Involvement of the LPS-LPB-CD14-MD2-TLR4 inflammation pathway in HIV-1/HAART-associated lipodystrophy syndrome (HALS)

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Objectives: A relationship between obesity and intestinal bacterial translocation has been reported. Very little information is available with respect to the involvement of the bacterial translocation mechanistic pathway in HIV-1/highly active antiretroviral therapy (HAART)-associated lipodystrophy syndrome (HALS). We determined whether lipopolysaccharide (LPS)-binding protein (LBP), cluster of differentiation 14 (CD14), myeloid differentiation protein 2 (MD2) and toll-like receptor 4 (TLR4) single-nucleotide polymorphisms and LPS, LBP and soluble CD14 (sCD14) plasma levels are involved in HALS.

Patients and methods: This cross-sectional multicentre study involved 558 treated HIV-1-infected patients, 240 with overt HALS and 318 without HALS. Anthropometric, clinical, immunovirological and metabolic variables were determined. Polymorphisms were assessed by genotyping. Plasma levels were determined by ELISA in 163 patients (81 with HALS and 82 without HALS) whose stored plasma samples were available. Student’s t-test, one-way ANOVA, two-way repeated measures ANOVA, the χ² test and Pearson and Spearman correlation analyses were carried out for statistical analysis.

Results: LBP rs2232582 T→C polymorphism was significantly associated with HALS (P=0.01 and P=0.048 for genotype and allele analyses, respectively). Plasma levels of LPS (P<0.009) and LBP (P<0.001) were significantly higher and sCD14 significantly lower (P<0.001) in patients with HALS compared with subjects without HALS. LPS levels were independently predicted by triglycerides (P<0.001) and hepatitis C virus (P=0.038), LBP levels by HALS (P<0.001) and sCD14 levels by age (P=0.008), current HIV-1 viral load (P=0.001) and protease inhibitor use (P=0.018).

Conclusions: HALS is associated with LBP polymorphism and with higher bacterial translocation.

Keywords: genetics, microbial translocation, inflammation
Introduction
After years of intensive research, the pathogenesis of HIV-1/highly active antiretroviral therapy (HAART)-associated lipodystrophy syndrome (HALS) is not completely known. Studies have sought candidates for the factors involved, among them being the type of antiretroviral drugs used, disturbances in cytokine and adipokine synthesis, mainly produced by adipose tissue, and immune activation. Noteworthy is that some morphological traits and pathological mechanisms in HALS are reminiscent of obesity. Recent information suggests that there is a relationship between immune activation and obesity or metabolic disturbances in the general population. In this way, a close relationship exists between LPS, LBP and sCD14 (sCD14) plasma levels and immune activation, which is tempered but not fully reverted with successful HAART. An increase in cytokine synthesis, mainly produced by adipose tissue, inflamma-
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Assessment of HALS
All patients were given a full physical examination to assess the type (lipoatrophy, lipohypertrophy or mixed) and degree (slight, moderate or severe) of lipodystrophy. Criteria for lipoatrophy were one or more of the following: loss of fat from the face, arms and legs, prominent veins in the arms and legs and a thin bottom. Lipohypertrophy was defined by the presence of one or more of the following criteria: increase in abdominal perimeter, breast and/or neck fat deposition. We defined mixed lipodystrophy as being when at least one characteristic of lipoatrophy and one of lipohypertrophy were concomitantly present in a given patient. Lipodystrophy was categorized in accordance with a previously validated scale: nil (0), slight (1), moderate (2) and severe (3). Doubtful cases were excluded. This categorization was evaluated for the face, arms, legs, buttocks, abdomen, neck and breasts. The sum of the values corresponding to each body area indicated the degree of lipodystrophy: nil (0), slight (1–6), moderate (7–12) and severe (13–18). In this study we included only extreme lipodystrophy phenotypes (nil versus severe cases) in order to avoid superposition between groups. To objectively assess the distribution of visceral adipose tissue and subcutaneous adipose tissue, a single-slice CT scan was performed at the L4 level in the 558 patients included in this study. The surface of adipose tissue was measured in cm².

Laboratory methods
Blood measurements
HIV-1 infection-related parameters and plasma glucose, insulin, homeostasis model assessment for insulin resistance (HOMA-IR), total cholesterol, high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc) and triglycerides were measured as previously described.

Assessment of polymorphisms
All the single-nucleotide polymorphisms (SNPs) were validated by genotyping (KBioscience, Herts, UK) and were as follows: LBP, rs2232582 (T→C); rs2232596 (G→A) and rs2232618 (T→C); CD14, rs2569190 (A→G); TLR4, rs4986790 (A→G, Asp299Gly); rs4986791 (C→T, Thr399Ile); rs1927911 (C→T) and rs11536889 (G→C); MD-2, rs11465996 (C→G).

