LL-37 Concentrations and the Relationship to Vitamin D, Immune Status, and Inflammation in HIV-Infected Children and Young Adults

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Abstract

Antimicrobial peptide LL-37 is produced in response to active vitamin D to exert immunomodulatory effects and inhibits HIV replication *in vitro*. To date, no studies have investigated LL-37 in HIV-infected patients. This study sought to investigate LL-37 and the relationship to 25-hydroxyvitamin D [25(OH)D] and HIV-related variables in this population. HIV-infected subjects and healthy controls ages 1–25 years old were prospectively enrolled in this cross-sectional study. Fasting plasma LL-37 and 25(OH)D concentrations were measured in duplicate with ELISA. HIV⁺ subjects (36 antiretroviral therapy (ART)-experienced subjects; 27 ART-naïve subjects) and 31 healthy controls were enrolled. Overall, 93% were black and the median age was 20 years. There was no difference in median (interquartile range) LL-37 between the HIV-infected group and controls [58.3 (46.4,69.5) vs. 51.3 (40.8,98.2) ng/ml, respectively; $p=0.57$]; however, the ART-experienced group had higher concentrations than the ART-naïve group [66.2 (55.4,77.0) vs. 48.9 (38.9,57.9) ng/ml, respectively; $p<0.001$]. LL-37 was positively correlated with 25(OH)D in controls, but not in HIV-infected groups, and was positively correlated with current CD4 and Δ CD4 (current-nadir) in the ART-experienced group. After adjustment for age, race, sex, and HIV duration, the association between LL-37 and CD4 remained significant. These findings suggest that HIV and/or HIV-related variables may alter the expected positive relationship between vitamin D and LL-37 and should be further investigated.

Introduction

THE PREVALENCE OF VITAMIN D DEFICIENCY in the HIV-infected population, as determined by blood concentrations of 25-hydroxyvitamin D [25(OH)D], is very high, and is widespread among HIV-infected adults and children.^{1–3} We and others have demonstrated >90% of subjects with HIV have concentrations <30 ng/ml, which has been suggested to be optimal for vitamin D status.^{2,4–6} While the causes of vitamin D deficiency in HIV are multifactorial, including inadequate diet and darker skin pigmentation, certain commonly prescribed antiretroviral medications, particularly efavirenz, have been shown to interfere with normal vitamin D metabolism.^{2,4,5}

Vitamin D is a steroid hormone that has long been known to play a critical role in the immune system, including effects

on innate immunity, adaptive immunity, and levels of inflammatory cytokines, including interleukin-6 (IL-6).^{7–10} In non-HIV populations, an individual's vitamin D status affects their susceptibility to tuberculosis, the disease severity, and their response to treatment.^{11,12} Clinical studies have also shown that children with vitamin D deficiency and rickets are predisposed to respiratory infections and pneumonia, and vitamin D supplementation reduces this risk.^{13–16} Moreover, vitamin D deficiency has been shown to negatively impact HIV disease progression and mortality,^{17,18} whereas increased vitamin D concentrations inhibit HIV replication.¹⁹

More recently, studies have shown that vitamin D's effects on the immune system derive from its upregulation of the antimicrobial peptide cathelicidin, LL-37.^{19–22} In response to endogenously produced 1,25-dihydroxyvitamin D [1,25(OH)₂D], cells produce LL-37, which in turn inhibits bacteria through the

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disruption of the cellular membrane, but also has broad antimicrobial activity against gram-positive and gram-negative bacteria as well as certain viruses and fungi. LL-37 also exerts chemotactic, immunomodulatory, and angiogenic effects, and affects serum levels of proinflammatory cytokines. Moreover, LL-37 has been shown to increase autophagy in macrophages to enhance innate immunity against viral infections,²¹ and *in vitro* LL-37 inhibits HIV replication in peripheral blood mononuclear cells including in CD4⁺ T cells.^{19,22}

