

## ABSTRACT

**BACKGROUND:** Chemokine ligand 3-like 1 (CCL3L1), as a member of the chemokine family, is encoded by a variable copy-number gene and plays an important role HIV-1/AIDS susceptibility. Individuals with more copies of CCL3L1 than their population median have been found to be less susceptible to HIV infection. We investigated whether lower CCL3L1 gene copy number than population median is associated with HIV-1 infection among intravenous drug users (IDU).

**METHODS:** Blood samples were collected from 374 ancestrally Caucasian intravenous drug users (mean age 26 years). Of these 208 were HIV positive (all ARV naive); 285 HCV positive; 57 HBV positive. The CCL3L1 gene copy-number was measured by real-time PCR (TaqMan) using primers as described elsewhere and confirmed at two copies per diploid genome for each sample.

**RESULTS:** In HIV-pos and HIV-neg subjects the CCL3L1 copy numbers ranged between 0 & 5 and 0 & 6, respectively with the median value of 2 in both populations. More HIV-pos (85.1%) or those co-infected with HCV (88%) had 0-2 CCL3L1 gene copies than HIV-neg (73.5%), HCV mono-infected (67%) or HIV mono-infected (71%) (OR=2.06 with 95% CI: 1.23-3.44 HIV-pos vs HIV-neg; OR=3.52 (95% CI: 1.93-6.4) HIV-pos/HCV-pos vs HIV-neg/HCV-pos; OR=0.3 (95% CI: 0.11-1.03 HIV-pos/HCV-pos vs HIV-neg/HCV-neg) ). The number of subjects with CCL3L1 copy numbers of 0-2 in HCV-pos and HIV-neg population irrespective of HIV status was similar.

**CONCLUSION:** In the high risk IDU population the subjects who display smaller number of copies of CCL3L1 gene than their population median have increased risk of acquiring HIV-1, indicating that this chemokine may play an essential role in protective immunity.

## INTRODUCTION

Segmental copy-number polymorphisms represent a significant component of human genetic variation and are likely to contribute to disease susceptibility. Chemokine ligand 3-like 1 (CCL3L1) is a member of the chemokine family and encoded by a variable copy-number of genes. It is a natural ligand of CCR5 and thereby plays an important role HIV-1/AIDS susceptibility. Previous studies have shown that low gene copy-number of CCL3L1 is associated with lower chemokine concentrations, a higher proportion of CCR5-expressing CD4+ cells, an increased risk of HIV acquisition, higher viral loads and accelerated rate of disease progression [1]. Individuals with greater gene-copies of CCL3L1 than their population median have been found to be less susceptible to HIV infection than those with higher than population median of gene-copies [1].

CCL3 and other chemokines may also play an important role in the development of inflammation in HCV infection as some previous studies have shown an increase in CCL3 levels in serum and in the liver [2, 3, 4]. Whether in HCV infections CCL3L1 gene-copy number is correlated with the pathogenesis of the disease is not known.

Also, previous studies when looking at the correlations between CCL3L1 gene-copy numbers and prevalence of the HIV infection did not distinguish between various populations (MSM, perinatally or heterosexually acquired HIV infections, IDU) and thus the role of such important risk factors has not been studied.

We aimed to study the CCL3L1 gene copy numbers in IDU population in Estonia and to describe associations between these gene copy numbers and presence of HIV-1, HCV and HBV infection.

## SUBJECTS AND METHODS

**Subjects.** A total of 374 ancestrally Caucasian IDUs (mean age 26 years; 301 men and 55 women) from various syringe exchange programs and Estonian prisons were included.

**Infection detection.** HIV, HCV and HBV testing was done at the Estonian HIV Central Reference Laboratory. HIV antibody screening was conducted with the use of the enzyme-linked immunosorbent assay (ELISA). Positive results were confirmed with the use of the HIV immunoblot assay. HCV and HBV antibody testing was conducted with the use of the respective commercial ELISA test kits (Table 1).