LPS, LBP and sCD14 plasma levels
Circulating LPS has been proposed as a direct marker of bacterial translocation and LPS levels were determined with a commercial Limulus Amoeboocyte Lysate (LAL) kit (Lonza, Spain). Plasma LBP levels were measured using the Human LBP ELISA kit (HyCult Biotechnology bv; PB Uden, The Netherlands). Plasma sCD14 was assayed with a commercially available ELISA kit on sera diluted 0.01% in duplicate wells (R&D Systems, Abingdon, UK).

Statistical analyses
Statistical analyses were carried out using the SPSS/PC+ statistical package (version 17; SPSS, Chicago, IL, USA). Prior to the statistical analyses, normal distribution and homogeneity of the variances were tested.
Normally distributed data are expressed as mean ± SD, whereas variables with a skewed distribution are expressed as IQR or transformed into a decimal logarithm. Categorical variables are expressed as number (%). Qualitative variables were analysed using the χ² test or Fisher's exact test as necessary. Student's t-test and one way ANOVA with the post hoc Bonferroni test were used to compare continuous variables between two groups and more than two groups, respectively; to compare variables that did not have a Gaussian distribution we used the Mann–Whitney U-test and Kruskal–Wallis test. CD4+ T cell variation or recovery and the HIV-1 plasma viral load between two timepoints, pre-HAART and current, were analysed using two-way repeated measures ANOVA. Associations between quantitative variables were evaluated using Pearson correlation analysis or the Spearman correlation for non-normally distributed variables. The independence of associations was evaluated by linear regression analysis. In all statistical tests, a P value < 0.05 was considered significant.

Results

Characteristics of study population

Table 1 and Table S1 (available as Supplementary data at JAC Online) show demographic, clinical and metabolic characteristics of the patients studied categorized according to the presence or absence of HALS. All patients with HALS had marked lipoatrophy that was severe enough to be treated with facial implants and most had also lipohypertrophy.

Genetic association study

Data are shown in Table 2. Distributions of genotypes and allele frequencies of all SNPs assessed were in accordance with the expected Hardy–Weinberg equilibrium. There was an association between HALS and the polymorphism of the PTPN2 gene (rs564288 C>T).

Table 1. Demographic and clinical characteristics of the patients studied, categorized according to the presence or absence of HALS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-HALS (n=318)</th>
<th>HALS (n=240)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43.5 ± 9.5</td>
<td>45.5 ± 9.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>227 (71.4)</td>
<td>171 (71.3)</td>
<td>1</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.9 ± 3.1</td>
<td>23.6 ± 2.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Waist–hip circumference ratio</td>
<td>0.89 ± 0.09</td>
<td>0.93 ± 0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Subcutaneous abdominal fat tissue (cm²)a</td>
<td>128.9 ± 61.3</td>
<td>50 ± 30.7</td>
<td>0.004</td>
</tr>
<tr>
<td>Visceral abdominal fat tissue (cm²)a</td>
<td>37.2 ± 27</td>
<td>110.2 ± 68</td>
<td>0.01</td>
</tr>
<tr>
<td>HCV infection, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homosexual</td>
<td>111 (34.9)</td>
<td>76 (31.7)</td>
<td>0.4</td>
</tr>
<tr>
<td>heterosexual</td>
<td>93 (29.2)</td>
<td>81 (33.8)</td>
<td>0.35</td>
</tr>
<tr>
<td>injection drug user</td>
<td>114 (35.8)</td>
<td>77 (32.1)</td>
<td>0.35</td>
</tr>
<tr>
<td>other/unknown</td>
<td>0</td>
<td>6 (2.5)</td>
<td>0.004</td>
</tr>
<tr>
<td>Duration of HIV infection (years)</td>
<td>11.7 ± 5.7</td>
<td>13.7 ± 5.9</td>
<td>0.002</td>
</tr>
<tr>
<td>AIDS, n (%)</td>
<td>85 (26.7)</td>
<td>118 (49.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4 cell count (cells/mm³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-HAART</td>
<td>361 ± 267</td>
<td>346 ± 257</td>
<td>0.68</td>
</tr>
<tr>
<td>current</td>
<td>474 ± 297</td>
<td>560 ± 308</td>
<td>0.04</td>
</tr>
<tr>
<td>Log plasma HIV-1 load (copies/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-HAART</td>
<td>4.4 ± 1.2</td>
<td>4 ± 1.5</td>
<td>0.03</td>
</tr>
<tr>
<td>current</td>
<td>2.6 ± 1.2</td>
<td>2.2 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current plasma HIV-1 load &lt;200 copies/mL, n (%)</td>
<td>208 (65.4)</td>
<td>189 (78.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Exposure to NRTI before HAART, n (%)</td>
<td>226 (71.1)</td>
<td>198 (82.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Duration of HAART (months)</td>
<td>81 (37–117)</td>
<td>66 (44–126)</td>
<td>0.9</td>
</tr>
<tr>
<td>NNRTI consumption, n (%)</td>
<td>300 (94.3)</td>
<td>239 (99.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cumulative time on NNRTI (months)</td>
<td>128 (72–168)</td>
<td>159 (98–180)</td>
<td>0.05</td>
</tr>
<tr>
<td>Cumulative time on NNRTI, n (%)</td>
<td>220 (69.2)</td>
<td>168 (70)</td>
<td>0.8</td>
</tr>
<tr>
<td>Cumulative time on NNRTI (months)</td>
<td>21 (11–36)</td>
<td>26 (15–54)</td>
<td>0.008</td>
</tr>
<tr>
<td>PI consumption, n (%)</td>
<td>251 (78.9)</td>
<td>217 (90.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cumulative time on PI (months)</td>
<td>47 (27–88)</td>
<td>55 (32–84)</td>
<td>0.15</td>
</tr>
<tr>
<td>Zidovudine consumption, n (%)</td>
<td>225 (70.8)</td>
<td>186 (77.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cumulative time on zidovudine (months)</td>
<td>45 (24–80)</td>
<td>41 (10–61)</td>
<td>0.03</td>
</tr>
<tr>
<td>Stavudine consumption, n (%)</td>
<td>156 (49.1)</td>
<td>196 (81.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cumulative time on stavudine (months)</td>
<td>37 (19–72)</td>
<td>39 (24–60)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or as median and IQR; however, qualitative variables are expressed as n (%).