There are few studies that have evaluated LL-37 *in vivo* in non-HIV populations,^{23–25} and to date, there are no published studies in HIV-infected individuals despite the *in vitro* data suggesting that LL-37 inhibits HIV replication. Investigating LL-37 in the HIV-infected population, particularly in HIV-infected youth, where opportunity exists to optimize health earlier in life, is important. Thus, the purpose of this study was to (1) determine plasma LL-37 concentrations in HIV-infected children and young adults, (2) examine the relationship between plasma LL-37 and 25(OH)D concentrations, and (3) investigate the relationship between LL-37 and HIV-related variables, especially CD4 cell counts and CD4 restoration after antiretroviral therapy (ART). Secondary objectives were to (1) explore the relationship between LL-37 and inflammatory markers that are known to be elevated in HIV, and (2) compare the results to a healthy control group.

Materials and Methods

Study design/population

Individuals ages 1–25 years with documented HIV-1 infection who obtained their medical care through the Grady Health System in Atlanta, GA were eligible for the original, prospective, cross-sectional study. Subjects were drawn from this larger, parent study investigating the vitamin D status of HIV-infected youth and its relationship with cardiac biomarkers, inflammation, and immune restoration.⁴ This current analysis included all ART-naïve subjects from the parent study. Antiretroviral therapy-experienced subjects and healthy controls with plasma 25(OH)D concentrations in the upper and lower quartiles were then chosen from the parent study in similar numbers to the ART-naïve subjects. This allowed for the widest range of 25(OH)D to evaluate the relationship between LL-37 and vitamin D.

Patients with acute illnesses were eligible after complete resolution of symptoms for ≥ 1 month, while those with active inflammatory conditions or medication use known to affect inflammation were not eligible. Eligible HIV-infected patients were recruited during their regular clinic visits over a 10-month period of time (June–March). Over 95% of approached patients consented to study participation.

Healthy controls were chosen over this same time period from a larger convenience sample with similar socioeconomic demographics, which included relatives of HIV-infected patients and HIV-negative patients seen at the clinic. Controls were recruited with advertisement flyers hung in the HIV clinic and by word of mouth and selected so that the overall group matched the HIV-infected subjects in age, sex, and race. Controls were eligible if they self-reported to be free of chronic disease and had no recent or current infection. Potential subjects ≥ 13 years of age were screened for HIV infection before enrollment with the OraQuick Advance Rapid HIV Test (OraSure Technologies, Inc, Bethlehem,

PA). Controls < 13 years of age were assumed to be HIV uninfected unless they were considered at high risk for having or contracting HIV. Exclusion criteria for controls were the same as for the infected group.

The study was reviewed and approved by the Institutional Review Boards of Emory University and Grady Health Systems. Written informed consent was provided by all participants (and/or their legal guardian, if applicable). Subjects between the ages of 6 and 10 years gave verbal assent and those between 11 and 16 years gave written assent.

Clinical assessments

All HIV-infected subjects and controls (or guardians) completed questionnaires providing relevant demographic and medical information. In addition, an extensive chart review was conducted for the HIV⁺ subjects, including known duration of HIV infection, detailed ART history, past and current medical diagnoses, current medications, and nadir CD4 count.

Laboratory assessments

Subjects fasted for ≥ 8 h prior to blood sampling. Plasma was extracted and stored at -80°C until analysis without prior thawing. Plasma concentrations for LL-37 and 25(OH)D were both assessed with a specific enzyme-linked immunosorbent assays (ELISA) (Hycult Biotechnology, Uden, Netherlands and IDS, LTD, Fountain Hills, AZ, respectively) as per the manufacturer's product manual and tested in duplicate.^{24,25} The calculated means of the measurements were used in the analysis. Median intraassay and interassay coefficients of variation (CV) for both LL-37 and 25(OH)D were $< 12\%$. Quality control for 25(OH)D was ensured by participation in the vitamin D external quality assessment scheme (DEQAS, site 606). Laboratory personnel were blinded to clinical information.

