The Effect of Rosuvastatin on Markers of Immune Activation in Treatment-Naive Human Immunodeficiency Virus-Patients

Robyn G. M. Weijsma,1 Eric R. A. Vos,1 Jaap Ten Oever,2 Muriel Van Schilfgaarde,3 Lea M. Dijksman,4 André Van Der Ven,2 Guido E. L. Van Den Berk,1 Kees Brinkman,1 Jos P. H. J. Frissen,1 Anja Leyte,3 Ineke W. E. M. Schouten,1 Mihai G. Netea,2 and Willem L. Blok1

1Department of Internal Medicine, Onze Lieve Vrouwe Gasthuis, Amsterdam, 2Department of Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, and 3Departments of Haematology and Clinical Chemistry, and 4Teaching Hospital, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands

Background. Immune activation has been implicated in the excess mortality in human immunodeficiency virus (HIV)-infected patients, due to cardiovascular diseases and malignancies. Statins may modulate this immune activation. We assessed the capacity of rosuvastatin to mitigate immune activation in treatment-naive HIV-infected patients.

Methods. In a randomized double-blind placebo-controlled crossover study, we explored the effects of 8 weeks of rosuvastatin 20 mg in treatment-naive male HIV-infected patients (n = 28) on immune activation markers: neopterin, soluble Toll-like receptor (TLR)2, sTLR4, interleukin (IL)-6, IL-1Ra, IL-18, d-dimer, highly sensitive C-reactive protein, and CD38 and/or human leukocyte antigen-DR expression on T cells. Baseline data were compared with healthy male controls (n = 10). Furthermore, the effects of rosuvastatin on HIV-1 RNA, CD4/CD8 T-cell count, and low-density lipoprotein cholesterol were examined and side effects were registered.

Results. T-cell activation levels were higher in patients than in controls. Patients had higher levels of circulating IL-18, sTLR2, and neopterin (all P < .01). Twenty patients completed the study. Rosuvastatin increased the CD4/CD8 T-cell ratio (P = .02). No effect on other markers was found.

Conclusions. Patients infected with HIV had higher levels of circulating neopterin, IL-18, sTLR2, and T-cell activation markers. Rosuvastatin had a small but significant positive effect on CD4/CD8 T-cell ratio, but no influence on other markers of T-cell activation and innate immunity was identified (The Netherlands National Trial Register [NTR] NTR 2349, http://www.trialregister.nl/trialreg/index.asp).

Keywords. HIV-1; 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; immune activation; statin.

Despite the enormous success of highly active antiretroviral therapy (ART), life expectancy of patients infected with human immunodeficiency virus (HIV) still lags behind the general population [1–3]. In the current treatment era, excess mortality is, to a large extent, due to non-acquired immune deficiency syndrome (AIDS)-defining diseases, such as malignancies and cardiovascular diseases. Several factors may be held responsible: for example, increased prevalence of smoking in HIV-patients, toxicity of ART, and socioeconomic factors [4, 5]. Moreover, several studies have suggested that persistent immune activation and coagulation may play an important role in the pathogenesis of these complications [6, 7].

In patients infected with HIV, elevated plasma levels of lipopolysaccharide (LPS), neopterin (a soluble marker for activation of monocytes), and of several proinflammatory and anti-inflammatory cytokines such as interleukin (IL)-6 and IL-10 have been reported [6, 8]. In addition, increased concentrations of IL-6 and d-dimer have been found to correlate with mortality in patients infected with HIV [9].

It has been postulated that modulating this inflammation and immune activation may counter the increased risk of non-AIDS-defining diseases [6]. Two recent studies stated that the use of statins reduces all-cause mortality in patients infected with HIV [10, 11]. Statins, or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are lipid-lowering agents. There is an ongoing debate on the possible pleiotropic effects of statins. Hodgkinson et al [12] reported that statins have an inhibitory effect on the LPS/Toll-like receptor (TLR)4 inflammatory response. In the JUPITER study, the use of rosuvastatin resulted in a significant decrease in highly sensitive C-reactive protein (hsCRP) levels and reduced the number of vascular events in patients with elevated hsCRP, indicating an anti-inflammatory effect [13]. Several studies have investigated the effects of statins in patients infected with HIV. Ganesan et al [14] reported
a reduction of T-cell activation levels after 8 weeks of atorvastatin. Others demonstrated a similar decline in T-cell activation after 48 weeks of rosvuastatin in patients treated with ART [15].

