Bile-Salt Stimulated Lipase Polymorphisms do not Associate with HCV Susceptibility

Running title: BSSL polymorphisms and HCV susceptibility

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ABSTRACT

Bile-salt stimulate lipase (BSSL) is a glycoprotein found in human milk and blood that can potently bind DC-SIGN. The BSSL gene is highly polymorphic with a variant number of O-linked glycosylated 11 amino acid repeats at the C-terminus of the protein, encoded in exon 11 of the gene. It has been shown that certain BSSL genotypes associate with decreased HIV-1 transmission in vitro and decreased HIV-1 disease progression. The protein forms dimers and individuals possessing one high (typically 14-21) and one low (typically 7-11) number of repeat domains has been shown to have stronger binding of BSSL to DC-SIGN and HIV-1 inhibitory activity in vitro. Since we previously demonstrated that SNPs within the DC-SIGN gene can associate with risk of HCV sexual transmission and which can be linked to diminished DC-SIGN gene expression we aimed to identify whether BSSL polymorphisms associated similarly through differential binding to DC-SIGN. DNA was isolated from the HIV-1 infected MSM cohort (MOSAIC) composed of HCV multiple exposed uninfected (MEU) (N=30) and multiple exposed HCV infected (MEI) (N=32) individuals and from the Amsterdam cohort studies (ACS) intravenous drug using (IDU) cohort (22 MEI and 40 MEU). The numbers of repeats in exon 11 were determined by PCR with repeat distributions compared between MEI and MEU. No statistical significant difference in the copy number of exon 11 repeats, or combinations of, in the BSSL gene was observed when comparing HCV infected MEI with MEU, thus the exon 11 repeat copy number in the BSSL gene does not affect HCV susceptibility.

Keywords:

BSSL; HCV; DC-SIGN; polymorphism

Through prospective and retrospective studies several risk factors for acquiring Hepatitis C Virus (HCV) infection have been identified. In Western countries the main risk factors for acquiring HCV are injecting drugs, receiving blood transfusions or blood products before screening and high-risk sexual behavior among men who have sex with men (MSM) (Alter, 2007). However, despite high-risk behavior some individuals remain uninfected. Several studies have shown that among injecting drug users (IDU) ultimately 10-20% do not seroconvert, suggesting a biological reason why those multiple exposed uninfected (MEU) individuals are less prone to contract HCV (Sutton et.al., 2006; Hagan et.al., 2008). During IDU exposure HCV enters the blood stream and therefore interacts with a large array of different cell types whilst during MSM transmission the first cells of interaction are those residing within or directly below the mucosal surface, including dendritic cells (DCs) and macrophages. These cells express a large array of molecules, such as C-type lectins, differentially expressed at the cell surface which can interact with a large array of pathogen and pathogen antigens (Solano-Galvez et.al., 2019). Concerning HIV-1 transmission the molecule that has garnered the most interest is DC-SIGN [Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-Intergin (CD209/CLEC4L)] expressed on specific DC sub-sets and which can support viral capture and transfer to $CD4^+$ lymphocytes *in vitro* (Solano-Galvez et.al., 2019; Soilleux et.al., 2002; Geijtenbeek et.al., 2000). DC-SIGN has also been shown to be expressed on an array of stimulated macrophages and predicted to be involved with pathogen signalling and modulation of host responses (Pohlmann et.al., 2001). HCV through the E2 envelope glycoprotein has also been described to interact with DC-SIGN but with very little known as to the significance for transmission or disease (Lozach et.al., 2003; Pöhlmann et.al., 2003).