Biomarkers that we have previously shown to be elevated in HIV-infected patients were selected.^{26,27} Plasma levels of the proinflammatory cytokines, soluble tumor necrosis factor- α receptors I, II (sTNFR-I, -II), and IL-6 were measured using Luminex (IL-6: Millipore, Billerica, MA; sTNFR-I, II: Invitrogen, Carlsbad, CA), and high sensitivity C-reactive protein (hsCRP) was measured by ELISA (Cayman Chemical, Ann Arbor, MI). Cellular adhesion molecules, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1), were measured using Luminex (Millipore, Billerica, MA). Protocols were per the manufacturers' product manuals. Median intraassay and interassay CVs were all $< 10\%$.

CD4 counts, CD8 counts, and HIV-1 RNA were recorded from HIV-infected subjects' medical records as markers of HIV disease.

Statistical methods

Demographics, clinical characteristics, and laboratory parameters are described by HIV status. Continuous measures are described by medians/interquartile range, and nominal variables are described with frequencies/percentages. Non-parametric tests (Kruskal–Wallis for medians; chi-squared for categorical variables) were used to assess differences between groups. Spearman coefficients were used to assess

TABLE 1. DEMOGRAPHICS AND CLINICAL CHARACTERISTICS BY STUDY GROUP

<i>Median (IQR) or no. (%)</i>	<i>HIV⁺ ART-Exp. (N=36)</i>	<i>HIV⁺ ART-naive (N=27)</i>	<i>Healthy controls (N=31)</i>
Age, years	17.9 (4.6, 20.2)	20.2 (19.1, 21.0)	21.0 (18, 23)
Male sex	21 (58%)	23 (85%)*	16 (52%)
Black race	34 (94%)	26 (96%)	27 (87%)
Body mass index, kg/m ²	21.4 (20.0, 25.3)	20.6 (19.9, 23.3)	23.9 (21.0, 27.5)
25(OH)D, ng/ml	15.5 (10.8, 19.6)	14.0 (12.4, 18.4)	15.4 (11.9, 21.9)

* $p < 0.05$.

IQR, interquartile range; ART, antiretroviral therapy; Exp., experienced; 25(OH)D, 25-hydroxyvitamin D.

correlations with LL-37. Multivariable linear regression models were then performed to determine variables independently associated with LL-37. Variables for the regression models were chosen either based on the results of the univariate analysis and/or those likely to affect LL-37. p values < 0.05 were considered significant. All analyses were carried out using SAS, v.9.2 (The SAS Institute, Cary, NC).

Results

Sixty-three HIV-infected subjects (27 ART-naïve and 36 ART-experienced subjects) and 31 healthy controls were enrolled. Table 1 shows the demographic, clinical, and laboratory characteristics of the three groups. Groups were similar with regards to age, race, sex, and body mass index. The ART-naïve group had significantly more males compared to the other two groups ($p < 0.01$). All three groups had similar plasma concentrations of 25(OH)D, and almost all subjects were in the vitamin D deficient range (< 20 ng/ml) as per the Institute of Medicine's guidelines.²⁸ Table 2 shows the HIV-related characteristics for both HIV-infected groups. The minimum length of time of ART exposure in the ART-

experienced group was 6 months. No HIV-infected subjects were coinfecting with hepatitis C.

There was no difference in plasma concentrations of LL-37 between the HIV-infected group as a whole compared to the healthy controls [median (interquartile range (IQR)) HIV: 58.3 (46.4, 69.5) ng/ml; controls: 51.3 (40.8, 98.2) ng/ml; $p = 0.57$]. However, as Fig. 1 illustrates, when the HIV-infected group was evaluated separately by ART status, the ART-experienced group had a statistically significantly higher LL-37 plasma concentration compared to the ART-naïve group ($p < 0.001$). When adjusted for age, sex, race, and HIV duration, this difference did not remain statistically significant ($p = 0.52$). The median plasma LL-37 concentration in each of the HIV-infected groups was still similar to the controls even when separated by ART status.