aAssessed by CT scan at the L4 level.
between the LBP rs2232582 T→C SNP and HALS, carriers of the T allele being significantly overrepresented in HALS patients in both genotype and allele analyses. There was a marginal but significant association between the CD14 rs2569190 A→G SNP and HALS, but in the genotype analysis only. LBP and sCD14 plasma levels were not influenced by LBP and CD14 polymorphism, respectively, either in the whole cohort or in the HALS and non-HALS subsets.

**Plasma levels of LPS, LBP and sCD14**

**HALS study**

Figure 1 shows the values of circulating LPS, LBP and sCD14 in the assessed patients categorized according to the presence or absence of lipodystrophy. Patients with HALS had significantly higher LPS and LBP levels and significantly lower circulating sCD14 levels compared with patients without HALS.

**Correlations with metabolic and immunovirological parameters**

We assessed the correlations between LPS, LBP and sCD14 and total cholesterol, LDLc, HDLc, triglycerides, glycaemia, insulin, HOMA-IR, pre-HAART and current plasma viral load, pre-HAART and current CD4+ T cell count, and CD4+ T cell gain due to HAART. Each correlation was assessed in the whole cohort and in the subsets with and without HALS separately. Correlations with metabolic data are shown in Tables S2, S3 and S4 (available...
as Supplementary data at JAC Online). Correlations with immunovirological data are shown in Tables S5, S6 and S7 (available as Supplementary data at JAC Online).

Relationship between LPS, LBP and sCD14

In the whole cohort, there was a correlation between plasma LPS and LBP levels ($r=0.28$, $P<0.001$) and between plasma LBP and sCD14 levels ($r=-0.21$, $P=0.008$). In patients with HALS there was a correlation between plasma LBP and sCD14 ($r=0.24$, $P=0.03$), and in the non-HALS subset there was a correlation between plasma LPS and LBP ($r=0.33$, $P=0.002$).

Regression analyses

To investigate the strength of the associations, we constructed linear regression analyses considering the levels of LPS, LBP and sCD14 as dependent variables and including the above-mentioned bivariate correlations, adjusting for age and gender. For LPS, the model had a multiple correlation coefficient of $R=0.73$ and plasma LPS levels were mainly predicted by triglycerides ($B=0.05; P<0.001$) and hepatitis C virus (HCV) ($B=-0.09; P=0.04$). For LBP, the model had a multiple correlation coefficient of $R=0.64$ and plasma LBP levels were independently predicted by the presence of HALS ($B=0.74, P<0.001$). For sCD14, the model had a multiple correlation coefficient of $R=0.61$ and plasma sCD14 levels were predicted by age ($B=-0.01; P=0.008$), PI consumption ($B=-0.41, P=0.018$) and current viral load ($B=0.11; P=0.001$).

