Characterization of DC-SIGN binding glycoproteins and the role in HIV-1 infection
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HIV-1 disease progression is associated with bile-salt stimulated lipase (BSSL) gene polymorphism

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ABSTRACT

Objective: BSSL (bile-salt stimulated lipase) binds to DC-SIGN and CXCR4 and in vitro DC-SIGN binding properties of BSSL are associated with a polymorphism in the repeated motif in exon 11 of the BSSL gene. This study investigates the relation between identified BSSL polymorphisms and HIV-1 infection and disease course.

Design: Relation between BSSL polymorphism and HIV-1 transmission and disease course was studied in a cohort of men having sex with men including 334 seropositive and 48 high risk seronegative individuals participating in the Amsterdam Cohort Studies on HIV infection and AIDS.

Methods: We used PCR to determine the number of repeats encoded by BSSL exon 11 for the men included in our cohort. Pearson Chi-square test was used to determine the relation of BSSL genotype distributions between selected groups. The relation between BSSL genotype and disease progression and the emergence of CXCR4-using variants was studied using Kaplan Meier and multivariate Cox proportional hazard analysis. We analyzed the association of BSSL genotype with CD4 cell count, both pre-infection and post-infection at viral setpoint. In addition, we evaluated a possible association of the BSSL genotype with the setpoint viral load.

Results: We found a correlation between the homozygosity for the high (H) repeat number BSSL genotype (HH) and high CD4 cell numbers prior to infection (p=0.007). In HIV-1 patients, slow disease progression was linked to the HH BSSL genotype (p=0.049, RH=0.737) as was the delayed emergence of CXCR4-using HIV-1 variants (p=0.007, RH=0.515). The BSSL genotype that was most prevalent in patients with slow disease progression (HH) was also dominant in high risk seronegative individuals.

Conclusion: Our research identifies BSSL as a marker for HIV-1 disease progression and CD4 cell homeostasis.
INTRODUCTION

Millions have been infected worldwide with human immunodeficiency virus type-1 (HIV-1) but major differences exist between individuals in infection risk and rate of disease progression. These differences can be attributed in part to polymorphisms in host genes encoding HIV-1 co-receptors such as CCR5 and CCR2 and their ligands (Paxton & Kang, 1998; Smith et al., 1997; Winkler et al., 1998; Liu et al., 1999; Berger et al., 1999). In addition, carrying human leukocyte antigen (HLA) variants B27 or B57 is associated with slower HIV-1 disease progression probably through HIV-1 specific CD8+ T-lymphocyte responses (Gao et al., 2005). Knowledge of host genetic polymorphisms that affect HIV-1 pathogenesis has helped with the successful development of antiretroviral drugs and ways to modulate beneficial immune responses. Due to therapy failure and incomplete virus suppression, there is a need for identification of novel drug targets for the treatment of HIV-1 infection.

Among cell types targeted by HIV-1 are dendritic cells (DCs), which play a central role in the protection against infections and the activation of anti-microbial immune responses (Haase, 2005). DCs capture micro-organisms aimed at degrading these pathogens and present their antigens to resting T-lymphocytes thereby activating adaptive immunity. Interaction between HIV-1 and DCs in all likelihood will occur at sites where mucosa is breached when individuals are exposed to HIV-1. DCs efficiently capture HIV-1 but captured HIV-1 partly escapes from degradation and fully infectious virus particles are subsequently presented to CD4+ T-lymphocytes, thereby efficiently infecting these target cells (trans-infection) (Geijtenbeek et al., 2000).

