brazilian population. All patients were under a normal diet.

Methods

Parameters evaluated in the whole HIV group were the β-chemokine RANTES and MIP-1α plasma levels, serum apolipoprotein AI (apo AI), B (apo B), and E (apo E), and the lipid profile, which comprised triglycerides, total cholesterol (TC), HDL cholesterol (HDL-C), VLDL cholesterol (VLDL-C), and LDL cholesterol (LDL-C). Genotyping of CCR5 and apolipoprotein E was carried out. The immunological and virological profiles of the group were assessed by CD_3^+/CD_4^+ and CD_3^+/CD_8^+ counts and by viral load quantification.

Venous blood was sampled by venipuncture in EDTA-containing tubes, between 7:00 and 9:00 A.M., after a 12 hour-fast, to obtain serum for the biochemical variables and plasma for the CD_4^+ and CD_8^+ T-lymphocyte counts, the viral load and the β chemokine quantifications. All apolipoproteins were measured by nephelometry, on a Behring Nephelometer Analyser, with Behring reagents (Berhrengwert AG, Marburg, Germany). Triglycerides and TC were determined by enzymatic colorimetric assay (Sera Pack, Bayer, USA). HDL-C levels were measured after selective solubilization of HDL, followed by enzymatic assay. All of these assays were performed in an automatic clinical chemistry analyzer (RA-100, Techinicon). LDL-C was calculated by using the Friedwald equation, whenever the triglyceride levels were equal to or less than 400 mg/dL [22]. Plasma levels of RANTES and MIP-1α were determined by enzyme immunoassay (ELISA), according to manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN, USA). Concentrations of chemokines were calculated from a standard log-log transformation curve and by linear regression.

The HIV-1 RNA load was determined from plasma that had been kept frozen at -80ºC, by using the Nuclisens assay (Organon Teknika, Boxtel, The Netherlands), using the method specified by the manufacturer. The dynamic range of this assay is 1.9 log to 6.0 log RNA/mL of plasma, and the sensitivity is 1.9 log/mL. The CD_3^+/CD_4^+ and CD_3^+/CD_8^+ cells were counted by flow cytometry (Beckton-Dickinson Immunocytometry Systems, San Jose, California, USA).

DNA was extracted from blood lymphocytes by using a salting-out procedure [23], modified in our laboratory, and the genotype determination was performed as follows:

PCR genotyping of the CCR5 allele

Genotypic analysis of the CCR5 alleles was performed using the oligonucleotide primers reported by Rubbert et al. [24], which cover nucleotides 533-553 and 685-712 of the CCR5 gene in PCR procedures. Using this set of primers, the wild-type CCR5 allele gives rise to a 180 bp PCR product, while the deleted allele is 148 bp. Genomic DNA (50-100 ng) from each individual was amplified in a total volume of 20 μL in a buffer containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each primer, 1 U Taq DNA polymerase (Invitrogen Life Technologies), 20 mM $(NH_4)_2SO_4$ and 0.1% Tween 20. Cycling conditions were 94ºC for 1 min, 55ºC for 2 min and 72ºC for 2 min, for 35 cycles. The reaction products were run on 3% agarose gels, and DNA bands were stained with ethidium bromide.

PCR genotyping of the apolipoprotein E gene

The apo E polymorphism was detected after DNA amplification by PCR, with primer sequences for *Hha* I polymorphism at the apo E gene (exon 4), described by Hixon & Vernier [25]. The amplification reaction for 50 μL contained 300 ng of genomic DNA, 1 X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl),

2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 30 pM of each primer, 11.6% DMSO and 1.5 U of Taq DNA Polymerase (Invitrogen Life Technologies). The amplification protocol consisted of an initial denaturation step at 95ºC for 6 minutes, followed by 30 cycles at 95ºC for 60 seconds, 64ºC for 50 seconds, and 72ºC for 90 seconds, and a final extension step of 7 minutes at 72ºC. Ten microliters of the PCR product were digested with 5U of HhaI (CfoI) (Gibco, BRL) and incubated overnight at 37ºC. The digested DNA fragment was loaded onto a 15% non-denaturing polyacrylamide gel and electrophoresed for four hours to improve band resolution in 1X TBE buffer (pH 8.4). The gel was silver stained and the genotype was determined. Twenty-five percent of the samples had their genotype evaluated twice, in order to resolve any misinterpretations of the polyacrylamide gel.