Discussion

This is the first report to assess the perturbations of both circulating and gene polymorphisms of the members of the LPS–LPB–CD14–MD2–TLR4 complex together in treated HIV-1-infected patients with HALS. We found that the LBP rs2232582 C→T and CD14 rs2569190 A→G SNPs were associated with HALS. LBP and CD14 genotypes did not modulate the plasma levels of the molecules for which they respectively encode. In patients with HALS there was an excess of circulating LPS and LPB while in subjects without HALS circulating sCD14 levels were lower. The strongest independent predictors of plasma LPS were triglycerides and HCV. HALS was the unique independent predictor of circulating LBP levels and sCD14 levels were independently determined by age, PI consumption and plasma HIV-1 RNA levels.

Since not all treated HIV-1-infected patients develop HALS, a host genetic individual vulnerability has been proposed. In this study we have assessed the effect of LBP, CD14, MD2 and TLR4 genetic variants. The rationale behind this study is the involvement of these genes in obesity and HALS is reminiscent of obesity. Our data indicate that the LBP rs2232582 C→T SNP may be implicated in HALS, carriers of the T allele having greater prevalence of HALS; carriage of the C allele could therefore partially protect against HALS. Note that our finding of a positive association regarding LBP polymorphism is likely to be further replicated since it is based on double contrasts (genotype and allele analyses). We also found a marginal association between polymorphism in the CD14 gene and HALS, but in the genotype analysis only. No association with MD2 and TLR4 polymorphisms and HALS was observed, confirming previous investigations.

A question arises as to which pathophysiological mechanisms could explain the association between LBP genetic variants and HALS. We have shown here that HALS patients have high systemic LBP levels, but these do not seem to be under genetic control in our patients. Hence, the mechanistic pathways that could explain the association between LBP gene variants and HALS remain obscure and demand further research. Potential explanations are an impairment of LBP function by the T to C mutation and that patients with the TT genotype are simply much better able to collect LPS and bring it to TLR4, thus amplifying the effects of circulating LPS.

An additional finding in our study is that HALS patients have high circulating LPS and LBP and low sCD14 levels. Inflammation biomarkers such as LPS and LBP have been studied in the HIV-1 setting but reports have focused on immunopathogenesis and immune activation and on several clinical outcomes and co-morbidities. Untreated HIV-1-infected patients have subclinical inflammation and the inflammatory parameters tend to improve when on HAART, but normal levels are usually not reached, even in patients who achieve virological suppression. Innovative investigations from Koethe et al. showed that HIV-1-infected individuals on HAART who were overweight had lower sCD14 plasma levels compared with infected patients of normal weight. Our data agree with this, since we found that our HALS subset also had significantly lower circulating sCD14 levels. The reason for the decrease in sCD14 in these patients is not clear but it could perhaps be related to CD14 down-regulation as a protective mechanism against excess of LPS and LBP. New information from our study is that individuals with an extreme HALS phenotype have high intestinal bacterial translocation, which is highlighted by the fact that the most robust independent determinant of LBP levels in our study was the presence of HALS. The origin of the increased LBP overproduction in these patients seems to be the...
adipose tissue, as has been demonstrated in obese subjects and in a small cohort of HIV-1-infected patients with HALS \cite{16,17} and this reinforces the strong interactions between adipose tissue and immune function in HIV-1-infected subjects.\cite{35,36} This is highlighted by the finding that in our HALS patients plasma levels of both LPS and LBP were positively correlated with pre-HAART HIV-1 viral load and negatively correlated with CD4+ T cell count. This could reflect a higher degree of inflammation and of immune activation in these patients.

Overall, it should be noted that there was a discrepancy in the presence of an association between rs2232582 T→C and HALS, an association between LBP levels and HALS, but the lack of association between rs2232582 T→C and LBP. Despite this inconsistency, our findings may stimulate further research following this line. It could be useful to assess the relationship between the polymorphisms (and the circulating molecules) in the LPS pathway and downstream inflammatory mediators such as IL-6 and TNF-α. Also, the assessment of the effect on adipose tissue biology related to peroxisome proliferator-activated receptor-γ (PPAR-γ), lipid storage and release, inflammatory cell infiltration, fibrosis, and cytokine/adipokine gene expression should be of interest.

Our study has some limitations. Firstly, the cross-sectional nature of our design provides associations but not causality. Secondly, our genetic association findings need further replication. We believe, however, that our results are of value given the large number of patients studied, the good categorization of extreme phenotypes and the fact that our positive association findings are based on two genetic contrasts. Finally, we acknowledge that the multiple comparisons performed in this study make some of the marginal associations found less likely to be real.

In summary, when taken together our data suggest that genetic variability within the LBP gene may be associated with HALS. There is a strong relationship between HALS and systemic inflammation, and the presence of HALS predicts circulating LBP levels independently. These findings may open a new and interesting mechanistic pathway for investigating the causes of HALS.

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Transparency declarations
None to declare.

Supplementary data
Figure S1 and Tables S1 to S7 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References