DC-specific intercellular adhesion molecule-3 grabbing non integrin (DC-SIGN) is the major HIV-1 binding receptor expressed by immature DCs used for trans-infection. DC-SIGN forms tetramers that bind terminal fucoses and high mannose structures of “self” antigens and pathogen glycans and recognizes a wide range of micro-organisms (van Liempt et al., 2004; van Liempt et al., 2006; van Kooyk & Geijtenbeek, 2003; Guo et al., 2004). HIV-1 binds to DC-SIGN and uses the pathogen receptor for trans-infection but, in addition, DC-SIGN binding of HIV-1 results in modulation of DC immune signaling (Gringhuis et al., 2009). DC-SIGN is also required for the formation of the infectious synapse between DC and T-lymphocyte during trans-infection of CD4+ T-lymphocytes with HIV-1 and DC-SIGN binding of neutralized virus can result in removal of neutralizing antibodies (Arrighi et al., 2004; van Montfort et al., 2007). Human DC-SIGN gene polymorphisms associate with the efficiency of HIV-1 transmission and the rate of disease progression and decreased DC-SIGN expression in Rhesus Macaques is associated with accelerated disease progression (Liu et al., 2004; Koizumi et al., 2007; Yearley et al., 2008). Whether DC-SIGN ultimately limits or accelerates human disease progression remains unclear, but obviously factors that competitively bind with HIV-1 to DC-SIGN will influence its function.
Among molecules that bind to DC-SIGN are mucin 6 in seminal plasma and bile-salt stimulated lipase (BSSL, BSDL, CEL) as well as mucin 1 identified in human milk (Stax et al., 2009; Naarding et al., 2006; Saeland et al., 2009; Naarding et al., 2005). BSSL is a dimeric glycoprotein that is abundantly expressed in milk but, in addition, is also expressed in blood (Panicot-Dubois et al., 2007; McKillop et al., 1998). The presence of DC-SIGN blocking molecules in semen and breast milk may, at least in part, influence HIV-1 transmission. This could help explain the relatively low risk of HIV-1 infection during sex or breastfeeding (O’Brien et al., 1994; Varghese et al., 2002; Shapiro et al., 2009; Jin et al., 2010). We previously identified that breast milks derived from different mothers do not have equal DC-SIGN binding properties suggesting mother-dependent protection levels for HIV-1 transmission during breastfeeding (Naarding et al., 2006). In addition to DC-SIGN binding, BSSL from blood can bind to chemokine receptor CXCR4 (Panicot-Dubois et al., 2007; Aubert-Jousset et al., 2004) suggesting a potential supplementary role for BSSL in HIV-1 pathogenesis.

Previous studies in our lab demonstrated that the DC-SIGN binding capacity of milk is associated with variation in the BSSL repeat domain (chapter 3). Aim of the present investigation is to determine the relation between variation in the BSSL genotype (repeat domain) and HIV-1 infection and disease progression for men having sex with men (MSM) participating in the Amsterdam Cohort Studies on HIV infection and AIDS (ACS). Our research demonstrates that both slow HIV-1 disease progression and emergence of delayed CXCR4-using HIV-1 variants are correlated with homozygosity for the high (H) repeat number BSSL genotype (HH). The BSSL genotype that is dominant in patients with slow disease progression (HH) corresponds to the main genotype observed in high risk seronegative individuals. In addition, our study reveals that the BSSL HH genotype correlates with high CD4 cell numbers prior to infection. Our research provides new insights in the role of BSSL in HIV-1 pathogenesis with the potential for BSSL as a new target for anti-HIV-1 drug development.