In this study, we explored the baseline differences in soluble markers of immune activation and in T-cell activation status between HIV-infected subjects and controls, and we investigated the effects of rosvuastatin on these markers in HIV-infected subjects in a double-blind placebo-controlled crossover trial. Only ART-naive patients were selected, to exclude the effect of treatment. We postulated that the use of rosvuastatin decreases the concentrations of soluble markers of innate immunity with resulting modulation of T-cell activation. We chose to examine the effect of rosvuastatin, because this statin has been investigated in the JUPITER study [13] and is proven to be well tolerated in patients infected with HIV [16, 17].

**METHODS**

**Study Population**

We included ART-naive HIV-infected male patients from April 2011 until January 2013 in the Onze Lieve Vrouwe Gasthuis (OLVG) Hospital in Amsterdam. Male volunteers, confirmed HIV-negative, who underwent a wisdom tooth extraction for noninflammatory reasons more than 2 weeks before inclusion, were included as healthy controls. The inclusion criteria for HIV-patients were an age of 18 years or older, having CD4 cell counts on 2 occasions higher than 350 cells/mm³ and HIV-1 RNA higher than 1000 copies/mL. Patients were eligible if there was no evidence of acute HIV-infection, which was defined as the presence of a detectable HIV-1 viral RNA in the presence of a nonreactive HIV-1 or HIV-2 antibody assay or an indeterminate Western blot. Laboratory results at screening were aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤1.5 times the upper limit of normal (ULN), creatine kinase ≤3 times the ULN, absolute neutrophil count ≥1000/mm³, hemoglobin ≥7.5 mmol/L, platelet count ≥100 000/mm³, creatinine ≤2 times the ULN, amylase and lipase ≤1.25 times the ULN, and low-density lipoprotein (LDL) ≥1.0 mmol/L.

Exclusion criteria were active drug use or alcohol dependence, serious illness, including opportunistic infections or neoplasm, requiring systemic treatment and/or hospitalization 30 days before entry of the study.

To ensure rosvuastatin was well tolerated, participants were excluded if there was known hypersensitivity to this drug or its components, a history of myositis or rhabdomyolysis, the use of drugs that significantly interact with rosvuastatin, or the use of other lipid-lowering agents. To assure there was no confounding on the immune response, use of concurrent immune modulators or vaccinations within 6 weeks of study entry were exclusion criteria as well. All participants provided written informed consent.

**Study Design**

We completed a double-blind randomized placebo-controlled pilot study with a crossover design. The HIV-infected participants were assigned to 2 random groups: 1 received rosvuastatin 20 mg qd, and the other received placebo during 8 weeks. After this first cycle, both study groups discontinued all study-related medications for 4 weeks, referred to as the washout period. Next, subjects receiving placebo switched to rosvuastatin and vice versa for a period of 8 more weeks. Throughout the study, participants had scheduled visits at the clinic every 4 weeks. Blood was drawn at 4 weeks (screening) and 1 day (baseline) before start of medication and on week 4, 8, 12, 16, and 20 after the start of medication. Noninfected controls donated blood samples once for all the parameters.

The carryover effect is defined as the persistence of effect of a treatment applied in one period, in a subsequent period. To minimize this effect, a washout period of 4 times the action duration of a treatment has been recommended [18]. Because the half-life of rosvuastatin is 19 hours, we estimated a washout period of 4 weeks to be sufficient.

To ensure the double-blind character of the study, the treating physician was not informed on the results of blood tests during the study period, and the samples for the lipid panel were measured after completion of the study.

Because both immune activation and statin use may cause fatigue, a questionnaire estimating quality of life was filled out at baseline, week 8, and week 20. This contained the Medical Outcomes Study HIV Health Survey (MOS-HIV) questionnaire and a combined complaints list, which was derived from the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 and has been used in HIV-infected patients and statin users [19, 20].

