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ABBREVIATIONS

AA	–	African-Americans
AIDS	–	Acquired immunodeficiency syndrome
CCL3L1	–	CC chemokine ligand 3 like 1
CCL3L1	–	CC chemokine ligand 3 like 1 gene
CCL5	–	CC chemokine ligand 5
CCL5	–	CC chemokine ligand 5 gene
CCR5	–	CC chemokine receptor 5
CCR5	–	CC chemokine receptor 5 gene
CCR5-Δ32	–	32 base pair deletion in CCR5 gene
CD	–	cluster of differentiation antigen
CI	–	Confidence interval
CMI	–	Cell mediated immunity
CRF	–	Circulating recombinant form
CXCR4	–	CX chemokine receptor 4
DC	–	Dendritic cell
DNA	–	desoxyribonucleic acid
EA	–	European-Americans
ESN	–	Exposed HIV uninfected subject
GWAS	–	Genome wide association study
HBV	–	Hepatitis B virus
HCV	–	Hepatitis C virus
HESN	–	Highly exposed HIV seronegative individual
HH	–	Human haplotype
HIV	–	Human immunodeficiency virus
HLA	–	Human leukocyte antigen
HWD	–	Hardy-Weinberg Equilibrium
IDU	–	Intravenous drug user
IVDU	–	Intravenous drug use
IFN	–	Interferon
IL	–	Interleukin
MGB	–	Minor groove binder
MTCT	–	Mother-to-child transmission
NF-kB	–	Nuclear factor-κB
NGS	–	Next generation sequencing
NK	–	natural killer
OR	–	odd ratio
ORF	–	open reading frame
PBMC	–	Peripheral blood mononuclear cell
PCR	–	Polymerase chain reaction
qPCR	–	Quantitive real-time PCR
RNA	–	Ribonucleic acid
SDF-1	–	Stromal cell-derived factor 1

SNP – Single nucleotide polymorphism
Th – T-helper
TLR3 – Toll-like receptor 3
TLR3 – Toll-like receptor 3 gene
TNF – Tumor necrosis factor

1. INTRODUCTION

The injecting drug use and the spread of blood-borne viruses (e.g. human immunodeficiency virus (HIV) and hepatitis C virus (HCV)) due to contaminated syringes are a major concern worldwide. As of 2012, approximately 35 million people are infected with HIV and 150 million have persistent HCV infection (www.who.int). Despite the fact that 20% of HCV infected persons clear the virus spontaneously, HIV infection cannot be resolved by host's immune system.

However, despite multiple exposures to HIV, some people do not get infected and are called thus highly exposed HIV seronegative individuals (HESNs). They can be found among intravenous drug users (IDUs) sharing needles, haemophiliacs who repeatedly received contaminated blood products, infants born to HIV-infected mothers and individuals exposed through HIV positive sexual partners. The reasons behind this phenomenon are not fully understood and HESNs are under major interest in order to explain the resistance against HIV.

Many factors influence the susceptibility to HIV. The factors are, for example, the route of transmission, the viral load of the index subjects, the presence of other sexually transmitted diseases and immunological and genetic factors of the host. The best known genetic factor is the 32 base pair deletion in HIV co-receptor encoding gene (CC chemokine receptor 5) CCR5, which gives the resistance to HIV R5-tropic viruses (viruses that use CCR5 as a co-receptor). Also other polymorphisms in different genes (in targeted gene approach or in genome wide association studies) have been identified to influence the susceptibility to HIV. The associations between host genetic factors and susceptibility to HIV are relatively well described in subjects infected through sexual or mother-to-child transmission. Whether these associations are present in IDU populations, such as the majority of HIV positives in former Soviet Union, including Estonia, is poorly studied.

In Estonia, the infection rate of HIV and HCV among IDUs is very high [Uuskula et al., 2007]. The HCV epidemic started in the early 1990s and the HIV epidemic in 2000 when rare recombinant form CRF06_cpx entered into the IDUs population (www.terviseamet.ee). The high prevalence of these infections is still a problem today. Previous studies and doctoral theses on HIV infection in Estonia have mainly concentrated on the epidemiological aspects of the HIV epidemic [Rüütel, 2009] and HIV molecular epidemiology including HIV drug resistance and vaccine development [Adojaan, 2009; Avi, 2011]. The virus circulating in Estonia is relatively well described. However, little research has been conducted to evaluate how host genetic factors influence the susceptibility to HIV in the population predominantly consisting of subjects infected via intravenous drug use. Similarly to HIV in the Estonian HCV epidemic, the

characteristics of the virus are described [Žusinaite, 2005; Tallo, 2008] but the relationship between host factors and the susceptibility to HCV is unclear.

Bearing in mind the lack of knowledge of host genetic factors in HIV and HCV susceptibility to IDUs population, we first aimed to explore this area further.

2. REVIEW OF THE LITERATURE

2.1. HIV and HCV epidemics in the world

Approximately 35 million people were infected with HIV in the year 2012, and 2.5 million new infections occurred in 2011 (www.who.int). The virus causes an acquired immunodeficiency syndrome (AIDS), a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to appear. Now, when there is relatively effective treatment available, the cases of AIDS have dropped in developed countries but the HIV epidemic has a major impact in certain regions and risk groups. Despite the effective treatment, the virus is persistent for a lifetime with some exceptions. There is only limited evidence that HIV could be completely eliminated – a case of stem cell transplantation from *CCR5*-Δ32 homozygous subjects and in very early treatment initiation in a new born [Allers et al., 2011; Persaud et al., 2013].

Another virus that shares similar transmission route with HIV (blood borne virus) and causes worldwide epidemic is HCV. Over 150 million people have a persistent HCV infection being the main cause of acute and chronic liver diseases including chronic hepatitis, cirrhosis and hepatocellular carcinoma (www.who.int) [Liang et al., 2000]. Acute HCV infection is often asymptomatic or with non-specific and mild symptoms. Unlike HIV, up to 20% of HCV infected subjects will have spontaneous clearance of the virus, and of the remaining, the virus could be eliminated with treatment, however, up to 40% of treated persons will not be responsive to the therapy [Alter and Seeff, 2000].

A major concern is the co-infection with both HIV and HCV. Approximately 30% of HIV-positive subjects in the United States and Europe are co-infected with HCV [Alter, 2006]. The co-infection rate among IDUs is higher and exceeds 90% in most studies [Sherman et al., 2002a]. HIV/HCV co-infection results in a higher rate of morbidity and mortality of liver diseases. The influence of HIV infection on HCV disease includes a higher rate of viral persistence and increased HCV viral loads [Cribier et al., 1995; Matthews-Greer et al., 2001; Sherman et al., 2002b], a faster rate of progression to fibrosis, cirrhosis and hepatocellular carcinoma [Martinez-Sierra et al., 2003; Mohsen et al., 2003; Pol and Zylberberg, 1998]. In addition, persons co-infected with HIV/HCV have a worse treatment response to interferon-alpha (IFN-α)-based therapies compared to HCV monoinfected individuals [Chung et al., 2004; Perez-Olmeda et al., 2003; Torriani et al., 2004].

The influence of HCV on HIV infection is still arguable. More rapid HIV disease progression and an increased risk of progression to AIDS and death in HIV/HCV co-infected compared to HIV monoinfected patients have been demonstrated [De Luca et al., 2002; Piroth et al., 1998]. However, there is a number of studies not finding any correlation between HIV/HCV co-infection

and HIV disease progression, the development of AIDS and death [Haydon et al., 1998; Rockstroh et al., 2005; Staples et al., 1999; Sulkowski et al., 2002]

2.2. HIV and HCV epidemics in Estonia

In Estonia, the increased rates of HCV infection were highest at the beginning of the 1990s (Figure 1). Similar to other areas, the prevalence was especially high among IDUs ranging from 76% to 90% [Lõhmus et al., 2008]. Not surprisingly, HIV and HCV co-infections are also common – 96% of HIV positive IDUs in some areas in Estonia are co-infected with HCV [Uuskula et al., 2007]. The main HCV genotypes circulating in Estonia are 1 and 3 from which the most prevalent subtype is 1b and in lesser extent subtype 3a [Zusinaite et al., 2000; Tallo et al., 2007].

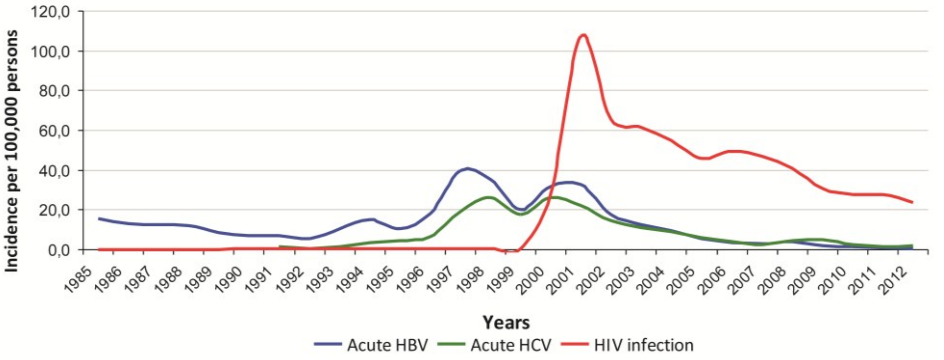


Figure 1. The prevalence of HIV, HBV and HCV infection in Estonia. Incidence per 100,000 population of HIV (red line), HBV (blue line) and HCV (green line) infection in Estonia between 1985-2010, as reported by the Estonian Health Board.

The first HIV positive subject in Estonia was diagnosed in 1988, but until 2000 the prevalence of the infection remained low (under 100 cases). The disease was mainly transmitted sexually (homo- or heterosexually) [Ustina et al., 2001]. The HIV epidemic suddenly erupted in August 2000 when HIV-1 was introduced to the IDUs population (Figure 1) [Laisaar et al., 2011]. After that, the number of patients infected with HIV-1 increased rapidly, reaching the highest prevalence of 105.3 per 100,000 inhabitants in European Union in 2001. Since 2001, the number of new infections is declining but Estonia is still one of the three countries in Eastern-Europe and Central Asia in which the estimated HIV prevalence is around 1% of adult population [Lai et al., 2009].

Contrary to other countries of the former Soviet Union in which subtype A1 is the most prevalent, the Estonian HIV-1 epidemic is stably caused by the rare HIV-1 CRF06_cpx and by its recombinants with the subtype A1 [Avi et al., 2009; Avi et al., 2010; Zetterberg et al., 2004]. The HIV-1 CRF06_cpx is a

recombinant form between subtypes A, G, K and J [Montavon et al., 1999; Oelrichs et al., 1998]. Before the Estonian outbreak, CRF06_cpx was spreading mainly in Mali, Niger and Burkina Faso, and only single strains had been described outside of Africa [Mamadou et al., 2002; Montavon et al., 1999; Oelrichs et al., 1998]. The routes how the virus was imported to Estonia are not defined.

The HIV epidemic is located in Northern and North-Eastern part of Estonia (capital Tallinn and Ida-Viru County, respectively). In the new concentrated epidemic in 2000–2001, over two-thirds of the infected subjects were young male IDUs (www.terviseamet.ee). This situation remained unchanged during the following years until 2009, when the proportion of IDUs started to diminish (unpublished data). Still, most new cases have been diagnosed among men (68%) and among people under 30 during the past decade [Laisaar et al., 2011]. Similar to other countries, the IDU epidemic is slowly moving to the general population; according to the Estonian HIV database approximately 50% of HIV cases occur among non-IDUs in 2010 (unpublished data). The HIV subtype structure, however, has been unchanged during the entire concentrated epidemic period [Avi et al., 2013].

2.3. Factors influencing the susceptibility to HIV

The resistance of HESNs to HIV is not fully understood and these subjects are under major interest to explore the reasons between the acquisition of HIV infection and host factors, and also other potential factors that could explain the resistance to HIV. However, the definition of HESNs is very broad and usually the term is defined based on the specific study population. For example, based on the various interval of sharing the used needles (receptive sharing) or having unprotected sex with HIV-positive subject. However, a number of studies have determined the associations between different host and viral factors and the susceptibility to HIV using HIV positive subjects and HIV negative healthy controls.