**RESULTS**

Highly exposed seronegative individuals and slow progressing seropositives have comparable BSSL genotype distributions. We genotyped the variable number of tandem repeat (VNTR) domain of the BSSL gene in a cohort of 334 HIV-1 seropositive men and 48 seronegative men with high risk behavior (high risk seronegatives, HRSN). We found that the number of repeats in this domain is highly variable ranging from 10 to 18 in seropositive individuals (Figure 2A+B). Furthermore we observed that 82% within this group carry at least one allele with 16 repeats, similar to the situation in previously genotyped HIV-1 negative mothers in a study on BSSL in human milk (chapter 3). BSSL repeat numbers in HRSN range from 5-17 with 85% having at least one allele with 16 repeats (Figure 2). As compared to seropositives, a trend was observed with HRSN having more frequently two alleles with 16 repeats (p=0.059).
We hypothesized that specific BSSL genotypes might provide a certain level of protection against HIV-1 infection. To test this we categorized individuals in this study based on number of repeats in the BSSL genotype. We defined 16 to 18 repeats as high (H) and less than 16 repeats as low (L) repeat numbers. Individuals were categorized in 3 groups having either two low (LL), one low and one high (LH) or two high repeat number alleles (HH). HH is the major genotype whereas the LL genotype reaches relatively low numbers in both HRSN and seropositives. HRSN and seropositive individuals were not statistically different for the HH genotype (Figure 1, p=0.190).

Disease progression was defined as the time between HIV-1 infection and diagnosis of AIDS or when CD4 counts dropped below 200 cells/µl. Within the group of 334 seropositive individuals we arbitrarily defined the 50 patients with fastest disease progression as fast progressors and 50 patients with the slowest disease progression as slow progressors (Table 1). Figure 1 demonstrates that HRSN men and seropositive men with slow disease progression have similar BSSL genotype distributions. The BSSL genotype distribution in HRSN individuals is significantly different from HIV-1 patients with fast disease progression (Figure 1, p=0.028), with a higher frequency of the HH genotype in these HRSN individuals.

**Figure 1:** BSSL genotype distribution in high risk seronegative (HRSN) individuals and HIV-1 positive individuals. Within the group of HIV-1 positives, 50 individuals with fastest (mean time to AIDS or CD4 cell count <200 cells/µl=2.18±0.2 year) and 50 with slowest (mean time to AIDS or CD4 cell count <200 cells/µl=13.94±0.7 year) disease progression were compared. HH genotype is significantly more prevalent in slow progressors when compared to fast progressors (p=0.015). BSSL genotype distribution in HRSN is similar to genotype distribution in 50 slowest progressors but significantly different compared to genotype distribution in 50 fastest progressors (p=0.028).

**BSSL number of repeats correlates with HIV-1 disease progression.** We compared the number of repeats in the VNTR domain of BSSL for fast progressors to that of slow progressors (Figure 2C+D). The distribution of genotypes is significantly different between the fast and slow progressors (p=0.036). Since the LL genotype reaches relatively low frequencies (Figures 1) we compared the major (HH) genotype with the combined LL with LH genotype (LL+LH) for Kaplan Meier survival - and Cox regression analysis. We observed a trend towards delayed disease progression in patients with the HH genotype (Figure 3A, Table 2, log rank p=0.056). Subsequent multivariate Cox proportional hazard analysis including survival markers CCR5-Δ32, HLA-B57 and HIV-1 viral load below 10^4.5 copies per ml plasma and CD4 levels above 500 cells per µl blood at viral setpoint demonstrated that the HH genotype is independently associated with slower disease progression (Table 2, p=0.049, RH=0.737). The effect of the HH genotype on disease progression appeared
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non-proportional and was observed from 6 years after seroconversion onwards. When Kaplan Meier and Cox proportional hazard analysis was performed after left-truncation of the first 6 years post-seroconversion, a strong association between the HH genotype and delayed disease progression was observed (p=0.007, RH=0.518, Table 3). Multivariate analysis demonstrated that the predictive value of the HH BSSL genotype for prolonged survival in this analysis was independent of the CCR5-Δ32 genotype (Table 3).

Table 1: Characteristics of HIV-1 positive groups including slow progressors, fast progressors and whole group.