Bile-salt stimulated lipase (BSSL), also known as Bile-salt stimulated cholesterol esterase, is a high molecular weight Lewis X (LeX)-containing glycoprotein that is expressed and excreted by pancreatic cells and, in some species, by lactating mammary glands and secreted in milk (Hernell et.al., 1994). Combined with bile salt it has a hydrolysing activity to digest long-chain triacylglycerols (Hernell

et.al., 1994). BSSL is found in human milk and blood and can potently bind DC-SIGN (Naarding et.al., 2005; Naarding et.al., 2006). Interestingly, human milk has been shown not to have the capacity to bind L-SIGN (Naarding et.al., 2005), a molecule known to bind the HCV envelope protein and aide in transmission of the virus (Lozach et.al., 2003). It has been shown that binding of human milk derived BSSL to DC-SIGN can prevent HIV-1 capture and transfer to $CD4^+$ cells (Naarding et.al., 2006). The interaction of HIV-1 with DC-SIGN has been postulated to be involved with risk of HIV-1 transmission across a mucosal surface as well as early viral dissemination (Geijtenbeek et.al., 2000)[6]. The BSSL protein is highly polymorphic amongst individuals with a large number of 11 amino acid repeats (ranging from 7-21) which are O-linked glycosylated and which are found as genetic repeats within exon 11 of the gene (Hernell et.al., 1994). It has been shown that individuals with a high and low copy number of the exon 11 repeat have stronger binding to DC-SIGN than individuals with similar number of repeats per allele (Stax et.al., 2011). Additionally, certain BSSL genotypes associate with decreased HIV-1 transmission but more strikingly with disease progression (Stax et.al., 2012). These results suggest that BSSL, either expressed at the mucosal surface or in plasma, can modulate HIV-1 transmission as well as subsequent viral replication. Additionally BSSL has immunological skewing properties that may influence responses mounted against a virus and which may therefore influence viral transmission as well as subsequent replication.

We recently reported that three polymorphisms within the DC-SIGN promoter associate with sexual transmission of HCV, suggesting that DC-SIGN may indeed play a role in HCV transmission (Steba et.al., 2017). The association was only found within the MSM cohort but not for those undergoing IDU exposure, indicating that different routes of exposure may have different biological mechanisms of transmission. Since BSSL can bind DC-SIGN we aimed to identify whether BSSL polymorphisms associate with risk of HCV infection. To this end, we used samples from two well-defined cohorts of injecting drug users (IDU) and HIV-1 infected men having sex with men (MSM) with acute HCV or HCV negatives with high-risk behaviour for acquiring HCV. We compared the genotype frequency of the

BSSL polymorphism between multiple exposed infected (MEI) and multiple exposed uninfected (MEU) individuals. The utilisation of both cohorts therefore allowed for the comparison between the different routes of infection and to identify whether associations with one cohort over the other could be identified for BSSL genotyping as with the DC-SIGN promoter genotypes.

A well-defined population was used to study the role of BSSL repeat number in relation to HCV susceptibility. 124 serum samples were collected from two cohorts (Table 1). 62 HIV-1 infected Western European MSM from the MSM Observational Study of Acute Infection with Hepatitis C (MOSAIC) cohort recruited from either the Academic Medical Center or at OLVG hospital in Amsterdam. Risk behavior data was available from behavioral questionnaires collected at 6 month intervals. Based on a recent study, sexual behavior with increased risk of acquiring HCV infection was defined as having reported any of the following: inconsistent condom use and anal intercourse with an HCV-infected sex partner; fisting; use of sex toys; rectal bleeding during or after sex; group sex (Vanhommerig et.al., 2015; Newsum et.al., 2017). MOSAIC risk scores were subsequently calculated, where a score of ≥2 was defined as high risk. The MOSAIC study was approved by the Institutional Review Board of the Academic Medical Center under assigned study numbers NL26485.018.09 and NL48572.018.14. Additionally, 62 samples were collected from the Amsterdam Cohort Studies (ACS) among IDU (van den Hoek et.al., 1988). Serum samples of Western European individuals were selected (40 MEU and 22 MEI), who started injecting before 1990, which was a period with high HCV incidence among drug users (up to 27.5/100 person years in the 1980s). Participants injected for ≥2 years and either seroconverted for HCV (MEI) or remained HCV seronegative (MEU) during follow-up. ACS participants complete a standardized questionnaire about their health, risk behavior, and socio-demographic situation every 4-6 months. Blood is drawn for laboratory testing and storage. The ACS study was approved by the Institutional Review Board of the Academic Medical Center at the University of Amsterdam and ethical committees/board of directors of each institute recruiting participants (assigned study numbers MEC 07/182 and MEC 09/040).