2.3.1. The route of transmission and the susceptibility to HIV infection

HIV can be transmitted by sexual, parenteral and mother-to-child route. Sexual transmission includes female-to-male, male-to-female and male-to-male transmission; parenteral transmission includes the transfusion of the infected blood or infected syringes in IDUs. Mother-to-child transmission (MTCT) could occur during pregnancy, during labour or postnatally [Galvin and Cohen, 2004; Royce et al., 1997]. The highest probability to acquire HIV infection is via routes that lack the first human defence mechanism – the mucosal barrier (e.g. through contaminated blood or syringes) [Shaw and Hunter, 2012]. When

infected blood products are used, the likelihood of viral transmission is near to 100% [Galvin and Cohen, 2004]. However, this route presently is very rare in the developed world. Similarly, the transmission rate in subjects using intravenous drugs and sharing the needles is very high compared to sexual transmission up to 20 times [Galvin and Cohen, 2004]. In addition to parenteral transmission, sharing used needles increases the probability of infection because the virus can be viable and proliferating up to four weeks depending on the storage temperature, the volume of residual blood and the viral load [Abdala et al., 1999; Uuskula et al., 2006]. Among sexual routes, the highest risk to get infected is unprotected rectal intercourse followed by vaginal and orogenital contact [Gray et al., 2001; Hladik and Hope, 2009; Powers et al., 2008; Wawer et al., 2005; Winkelstein et al., 1987]. Peripartum and perinatal mother-to-child transmission without ARV prophylaxis 25–45% but with prophylaxis can be diminished to less than one percent [Galvin and Cohen, 2004]. Several other factors such as the presence of sexually transmitted diseases, especially those that generate genital inflammation and ulcers, and pregnancy are described to increase the susceptibility to HIV infection, whereas circumcision decreases it up to 60% [Auvert et al., 2005; Bailey et al., 2007; Galvin and Cohen, 2004; Gray et al., 2007].

2.3.2. Viral factors associated with the susceptibility to HIV infection

For now there is no clear evidence that HIV-1 viral diversity (subtypes) influence HIV-1 transmission. Although the prevalence of CRF01_AE increased during 1995 to 1998 compared to subtype B among Thai IDUs [Hudgens et al., 2002], it is unclear whether other factors (host and epidemiological) have contributed to that. In Uganda and its neighbouring areas, subtype D has shown to have R5/X4 dual tropism more often than other HIV-1 subtypes, but presumably it does not influence viral transmissibility but rather affects viral pathogenesis [Church et al., 2010].

The HIV viral load of a donor is a major factor influencing its transmissibility. Studies on discordant couples have shown that viral load is in a positive correlation with the transmission rate and that subjects with plasma viral load of less than 1000 copy/mL rarely transmit the virus [Fideli et al., 2001; Quinn et al., 2000]. It is likely that similar trends are seen in all transmission routes. In addition, the stage of HIV infection has been associated with the acquisition of HIV. Namely persons with acute infection are more likely to transmit the virus than those in the other stages of infection [Brenner et al., 2007; Powers et al., 2008; Wawer et al., 2005]. This probably reflects the influence of viral load rather than anything else.

2.3.3. Host factors associated with the susceptibility to HIV infection

The analysis of host factors could be divided into immunological and genetic factors.

2.3.3.1. Immune factors associated with the susceptibility to HIV infection

The majority of studies exploring the associations between host immune factors and the susceptibility to HIV have been conducted in the sexually exposed HIV uninfected subjects (ESNs). Velilla et al (2005) showed in vitro that the peripheral blood monocytes of ESNs have a greater potential to undergo spontaneous apoptosis as well as HIV induced apoptosis compared with the cells of healthy controls [Velilla et al., 2005]. The higher expression of INF-α in ESNs as compared to the controls has also been demonstrated because INF-α induces apoptosis of CD4+ T cells and inhibits HIV replication [Hirbod et al., 2006]. In addition, a higher production of INF-γ by innate immune cells (e.g. NK cells and CD3+/CD56+ cells) in ESNs compared to healthy controls has been suggested to have a controlling effect in sexual and vertical transmission [Kuhn et al., 2001; Montoya et al., 2006]. Also, highly exposed IDUs had a higher lytic activity of NK cells and NK cells producing INF-γ, tumor necrosis factor alpha (TNF-α), CCL3, CCL4 and CCL5 than controls and HIV-1 seroconverters [Scott-Algara et al., 2003].

The level of innate soluble factors has been associated with the resistance to HIV infection. The level of CCL5 and CCL3 is elevated in cultures of HIV-specific CD4+ T cells from ESNs compared to healthy controls, and these chemokines were able to inhibit the replication of R5-tropic viruses [Furci et al., 1997]. Through the activation of these chemokines, the CD4+ T cells from ESNs were less susceptible to HIV than those from healthy controls[Paxton et al., 1996].

Immune activation is essential to have an effective immune response against pathogens including HIV but also to induce the viral replication in infected cells [Biasin et al., 2000]. Begaud et al showed a lower number of CD4+HLA-DR+ T cells in ESNs compared to healthy controls [Begaud et al., 2006]. This suggests that lower immune activation may contribute to HIV resistance. Baisin et al (2000) found that CD4+CD28+ and CD4+CD38+ T cells were augmented in ESNs compared to the controls. Also they showed that PBMCs from ESNs express higher levels of IL-6, IL-10, IL-12, INF-γ, TNF-α, TNF-β mRNA than PBMCs from controls. In ESNs, HIV-specific INF-γ-secreting CD8+ T cells in cervical vaginal washes were increased compared to controls [Biasin et al., 2000]. Recently, it has been demonstrated that CCR5 expression on CD4+ T cells is higher in HESNs than in unexposed uninfected subjects. In addition, HESNs had lower levels of naïve and CD28+ T cells and higher level of HLA-DR+ T cells compared to unexposed uninfected subjects [Suy et al., 2007].

The resistance to HIV infection is also associated with the production of HIV-1 specific immunoglobulin A in cervicovaginal secretions, saliva, serum, maternal milk and urethral swabs [Piacentini et al., 2008].

2.3.3.2. Host genetic factors and the susceptibility to HIV infection

In different association studies, several host genetic factors have been related to the risk of HIV infection. As presented in Table 1 the major attention has been on *CCR5* and its ligands.

Table 1. The associations between chemokines and chemokine receptors and the susceptibility to HIV-1

Gene	SNP/haplotype	Effect on susceptibility	Population or region; transmission	References
Chemokine receptors				
CCR5/ CCR5-CCR2	rs333 (Δ32) Δ32/Δ32	Complete resistance to HIV-1 R5 strains	Caucasian; various risk groups	[Dean et al., 1996; Samson et al., 1996]
	HHD/HHD	Increased risk of HIV/MTCT	Kenyan; MTCT	[John et al., 2001]
	HHE, HHE/HHE		Argentinean, Caucasian, AA; MTCT, sexual	[Mangano et al., 2001; Tang et al., 2002]
	HHF*2, HHF*2/HHF*2	Protective against HIV/MTCT	Asian, Argentinean; MTCT, sexual	[Louisirirotchanakul et al., 2002; Mangano et al., 2000]
	Heterozygocity of rs333 and rs1799987 (G303A)		Caucasian; sexual	[Hladik et al., 2005]
	rs1799987G, rs1799988T		Malawi; MTCT	[Pedersen et al., 2007]

Table 1. Continuation

Gene	SNP/haplotype	Effect on susceptibility	Population or region; transmission	References
Chemokines				
CCL5	rs2280789T (In.1.1T), rs2107538A (-471A) and rs2280788G (-96G)	Protective against HIV	Indians, Caucasians, Japanese, AA, EA; various risk groups	[Ahlenstiel et al., 2005; An et al., 2002; Liu et al., 1999; Rathore et al., 2008]
	rs2107538A	Increased risk of HIV	AA, EA, Asians; various risk group	[An et al., 2002; McDermott et al., 2000]
	3'-222C		AA, EA, Asians; various risk groups	[An et al., 2002]
	Homozygocity of rs2107538A and rs2280788C (-471A/A and -96C/C)		EA; sexual	[Gonzalez et al., 2001]
CCL3L1	Higher copy-number than population median	Protective against HIV/MTCT	EA, AA, African, Japanese; MTCT, various risk groups	[Gonzalez et al., 2005; Meddows-Taylor et al., 2006; Nakajima et al., 2007]
	ss46566437T, ss46566438T, *and* ss46566439C		AA ; various risk groups	[Modi et al., 2006]
CCL2-CCL17-CCL11	Hap 7 (31 kb) at 17q11.2-q12		EA; various risk groups	[Modi et al., 2003]
DC-SIGN	rs4804803C (–336C)	Increased risk of HIV	EA; various risk groups	[Martin et al., 2004]

AA – African Americans; EA – European Americans
Various risk groups – analyses comprised many different transmission routes (mucosal and parenteral, except MTCT) and in majority of the cases these routes were combined together not analysed separately.

The major attention that CCR5 and its ligands has gained is because HIV uses CD4 as the main receptor and CCR5 or another chemokine receptor, C-X-C chemokine receptor 4 (CXCR4), as the co-receptor to enter into T lymphocytes [Berger et al., 1999] (Figure 2). Based on which co-receptor is used, the viruses are determined to R5-tropic (using CCR5), X4-tropic (using CXCR4) or R5X4-tropic (using both co-receptors, also called as dual/mixed tropic) [Berger et al., 1998]. At the beginning of HIV infection R5-tropic viruses are prevailing.

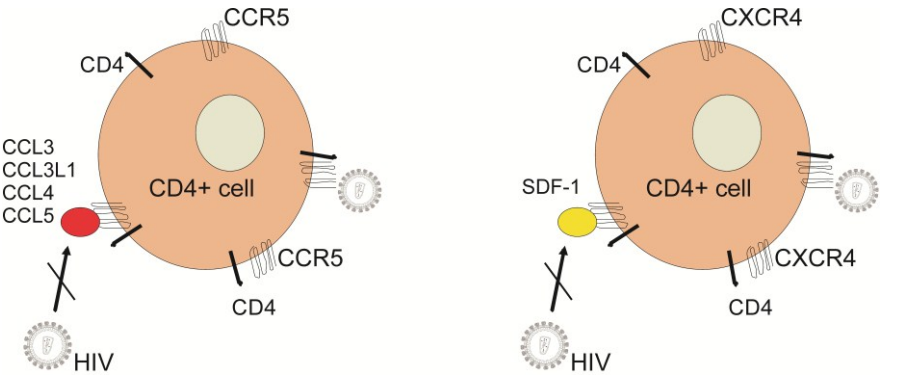


Figure 2. The usage of co-receptor by HIV-1 and main natural ligands for both co-receptors. The R5-tropic and X4-tropic virus are presented. The CCR5 ligands CCL3, CCL3L1, CCL4 and CCL5 (in red on the left panel) and CXCR4 ligand SDF-1 (in yellow on right panel) compete with HIV for CXCR4 and CCR5 occupation, respectively, blocking HIV entry to the cell (crossed arrow).

The host genetic factors that influence HIV susceptibility are mainly found in studies using targeted gene approach, and in lesser extent, in genome wide association studies (GWASs) [Limou et al., 2010]. In addition to the genes of CCR5 and its ligands, the attention has included also cytokines and HLA (Table 2).