<table>
<thead>
<tr>
<th></th>
<th>Age at seroconversion</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Time to AIDS</th>
<th>CD4 setpoint</th>
<th>Viral load setpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (year)</td>
<td>Range (year)</td>
<td>Male</td>
<td>Female</td>
<td>Caucasian</td>
<td>Non-caucasian</td>
</tr>
<tr>
<td>Fast progressors</td>
<td>36.8</td>
<td>22.9-53.6</td>
<td>50</td>
<td>0</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>Slow progressors</td>
<td>32.4</td>
<td>21.8-43.1</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>All seropositives</td>
<td>34.9</td>
<td>19.6-55.6</td>
<td>334</td>
<td>0</td>
<td>315</td>
<td>19</td>
</tr>
</tbody>
</table>
*SD: standard deviation

Figure 2: Allele frequency (z-axis) of BSSL repeat numbers for both alleles (x-axis and y-axis). The number of repeats in the BSSL gene is highly variable with most patients having at least 1 allele with 16 repeats. Genotype distribution in (A) seropositive individuals, (B) high risk seronegatives (HRSN), (C) 50 patients with fastest disease progression and (D) 50 patients with slowest disease progression.
Emergence of CXCR4-using variants is associated with the number of repeats in BSSL. DC-SIGN differentially enhances in-trans infection with CCR5 (RS) and CXCR4 using (X4) HIV-1 and BSSL interacts with CXCR4 (van Montfort et al., 2008; Panicot-Dubois et al., 2007). We hypothesized that size variation in the BSSL VNTR domain could influence the time to CXCR4-using variant emergence during disease progression. CXCR4-using viruses emerge significantly later in patients with the HH than in the other patients (log rank \( p=0.042 \), Figure 3B). Again the effect of the HH BSSL genotype was non-proportional and a protective effect was observed from 5 years after seroconversion onwards. Emergence of CXCR4-using variants was also studied using multivariate Cox proportional hazard analysis including other markers associated with disease progression and potentially the emergence of CXCR4-using variants (HLA-B57, viral load – and CD4 counts at viral setpoint). This analysis confirmed that the HH genotype associates independently with delayed emergence of CXCR4-using variants (\( p=0.007 \), RH=0.515, Table 4).

### Table 2: Multivariate Cox proportional hazard analysis from 2 years after seroconversion to AIDS or CD4 cell counts below 200 cells per µl blood for patients with the HH genotype when compared to the patients with non-HH genotypes (LL+LH). Patients with the HH genotype show a strong trend towards delayed disease progression (log rank \( p=0.056 \)).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number(^1)</th>
<th>Event(^2)</th>
<th>Crude p-value</th>
<th>RH(^3)</th>
<th>Adjusted p-value</th>
<th>RH(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSSL HH vs LL+LH</td>
<td>304</td>
<td>185</td>
<td>0.092</td>
<td>0.776</td>
<td>0.049</td>
<td>0.737</td>
</tr>
<tr>
<td>CCR5-∆32 genotype vs WT(^4)</td>
<td>303</td>
<td>184</td>
<td>&lt;0.001</td>
<td>0.405</td>
<td>&lt;0.001</td>
<td>0.428</td>
</tr>
<tr>
<td>CD4 cells &gt;500 cells per µl</td>
<td>296</td>
<td>182</td>
<td>&lt;0.001</td>
<td>0.477</td>
<td>0.002</td>
<td>0.615</td>
</tr>
<tr>
<td>Viral RNA load &lt;10^4 copies per ml</td>
<td>298</td>
<td>180</td>
<td>&lt;0.001</td>
<td>0.370</td>
<td>&lt;0.001</td>
<td>0.440</td>
</tr>
<tr>
<td>HLA-B57</td>
<td>304</td>
<td>185</td>
<td>0.003</td>
<td>0.288</td>
<td>0.039</td>
<td>0.418</td>
</tr>
</tbody>
</table>

\(^1\)number of individuals included in the analysis
\(^2\)number of individuals that reach the end point
\(^3\)relative hazard
\(^4\)wild type CCR5 genotype

Figure 3: Kaplan Meier analysis of patients with HH and non-HH (LL+LH) genotypes. (A) Using AIDS or CD4 cells below 200 cells/µl as an endpoint or (B) using first detection of CXCR4-using HIV-1 variants as an endpoint.
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We studied the relation between BSSL genotype and CD4 cell decline during disease progression, and the viral load and CD4 cell count at viral setpoint, but did not find significant differences between patients with different BSSL genotypes (data not shown). However, when evaluating the CD4 cell numbers prior to infection, we found that the HH genotype is associated with elevated levels of CD4 cells (p=0.007, Figure 4).