DNA was isolated from 200 µl serum with QIAamp DNA blood mini kit according to the manufacturers protocol (Qiagen). The number of BSSL exon 11 repeats was determined for each subject by PCR as represented (Figure 1). The DNA was amplified with ready-to-use PCR grade PCR Mix kit (Roche Diagnostics GmbH, Mannheim, Germany) in a volume of 25 µl as follows: 2.5 µl 10x PCR buffer, 0.20 µl Fwd primer (50 µM, Biolegio), 0.20 µl Rev primer (50µM, Biolegio), 2.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP (10mM each), 0.5 µl Bovine Serum Albumin (BSA) (5mg/ml), 0.25 µl FastStart Taq DNA polymerase (5U/µl), 13.35 µl Bakerwater, 5 µl template DNA. Amplification was accomplished under the following conditions: denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 30s, 60°C for 30s and 72°C for 1 min and a final extension step at 72°C for 10min. For 1 sample we did not retrieve good PCR results. Univariable logistic regression was used to estimate the odds ratio (OR) and corresponding 95% confidence interval between MEU and MEI and the χ^2 test was used to calculate p-values using SPSS software (IBM, version 20). P-values of <0.05 were considered statistically significant.

We investigated whether the BSSL repeat region polymorphism associates with susceptibility to HCV infection. Therefore, we analyzed the repeat length of BSSL exon 11 between MEI and MEU from our well-defined cohorts. A total of 20 different BSSL genotypes were identified within the study population where being homozygous for 16 repeats was most common. Genotype distribution between MEI and MEU were compared and the genotypes were equally distributed amongst MEI and MEU individuals. When the genotypes were split into high number of repeats (HH), low number repeats (LL) and one high and one low allele (LH) according to our previous designation (Stax et.al., 2011; Stax et.al., 2012) the distribution of BSSL repeat polymorphisms were not significantly different between the MEI and MEU individuals (LL+LH vs HH; OR: 1.3368 CI: 0.66-2.74 p=0.4295) (Table 2). It should be noted that within the MOSAIC study all individuals are HIV-1 positive whilst none are infected within the ACS (IDU) cohort (with only 3 seroconversions observed during follow up),

however since we are comparing between the MEI and MEU groups within each cohort the HIV-1 status should be irrelevant. Furthermore, we are comparing intrinsic genetic polymorphisms which are non-adaptive and therefore should not be influenced by HIV-1 infection, although modulation of induced immunity via BBSL binding DC-SIGN cannot be ruled out but again status is comparable between the groupings being compared. It should be stated that an array of variant genotypes were found within the study population; we identified 20 different BSSL genotypes within this study population and supporting what had been previously reported (Stax et.al., 2011). This variation within the study population of 124 individuals may have restricted the analysis, therefore we cannot exclude a possible association between BSSL genotype and HCV susceptibility if larger numbers were tested. However, since we utilized a well-defined population where genotypic associations have been previously found our results suggest that there is no association between BSSL repeat length and HCV susceptibility.

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Table 1 Patient characteristics