Table 2. The associations between different host genetic factors and the susceptibility to HIV-1

Gene	SNP/ haplotype	Effect on susceptibility	Population or region, transmission	Referencess
Cytokines				
IL-10RB	rs2266590A	Protective against HIV	AA; sexual, IVDU	[Shrestha et al., 2010b]
IL-20	rs2981572T		AA; sexual, IVDU	[Shrestha et al., 2010b]
IRF-1	rs17848395A (619A)	Protective against HIV	African; sexual	[Ball et al., 2007]
	rs17848424G (6516G)		African; sexual	[Ball et al., 2007]

Table 2. Continuation

Gene	SNP/ haplotype	Effect on susceptibility	Population or region, transmission	Referencess
HLA system				
HLA	HLA class I concordance between mother and child Mother's homozygosity	Increased risk of MTCT	Kenyan; MTCT	[Mackelprang et al., 2008]
	A*36	Increased risk of HIV	Zambian; sexual	[Tang et al., 2008]
	G*010108		Zimbabwean; sexual	[Matte et al., 2004]
	G*0105N, Cw*18	Protective against HIV	Zimbabwean; sexual	[Lajoie et al., 2006; Matte et al., 2004]
	E*0103		Zimbabwean; sexual	[Lajoie et al., 2006]
KIR	3DS1 homozygocity		Caucasian; sexual, IVDU	[Boulet et al., 2008]
Other				
APOBEC 3G	C40693T	Increased risk of HIV	Caucasian; sexual, IVDU	[Valcke et al., 2006]
APOBEC 3B	Δ3B/Δ3B		EA; various risk groups	[An et al., 2009]
TRIM5	rs16934386C		AA; Various risk groups	[Javanbakht et al., 2006]
	rs7127617C		AA; various risk groups	[Javanbakht et al., 2006]
	rs10838525A (R136Q)		EA; sexual	[Speelmon et al., 2006]
	rs10838525A (R136Q)	Protective against HIV	AA, Pumwani; sexual	[Javanbakht et al., 2006; Price et al., 2010]
	rs3740996T (H43Y)		AA; various risk groups	[Javanbakht et al., 2006]
TLR3	rs3775291T (Leu412Phe)		European; IVDU	[Sironi et al., 2012]
NLRP3	rs10754558G		Brazil , Italy; sexual, MTCT	[Pontillo et al., 2010]
CD4	868T	Increased risk of HIV/MTCT	Kenyan, Nairobi; sexual, MTCT	[Choi et al., 2010; Oyugi et al., 2009]

AA – African-American; EA – European-American

Various risk groups – analyses comprised many different transmission routes (mucosal and parenteral, except MTCT) and in majority of the cases these routes were combined together not analysed separately.

2.4. Factors influencing the susceptibility to HCV and viral clearance

The studies of HCV infection have mostly focused on the response to treatment and adverse effects, and not on the disease susceptibility, but there are some exceptions.

2.4.1. The route of transmission and the susceptibility to HCV

The route of HCV transmission is through blood products – blood transfusion in early years and contaminated needles among IDUs at present times [Alter et al., 1975; Shepard et al., 2005; Wasley and Alter, 2000]. Similar to HIV, the percentage of HCV infection (up to 85%) is very high among IDUs [Burt et al., 2007; Shepard et al., 2005; Tseng et al., 2007]. The possible reason for such efficient transmission is the long viability (more than 2 months) of the virus in the needles [Paintsil et al., 2010].

2.4.2. Viral factors associated with the susceptibility to HCV

Similar to other blood borne infections, the high viral load is also a significant contributing factor in HCV transmission. Compared to HIV, HCV is more infectious and thus more easily transmittable. When in IDUs the prevalence of HIV is around 60% then in the same population the HCV infection rate may reach up to 85% or even higher [Mathers et al., 2008; Nelson et al., 2011]. The viral escape mutations are the factors that contribute to HCV persistence so that the presence of them may be associated with a delay of the adaptive immune response [Bowen and Walker, 2005; Rehermann and Nascimbeni, 2005]. A study of two patients infected with the same strain showed that even persons sharing several HLA alleles, one person cleared the virus and the other one had a chronic infection. The first one had multispecific and vigorous T-cell responses but the other had viral mutations in CD8+ T-cell epitope [Tester et al., 2005]. Cox et al (2005) showed that no substitutions within CD8+ T-cell epitopes were observed in subjects who cleared the virus but substitutions existed in 60–75% in subjects who had persistent infection [Cox et al., 2005].

2.4.3. Host factors associated with HCV clearence

In contrast to HIV infection, HCV can be spontaneously cleared from the organism in approximately 20% of the cases [Villano et al., 1999]. HCV clearance has been evaluated in several studies. The susceptibility to HCV has gained less attention.

2.4.3.1. Immune factors associated with HCV clearence

In acute HCV infection, the HCV-specific CD4+ T cells are important in the controlling of the infection, and are crucial in HCV clearance [Diepolder et al., 1995; Gerlach et al., 1999; Lechner et al., 2000b; Missale et al., 1996; Rosen et al., 2002]. Persons who are able to clear the virus have broad and strong CD4+ T cell responses, while chronically infected subjects have poor and narrow responses [Day et al., 2002; Urbani et al., 2006]. In addition, an expression of Th1 cytokine profile from peripheral mononuclear cells (PBMCs) from persons with viral clearance, compared to Th2 profile from chronically infected persons suggests that Th1 is associated with a successful immune response in the early stages of the infection [Folgori et al., 2006; Tsai et al., 1997; Ulsenheimer et al., 2003]. The accumulation of virus-specific CD8+ T cells in the liver has been associated with the transient clearance of viral RNA from blood plasma [Cooper et al., 1999; Thimme et al., 2002]. Similar to CD4+ T cell responses, a broad and multispecific CD8+ T cell response is associated with viral clearance [Cooper et al., 1999; Cox et al., 2005; Cucchiarini et al., 2000; Deignan et al., 2002; Durante-Mangoni et al., 2004; Nascimbeni et al., 2011; Shoukry et al., 2003; Thimme et al., 2002; Wherry et al., 2003].

2.4.3.2. Genetic factors associated with HCV clearance and/or the susceptibility to HCV

The host genetic factors are believed to play a crucial role in HCV clearance and in some cases also in susceptibility. White American IDUs have higher chance to clear HCV than African-Americans [Villano et al., 1999], which refers to a genetic variation between these populations. In another study, a higher frequency of HCV persistence was observed among non-Hispanic blacks compared with non-Hispanic whites or Mexican Americans [Alter et al., 1999]. One suggested mechanism behind this phenomenon is the lower INF-γ production in the primary infection by African-Americans compared to European-Americans [Sugimoto et al., 2003]. No specific genetic factor has been found to explain these results but different SNPs have been associated with HCV clearance. For example, polymorphisms in 3' untranslated region of 2'-5'oligo-adenylate synthetase 1 gene, in the promoter region of dsRNA-dependent protein kinase, HLA class I genes and haplotypes of inducible nitric oxygen synthase gene and IL-10 gene have been associated with HCV clearance [Fanning et al., 2004; Knapp et al., 2003a; Knapp et al., 2003b; Mangia et al., 2004; Oleksyk et al., 2005; Yee et al., 2004]. Additional human genetic factors that influence HCV clearance are listed in Table 3.

In 2009, three GWASs identified SNPs in IL28B gene that were related to the outcome of interferon therapy such that the rare variant had a worse outcome compared to the common variant [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. The same SNPs were showing similar effect on HCV clearance [Thomas et al., 2009].

Table 3. The associations between host genetic factors and clearance/susceptibility to HCV

Gene	SNP/haplotype	Effect on susceptibility/ viral clearance	Population or region [references]*
CCR5	rs333 (Δ32) homozygocity	Increased risk of HCV	Caucasian [Woitas et al., 2002]
CCL3L1	Lower copy number than population median	Increased risk of chronic HCV	Caucasian [Grunhage et al., 2010]
IL28B	rs12798960C homozygocity	Decreased risk of HCV, spontaneous clearance	Caucasian, Egyptians [Pasha et al., 2013; Thomas et al., 2009]
KIR/HLA	*KIR2DL3/HLA-C1* homozygosity	Decreased risk of HCV, spontaneous viral clearance	Caucasian [Zuniga et al., 2009]
IL-10	–1082A homozygocity	Decreased risk of HCV	Egyptian [Pasha et al., 2013; Thomas et al., 2009]
	–1082G	Spontaneous viral clearance	Argentinean [Ramos et al., 2012]
TGF-β	–509T	Increased risk of HCV	Egyptian [Pasha et al., 2013; Thomas et al., 2009]
IL-4	+3C	Spontaneous viral clearance	Argentinean [Ramos et al., 2012]

*In the majority of listed studies the transmission of HCVis unknown, but in general the HCV infection has been transmitted by parenteral route

The following chapters will focus on the genetic diversity of chemokine receptor CCR5, its ligands CCL3 and CCL5 and TLR3. These molecules play a role in HIV entry into the cells and due to that it could be assumed that the genes encoding these molecules could interfere to the susceptibility to HIV infection. In addition, they are involved in the pathogenesis of HCV infection, which is a common co-infection among IDUs, the population where Estonian HIV epidemic situates.

2.5. Chemokines and chemokine receptors

Chemokines are chemoattractant cytokines produced by a variety of cell types including T cells, macrophages, NK cells, B cells, and masts cells. Chemokines are involved in the regulation of cell trafficking to areas of injury and in inflammatory and homeostatic processes. The specific effects of chemokines are

mediated through members of a family of 7-transmembrane-spanning, G-protein-coupled receptors [Murphy, 1994].

Some chemokine receptors serve as entries to the cell for pathogens like HIV that is using CCR5 and CXCR4 as the two main co-receptors for viral entry. For that reason the most studied host factors in the context of HIV are with CCR5 and its ligands. As discussed above, the genetic diversity of CCR5 and its ligands CCL3-CCL4 cluster and CCL5 (previously named as RANTES) has been demonstrated to influence the susceptibility to HIV and/or the disease progression (Table 1).

2.5.1. The genetic variability of CCR2-CCR5 and the susceptibility to HIV infection

CCR5 gene is located at 3q21.3 and includes two 5' untranslated exons (Exon1, Exon2a and Exon2b) and one exon (Exon3) that contains the entire coding sequence [Carrington et al., 1999; Mummidi et al., 1997] (Figure 5). The expression of CCR5 is restricted to the activated T cells and memory T cells, monocytes/macrophages, microglial cells and, to a lesser extent, B cells [Bleul et al., 1997; He et al., 1997; Wu et al., 1996; Wu et al., 1997].

The best known polymorphism in CCR5 gene is a 32 bp deletion in ORF (CCR5-Δ32) that in a homozygote state gives complete resistance to HIV-1 R5 [Samson et al., 1996]. The prevalence of *CCR5*-Δ32 allele varies between populations being highest in Scandinavian (*CCR5*-Δ32 homozygocity 2–3% and heterozygocity around 20%) and absent in African populations [Novembre et al., 2005]. In Estonia, about 3% of populations carries *CCR5*-Δ32 in the homozygous state and 21% in the heterozygous state [Adojaan et al., 2007; Kalev et al., 2000].

A minor HIV-1 co-receptor *CCR2* and *CCR5* are separated by only ~14 kb, partly explaining the near complete linkage disequilibrium between these two genes [Smith et al., 1997]. Polymorphism, a G-to-A transition at position 190, changes CCR2B codon 64 from valine to isoleucine, introducing a conservative amino acid change into the first transmembrane domain [Smith et al., 1997]. Studies have not determined a difference in HIV-1 co-receptor activity between the variant *CCR2B*-64I and *CCR2B*-64V, and excluded the possibility that *CCR2B*-64I exerts a dominant-negative effect on the expression and the activity of CCR5 [Lee et al., 1998; Mariani et al., 1999].

In addition, several polymorphisms within the *CCR5* promoter region and one in *CCR2* gene have been identified as being associated with the susceptibility to HIV infection [Gonzalez et al., 1999]. Based on seven SNPs in the cis-regulatory region of *CCR5* and the presence of the *CCR2* V64I and *CCR5* ORF Δ32, nine *CCR5* human haplotypes (HH) have been defined by the evolutionary analyses [Mummidi et al., 2000] (Figure 3). These *CCR5* HH are designated as HHA to HHG*2, with HHF*2 and HHG*2 denoting the haplotypes that bear the *CCR2*-64I and *CCR5*-Δ32 polymorphisms, respectively.