**Laboratory Methods**

Blood was drawn in the morning, after the appliance of a light tourniquet, into vacutainer tubes using a 21-gauge butterfly needle (BD Safety Lok Blood Collection Set, Plymouth, UK). On the same day, in the Haematology and Clinical Chemistry Laboratory of the OLVG Hospital, CD3⁺, CD4⁺, and CD8⁺ counts and expression of the T-cell activation markers were measured using anti-CD8 fluorescein isothiocyanate (SK1, immunoglobulin [Ig]G1), anti-CD3 phycoerythrin (HB7, IgG1), anti-CD3 peridinin chlorophyll (SK7, IgG1), and anti-human leucocyte antigen (HLA)-DR allophycocyanin (L243, IgG2a) from BD’s MultiTest reagent kit on blood collected in tubes containing 7.2 mg of K₂-ethylenediaminetetraacetic acid (EDTA) (BD, Plymouth, UK). Twenty microliters of monoclonal antibody mixture was added to a TruCOUNT FACS tube (BD) and mixed with 50 μL of EDTA whole blood. After an incubation period of 15 minutes, 1 mL lysing solution (BD) was added to lyse all erythrocytes. After another incubation period of 30 minutes, the samples were run on a FACS Calibur (BD). Gating was performed using CellQuestpro software (BD).
T-helper cells were defined as CD3⁺CD8⁻ cells, and cytotoxic T cells were defined as CD3⁺CD8⁺. Quadrants were set on distinct CD38 and HLA-DR-negative subpopulations.

The Microbiology Laboratory of the OLVG hospital used the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, version 2.0 (Roche Molecular Systems, Pleasanton, CA) for the quantification of HIV type-1 RNA in plasma with a quantitative range of 20–10 000 000 copies/mL.

The Laboratory of Experimental Internal Medicine of the Radboud University Medical Centre measured soluble TLR (sTLR)2 and sTLR4 by commercial enzyme-linked immunosorbent assay (ELISA) kits (USCN Life Science, Inc., Wuhan, China) with a lower limit of detection of 0.62 ng/mL and 1.3 ng/mL, respectively. Circulating IL-1Ra, IL-6, and IL-18 were measured using Luminex assays (Merck Millipore, Billerica, MA) with a lower detection limit of 9.8 pg/mL, 2.4 pg/mL, and 9.8 pg/mL, respectively. Competitive ELISA (IBL International, Hamburg, Germany) was used for the quantitative determination of neopterin and had a lower limit of detection of 1.35 nmol/L.

**Statistical Analysis**

Ganesan et al [14], studying 22 patients receiving atorvastatin 80 mg qd, found significant decreases in the proportion of lymphocytes expressing HLA-DR. Given these results and in view of the relative cholesterol-lowering potency of rosuvastatin compared with atorvastatin, we estimated that a sample size of 20 participants would be adequate to observe potential inhibitory effects on inflammatory markers.

All statistical analyses were performed using IBM SPSS Statistics, version 20. Measurements at baseline and screening were merged into 1 average value. Differences between patients and controls were assessed by a Mann–Whitney U test for continuous variables and Fisher’s exact test for the dichotomous variable race. A Spearman Rank test was used for analyzing correlations.

Subsequently, the effect of rosuvastatin on markers of immune activation in HIV-positive subjects was assessed. Both baseline measurements (ie, week 0 and 12) were not taken into account in the analyses due to the crossover design [21]. Continuous variables were primarily tested for normal distribution. Nonnormally distributed variables were natural log transformed. Parameters that had a distinctly nonnormal distribution were dichotomized for further analyses—based upon upper and lower bounds known from common practice or from 95% range of controls active in the trial. Thereafter, univariate analyses without correcting for covariates were conducted: continuous outcome variables were analyzed by means of linear mixed modeling (LMM) and dichotomous outcome variables by means of generalized estimating equations (GEE). Both LMM and GEE are constructive in analyses with correlated observations, ie, they are able to correct for the dependency of the observations. Moreover, both analyses are able to include incomplete cases due to the fact that missing outcomes are assumed to be missing at random. Nevertheless, logistic multilevel analysis (ie, LMM) results in an overestimation; therefore, GEE analysis was used for dichotomous outcome variables because its logistic analysis provides a more valid estimation, ie, smaller regression coefficients and standard errors due to its “population average” approach compared with the “subject-specific” approach in multilevel analysis. In LMM analyses, fixed effects for treatment-sequence (rosuvastatin-placebo vs placebo-rosuvastatin) and treatment (rosuvastatin vs placebo) were included as well as a random intercept for treatment on the individual level. An unstructured covariance structure was assumed. The possibility of a random slope was subsequently investigated by means of the maximum likelihood ratio test to achieve the most parsimonious model. In GEE analyses, an exchangeable correlation structure was assumed [21].