**Table 3:** Cox proportional hazard analysis from 6 years after seroconversion to AIDS or CD4 cell counts below 200 cells per µl blood for patients with the HH genotype when compared to the patients with non-HH genotypes (LL+LH). HH genotype is associated with slower disease progression (log rank \( p=0.005 \)).

<table>
<thead>
<tr>
<th></th>
<th>Crude</th>
<th>Adjusted</th>
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<tbody>
<tr>
<td></td>
<td>Number (^1)</td>
<td>Event (^2)</td>
</tr>
<tr>
<td>BSSL HH vs LL+LH</td>
<td>169</td>
<td>77</td>
</tr>
<tr>
<td>CCR5-(\Delta32) genotype vs WT(^4)</td>
<td>168</td>
<td>76</td>
</tr>
<tr>
<td>CD4 cells &gt;500 cells per µl</td>
<td>163</td>
<td>75</td>
</tr>
<tr>
<td>Viral RNA load &lt;10(^{+5}) copies per ml</td>
<td>167</td>
<td>76</td>
</tr>
<tr>
<td>HLA-B57</td>
<td>169</td>
<td>77</td>
</tr>
</tbody>
</table>

\(^1\) number of individuals included in the analysis
\(^2\) number of individuals that reach the end point
\(^3\) relative hazard
\(^4\) wild type genotype
\(^5\) Not significant

**BSSL genotype is associated with CD4 cell numbers in uninfected individuals.**

We studied the relation between BSSL genotype and CD4 cell decline during disease progression, and the viral load and CD4 cell count at viral setpoint, but did not find significant differences between patients with different BSSL genotypes (data not shown). However, when evaluating the CD4 cell numbers prior to infection, we found that the HH genotype is associated with elevated levels of CD4 cells (p=0.007, Figure 4).

**Table 4:** Multivariate Cox proportional hazard analysis from 2 years after seroconversion to emergence of CXCR4-using variant for patients with the HH genotype when compared to patients with non-HH genotypes (LL+LH). HH genotype is associated with delayed emergence of CXCR4 using HIV-1 variants (log rank \( p=0.042 \)).

<table>
<thead>
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<th></th>
<th>Crude</th>
<th>Adjusted</th>
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<tbody>
<tr>
<td></td>
<td>Number (^1)</td>
<td>Event (^2)</td>
</tr>
<tr>
<td>BSSL HH vs LL+LH</td>
<td>261</td>
<td>81</td>
</tr>
<tr>
<td>Viral RNA load &lt;10(^{+5}) copies per ml</td>
<td>258</td>
<td>81</td>
</tr>
<tr>
<td>HLA-B57</td>
<td>261</td>
<td>81</td>
</tr>
<tr>
<td>CD4 cell &gt;500 per µl</td>
<td>257</td>
<td>80</td>
</tr>
<tr>
<td>CCR5-(\Delta32) genotype vs WT(^4)</td>
<td>260</td>
<td>81</td>
</tr>
</tbody>
</table>

\(^1\) number of individuals included in the analysis
\(^2\) number of individuals that reach the end point
\(^3\) relative hazard
\(^4\) wild type genotype
\(^5\) Not significant
DISCUSSION

In this study we demonstrate that the rate of HIV-1 disease progression is associated with BSSL polymorphisms in a cohort of MSM. BSSL genes have either a high (H=16 to 18) or a low (L<16) number of repeats in their variable number of tandem repeat (VNTR) domain. Individuals can be categorized into having two low (LL), one low and one high (LH) or two high (HH) repeat number BSSL alleles. Our study reveals that uninfected individuals with the HH genotype have elevated CD4 cell numbers compared to individuals carrying the other genotypes. Once infected with HIV-1, MSM with the HH genotype progress to disease more slowly and show delayed development of CXCR4-using HIV-1 variants. The BSSL genotype distribution in slow progressors and HRSN is similar and significantly different from rapid progressors.