Because of its similarity to the chimpanzee *CCR5* sequence, the human *CCR5* HHA haplotype is classified as the ancestral *CCR5* haplotype [Mummidi et al., 2000] (Figure 3).

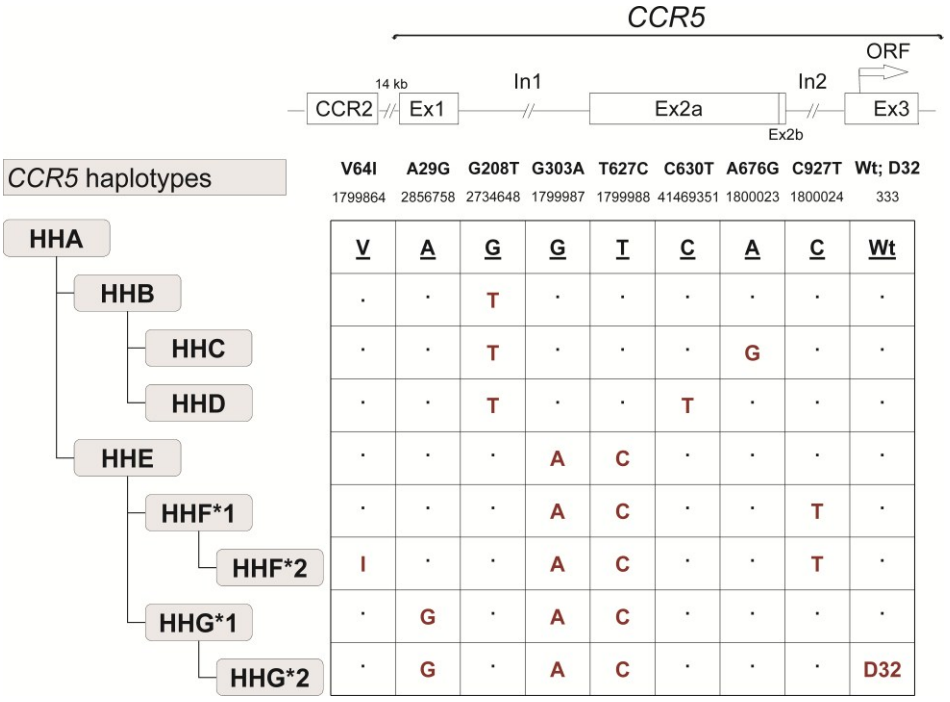


Figure 3. *CCR5* polymorphisms and haplotypes. Nucleotide variations relative to the ancestral sequence and the *CCR5* numbering systems are shown. Top numbering is based on GenBank accession numbers AF031236 and AF031237 bottom numbers are rs-numbers. Ex – exon; In – intron; ORF – open-reading frame; Wt – wild-type; D32 – 32-basepair deletion.

Both *CCR5* SNPs and haplotypes have been associated with the susceptibility to HIV and the disease progression in sexual and MTCT (Table 1).

2.5.2. *CCL3L1* and the susceptibility to HIV infection

The cluster of proinflammatory CC chemokines containg 16 genes is localized at 17q11.2-q12. The MIP-1α, and MIP-1β were renamed as CCL3 and CCL4. Four genes comprise the two closely related, paralogous pairs *CCL3-CCL3L* and *CCL4-CCL4L* [Modi, 2004] (Figure 4). Both of these pairs share 95% sequence identity at both the genomic and the amino acid levels.

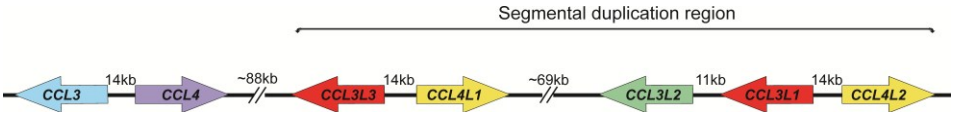


Figure 4. Organization of *CCL3-CCL4* cluster. Modified from PlosPathogens [Gornalusse et al., 2009]

Official symbols for CCL3L genes are *CCL3L1, CCL3L2* and *CCL3L3* and for CCL4L genes are *CCL4L1* and *CCL4L2*. *CCL3L1* and *CCL3L3* are separate genes, but they have three identical exons encoding identical proteins [Menten et al., 2002; Modi, 2004], and for that reason, they are denoted together as CCL3L1. CCL3L2 is a pseudogene containing a 5' truncation compared to CCL3L1 [Hirashima et al., 1992]. The non-allelic copies of CCL3 and CCL4 were designated as CCL3L (previously LD78β, AT 464.2, GOS19-2) and CCL4L (previously LAG-1, AT744.2) [Zlotnik and Yoshie, 2000].

Among all human, chemokine genes CCL3L and CCL4L are present in variable copy numbers. Individuals may vary in the total number of CCL3L and CCL4L genes and in their individual components. The *CCL3L* copy number varies from 0 to 14 and *CCL4L* from 0 to 10 and is strongly influenced by ethnicity. The highest copy number of *CCL3L-CCL4L* is in African populations (median 6 for *CCL3L* and 4 for *CCL4L*) and the lowest in European populations (median 2 for both *CCL3L* and *CCL4L*) [Colobran et al., 2008; Gonzalez et al., 2005].

Although CCL3, CCL3L, CCL4 and CCL4L bind to CCR5, the CCL3L1 is more effective in inducing intracellular Ca^{2+} signaling and chemotaxis through CCR5 compared to CCL3. Its binding affinity to CCR5 is higher, and the antagonism to HIV-1 entry through CCR5 is greater than CCL3 being also better than CCL5 [Aquaro et al., 2001; Menten et al., 1999; Nibbs et al., 1999; Xin et al., 1999]. CCL3 and CCL4 are expressed by monocytes/macrophages, T and B lymphocytes and dendritic cells (although they are inducible in most mature haematopoietic cells). Both of them are potent chemoattractants of these cells [Menten et al., 2002].

Studies have shown that the higher copy number of *CCL3L1* than the population median is associated with the decreased risk of HIV infection and has a beneficial effect in the disease progression [Gonzalez et al., 2005] (Table 1). This association was observed in different ethnic groups with various transmission routes – MTCT in Africans, sexual transmission in AA, EA and Hispanic-Americans, and blood transfusion in Japanese [Gonzalez et al., 2005; Kuhn et al., 2007; Meddows-Taylor et al., 2006; Nakajima et al., 2007]. However, several studies have not found any associations between the *CCL3L1* copy number and the acquisition of HIV or the disease progression [Bhattacharya et al., 2009; Rathore et al., 2009; Shao et al., 2007; Urban et al., 2009].

2.5.3. The genetic variability of CCL5 and the susceptibility to HIV infection

The CCL5 gene is located in chromosome 17 (17q11.2–q12) and consists of a promoter, three exons and two introns [Donlon et al., 1990]. HIV association studies have been concentrating mainly on three SNPs in *CCL5*. Two of these SNPs are located in the promoter region (G-471A and C-96G) and one in intron (TIn1.1C). The promoter -471A and -96G alleles enhance CCL5 production and intron In1.1C allele reduces CCL5 transcription [An et al., 2002; Bai et al., 2005; Liu et al., 1999; Nickel et al., 2000; Tanaka et al., 2006].

The CCL5 is an inflammatory chemokine, which mediates chemotactic activity in T cells, monocytes, dendritic cells, natural killer cells, eosinophils, and basophils [de la Rosa et al., 2003; Roth et al., 1995; Schall et al., 1990].

The *CCL5* SNPs have been associated with the different risk of HIV infection in many populations as shown in Table 1.

2.6. Toll-like receptors

TLRs are pattern-recognition receptors that activate innate immune response in humans and thus play a key role in immune response [Kawai and Akira, 2011]. The human TLRs family consists of 10 members (TLR1–TLR10), which can be classified to (i) TLRs that generally locate in intracellular compartment (e.g. endosomes) – TLR3, TLR7, TLR8 and TLR9 and (ii) TLRs that typically locate on the cell surface – TLR1, TLR2, TLR4, TLR5 and TLR6 [Beutler, 2004; Leulier and Lemaitre, 2008; Medzhitov, 2001; West et al., 2006]. Intracellular TLRs nucleic acid-based agonists are specialised to recognize viruses, whereas cell-surface TLRs detect products (e.g glycolipids, lipopeptides, flagellin), which are a part of a variety of organisms (bacteria, parasites and fungi [Kawai and Akira, 2006]. The TLR10 plays a role in the innate immune response to infection in intestinal epithelial cells [Regan et al., 2013].

In terms of blood borne infections such as HIV, HCV and HBV, the intracellular TLR members (TLR3, TLR7, TLR8 and TLR9) that recognize double-stranded RNA (dsRNA) and trigger immune responses against both RNA and DNA viruses by stimulating type I INFs and inflammatory cytokines, have been suggested to influence the course of these infections [Alexopoulou et al., 2001; Matsumoto et al., 2004]. Recently, in addition to TLR7 and TLR9, also TLR3 has gathered attention in HIV susceptibility [Breckpot et al., 2010; Mandl et al., 2008; Sironi et al., 2012]. It has been suggested that retroviral genome dimerises and forms a secondary structure, and it is likely that during this process a double-stranded RNA is formed [Greatorex, 2004; Russell et al., 2004; Watts et al., 2009]. Using a xenotropic murine leukemia virus-related virus as a model, Miyauchi et al., (2012) demonstrated that TLR3 is able to recognize retroviral genome and thus evoke antiviral response [Miyauchi et al.,

2012]. For that reason the diversity of *TLR3* has recently gained interest in the context of HIV infection.

2.6.1. The genetic variability of TLR3 and the susceptibility to HIV infection

TLR3 gene is located in chromosome 4 (4q35.1) and contains four exons. In European population, TLR3 gene comprises 12 segregating sites (without indels) and in exon 4, one non-synonymous mutation (rs3775291 C→T, also known as Leu412Phe).

TLR3 is expressed within the endosomal compartment of conventional dendritic cells, macrophages, T lymphocytes, fibroblasts and hepatocytes [Jiang et al., 2005; Lang et al., 2006; Muzio et al., 2000; Rudd et al., 2005; Tabiasco et al., 2006; Visintin et al., 2001]. In addition, TLR3 is expressed in epithelial cells as well as on their surface [Sha et al., 2004; Uehara et al., 2007].

Accordingly, a *TLR3* polymorphism rs3775291 C→T has been associated with an increased risk of enteroviral myocarditis and herpes simplex-1 encephalitis but provides protection against tick-borne encephalitis [Gorbea et al., 2010; Kindberg et al., 2011; Zhang et al., 2007]. Recently, a minor allele of *TLR3* rs3775291 (T) was shown to be associated with the resistance to HIV-1 infection in the Spanish (exposed by intravenous transmission) and Italian cohorts (exposed by sexual transmission) of HESNs such that T homozygosity was overrepresented among infected HESNs as compared to healthy volunteers [Sironi et al., 2012].

2.7. Associations between *CCR5, CCL3L1, CCL5* and *TLR3* and HCV susceptibility and viral clearance

In terms of HCV infection, the CCR5 is involved in the complex processes of HCV immune response. Studies have suggested that Th1-CCL5-CCR5 system might be important in the induction of immunity against HCV and thus influences the outcome of chronic HCV infection [Larrubia et al., 2008; Zeremski et al., 2007]. It is hypothesised that CCR5 interacts with its ligands to promote the recruitment of Th1 expressing cells into the liver and thus mediates the clearance of HCV infected hepatocytes [Kusano et al., 2000; Shields et al., 1999]. In addition, HCV itself down-regulates CCR5 expression via a direct interaction of HCV E2 envelope protein with the tetraspanin CD81 [Nattermann et al., 2004; Solari et al., 1997]. Nevertheless, HCV core protein and NS5A alters *CCL5* promoter activity [Soo et al., 2002] resulting in higher levels of CCL5. An increased binding of CCL5 to CCR5 decreases CCR5 surface density due to receptor internalization [Solari et al., 1997] and possibly through that affects the Th1-CCL5-CCR5 system. However, the influence of the genetic variation of CCR5 and its ligands is poorly studied.