	MEI		MEU	
Characteristics	**MOSAIC**	**ACS**	**MOSAIC**	**ACS**
n (total=124)	32	22	30	40
Mean age ± SD	43.0 ±6.9	52.0 ±6.9	48.5 ±7.9	52.8 ±7.2
% Male gender	32 (100%)	11 (50%)	30 (100%)	29 (72.5%)
% Dutch Nationality	28 (87.5%)	19 (86.4%)	29 (96.7%)	37 (92.5%)
% HIV positive at entry	32 (100%)	0 (0%)	30 (100%)	0 (0%)
HIV seroconversion during follow-up	n.a	3 (13.6%)	n.a	0 (0%)
Median start date of Follow-up (IQR)	22/2/2011 (4/2/2010-2/8/2011)	23/2/1988 (15/1/1987-08/02/1992)	14/2/2011 (19/5/2010-20/12/2011)	20/10/1992 (12/09/1988-22/04/1998)
Median time of follow-up ± SD	4.01 ± 1.80	14.96 ± 5.65	3.78 ± 1.30	14.31 ±5.62
Mean duration IDU in years	4 IDU in last 6 months (no duration)	7.21 ±3.42	n.a	8.45±4.83
% Reported sharing of needles[§]	0 (0%)	15(75%)	0 (0%)	22 (55%)
Having an HCV-infected sex partner*	7 (21.9%)	n.a	1 (3.3%)	n.a
Fisting[§]				
With steady partner	9 (28.1%)	n.a	5 (16.7%)	n.a
With casual partner(s)	10 (31.3%)	n.a	8 (26.7%)	n.a
Use of sex toys[§]				
With steady partner	13 (40.6%)	n.a	12 (40%)	n.a
With casual partner(s)	15 (46.9%)	n.a	4 (13.3%)	n.a
Rectal bleeding during or after sex[§]				
With steady partner	7 (21.9%)	n.a	10 (33.3%)	n.a
With casual partner(s)	15 (46.9%)	n.a	8 (26.7%)	n.a
Groupsex[§]	24 (75%)	n.a	23 76.7%)	n.a
Rectal bleeding during or after sex*	17 (53.1%)	n.a	5 (16.7%)	n.a
CD4 count last negative moment(cases)/last visit (controls)*	523±138	n.a	621±222	n.a
CD4 count nadir	277±160	n.a	269±179	n.a
Baseline Mosaic Risk score (medium)*[#]	2.9	n.a.	1,1	n.a.

Characteristics of MOSAIC and ACS multiple exposed infected (MEI) and multiple exposed uninfected (MEU) participants

Table 2 BSSL repeat number characteristics

BSSL repeats ^^	MOSAIC		ACS		MOSAIC + ACS	
	MEI	**MEU**	**MEI**	**MEU**	**MEI**	**MEU**
HH N (%)	14 (44%)	18 (60%)	11 (50%)	19 (49%)	25 (46%)	37 (54%)
LL N (%)	3 (9%)	2 (7%)	5 (23%)	4 (10%)	8 (15%)	6 ((%)
HL N (%)	15 (47%)	10 (33%)	6 (27%)	16 (43%)	21 (39%)	26 (38%)

^^= high number alleles (HH), low number alleles (LL) one high and one low allele (LH)

Distribution of BSSL repeat numbers amongst MEI and MEU individuals. Genotypes were split into two groups where 16 to 18 repeats were defined as high (H) and less than 16 repeats as low (L). People who were heterozygous were defined as Low High (LH). The table presents the number of people (N) harboring a certain repeat number.

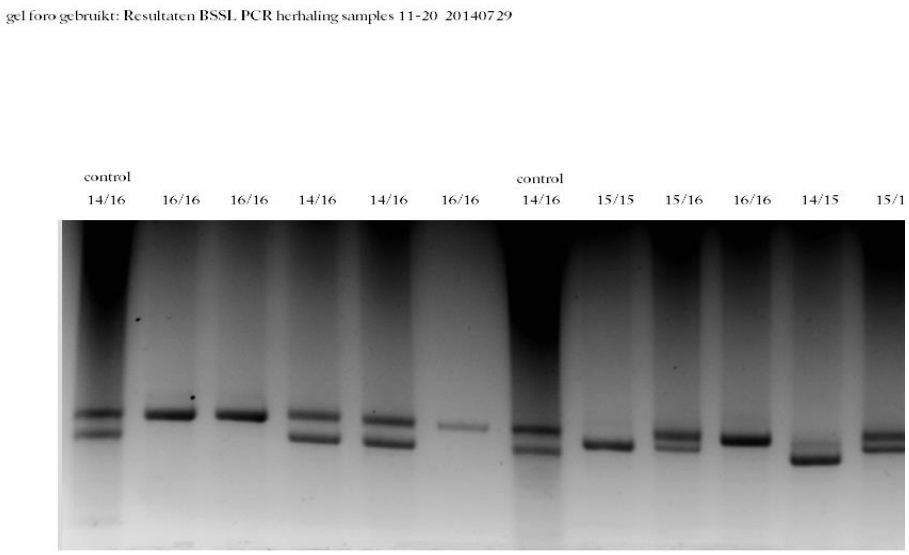

Figure 1 Typical agarose gel of PCR resultant products enabling for the identification of the number of BSSL repeats. Control samples were used to enable for the accurate determination of the repeat numbers in unknown samples.