**Table 1. Study population according their HIV and HCV or HBV status**

	HCV+	HCV-
HIV+	177	31
HIV-	108	57
	285	88

	HBV+	HBV-
HIV+	47	160
HIV-	9	153
	56	313

**Cell line cultivation and DNA isolation.** The A431 cells were courtesy from professor Ilmo Virtanen (University of Helsinki, Institute of Biomedicine). Cells were cultured in IMDM medium (Isocove modified Dulbecco medium) supplemented with 10% fetal calf serum, 100 mM streptomycin, 100 mM penicillin and cultivated in 5% CO<sub>2</sub> at 37 °C for 2 to 3 weeks. PBMCs were purified by density gradient centrifugation on a Ficoll/sodium diatrizoate solution in BD Vacutainer CPT Cell Preparation tube (Becton Dickinson). The DNA from PBMCs and A431 cells were extracted using Qiagen QIamp DNA Mini kit according to manufactures protocol.

**Analyses of CCL3L1 gene copy number.** The CCL3L1 gene copy number was measured by real-time PCR (TaqMan) using ABI/Prism 7500 Sequence Detector System as described elsewhere [1]. Primer and probe sequences are presented in Table 2.

**Table 2. Primers and probes [1]**

Gene	Primers	Probes
CCL3L1	5'-tctccaagctctccaaga-3' 5'-ctgagccaccctctaccag-3'	5'-FAM-aggccgcaggctgtgtga-TAMRA-3'
Beta-globin	5'-ggcaaccclaaagggaagga-3' 5'-ggtaggaagcctctcaactca-3'	5'-VIC-caltgcaagaagaactcgtcggt-TAMRA-3'

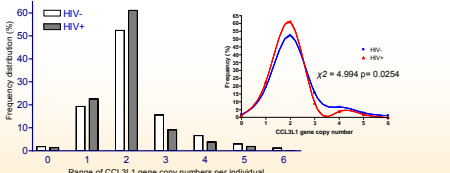
**Statistical analyses.** Standard procedures in the STATA 6.0 (Stata Corporation, College Station, Texas, USA) were used for descriptive and comparative statistics. Differences in gene copy numbers and related variables were assessed by  $\chi^2$  or Fischer tests and logistic regression. A logistic regression model was developed to examine the relationship between HIV-status and individual variables, while controlling for the effect of other variables like gene copy number, HCV, HBC, age and gender.

## RESULTS

**CCL3L1 and HIV infection.** The CCL3L1 gene copy numbers in HIV+ and HIV-neg populations were presented in Table 3 and on Figure 1.

**Table 3. CCL3L1 gene copy numbers in the study population (n=374). SD – standard deviation, IQR – interquartile range (the 25-75th percentile)**

HIV-1 status	Sample size (n)	Mean	SD	Median	Range	IQR
All subjects	374	2.072	0.948	2	1-6	2-2
HIV+	208	1.971	0.839	2	1-5	2-2
HIV-	166	2.199	1.057	2	1-6	2-3

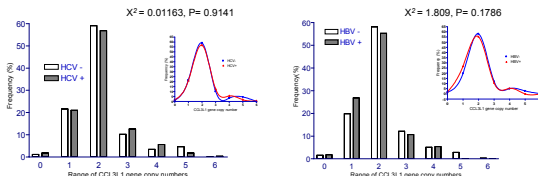


**Figure 1. Histograms and the cubic-spline frequency curves that show the distribution of the CCL3L1 copy numbers in HIV+ versus HIV- subjects is different.**

**More HIV-positive subjects (85.1%) had 0-2 CCL3L1 gene copies than HIV-negative subjects (p=0.025)**

When the copy number of CCL3L1 gene was dichotomized at the population specific median for comparison of HIV+ and HIV- groups, it was possible to see important difference, which occurs between subjects who had 2 and less copies and subjects who had 3 and more copies of CCL3L1 (OR = 2.059; 95% CI = 1.231-3.443; p = 0.006; Table 4, univariate unadjusted analysis). It means that subjects who possess 0-2 copies of CCL3L1 gene are at higher risk of acquiring HIV compared to subjects possessing 3 and more copies of CCL3L1 gene.