The *CCR5*-Δ32 homozygosity has been associated with the increased risk of HCV infection by Woitas et al., 2005 with contrary results from other studies [Glas et al., 2003; Poljak et al., 2003; Ruiz-Ferrer et al., 2004]. No other associations between *CCR5* SNPs and HCV acquisition have been described. The presence of G303A (rs1799987) regardless of other mutations (including HHE-HHG*2) was more frequent among persons with the sustained virological response to interferon-ribavirine therapy than non-responders [Konishi et al., 2004]. A similar effect has also been demonstrated in patients with HHE homozygosity [Dorak et al., 2002].

The *CCL5* polymorphisms have not been correlated with HCV acquisition. However, they have been associated with the outcome of HCV treatment. *CCL5* -471A has been associated with less severe hepatic inflammation and milder portal inflammation in Caucasians [Hellier et al., 2003; Promrat et al., 2003]. The possession of In1.1C and 3'-222C and the combination of -471A/In1.1C/3'222C are related to the worse response to interferon-ribavirin therapy in patients infected with HCV genotypes 1 and 4 [Wasmuth et al., 2004].

The *TLR3* polymorphism has been the focus in HCV infection because this receptor recognizes also HCV. However, until now no associations between TLR3 polymorphisms and susceptibility to HCV chronic infection has been found [Askar et al., 2009].

2.8. Summary of literature

Some individuals who, despite multiple exposures to HIV and HCV (hemofiliacs, commercial sex workers, IDUs who share needles etc.), will not get infected – they are defined as HESNs. This group of people has been an interest of research for a few decades in order to identify factors that influence the susceptibility to HIV. The great interest has been focused on the human genetic factors such as HIV co-receptor CCR5 and its ligands genes. An excellent example of discovery was the identification of Δ32 homozygocity, which gives the complete resistance against HIV R5-tropic viruses [Samson et al., 1996]. Still, this does not explain the HESNs phenomenon entirely because most of them are not *CCR5*-Δ32 homozygots.

Other polymorphisms in *CCR5*, its ligands and also other genes have been associated with the susceptibility to HIV (Table 1). However, the majority of genetic studies have been conducted among populations exposed by hetero- or homosexual contact, or children born to HIV-positive mothers, in which the transmission rate is much lower than when viruses are directly injected into the bloodstream.

In addition to HIV, IDU populations are often affected by HCV and HBV. The CCR5 gene family has also been demonstrated to play a role in HCV infection but in a lesser extent. Still, association studies between HCV and

genetic factors are mainly conducted in the chronically infected subjects looking at the role of genetic factors in response to treatment. Much less attentions is paid to the susceptibility to infection or spontaneous clearance, which is characteristic to HCV but not to HIV. Taken together, whether or how *CCR5* and its ligands' polymorphisms influence susceptibility to HIV and/or HCV in IDUs is limited or unknown.

The population of Estonian IDUs (both HIV positive and negative) gives a unique opportunity to explore these associations for several reasons. Firstly, Estonian IDUs are homogeneous – relatively young, Caucasian, males co-infected with HCV [Uuskula et al., 2007]. Secondly, they are infected with monophyletic HIV-1 CRF06_cpx viruses, which provide the possibility to evaluate associations between host genetic factors and the susceptibility to HIV with minimal viral heterogeneity [Avi et al., 2011; Avi et al., 2009; Avi et al., 2010; Zetterberg et al., 2004]. Thirdly, the high prevalence of HCV enables investigations in the associations between host genetic factors and HCV and/or HIV/HCV co-infection.

As described above, CCR5 with its ligands are playing a crucial role in HIV infection participating in the viral entry into cells. In addition, CCR5 and its ligands are involved in the course of HCV infection, and TLR3 interacts with these viruses by recognising HCV and probably also HIV. Thus, this thesis concentrates on the associations between CCR5 and its ligands (CCL3L1 and CCL5) and TLR3 gene variability and susceptibility to HIV and/or HCV infection.

3. AIMS OF THE RESEARCH

The general aim of this thesis was to assess the associations between host genes encoding innate and adaptive immunity, and the acquisition of HIV and/or co-infection with HCV in the IDUs of Caucasian origin.

The study had the following objectives:

1. To determine whether and how *CCR5* haplotypes/haplotype pairs are related to the susceptibility to HIV and/or HCV infection among IDUs
2. To identify whether and how the *CCL3L1* gene copy number is associated with the susceptibility to HIV and/or HCV infection among IDUs
3. To determine whether and how *CCL5* haplotypes/haplotype pairs are related to the susceptibility to HIV and/or HCV infection among IDUs
4. To evaluate whether and how *TLR3* polymorphisms are associated with the susceptibility to HIV and/or HCV infection in IDUs

4. MATERIALS AND METHODS

Altogether, the thesis included four studies conducted in Tallinn and Ida-Viru County in 2006 and 2007, and in Tallinn in 2010. These two regions are the epicentres of Estonian HIV epidemic. Two cross-sectional studies were conducted in two syringe exchange programmes and in three Estonian prisons in 2006 and 2007, and in one syringe exchange programme in 2010. The studies are detailed in Table 4.

4.1. Study design and population

All studies were conducted in IDUs populations and two recruitments were used – one for CCL3, CCL5 and CCR5 studies and another for TLR3 study (Table 4). Subjects in the syringe exchange programmes were recruited by using respondent driven sampling (RDS) [Malekinejad et al., 2008].

RDS is a sampling method for the recruitment of most-at-risk populations (hidden populations) for behavioural and biological HIV studies. It is a chain-referral sampling that begins with a specifically selected set of individuals (called seeds) from the target population. The seeds will recruit subjects from their social network (recruited participants) who will recruit the next ones. Seeds and recruited participants will be interviewed and, if necessary, biological samples are collected. The process of recruitment produces several waves of recruits and finally the subjects will reach to the equilibrium, which indicate that final samples are not biased by the selection of seeds.

In the current thesis, two RDS were conducted and in both six seeds were used. The recruitment of IDUs was conducted by the Department of Public Health in the University of Tartu and Estonian National Institute for Health Development. In addition, subjects were recruited from prisons and were invited by their treating physician.

All study subjects donated blood and filled in a questionnaire that included demographic data (date of birth, gender, nationality, risk behaviours, route of transmission and duration of IVDU). The subjects recruited in the syringe exchange programmes admitted an active use of intravenous drugs. The subjects from prisons were determined as IDUs if the previous use of intravenous drugs was reported. The duration of IVDU was defined as time (in full years) between the first episode of using intravenous drugs and the date of data collection. The truncated age and the duration of IVDU were measured in full years.

The blood donors were recruited in Tallinn and Ida-Viru County in 2010. In total, the blood sample leftovers of 500 (all HIV, HBV and HCV negative) blood donors were collected so that half of them were from Tallinn and half of them from Ida-Viru Blood Centre. The demographical data of blood donors was not available.

Table 4. The characteristics of the study populations of the thesis and the successfulness of genotyping

Study name	Recruited study population; No of recruited IDUs (sampling period)	Successfully genotyped; No of IDUs/blood donors	Primary aim	Publication
CCR5 study	IDUs from syringe exchange programmes & prisons; 374 (October 2006–May 2007)	369/500	To determine the associations between *CCR5* haplotypes and HIV and/or HCV seropositivity	2
CCL3L1 study		374/ND	To evaluate the associations between *CCL3L1* copy number and HIV seropositivity	1
CCL5 study		368/ND	To determine the associations between *CCL5* haplotypes and HIV and/or HCV seropositivity	4
TLR3 study	IDUs from syringe exchange programmes & blood donors; 345 (November–December 2011)	345/497	To determine the associations between *TLR3* variability and susceptibility to HIV	3

There was two recruitments (second column) – one indicated in grey and another colourless; ND – not done

In the TLR3 study, 173 of 345 IDUs were HIV seronegative; 88 of them reported using previously used syringes (receptive sharing) and 85 did not admit to the repetitive usage. Latter were called unexposed and were excluded from further analysis. The median of receptive sharing was at least once a month. Based on that, the HESNs were defined as IDUs who had shared syringes at least once a month during last six months (n = 20) and non-HESNs as those who has shared syringes less frequently (n = 68).

Based on similar categories, the sub-groups of HCV seronegative IDUs were determined. HCV negative highly exposed IDUs were defined as IDUs who were HCV seronegative, and reported receptive sharing at least once a month during last six months. HCV negative non-highly exposed were defined as IDUs who were HCV seronegative, and reported receptive sharing less than once a month during last six months.

4.2. Ethical consideration

All study protocols have been approved by the Tallinn Medical Research Ethics Committee and/or the Research Ethics Committee of the University of Tartu and the University of Texas Health Science Center in San Antonio. All subjects signed an informed consent. Blood donors agreed with using their blood leftovers anonymously for research purposes.

4.3. Blood sampling and processing

From all study subjects, except blood donors, appoximately 8–16 ml blood was taken via venepuncture into EDTA tubes (BD Diagnostics, New Jersey, NJ, USA) in the syringe exchange programmes and prisons. The samples were transported to our laboratory in the Department of Microbiology in the University of Tartu within 24 hours and were centrifuged at 3000 rpm for 10 min. Plasma was extracted and divided into several aliquots and stored at –80 ^{o}C. The remaining fraction (cell fraction) of the sample was also divided into the aliquots and stored at -80 ^{o}C for DNA extraction.

The leftover blood samples (approximately 2 ml) of the blood donors were collected into EDTA tubes (BD Diagnostics, New Jersey, NJ, USA), stored at +4 ^{o}C and after confirming their negativity of all three infections they were sent to our laboratory in the Department of Microbiology in the University of Tartu and stored at -80 ^{o}C for DNA extraction.

The genomic DNA was extracted from 200 µl of the stored cell fraction or of the whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The DNA quality and quantity were assessed by NanoDrop 3000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and DNA was stored at -80 ^{o}C.

In the CCL3L1 study, the genomic DNA from cell line A431 (courtesy of professor Ismo Virtanen, University of Helsinki, Institute of Biomedicine) were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

4.4. HIV, HBV and HCV antibody testing

HIV antibody testing was performed in the Estonian Central HIV Reference Laboratory using a fourth generation enzyme-linked immunoassay (Vironistica HIV Uniform II Ag/Ab, BioMerieux, Marcy Etoile, France); all positive results were confirmed by an immunoblotting assay (INNO LIA HIV I/II Score Westernblot, Microgen Bioproducts Ltd, Surrey, UK). HCV and HBV antibody and antigene testing was performed in the laboratory of the Estonian National Institute for Health Development. The presence of HCV antibodies was tested with the ETI-AB-HCVK-3 anti-HCV test (DiaSorin, Vercelli, Italy). HBV seropositivity was assessed by ETI-MAK-4 HBsAg (DiaSorin, Saluggia, Italy) and ETI-AB-COREK Plus (anti-HBc core) (DiaSorin, Saluggia, Italy).

4.5. The determination of CCL3L1 gene copy number

The determination of CCL3L1 gene copy number was performed in the Department of Microbiology, University of Tartu by Maarja Sadam [Sadam, 2008]. CCL3L1 gene copy number was estimated as described by Gonzalez *et al* (2005).

Real-time PCR was carried out on ABI/PRISM 7500 Sequence Detector System detecting the emitted FAM from the probe for *CCL3L1* and VIC from the probe for the *β-globin* during amplification. Real-time PCR was performed in 50 µL using 1x Universal Master Mix, 0.25 nM of appropriate probe, 0.3 nM of appropriate primer pairs and 2–10 ng genomic DNA and the following programme on ABI/PRISM 7500: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec, 60 °C for 1 min. The primers and probes for *CCL3L1* and for *β-globin* are presented in Table 5. The primers were purchased from TAGC (Copenhagen, Denmark) and the rest of the reagents from Applied Biosystems (Carlsbad, CA, USA).