Since differences in DC-SIGN binding properties of human breast milk link to differently sized BSSL forms (chapters 2 and 3), we hypothesized that BSSL expressed in blood behaves in a similar fashion. We demonstrate here that variation in the number of repeats encoded by the BSSL gene is indeed linked to disease progression in HIV-1 infected MSM. This could be the result of differences in DC-SIGN binding by differently sized BSSL forms. The HH genotype is associated with decreased DC-SIGN binding capacity of human milk (chapter 3) and in the present study we found that this low DC-SIGN binding genotype associates with slower disease progression. This could imply that higher DC-SIGN availability for HIV-1 capture limits disease progression in HIV-1 infected individuals similar to the situation with high DC-SIGN expression in Rhesus macaques. But care should be taken when translating the DC-SIGN binding properties of BSSL from milk into those of BSSL in blood since post-translational modifications such as glycosylation, essential for DC-SIGN binding, may differ significantly.

In addition to an effect of the BSSL genotype on disease progression, we observed a delay in the emergence of CXCR4-using HIV-1 variants is associated with the HH genotype. Both disease progression and emergence of CXCR4-using variants are delayed in patients with the HH genotype, although this might in part reflect the relation between the emergence of CXCR4-using variants and disease progression. Much of the damage related to HIV-1 infection including rapid depletion of CD4 cell numbers in the gut occurs early in
infection whereas clinical manifestation can be detected years later. This may explain why differences in CD4 cell numbers between carriers of different BSSL genotypes already exist prior to seroconversion while the effects of these genotypes on the emergence of CXCR4-using variants and disease course are only observed 6 years after infection. Furthermore, if BSSL indeed interacts with CXCR4 resulting in differential emergence of CXCR4-using variants it is most likely that the effects appear later in disease.

DC-SIGN differentially enhances trans-infection with R5 and CXCR4-using virus and the formation of infectious synapses between DCs and T-lymphocytes and subsequent T-cell stimulation (van Montfort et al., 2008; Arrighi et al., 2004). Furthermore, CXCR4 not only acts as co-receptor for CXCR4-using HIV-1 infection but is also involved in the regulation of T-cell migration, proliferation and differentiation (Berger et al., 1999; Moser & Loetscher, 2001; Wu & Yoder, 2009). BSSL interacts with both DC-SIGN and CXCR4 although not via the same structural domain (Naarding et al., 2006; Panicot-Dubois et al., 2007). Differences in BSSL genotypes in all likelihood translate into BSSL proteins with different DC-SIGN or CXCR4 binding properties and related modulation of DC-SIGN and CXCR4 roles in disease progression and CXCR4-using variant emergence. However, more research is needed to identify whether disease progression and emergence of CXCR4-using variants are similarly influenced by these interactions. In addition, differences in the interaction between BSSL and CXCR4 in individuals with different BSSL genotypes might potentially influence CD4 cell homeostasis.

The interaction between BSSL and CXCR4 was proposed to be mediated through the V3 like domain of BSSL which is not located in the VNTR region (Aubert-Jousset et al., 2004). This suggests that the potential influence of BSSL size variation on the interaction between BSSL and CXCR4 could be indirect, for example by influencing the accessibility of the V3 like domain to CXCR4. In addition, there may also be structural constraints that influence plasma concentrations of the different BSSL forms. In vitro studies with differently sized BSSL proteins should provide a better insight in the role of the VNTR in the interaction between BSSL and CXCR4.