Next, in a univariate fashion, variables that may be associated with plasma LL-37 concentrations were investigated (Table 3). Plasma concentrations of LL-37 were positively correlated with 25(OH)D in the healthy control group, but not within either of the HIV groups. In the ART-experienced group, plasma concentrations of LL-37 were positively correlated with current CD4 counts and with Δ CD4 (current

TABLE 2. CHARACTERISTICS OF THE HIV-INFECTED GROUP

<i>Median (IQR) or no. (%)</i>	<i>ART-Exp. (N=36)</i>	<i>ART-naive (N=27)</i>
Known years of HIV	14 (5, 17)	2 (1, 3)
Perinatally infected	27 (75%)	1 (4%)
Current CD4, cells/mm ³	431 (263, 646)	297 (244, 476)
Current CD4 %	28 (17, 32)	19 (14, 26)
CD4 nadir, cells/mm ³	184 (77, 337)	296 (204, 480)
Δ CD4 (current-nadir) ^a	229 (103, 342)	—
Current CD4/CD8 ratio count ^b	0.41 (0.21, 0.55)	0.65 (0.22, 1.07)
HIV-1 RNA < 80 copies/ml	29 (81%)	0 (0%)
HIV-1 RNA copies/ml (if > 80)	5,980 (2,878, 15,018) [N=8]	15,480 (6,678, 29,378) [N=27]
Currently on ART	30 (83%)	—
Currently on EFV	13 (36%)	—
Cumulative NRTI duration, months	103 (46, 135)	—
Cumulative EFV duration, months	13 (0, 39)	—
Cumulative PI duration, months	83 (10, 112)	—

^a Δ CD4 cell count = current – nadir CD4 cell count.^bCurrent CD4/CD8 % ratio similar. p -values were not calculated between groups, as groups are expected to be significantly different from one another.

ART, antiretroviral therapy; Exp., experienced; EFV, efavirenz; NRTI, nucleoside/nucleotide analogue reverse transcriptase inhibitor; PI, protease inhibitor.

FIG. 1. Box and whisker plots for LL-37 concentrations by antiretroviral treatment (ART) status. Box and whisker plots show the LL-37 concentrations for HIV-infected, ART-experienced subjects (**A**), HIV-infected, ART-naive subjects (**B**), and healthy controls (**C**). Each HIV group was compared to each other and to the healthy controls. The ART-experienced group had statistically higher LL-37 concentrations compared to the ART-naive group [median (IQR) ART-exp.: 66.2 (55.4, 77.0) ng/mL; ART-naive: 48.9 (38.9, 57.9) ng/mL; $p < 0.001$]. There was no difference between the ART-experienced group and the healthy controls [median IQR controls: 51.3 (40.8, 98.2) ng/mL; $p = 0.68$]. ART, antiretroviral therapy; Exp., experienced; IQR, interquartile range.

TABLE 3. SIGNIFICANT CORRELATIONS BETWEEN LL-37 AND VARIABLES OF INTEREST

Variable	ART-Exp.	ART-naive	Controls
	R^a p	R p	R p
25(OH)D	-0.24 0.15	0.02 0.93	0.43 0.02
Age	-0.35 0.04	0.11 0.59	-0.14 0.14
CD4 count	0.37 0.02	0.09 0.63	— —
Δ CD4 ^b	0.40 0.02	— —	— —
IL-6	0.52 <0.01	0.62 <0.01	0.33 0.08
sTNFR-I	0.39 0.02	0.29 0.15	0.20 0.29
sVCAM-1	0.03 0.87	-0.12 0.53	0.36 0.04

^a R = Spearman correlation coefficient.

^b Δ CD4 cell count = current – nadir CD4 cell count.

Blank spaces indicate nonapplicable correlations; body mass index, CD4 nadir, CD4/CD8 ratio, CD4/CD8 % ratio, HIV-1 RNA, hsCRP, sTNFR-II, and sICAM-1 were also tested but were not significant in any group.

Exp., experienced; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6; sTNFR-I, soluble tumor necrosis factor- α receptor-I; sTNFR-II, soluble tumor necrosis factor- α receptor-II; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1.

– nadir). In both HIV groups, plasma concentrations of LL-37 were strongly positively correlated with plasma levels of IL-6, but not with the other selected inflammatory markers. This relationship was heavily driven by LL-37 values $>100 \mu\text{g/mL}$. With all HIV-infected subjects considered together, the correlation decreased but remained statistically significant when these extreme values were eliminated (from $R = 0.58$; $p < 0.01$ to $R = 0.30$; $p = 0.02$). Interleukin-6 was not correlated LL-37 in the healthy controls.