The cycle number at which the fluorescence reached a fixed threshold [cycle threshold (Ct)] was determined. Six serial 1:2 dilutions (20–0.625 ng/reaction) of A431 cells genomic DNA (having two copies of *CCL3L1* per diploid genome) were used to generate standard curves of Ct value for *β-globin* and *CCL3L1* gene. For each test sample, the *β-globin* and *CCL3L1* were amplified in duplicate and the Ct was determined and converted into template quantity using the standard curves. For every individual sample the intervariability was assessed by formula: $V_{i(\%)} = 100*|(R_{i1}–R_{i2})/R_i|$ where V_i was the sample intervariability, R_{i1} and R_{i2} the sample i quantity and R_i the mean of duplicates. All samples where intervariability was more than 15% were reanalysed by the real-time PCR. *CCL3L1* copy number was the ratio of the mean template quantity for *CCL3L1* ($R_{iCCL3L1}$) to the mean template quantity for *β-globin* (R_{iglob}) multiplied by two [*CCL3L1* copy number = $R_{iCCL3L1}/ R_{iglob}*2$]. Afterwards, the *CCL3L1* copy number was rounded to integer.

4.6. The determination of *CCR5, CCL5* polymorphisms and haplotypes and *TLR3* polymorphism

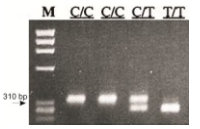
The determination of *CCR5* and *CCL5* SNPs were performed in the prof Sunil K Ahuja' laboratory in the Departments of Medicine, University of Texas Health Science Center, San Antonio, Texas, USA. Seven SNPs in *CCR5* and the presence of *CCR2*-V64I and *CCR5*-Δ32, three SNPs in *CCL5* and one SNP in *TLR3* were determined using TaqMan Allelic Discrimination assay (Table 5). The real-time PCR was performed in 8 µL using 1x Universal Master Mix, 0.25 nM of appropriate probe, 0.3 nM of appropriate primer pairs or SNP assays and approximately 2–5 ng genomic DNA. The sequences of primer and probes are listed in Table 6. The real-time PCRs were carried out on ABI/PRISM 7900 Sequence Detector System using the following programme: 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 sec, 60 °C for 1 min. All

reagents were from Applied Biosystems (Carlsbad, CA, USA). Custom made real-time PCR primers and probes are designed by professor Ahuja's laboratory at the University of Texas Health Science Center at San Antonio.

Table 5. Primers and probes used in the studies

Gene or gene and SNP (rs-number) Refs	**Primers and sequences (5'→3')**	**Probes sequences and fluorescents dyes (5'→3')**
CCL3L1 [Gonzalez et al., 2005]	Forward: TCTCCACAGCTTCCTAACCAAGA Reverse: CTGGACCCACTCCTCACTGG	FAM-AGGCCGGCAGGTCTGTGCTGA-TAMRA
β-globin [Gonzalez et al., 2005]	Forward: GGCAACCCTAAGGTGAAGGC Reverse: GGTGAGCCAGGCCATCACTA	VIC-CATGGCAAGAAAGTGCTCGGTGCCT-TAMRA
***CCR2* V64I** (rs1799864) [Catano et al., 2011]	Forward: TCTTTGGTTTTGTGGGCAACATG Reverse: AGGTAAATGTCAGTCAAGCACTTCA	64V: VIC-CTGGTCGTCCTCATC-MGBNFQ 64I: 6FAM-CTGGTCATCCTCATC-MGBNFQ
***CCR5* A29G** (rs2856758) [Catano et al., 2011]	Forward: TCATGTGGAAAATTTCTCATAGCTTCAGA Reverse: GAGGACTCACACTATGCCAGATAC	29A: VIC-AGTGAAGAATCCTGCCACC-MGBNFQ 29G: 6FAM - TGAAGGATCCTGCCACC-MGBNFQ
***CCR5* G208T** (rs2734648) [Catano et al., 2011]	Forward: CCGTGAGCCCATAGTTAAAACTCTT Reverse: CACAGATGCTCACCACCCAATATTA	208G: VIC-CAACAGGTTGTTTCCGT-MGBNFQ 208T: 6FAM-ACAACAGGTTTTTTC CGT-MGBNFQ
***CCR5* G303A** (rs1799987) [Catano et al., 2011]	Forward: GGGTGGTGAGCATCTGTGT Reverse: GCCAACTTAAACCAACTTTAAATGTAGAGG	303G: VIC-CCTGTGCCCCCTTT-MGBNFQ 303A: 6FAM-CCCTGTGTCCCCTTT-MGBNF

Table 5. Continuation

Gene or gene and SNP (rs-number) Refs	**Primers and sequences (5'→3')**	**Probes sequences and fluorescents dyes (5'→3')**
***CCR5* T627C** (rs1799988)	Commercial probes and primers (Applied Biosystems)	
***CCR5* C630T** (rs41469351) [Gonzalez et al., 1999]	Forward: GGTTAATGTGAAGTCCAGG ATCC Reverse: AACAGTTCTTCTTTTTAAGT TGAGCTTAAA ATAAGCTAGAGAATAGATC TCTGGTTT	RFLP* Restrictase Dra I 
***CCR5* A676G** (rs1800023) [Catano et al., 2011]	Forward: CCAGAGATCTATTCTCTAGC TTATTTTAAGC Reverse: TGTATTGAAGGCGAAAAGAA TCAG	676A: VIC-CAACTTAAAAAG AAGAACTGT- MGBNFQ 676G: 6FAM - AACTTAAAAGGAAGA ACTGT-MGBNFQ
***CCR5* C927T** (rs1800024) [Catano et al., 2011]	Forward: CCTGTTAGTTAGCTTCTGAG ATGAGTAAA Reverse: CCAAACTGTGACCCTTTCCTT ATCT	927C: VIC-TTTGCCAAATGTCTTCT-MGBNFQ 927T: 6FAM - TTTGCCAAATATCTTCT-MGBNFQ
***CCR5* Δ32** (rs333) [Catano et al., 2011]	Forward: CATTACACCTGCAGCTCTCA TTTT Reverse: CGAGTAGCAGATGACCATG ACAA	Wild type: 6FAM-CATACAGTC AGTATCAATTC-MGBNFQ Δ32: VIC-CCATACATTAAA GATAGTC-MGBNFQ
CCL5 C-96G (rs2280788)	Custom made probes and primers (Applied Biosystems)[#]	
CCL5 G-471A (rs2107538)	Custom made probes and primers (Applied Biosystems) [#]	
CCL5 TIn1.1C (rs2280789)	Custom made probes and primers (Applied Biosystems) [#]	
***TLR3* rs3775291**	Commercial probes and primers (Applied Biosystems)	

*****RFLP-PCR was carried out as described in Gonzalez *et al* (2005); [#] designed in the prof Sunil K Ahuja' laboratory in the Departments of Medicine, University of Texas Health Science Center, San Antonio, Texas, USA (sequences available upon request).

The *CCR5* haplotypes were defined as shown in Figure 5 [Gonzalez et al., 1999; Mummidi et al., 2000]. In *CCL5* three SNPs were detected – G-471A (rs2107538) and C-96G (rs2280788) in the promoter region and TIn1.1C (rs2280789) in intron. We focused on there variations as various previous studies had indicated the role of these polymorphism on HIV susceptibility and/or *CCL5* expression (Table 1) [An et al., 2002; Bai et al., 2005; Liu et al., 1999; Nickel et al., 2000; Tanaka et al., 2006]. Additionally, these three SNPs are located in one haploblock based on the genotype data from Caucasian population from HapMap.org (Figure 5).

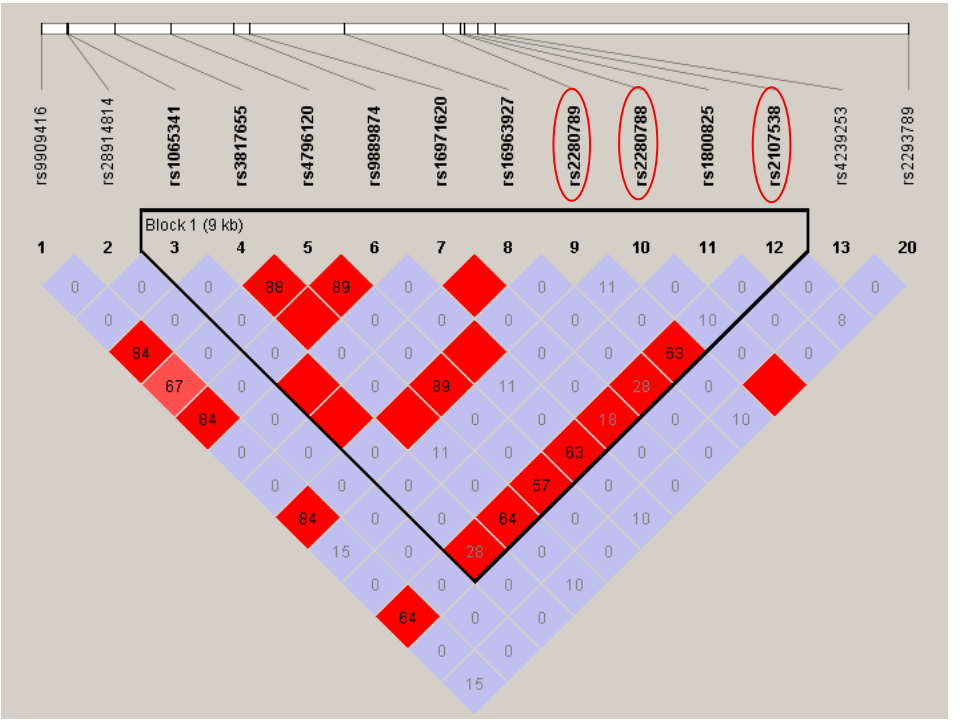
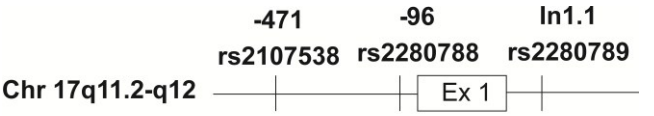


Figure 5. The location of *CCL5* rs2107538, rs2280788 and rs2280789 (indicated in red circles) in the haploblock according to Haploview analysis in Caucasian population. Numbers in the blocks indicate r^2 values.

Using HaploView programme, four *CCL5* haplotypes (A to D) were defined by these 3 SNPs in Caucasian population as demonstrated in Figure 6. The similar haplotypes were also identified from other populations previously (named accordingly as haplotype I to IV) [Rathore et al., 2008].



CCL5 haplotypes	G -471 A	C -96 G	T In1.1 C
Haplotype A	G	C	T
Haplotype B	A	C	T
Haplotype C	A	C	C
Haplotype D	A	G	C

Figure 6. Schematic figure of *CCL5* single nucleotide polymorphisms and *CCL5* haplotypes. On the upper panel schematic figure of CCL5 gene with chromosomal location and positions of SNPs with rs-numbers are shown. In the table, letters in three right side columns indicate nucleotide.

4.7. Statistical analysis

The Pearson's goodness-of-fit chi-square (degree of freedom = 1) was used for the analyses of the Hardy-Weinberg equilibrium. STATA 6.0 (Stata Corporation, College Station, Texas, USA) was used for the statistical analyses in the CCL3L1 study, the programme R versions 2.8.1 (www.r-project.org, last accessed 1st of July, 2011) was used in the CCR5 study and 2.13.1 (www.r-project.org, last accessed 1st of January, 2012) in the TLR3 and CCL5 study.