Statistical analysis

Differences between apolipoprotein concentration, lipid profile, and β-chemokines in the different groups of individuals divided according to the apo E genotype were compared by using the independent Student's *t*-test. The values were calculated as mean ± standard deviation (SD) and 95% confidence intervals (C.I.). Relationships between continuous variables were assessed by the Pearson correlation coefficient. P values of < 0.05 were considered statistically significant.

Results

Of the 24 patients enrolled in this study, 17, 5 and 2 had the apo E3/3, E3/4 and E2/4 genotypes, respectively. All the individuals were homozygous for the CCR5 allele.

We analyzed differences in the group divided according to their apo E genotype, examining only the apo E3/3 and apo E3/4 genotypes. The two individuals carrying the E2/4 genotype were excluded from this statistical approach, as they could not be pooled with any other group. The biochemical variables, the chemokines and the immunological and virological profiles did not differ significantly among the apo E genotypes (Table 1).

A positive correlation was found between apo E and CD_8^+ counts ($r = 0.57$, $p = 0.035$); apo E and viral load ($r = 0.48$, $p = 0.018$); MIP-1α and triglycerides ($r = 0.42$, $p = 0.039$) and MIP-1α and VLDL ($r = 0.42$, $p = 0.040$). A significant inverse correlation was found between viral load and CD_4^+ counts ($r = -0.20$, $p = 0.05$) and RANTES and CD_4^+ counts ($r = -0.44$, $p = 0.029$) (Figure 1). In addition to these values, some tendencies appeared for other parameters. For example, borderlines p-values were found for the following correlations: viral load and RANTES ($r = 0.36$, $p = 0.08$); apo E and MIP-1α ($r = 0.34$, $p = 0.09$) and cholesterol and CD_8 ($r = 0.36$, $p = 0.08$).

Discussion

In a previous study, we examined the CCR5 genotype frequency and the plasma levels of RANTES and MIP-1α in Brazilian HIV-infected individuals. We found increased levels of RANTES and decreased levels of MIP-1α during HIV infection [26]. Because chemokines and apo E genotypes have been implicated in the pathogenesis of atherosclerosis, we have now investigated the correlation between biochemical variables and β-chemokines (MIP-1α and RANTES) during HIV infection and the possible relationship between the E3/4 and E3/3 genetic variants of apolipoprotein E in these patients.

Our results for genotype and allelic frequency are similar to those of other studies in the Brazilian population [27-28]. We did not find differences in the mean values for RANTES and MIP 1α in the different apo E genotypes. However, we could not compare these results with previous studies, as the levels of these two β chemokines have not previously been compared among apo E genotypes. Thus, in view of existing knowledge on the participation of monocytes and proinflammatory cytokines in the pathogenesis of atherosclerosis, we hypothesize that these CC chemokines play a role in the recruitment and activation of monocytes/macrophages in this disease.

Table 1. Immunological and virological profile, chemokines and apolipoprotein levels, differentiated by apolipoprotein E genotype in Brazilian HIV-infected patients

Apolipoprotein E genotype	E3/3 (n=17) Mean ± S.D. (95% C.I.)	E3/4 (n=5) Mean ± S.D. (95% C.I.)
CD_4 (cells/mm^3)	427.49 ± 242.80 (306.65–548.13)	332.25 ± 254.28 (-72.37–736.87)
CD_8 (cells/mm^3)	909.78 ± 420.78 (700.53–1119.03)	726.75 ± 286.06 (271.57–1181.93)
Viral Load (log RNA/mL)	3.31 ± 1.24 (2.69–3.92)	3.28 ± 1.80 (0.42–6.14)
MIP-1α (pg/mL)	25.94 ± 6.43 (22.74–29.13)	26.05 ± 3.51 (20.47–31.63)
RANTES (ng/mL)	23.63 ± 17.54 (14.91–32.35)	67.76 ± 75.82 (-52.89–188.41)
Apo AI (mg/L)	1.23 ± 0.25 (1.11–1.36)	1.31 ± 0.22 (0.96–1.65)
Apo B (mg/L)	0.96 ± 0.26 (0.83–1.09)	0.98 ± 0.44 (0.27–1.68)
Apo B/AI	0.80 ± 0.23 (0.69–0.92)	0.75 ± 0.35 (0.19–1.31)
Apo E (mg/L)	0.05 ± 0.02 (0.04–0.06)	0.04 ± 0.01 (0.03–0.06)
TG (mg/dL)	173.71 ± 86.49 (130.69–216.72)	122.50 ± 74.40 (4.12–240.88)
TC (mg/dL)	172.82 ± 40.86 (152.51–193.14)	178.75 ± 42.60 (110.96–246.54)
HDL-C (mg/dL)	46.29 ± 18.40 (37.14–55.45)	48.75 ± 12.84 (28.32–69.18)
VLDL-C (mg/dL)	34.74 ± 17.30 (26.14–43.34)	24.50 ± 14.88 (0.82–48.18)
LDL-C (mg/dL)	91.79 ± 35.01 (74.38–109.20)	105.50 ± 34.06 (51.30–159.70)