Furthermore, multivariate analyses were conducted to investigate confounding of covariates age (years) and race (Caucasian vs other). Again, the unstructured and exchangeable working correlation structures were used in LMM and GEE, respectively.

Finally, LMM was performed to assess the quality of life of the participants during the trial measured by using the validated MOS-HIV questionnaire and complaints list.

All analyses aforementioned were performed on an intention-to-treat basis. Regression coefficients (B) for normally distributed continuous variables, geometric mean (GM) ratios for natural log-transformed continuous variables, or odds ratios (OR) for dichotomous variables, and 95% confidential intervals were obtained. All P values of <.05 were considered to be statistically significant.

**RESULTS**

**Study Population**

Of the 35 patients screened, 28 met the study criteria and were randomized. During the first cycle, 4 subjects in the placebo group dropped out due to start of ART or for personal reasons. During the second cycle, 2 subjects in the statin group dropped out due to subjective side effects or lost to follow-up. In the placebo group, 2 subjects dropped out due to start of ART. Twenty participants completed both cycles (Figure 1).

Table 1 shows the baseline characteristics. Patients were significantly older than controls (median 40 vs 27 years, P < .01). The median duration of established HIV infection was 1.8 years, and the median HIV-1 RNA was 30,750 copies/mL. Furthermore, at baseline, patients had a significantly lower CD4 count (480 vs 1080 cells/μL, P < .01), a higher CD8 count (1010 vs 530 cells/μL, P < .01), and lower CD4/CD8 T-cell ratio (0.5 vs 1.9, P < .01). Cholesterol levels were similar between both groups.

**Immune Activation Markers Between Human Immunodeficiency Virus Patients and Controls**

Patients had higher median T-cell activation levels than controls (eg, HLA-DR⁺CD38⁺ % of CD8⁺; 33.4 vs 5.6, P < .01) (Table 2).
Only the CD38+ percentage of CD4+ cells was not significantly different. In addition, IL-18, sTLR2, and neopterin levels were significant higher in patients compared with controls (322.5 vs 322.5 pg/mL [P < .01], 3.4 vs 2.0 ng/mL [P < .01], and 19.5 vs 5.4 nmol/L [P < .01], respectively). The hsCRP levels were higher in patients, but this difference did not reach the level of statistical significance (1.3 vs 0.9 mg/L, P = .07). We did not observe a difference in leukocyte count, d-dimer, IL-1Ra, IL-6, and sTLR4 levels. In HIV-patients, age correlated negatively with neopterin (R = −0.33, P < .01), sTLR2 (R = −0.25, P < .01), HLA-DR+ % of CD8+ T cells (R = −0.26, P < .01), and HLA-DR+CD38+ % of CD8+ T cells (R = −0.24, P < .01). No correlation was found between age and IL-18 (R = −0.85, P = .31) and other investigated markers of T-cell activation (data not shown).
During rosuvastatin treatment, LDL cholesterol plasma level decreased significantly compared with placebo treatment ($B = -0.94, P < .01$) (Table 3). The CD4 counts increased during statin treatment, but this did not reach the level of significance ($B = 0.92, P > .08$). CD4/CD8 T-cell ratio showed a small but significant increase ($GM ratio = 1.11, P = .02$). No change in HIV-1 RNA (GM ratio = 0.97, $P = .77$) nor on CD8 count (GM ratio = 0.95, $P = .24$) was found. Moreover, we did not observe an effect on T-cell activation markers (eg, HLA-DR+CD38+ % of CD8+; GM ratio = 1.00, $P = .95$).

Likewise, no treatment effect was found on levels of leukocytes (GM ratio = 1.04, $P = .39$), hsCRP (GM ratio = 0.02, $P = .69$), d-dimer (OR = 0.80, $P = .61$), sTLR2 (GM ratio = 0.89, $P = .25$), nor on neopterin ($B = 0.7$ nmol/L, $P = .75$).

During statin use, ALT and AST levels increased significantly (GM ratio = 1.49, $P < .01$ and GM ratio = 1.26, $P < .01$, respectively). Creatine kinase and creatinine did not show any change (GM ratio = 1.19, $P = .29$ and GM ratio = 1.01, $P = .37$, respectively).
patients infected with HIV displayed substantially increased T-cell activation compared with healthy controls. In contrast to other studies, we did not find increased concentrations of IL-6 or hsCRP (for the latter, the difference just failing statistical significance). This discrepancy may be due to the small sample size. However, other markers of monocyte/macrophage activation were increased in patients compared with controls, among which were neopterin, IL-18, and sTLR-2.