The differences in the CCL3L1 gene copy numbers between the study groups were assessed by the Chi-square tests. The distribution of *TLR3* rs3775291 and *CCR5* and CCL5 haplotypes between the study groups were evaluated by the Fisher's exact tests. Next, the univariate and multivariate logistic regression analyses were performed. The univariate logistic regression analyses were used to determine (i) the associations between host genetic factors (*CCL3L1* gene copy number, *CCR5* and *CCL5* haplotypes and *TLR3* T allele) and HIV or HCV serostatus; (ii) the associations between co-variates (age, gender, HCV and HBV infection and the duration of IVDU) and HIV or HCV serostatus. The multivariate logistic regression models were used to determine the independent effects of the host genetic factors and HIV or HCV serostatus.

In the CCL3L1 study, in addition to the abovementioned analyses the following analyses were carried out. Step-wise multivariate logistic regression models were conducted to analyse associations between *CCL3L1* copy number and HIV and HCV serostatus. In these analyses, *CCL3L1* copy number and co-variates were included sequentially. Interactive multivariate logistic regression models were built to assess the interactions between the duration of IVDU and *CCL3L1* copy number.

5. RESULTS AND DISCUSSION

The overview of the recruited and successfully genotyped subjects is presented in Table 5. The rates of successful genotyping were: 100% for *CCL3L1*, *CCR5* A29G (rs2856758), *CCR5* G208T (rs2734648), *CCR5* G303A (rs1799987), *CCR5* T627C (rs1799988) *CCR5* C630T (rs41469351), *CCR5* C927T (rs1800024) and *CCR5* Δ32 (rs333); 99.7% for *CCL5* C-96G (rs2280788); 99.6% for *TLR3* rs3775291; 99.5% for *CCR2* V64I (rs1799864), *CCR5* A676G (rs1800023), *CCL5* G-471A (rs2107538) and *CCL5* TIn.1.1C (rs2280789). All studied SNPs were in the Hardy-Weinberg Equilibrium (HWE) in IDUs and blood donors.

5.1. The effect of chemokine receptor and chemokine polymorphisms on HIV and HCV serostatus

These studies describe the distribution of *CCR5* and *CCL5* haplotypes and *CCL3L1* gene copy numbers and the associations between *CCR5/CCL5* haplotypes, *CCL3L1* gene copy numbers and HIV and/or HCV serostatus.

5.1.1. Population characteristics in CCR5, CCL3L1 and CCL5 studies

As shown in Table 6, the study population was relatively young and predominantly male. Approximately half of the subjects were HIV seropositive and three-quarters were HCV seropositive.

Table 6. The main characteristics of the study population for CCR5, CCL3L1 and CCL5 studies

	IDUs n=374[a,b]
Males, n (%)	300 (80)
Age in years, median (IQR)	26 (22–29)
Duration of IVDU (years), median (IQR)	8 (4–12)
HIV seropositive, n (%)	208 (56)
HCV seropositive, n (%)	285 (76)
HBV seropositive, n (%)	56 (15)

[a]In CCR5 and CCL5 study, 373 persons were in the study population.
[b]The population consists of IDUs from the syringe exchange programmes and from prisons.
IQR – interquartile range

The details of the distribution of HIV, HCV and HBV serostatus are presented in Table 7. There were 27% and 7% of subjects HCV or HIV monoinfected, respectively. Approximately one-third (35%) was dually infected with HIV and HCV and 12% were seropositive for HIV, HCV and HBV.

Table 7. HIV, HCV and HBV serostatus among IDUs

HIV	HCV	HBV	Total = 374 n (%)
+	+	+	44 (12%)
+	+	–	133 (36%)
+	–	+	4 (1%)
–	+	+	8 (2%)
+	–	–	27 (7%)
–	+	–	100 (27%)
–	–	+	1 (0%)
–	–	–	56 (15%)

+ seropositive; – seronegative

Altogether, 27% of subjects were from prisons and the rest from the syringe exchange programme. As presented in Table 8, IDUs from prisons and the syringe exchange programmes were similar in terms of gender, age and the prevalence of HCV seropositivity. From prison, only HIV positive subjects were recruited.

Table 8. The IDUs populations recruited in CCR5, CCL3L1 and CCL5 study – prison vs. syringe exchange programmes

	IDUs from prisons N = 103	IDUs from syringe exchange programmes N = 270	p-value
Age in years, median (IQR)	25.5 (22–29.5)	26 (22–29)	0.821
Males, %	79.6	86.3	0.152
HIV seropositive, %	100	38.9	p<0.001
HCV seropositive, %	70.0	78.5	0.108

As expected in the IDU population, the presence of HCV and HBV was associated with the HIV seropositivity and the presence of HIV and HBV with the HCV seropositivity (Table 9). The duration of IVDU was associated with the HCV or HIV seropositivity, each additional year of IVDU increased the odds of HCV and HIV seropositivity. Age and gender did not affect HIV serostatus but gender was associated with HCV serostatus such that females had lower odds of acquiring HCV infection.

Table 9. The associations between co-variates and HCV or HIV serostatus in an univariate logistic regression analysis

Co-variates[a]	*Outcome HIV seropositivity*			*Outcome HCV seropositivity*		
	OR	***95% CI***	***p***	***OR***	***95% CI***	***p***
Gender						
Male*	1.0			1.0		
Female	1.08	0.60–1.94	0.797	0.52[d]	0.27–0.99	0.046
Age (years)[b]						
<26*	1.0			1.0		
≥26	1.10	0.70–1.72	0.691	1.18	0.69–2.02	0.538
HIV serostatus						
HIV–*	NA			1.0		
HIV+	NA			3.01	1.83–4.96	<0.001
HCV serostatus						
HCV–*	1.0			NA		
HCV+	3.01	1.83–4.96	<0.001	NA		
HBV serostatus						
HBV–*	1.0			1.0		
HBV+	5.10	2.42–10.75	<0.001	3.57	1.38–9.25	0.009
Duration of intravenous drug use (years)[c]						
1 year*	1.0			1.0		
Increase with each year	1.08	1.01–1.15	0.025	1.24	1.13–1.36	<0.001

* – reference group

[a] The table represents the associations between co-variates and HIV or HCV infection among 374 IDUs.

[b] The age is splitted on the median of 26 years.

[c] Continuous variable in full-years. Reference is one year, every additional year gives 1.08 and 1.23 times odds of acquiring HIV or HCV, respectively.

[d] This association was significant only in CCL3L1 study.

OR – odds ratio; CI – confidence interval; p – significance value, NA – not applicable

5.1.2. The distribution of *CCR5* haplotypes and haplotype pairs in IDUs and blood donors

The distribution of *CCR5* haplotypes between IDUs and blood donors was similar (Figure 7). The most prevalent *CCR5* haplotype was HHE (56.4% in blood donors and 55% in IDUs) followed by HHC (46.6% and 49.3%, respectively) and HHF*2 in blood donors (26.4%) and HHG*2 in IDUs (20.1%).

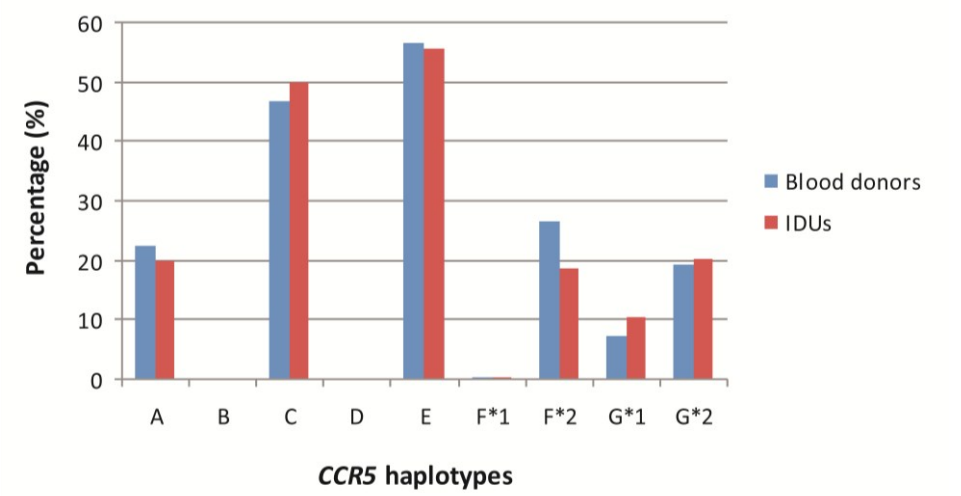


Figure 7. The distribution of *CCR5* haplotypes in blood donors and IDUs. The bars show the percentage of IDUs who carry at least one specific *CCR5* haplotype. Blue bars indicate the blood donors and red bars the IDUs.

In total, the 22 different *CCR5* haplotype pairs were present, of which HHC/HHE was most prevalent in both populations (Figure 8). The overall distribution of *CCR5* haplotype pairs between IDUs and blood donors was similar.

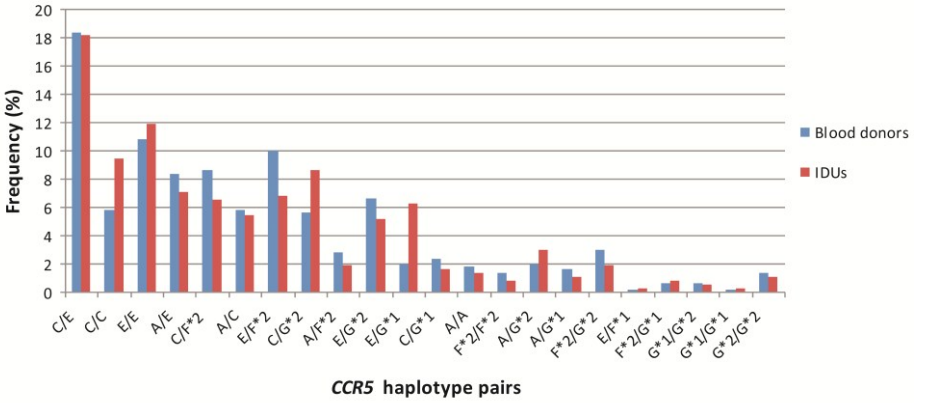


Figure 8. The distribution of *CCR5* haplotype pairs in blood donors and IDUs. Blue bars indicate the blood donors and red bars the IDUs. Haplotype pairs are presented without HH.

The relatively high frequency of HHG*2 (Δ32 bearing haplotype; ~20%) (Figure 7) is characteristic for North-European, especially Scandinavian, populations [Libert et al., 1998]. In our study, the prevalence of *CCR5*-Δ32

homozygosity was 1.1% (95% CI 0.3% – 2.8%) and 1.4% (95% CI 0.6% – 2.9%) (Figure 12, G2/G2) and heterozygosity 19.3% (95% CI 15.3% – 23.6%) and 20.1% (95% CI 16.6% – 23.8%) in IDUs and blood donors, respectively. Our results on *CCR5*-Δ32 homozygocity were comparable to the previous Estonian study where the prevalence of *CCR5*-Δ32 homozygocity was 3.2% (95% CI 1.8% – 5.1%) [Kalev et al., 2000]. The *CCR5*-Δ32 heterozygocity was similar in our and in Kalev *et al* (2002) study (23.2%; 95% CI 19.6% – 27.1%). The fact that the frequency of *CCR5*-Δ32 homozygosity is the highest in Northen Europe is well described and that there is a North to South gradient is also known; the frequency is around 3% in Scandinavia, 2% in France, Italy 1% and less than 1% in Spain [Libert et al., 1998; Lucotte and Mercier, 1998]. The *CCR5*-Δ32 in the homozygous state is absent in the African populations [Philpott et al., 2003]. Consistent with other studies on Caucasian populations, the HHB and HHD were not observed by us in Estonian populations [Gonzalez et al., 1999; Gonzalez et al., 2001].