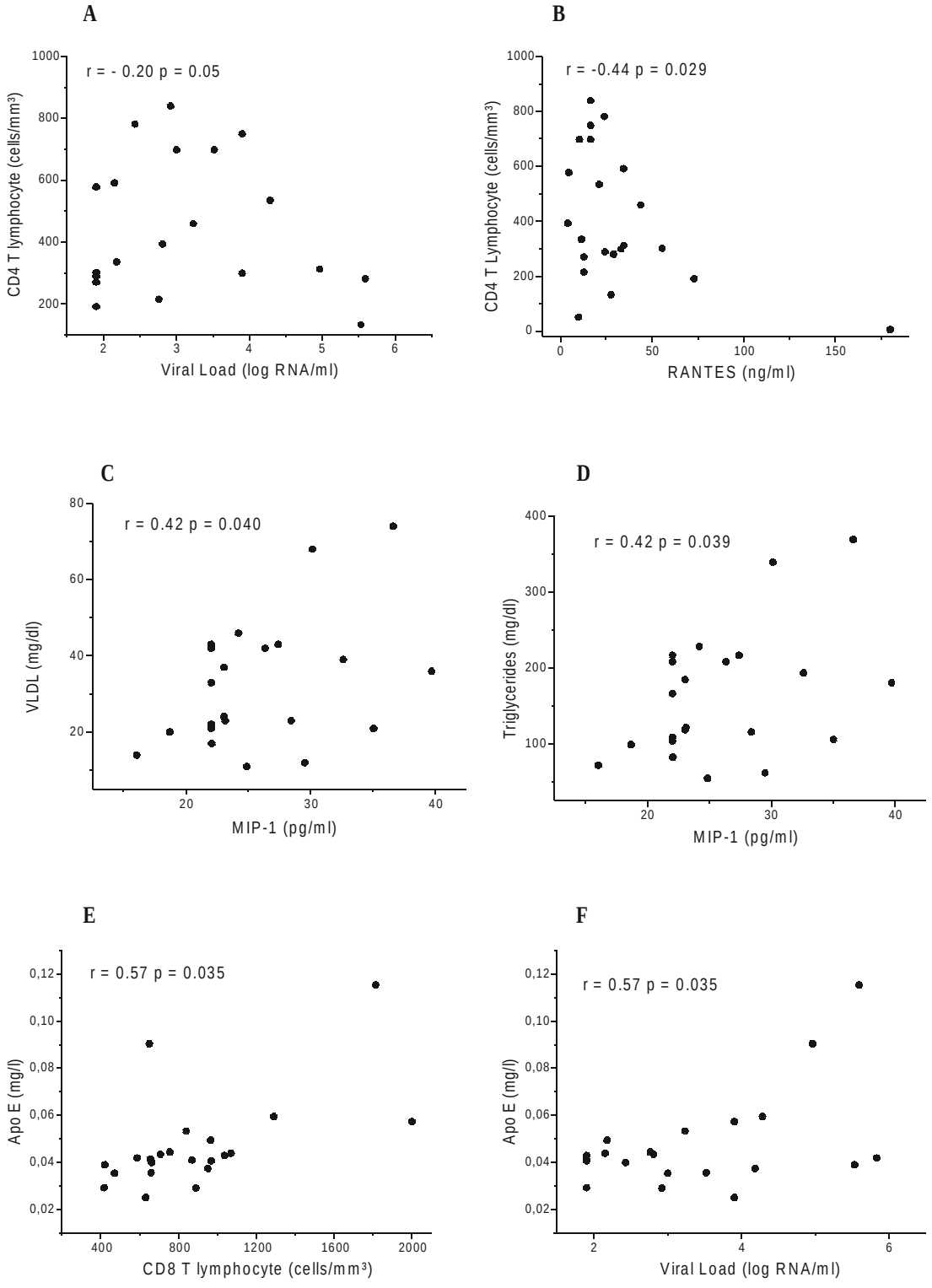
S.D. = standard deviation; C.I. = confidence interval; TG = triglycerides; TC = total choleserol; HDL-C = HDL cholesterol.