Increased concentrations of neopterin have been described before in patients infected with HIV and were associated with other markers of monocyte activation in these patients [22]. The increased concentrations of IL-18 in our patients is in accordance with the findings of other studies, in which IL-18 concentrations correlated positively with HIV viral load and negatively with CD4 cell counts [23, 24]. We are the first to describe elevated concentrations of sTLR-2 in patients infected with HIV. Soluble TLR-2 correlates positively with other inflammatory markers such as tumor necrosis factor-α, IL-6, and IL-1Ra [25]. Taken together, these findings confirm that our group of ART-naive HIV-patients showed substantial immune activation.

We did not find an effect of rosvastatin on any of the soluble markers of inflammation measured. Very few other studies have investigated the effect of statins on soluble markers of monocyte activation or inflammation in patients infected with HIV. In an uncontrolled study, a reduction in CRP concentration was observed after 45 days of pravastatin or rosuvastatin in patients treated with ART [26]. Eckard et al [27], investigating patients treated with ART, did not find an effect on hsCRP concentrations after 24 weeks of 10 mg rosuvastatin, but the same group reported a decrease of soluble CD14 [28]. These effects seemed to be more pronounced after 48 weeks of rosuvastatin [15]. Thus, the lack of an effect on soluble markers of inflammation in our study may be due to the relatively short duration of treatment.

Although we found a significant activation of CD8 cells in our patients at baseline, we did not observe a reduction of T-cell activation after 8 weeks of rosuvastatin treatment. However, we did observe a small but significant increase in CD4/CD8 ratio, which may be regarded as an indirect sign of decreased T-cell activation. Our study design was similar to the study by Ganesan et al [14], who treated ART-naive HIV-patients with 8 weeks of 80 mg atorvastatin and found significant reductions in circulating proportions of CD4⁺HLA-DR⁺, CD8⁺HLA-DR⁺, and CD8⁺HLA-DR⁻CD38⁺ T cells. They did not report on changes of CD4/CD8 ratio. The differences between our findings and the study of Ganesan et al [14] remain speculative. Possible explanations may include the fact that rosvastatin is a hydrophilic statin, whereas atorvastatin is lipophilic. Direct inhibition of T-cell activation in vitro has been reported with atorvastatin [29]. In vivo, the effects of statins on T-cell activation in non-HIV-patients have shown divergent results [30–32]. Of course, we cannot exclude the possibility that statins have only marginal effects on inflammatory markers in patients infected with HIV, and that small differences between studies are merely the result of biological variation between the patients in the various studies.

Rovastatin treatment resulted in well known side effects, such as flu-like symptoms and elevation of liver enzymes. Only 1 patient had to discontinue the study due to side effects.

Our study may have been underpowered due to a high number of patient withdrawal of an already small sample size. Therefore, we consider our study as being exploratory. In this respect, it is noteworthy that a substantial number of subjects withdrew from the study during the placebo phase because their CD4 cell count dropped and they had to start ART. As mentioned before, although the significant decline in LDL cholesterol suggests adequate rosvastatin intake, 8 weeks may be too short a period for a detectable effect on immune activation markers. Future research may investigate the effects of statins on markers of inflammation for a longer time period and in a larger study population. Possible differential effects of hydrophilic versus lipophilic statins may also be of interest in future studies. The fact that we chose to study ART-naive HIV-infected subjects to exclude the effect of ART on immune activation has its limitations. In future studies, an additional effect of statins on immune activation in ART-treated patients may be harder to detect.

**CONCLUSIONS**

In conclusion, this small group of treatment-naive HIV-infected patients had higher levels of T-cell activation markers and higher circulating neopterin, IL-18, and sTLR2 levels compared with controls. Eight weeks of rosvastatin had a small but significant positive effect on CD4/CD8 T-cell ratio, but no influence on other markers of T-cell activation and innate immunity was identified.

**Acknowledgments**

We greatly appreciate the help of Lucy Schrijnders-Gudde and Sanne Houba.

**Financial support.** This study was funded by the Internal Medicine Research Foundation Onze Lieve Vrouwe Gasthuis. M. G. N. was supported by...
a European Research Council Starting Grant (no. 310372). J. T. O. was supported by the European Regional Development Fund (Province of Gelderland project 2009–20103).

**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

**References**