Two multivariable linear regression models were then constructed to further investigate the relationship between LL-37 and current CD4 and Δ CD4, respectively. Variables that are known to affect plasma concentrations of 25(OH)D and may also affect LL-37 were entered into each model with either current CD4 count or Δ CD4. After adjustment for age, race, sex, and HIV duration, the relationship between LL-37 and current CD4 count remained significant ($p = 0.03$); however, the relationship between LL-37 and Δ CD4 did not ($p = 0.09$).

Discussion

This study sought to evaluate plasma LL-37 concentrations for the first time within an HIV-infected population and investigate the relationship with plasma concentrations of 25(OH)D and HIV-related variables. LL-37 concentrations were higher in ART-experienced HIV-infected subjects compared to ART-naive, HIV-infected subjects. In the ART-experienced group, LL-37 concentrations were associated

with both current CD4 counts and immune restoration after ART. Although LL-37 was positively correlated with 25(OH)D in the controls, as would be expected by known pathways, the same relationship was not seen in either HIV group. LL-37 was positively correlated with the proinflammatory cytokine IL-6 in both HIV groups.

Although vitamin D has long been known to play a critical role in the immune system, the precise mechanism has only recently been elucidated. One of the most important developments came from the study by Liu *et al.*, showing that vitamin D-deficient human macrophages have less effective mRNA cathelicidin expression upon toll-like receptor stimulation.²⁰ In response to endogenously produced 1,25(OH)₂D, macrophages produce the antimicrobial peptide cathelicidin, LL-37, which increases autophagy in macrophages to enhance innate immunity. This response is particularly important in controlling microbes such as *Mycobacterium tuberculosis*, but also appears to inhibit HIV replication as well, including in CD4⁺ lymphocytes.^{11,12,21,22}

Another more recent study showed that the interaction between vitamin D, LL-37, and HIV may be more complicated than previously thought. Campbell *et al.* demonstrated that exposing macrophages with 1,25(OH)₂D *in vitro* induces autophagy and inhibits HIV-1 replication.¹⁹ However, exposing macrophages with LL-37 at the concentrations found to be elicited by macrophages in response to physiological concentrations of 1,25(OH)₂D *in vitro*, and in the absence of 1,25(OH)₂D, LL-37 is unable to induce autophagy and actually enhances HIV-1 replication. Conversely, at concentrations of LL-37 higher than those produced by macrophages *in vitro* in response to physiological concentrations of 1,25(OH)₂D, but at concentrations found in the plasma of healthy individuals, they observed the inhibition of HIV-1. The authors suggest that perhaps other peptides are at play in addition to just LL-37 in controlling macrophage autophagy and HIV replication.

This latter study may explain why we did not observe the expected relationship between 25(OH)D and LL-37 in the HIV groups. There may be additional peptides at play that obscure this pathway, particularly in the setting of HIV infection. However, we did observe a link between LL-37 and both current CD4 counts and Δ CD4 in HIV-infected subjects who have been on ART. This is consistent with the *in vitro* data that show that LL-37 inhibits HIV replication, including in CD4⁺ cells. Studies evaluating the relationship between vitamin D status and CD4 counts have been conflicting.^{2,4,29–34} Thus, plasma concentrations of LL-37 may be a more direct link to CD4 counts than circulating concentrations of 25(OH)D.

Although we demonstrated a link between LL-37 and both current CD4 counts and Δ CD4 count in univariate analysis, only the current CD4 count remained significant after adjustment for age, sex, race, and HIV duration. Statistically, one typically adjusts for variables that are associated with the outcome (in this case, LL-37). However, it is unknown what affects LL-37 *in vivo*. Thus, we chose variables that are known to affect plasma concentrations of 25(OH)D. With this approach, Δ CD4 did not remain significant. This is likely due to the small sample size and the relatively large number of variables we included in the regression; however, it may be due to the fact that we have adjusted for the wrong variables. For example, genetic variants may also play a role.³⁵ As a

result, more studies are needed to further evaluate the relationship between LL-37, CD4 counts, immune restoration after ART, and disease progression in HIV-infected subjects. In particular, well-designed, randomized, placebo-controlled vitamin D supplementation studies in subjects with a range of CD4⁺ cell counts that include LL-37 assessments are needed to answer some of these critical questions.