During HIV infection, higher levels of both RANTES and MIP-1α are associated with HIV cell-resistance, as a result of simple competition with CCR5 (its natural receptor) on the surface of macrophages. This leads to a diminished rate of viral entry or fusion into the host's HIV-trophic macrophage cells (18-19). This resistance is exacerbated in individuals carrying the Δ32 genotype at the CCR5 gene (deletion of 32 bp at exon 4), because these individuals have defective binding domains at the CC receptor on the macrophage surface [29-30]. The heterozygous genotype for CCR5 is responsible for intrinsic differences in the plasma chemokine levels. Patients who were heterozygous for the 32 bp deletion allele had significantly higher levels of RANTES production from their CD_4^+ lymphocytes when compared to patients who did not carry the Δ32CCR5 allele (31). Although some reports have not associated the CCR5Δ32 with increased risk of coronary endothelial dysfunction or atherosclerosis [32], others have indicated that homozygosis for the CCR5 mutant allele is protective in later stages of CAD [33-34].

The correlations found in our study contribute to our understanding of dyslipidemia in HIV-infected individuals. These findings open new avenues for further molecular studies in this population and for better knowledge of HIV pathogenesis and its consequences. The positive correlations found between MIP-1α and triglycerides, and between MIP-1α and VLDL, and the tendency for a correlation between apo E and MIP-1α, allow us to infer that the triglyceride levels during HIV infection are associated with the levels of this β chemokine. High levels could indicate a propensity for leukocyte attraction and inflammation.

Increases in triglyceride levels in HIV and AIDS have been observed, both before and after the introduction of protease inhibitor drugs and HAART [1-3]. Because of the important side effects of HAART therapy on the HIV-infected patient lipid metabolism, we chose a cohort in a low-level protease-inhibitor

Figure 1. Correlations among the biochemical variables, T lymphocytes and chemokines in HIV-infected patients. (A) viral load and CD4 T-lymphocytes, (B) RANTES and CD_4 T-lymphocytes, (C) MIP-1α and VLDL, (D) MIP-1α and triglycerides, (E) CD8 T-lymphocytes and apolipoprotein E, and (F) viral load and apolipoprotein E.



regimen of therapy, to study the correlation between chemokine and biochemical variables; i.e., our results are derived from a population without the adverse effects of protease inhibitors. The correlations between apo E and viral load, and between apo E and CD_8^+, show that the increase in apo E that has been previously observed in HIV and AIDS [3] occurs to a greater degree as the infection advances. It also means that apo E can be considered an additional indirect marker for disease progression, as is the case for apo AI, cholesterol, and triglycerides [35]. The inverse correlation between CD_4 T-lymphocyte counts and RANTES concentrations may have clinically- and immunologically-relevant consequences in HIV-infected patients. It is also possible that RANTES has a direct influence on the nature of the inflammatory response.

There is data suggesting that MCP-1 (monocyte chemoattractant protein), another CC chemokine, is involved in the pathogenesis of atherosclerosis. MCP-1 is a natural ligand for CCR2, which is expressed by monocytes. It is present at high levels in macrophage-rich areas of atherosclerotic plaques [36]. Experiments using mice that lack CCR2 or MCP-1 develop severe atherosclerosis [37,38]. On the other hand**,** to our knowledge, no *in vivo* data exists on associations between CC chemokine and lipid levels and apo E genotypes in HIV-infected individuals. Chemokine receptor expression in specific leukocyte populations, together with chemokine expression, play a key role in determining the classes of leukocytes recruited in a given pathophysiological process [21]. The CCR2 gene is closely linked to CCR5, and strong linkage disequilibrium has been reported for the 32 bp mutation and polymorphism in the CCR5 regulatory region [39]. It has been suggested that the CCR2 mutation is also associated with lower levels of CCR5 expression [40]. More studies are required to elucidate the role of β-chemokines in atherosclerois and in HIV infection, regarding not only involvement in the pathophysiology of the virus (their role in competing with the CCR5 receptor), but also in the pathological appearance of atherosclerosis (a putative role in enhancing leukocyte migration in areas of inflammation).

In summary, HIV infection has an intimate relationship with a host's response mechanism, which aims at the clearance of the infectious agent. As an intrinsic part of such a response, the HIV-infected patients are exposed to disturbances in their metabolism, including serum lipid and apolipoprotein levels. In addition, many contributing factors associated with atherosclerosis, such as increased plasma cholesterol, hypertension, and diabetes, directly influence chemokine release. The interplay of the common risk factors for CAD and cytokines, chemokines, growth factors and other components of the atherosclerotic reaction in the HIV-infected individuals needs to be elucidated.

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