The BSSL genotype distribution in HRSN was similar to that of the slow progressors with a relative abundance of the HH genotype. In contrast, the HH genotype was significantly more abundant in the HRSN group when compared to seropositive men with fast disease progression. We suggest that the HH genotype provides partial protection against infection possibly related to heightened peripheral blood CD4 cell numbers in carriers of this genotype.

Taken together, we have identified BSSL as a marker for HIV-1 disease progression, emergence of CXCR4-using viruses and CD4 cell count in blood of uninfected individuals. Further investigation should be aimed at characterizing the effects of differently sized BSSL proteins on the interaction between DC-SIGN and serum derived BSSL. In addition, the binding between BSSL and CXCR4 should be further characterized including the effect of such binding on infection with CXCR4-using variants, CXCR4 signaling and CD4+
T-lymphocyte proliferation and migration. These studies may ultimately increase our understanding of the role of BSSL in HIV-1 pathogenesis.

**MATERIAL AND METHODS**

**Study participants.** Study participants were 335 HIV-1 seropositive men and 48 men with high risk behavior but not infected with HIV-1 participating in the Amsterdam cohort studies on HIV infection and AIDS as previously described (van Manen et al., 2008). PCR failed with one of the HIV-1 positive individuals and this sample was therefore excluded from further analysis. Kaplan Meier and Cox regression analysis was terminated when the minor genotype LL reached n=10. Since the introduction of HAART in the Netherlands in 1996 will likely influence disease course and emergence of CXCR4-using variants in infected individuals the censor data were set at 1-1-1996 thereby excluding post-HAART era data points. The ACS has been conducted in accordance with the ethical principles set out in the declaration of Helsinki and written informed consent is obtained prior to data collection. The study was approved by the Amsterdam Medical Center institutional medical ethics committee.

**Determination of disease progression.** The rate of disease progression was defined as the time between seroconversion and AIDS diagnosis or the time point CD4 cell number fell below 200 cells/µl according to the 1993 CDC definition.

**Viral load and CD4 cell count.** Viral load and CD4 cell counts from individuals in the study population were described previously (van Manen et al., 2009). A value for CD4 cell count pre seroconversion was determined only when three or more points were available. CD4 cell count pre seroconversion was defined by the mean value of all the available measurements when HIV-1 negative.

**Genotyping PCR.** Primers specific for BSSL were designed (forward primer: ACC AAC TTC CTG CGC TAC TGG ACC CTC, reverse primer: TGA TAC CAA GGC TCA TGG GAC GCT AAA AC) containing a FAM label for detection. After initial denaturation for 4' at 94° C the following PCR program was run for 35 cycles: 30'' 94° C, 3' 60° C, 1' 72° C followed by an extended elongation for 7' at 72° C. The PCR product was mixed with the Genescan™ – 1200 LIZ® Standard (Applied Biosystems; catalog#: 4379950) and the ABI 3100 capillary sequencer (Applied Biosystems) in Genescan mode was used for PCR product size determination. Data was analyzed using Genemapper software (Applied Biosystems).

**Statistics.** Kaplan Meier and multivariate Cox proportional hazard analysis were performed to study the relation between BSSL genotype and disease progression and the emergence of CXCR4-using variants. Pearson Chi-square exact test was used to study the relation of BSSL genotype distributions between selected groups. The effect of the genotype on CD4 cell count pre seroconversion, CD4 setpoint and viral load setpoint was analyzed using
a non-parametric ANOVA (rank transformed values). All Kaplan Meier, Cox proportional hazard analyses and Pearson Chi-square exact tests were carried out using SPSS (release 15, SPSS Inc.) and all ANOVA analyses using Graphpad Prism software version 5. P-value < 0.05 was considered statistically significant.

**ACKNOWLEDGEMENTS**

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**REFERENCE LIST**