It is unclear in this current study as to why the relationship between LL-37 and current CD4 counts, as well as increased LL-37 concentrations, was seen in ART-experienced, HIV-infected subjects compared to ART-naïve subjects (although this did not remain significant after adjusting for several variables, including HIV duration). One explanation may be that other factors specific to one of the HIV groups, such as the degree of immune activation, inflammation, and/or the addition of ART, change the concentrations of LL-37 and/or alter its function. For example, since certain antiretrovirals interfere with vitamin D metabolism,^{2,4,5} perhaps there is an alternate, compensatory pathway to produce adequate concentrations of LL-37. We did assess the relationship between LL-37 and CD4/CD8 ratio as a crude marker of immune activation³⁶; however, no significant relationship was seen. In future studies, evaluating additional immune activation markers, such as those expressed from monocytes/macrophages, would be important. Although there was no significant difference between the ART-experienced, HIV-infected subjects and the healthy controls, this appeared to be a function of a few outliers in the latter group. Additional studies are needed to determine if the increased plasma concentrations of LL-37 in ART-experienced, HIV-infected subjects are real and what specific causes may be at play.

There was a strong relationship between plasma concentrations of LL-37 and IL-6, but not the other measured inflammatory markers, in both the HIV groups. This was partly driven by the LL-37 outliers, but remained significant even when they were removed. Interleukin-6 is an immune protein with a wide range of functions from key roles in acute-phase protein induction to B and T cell growth and differentiation. Many of the cells that produce IL-6 also produce LL-37, including T cells, macrophages, and epithelial cells.^{37–39} Interleukin-6 has also been shown to be elevated in HIV infection²⁶. Thus, it is reasonable to think that LL-37 and IL-6 interact with one another or one affects the other in HIV. In fact, this relationship has been previously demonstrated in other populations⁴⁰ and *in vitro*.⁴¹ This is an important finding that deserves further investigation, including evaluating functional links between IL-6 and LL-37.

There are several important limitations to this study. Foremost, this was a cross-sectional study with a relatively small number of subjects, which limits the conclusions one can draw from these data. Given the small number of subjects in the ART-experienced group, it was impossible to evaluate the effects of specific antiretrovirals or ART classes on LL-37 concentrations. In addition, the subjects spanned a wide age range, including many younger and perinatally infected subjects. However, this population may be the ones who would benefit the most from interventions that would maximize immune restoration induced by ART and maintain CD4 counts, given the number of decades that they will live with HIV. Likewise, the majority of subjects were black. But, again, this population is at high risk for vitamin D deficiency and may benefit the most by optimizing vitamin D status, and

thus LL-37 concentrations. Importantly, the normal plasma concentrations for LL-37 are not currently known, and LL-37 measured in plasma may not reflect tissue or intracellular concentrations. Finally, the investigation between LL-37 and 25(OH)D maybe be incomplete since all subjects had sub-optimal concentrations of 25(OH)D.

These exploratory data are novel and shed light onto the role that LL-37 may play in HIV. LL-37 concentrations were higher in ART-experienced compared to ART-naïve subjects, and, notably, were associated with CD4 counts and immune restoration after treatment in this former group. Importantly, LL-37 was associated with the proinflammatory cytokine IL-6 in both HIV groups but not in controls, whereas LL-37 was associated with 25(OH)D in healthy controls but not in either HIV group. These data suggest that HIV infection and/or HIV-related variables may modify the expected relationship between vitamin D and LL-37. Likewise, LL-37 may affect the degree of inflammation seen in HIV-infected patients, which is known to alter the risk of many long-term HIV-related complications. More research is urgently needed to better characterize the role of LL-37 within the HIV population.

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