## CCL3L1 gene copy number and HBV and HCV infection.



**Figure 2. Histograms and the cubic-spline frequency curves about the distribution of CCL3L1 gene copy numbers in the study populations stratified by HCV or HBV status. No difference was found.**

**No differences in CCL3L1 gene copy numbers between HCV or HBV positive or negative subjects was found.**

## CCL3L1 and co-infection

In the univariate analysis, HBV status was the strongest risk factor for the HIV+ persons. The age group 21-30 years had a significant relative to age group aged  $\geq 20$ . HCV seropositivity was also a significant factor. Gender was a weak risk factor (Table 4).

In a multivariate logistic regression analysis including all the variables except gender (was a weak risk factor in a univariate analysis), the results were a little bit different, as ORs for HBV and age group  $\geq 31$  years became insignificant. By adjusted logistic model positive HCV status became the strongest risk factor. Also, odds of low CCL3L1 gene copy numbers increased with adjustment - from 2.059 to 2.705. Above mentioned differences between crude and adjusted ORs can be explained by some kind of confounding between variables as CCL3L1 copy number, age or HBV status. There was no evidence of any interaction between these three variables.

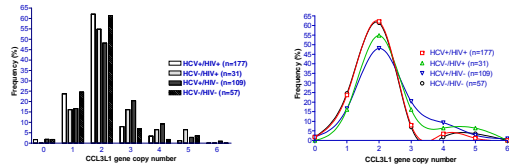
**Table 4. ORs and 95% CI for the association between CCL3L1 gene copy number, gender, age, HCV and HBV status and acquiring HIV infection among IDUs in Estonia.**

Variables	Unadjusted			Adjusted		
	OR	95% CI	p	OR	95% CI	p
<b>CCL3L1 copy</b>						
0-2	2.059	1.231-3.443	0.006	2.705	1.450-5.048	0.002
3-6†	1.0			1.0		
<b>Gender</b>						
Female†	1.0			1.0		
Male	0.926	0.515-1.663	0.797			
<b>Age (years)</b>						
$\leq 20$ †	1.0			1.0		
21-30	3.692	1.657-8.226	0.006	3.022	1.294-7.057	0.011
$\geq 31$	2.574	1.020-6.496	0.045	2.176	0.814-5.811	0.121
<b>HCV status</b>						
HCV-†	1.0			1.0		
HCV+	3.013	1.830-4.961	0.000	3.673	1.820-7.412	0.000
<b>HBV status</b>						
HBV-†	1.0			1.0		
HBV+	4.994	2.366-10.538	0.000	1.998	0.833-4.971	0.121

We found strong interaction between HCV status and CCL3L1 gene copy number on the HIV seropositivity by likelihood ratio test for interaction ( $\chi^2 = 13.10$ , p = 0.0003). It tells that there should be a HCV-stratum specific exposure effect. Therefore, we performed a logistic regression to examine the association between exposure (in this case CCL3L1 gene copy number) and outcome (HIV seropositivity) variables, separately for each level of the effect modifier (HCV status). Obtained results show that lower CCL3L1 gene copy number in HIV+ subjects tended to be associated with HIV-seropositivity, while the same is not true among the subjects without HCV infection (Table 5; Figure 3).

**Table 5. Odds and confidence intervals of HIV status related to interacted CCL3L1 gene copy numbers HCV status**

	OR	95% CI	P
<b>HCV-</b>			
CCL3L1 0-2 copies	0.342	0.113-1.036	0.058
CCL3L1 3-6 copies	1.0		
<b>HCV+</b>			
CCL3L1 0-2 copies	3.522	1.934-6.416	<0.001
CCL3L1 3-6 copies	1.0		



**Figure 3. Frequency of CCL3L1 gene copy numbers among HIV mono- and co-infected subjects (related to HCV).**

**More HIV+&HCV+ co-infected (88%) subjects had 0-2 CCL3L1 gene copies than HIV- mono-infected (67%) or HIV mono-infected (71%) (p<0.001; p=0.034).**

## CONCLUSIONS

In the high risk IDU population the subjects who display smaller number of copies of CCL3L1 gene than their population median have increased risk of acquiring HIV-1, indicating that this chemokine may play an essential role in protection.

Exposure to HCV co-infection may be additional risk factor which is associated with increased risk of acquiring HIV infection due to low CCL3L1 gene copy number.

## ACKNOWLEDGEMENTS

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