5.1.3. Associations between *CCR5* haplotypes and HIV serostatus

Of all *CCR5* haplotypes, only the frequency of HHF*2 was different between HIV seropositive and HIV seronegative IDUs being over-represented in latter as compared with the former (23.5% vs 15.0%; $p<0.05$). The univariate logistic regression showed that persons with HHF*2 had 1.8 times reduced odds for HIV seropositivity (OR = 0.57, 95% CI 0.34–0.98, P=0.041) than those without HHF*2 suggesting a beneficial role of HHF*2 in the susceptibility to HIV infection. However, after the adjustment for co-variates (HCV and HBV serostatus, the duration of IVDU) or other *CCR5* haplotypes the association did not remain significant (OR = 0.65; 95% CI 0.34 – 1.24).

The possession of HHF*2 protected the Kenyan and the Argentinean but not the Malawian children of MTCT of HIV [Mabuka et al., 2009; Mangano et al., 2001; Pedersen et al., 2007]. Furthermore, HHF*2 has been related with the decelerated progression of HIV infection to AIDS and preventing death in heterosexually infected African American adults and in perinatally infected children [Gonzalez et al., 1999; Ioannidis et al., 2001; Malhotra et al., 2011; Mangano et al., 2001; Mangano et al., 2000].

Despite the abovementioned findings, the role of HHF*2 in HIV infection is still controversial. There are a number of studies in various populations (mainly infected via heterosexual route) in which no associations between HHF*2 and the susceptibility to HIV/AIDS has been found [Hladik et al., 2005; Kageyama et al., 2001; Nguyen et al., 2004; Pedersen et al., 2007; Ramaley et al., 2002; Tan et al., 2010]. Furthermore, there is a study in which the detrimental effect of HHF*2 was detected [Ma et al., 2005]. In that study, *CCR2*-V64I allele (i.e. HHF*2) increased the risk of HIV seropositivity among men but not among women in Cameroon. Previous studies together with our data indicate that the

effect of HHF*2 is likely not independent and may depend on other factors such as population characteristics, the route of transmission and co-infections.

Consistent with previous studies, the *CCR5*-Δ32 homozygotes (HHG*2/ HHG*2) were found only in HIV negative subjects [Dean et al., 1996; Samson et al., 1996], but after the adjustment for co-variates (HCV, HBV serostatus, the duration of IVDU) it did not remain significant.

5.1.4. *CCR5* haplotypes and HCV serostatus

At first we analysed the associations between *CCR5* haplotypes and HCV serostatus, which was followed by the analyses of *CCR5* halpotypes and HIV/HCV co-infection. Finally, we included the haplotype pairs.

Comparing the distribution of *CCR5* haplotypes among HCV seropositive vs HCV seronegative IDUs, we observed that the only haplotype overrepresented in HCV seronegative compared with HCV seropositive subjects was HHG*1 (20.7% vs. 7.5%; p<0.001). In the univariate analysis, the possession of HHG*1 was associated with the decreased odds of HCV seropositivity (Table 10). In the multivariate logistic regression analyses, after including all other *CCR5* haplotypes and for co-variates (HIV and HBV serostatus, the duration of IVDU) into the analyses, the possession of HHG*1 remained associated with the reduced odds of HCV seropositivity compared with those lacking this haplotype (Table 10). The HIV and HBV serostatus and the duration of IVDU were included as co-variates because they were associated with HCV serostatus (**Artcle 2, Table 2**).

Table 10. Associations between *CCR5* HHG*1 and HCV seropositivity before and after the adjustment for other *CCR5* haplotypes and co-variates compared with *CCR5* non-HHG*1

Variable[a]	Outcome: HCV seropositivity OR (95% CI; p)
1. HHG*1	0.37 (0.17–0.84; 0.017)
2. HHG*1 adjusted for all other *CCR5* haplotypes	0.37 (0.16–0.82; 0.015)
3. HHG*1 adjusted for HIV and HBV serostatus and the duration of IVDU	0.07 (0.03–0.20; <0.0001)

[a]reference group is *CCR5* non-HHG*1

5.1.5. *CCR5* haplotypes and HIV/HCV co-infection

Bearing in mind the significant correlation between HCV and HIV serostatus (Table 9), we conducted an analysis between HHG*1 and HIV/HCV co-infection status where the co-infection with HIV was included as a potential confounder. We evaluated the distribution of HHG*1 according to HCV and HIV serostatus. As presented in Figure 9, the *CCR5* HHG*1 was significantly over-represented in subjects who were both HIV and HCV seronegative compared with subjects who were HCV seropositive only, HIV seropositive only or were dual seropositive (HIV+ and HCV+).

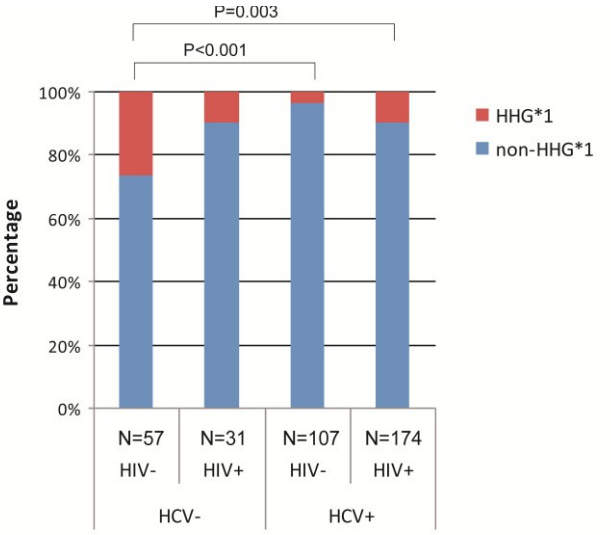


Figure 9. The distribution of *CCR5* HHG*1 in IDUs population stratified by HIV/HCV co-infection status. Red indicates IDUs who carry at least one HHG*1 allele and blue indicates IDUs who do not carry HHG*1.

After controlling co-variates (the duration of IVDU, HBV, *CCL3L1* copy number) in individuals who were both HCV and HIV seronegative (reference group), the odds of possessing a HHG*1 was decreased by 12 times (OR = 0.08; 95% CI 0.02–0.29) and six times (OR = 0.18; 95% CI 0.06–0.54) in IDUs who were HCV monoinfected, and HCV/HIV dual-infected, respectively (**Article 2, Table 3,** models 1 and 3) suggesting that HHG*1 mainly influences the susceptibility to HCV.

We did not analyse *CCR5* HHF*2 because it was not significant in the multivariate analyses (see chapter 5.1.3).

5.1.6. *CCR5* HHG*1-containing haplotype pairs and HCV serostatus

Next we determined which specific HHG*1-containg haplotype pair contributed to the reduced seropositivity of HCV. We evaluated two of the most common HHG*1-containing haplotype pairs – HHE/HHG*1 and HHC/HHG*1 (Figure 8). Of these, HHE/HHG*1 was more prevalent among the HCV seronegative than among the HCV seropositive IDUs (22.5% vs 2.6%; $p<0.001$). The odds of being HCV seropositive were decreased in IDUs who carried HHE/HHG*1 (OR = 0.11; 95% CI 0.04–0.29) compared with IDUs not having this haplotype pair. This association remained significant after the adjustment for co-variates (the duration of IVDU, HBV, *CCL3L1* copy number) (OR = 0.02; 95% CI 0.00–0.16).

In the context of co-infections, the HHE/HHG*1 was over-represented in subjects who were seronegative for both HIV and HCV compared with subjects who were seropositive for HCV and/or HIV (Figure 10). After controlling the same co-variates (the duration of IVDU, HBV, *CCL3L1* copy number), the odds of possessing HHE/HHG*1 was decreased 50 and 14 times in IDUs who were HCV monoinfected (OR = 0.02; 95% CI 0.00–0.20 and OR = 0.07; 95% CI 0.01–0.32) and HCV/HIV dual-infected, respectively, compared with IDUs who were seronegative for both HIV and HCV. The odds of the possession of HHE/HHG*1 was not significant comparing HIV monoinfected IDUs with HIV/HCV dualnegative IDUs (**Article 2, Table 3**, models 4 and 6).

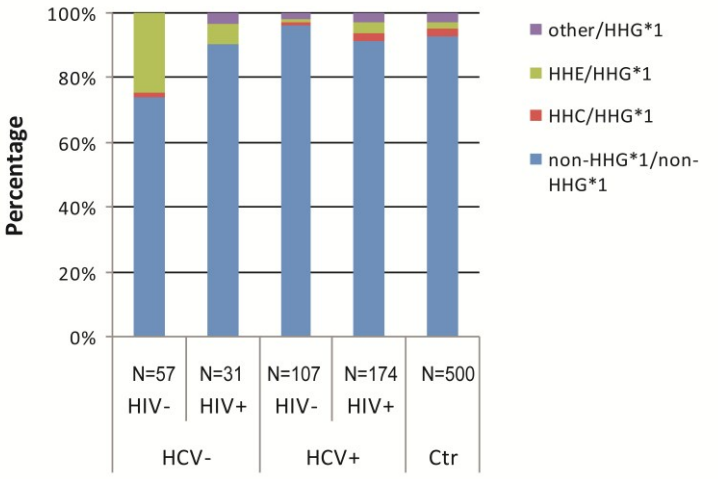


Figure 10. The distribution of *CCR5* HHG*1-containing haplotype pairs in IDUs and blood donors (named as Ctr.). Blue indicates the IDUs who do not carry HHG*1 in either allele, red indicates the IDU carrying HHC/HHG*1, green indicates the IDUs carrying HHE/HHG*1 and purple indicates the IDUs who carry *CCR5* haplotype pair that consists of HHG*1 and other haplotype (except HHC or HHE).

5.1.7. *CCR5* HHG*1 and HHE/HHG*1 in HCV and HIV seronegative IDUs vs. blood donors

The abovementioned findings showed that HHG*1 (including HHE/HHG*1) is associated with the resistance of acquiring HCV or HCV/HIV infection in IDUs. Based on that, the prevalence of HHG*1 or HHE/HHG*1 among HCV/HIV double negative IDUs should be over-represented compared with blood donors. These analyses showed that the prevalence of HHG*1 was 26.3% vs. 7.4% ($p<0.0001$) comparing HCV/HIV double negative IDUs to blood donors, respectively, and HHE/HHG*1 haplotype pair 24.6% vs 2.0% ($p<0.0001$; Figure 10).

To the best of our knowledge, the associations between *CCR5* haplotypes and HCV infection have been poorly studied despite the role of CCR5 in the pathogenesis of HCV infection. Previously, the study by Woitas et al., (2005) in patients with haemophilia demonstrated that subjects with *CCR5*-Δ32 homozygocity (HHG*2/HHG*2) had an increased risk of HCV infection compared to healthy controls and HIV and HCV/HIV infected subjects. In this study, we and others [Glas et al., 2003; Poljak et al., 2003; Ruiz-Ferrer et al., 2004] in HCV seropositive and chronically infected HCV positive subjects were not able to replicate the findings of Woitas et al. although one should note that the study populations in these studies were different. Retrospectively, one could speculate that the high frequency of HHG*2/HHG*2 in the HCV positive individuals in Woitas study did not represent the increased susceptibility to HCV but rather reflected the resistance of HIV in individuals who were exposed to both viruses [Promrat and Liang 2003]. While associations between the acquisition of HCV and the genetic variability of CCR5 are limited, the role of *CCR5* polymorphisms in response to interferon therapy is better described suggesting *CCR5* contribution to the pathogenesis of HCV infection [Dorak et al., 2002; Hellier et al., 2003; Konishi et al., 2004; Promrat et al., 2003].

5.1.8. The distribution of CCL3L1 gene copy number in IDUs

As presented in Figure 11, the CCL3L1 gene copy number of IDUs varied from zero to six with the population median of two being consistent with other studies in Caucasian populations [Gonzalez et al., 2005; Grunhage et al., 2010; Shostakovich-Koretskaya et al., 2009]. In African populations, however, higher gene copy numbers have been demonstrated with the population average of six (range 0 to 11) [Gonzalez et al., 2005].

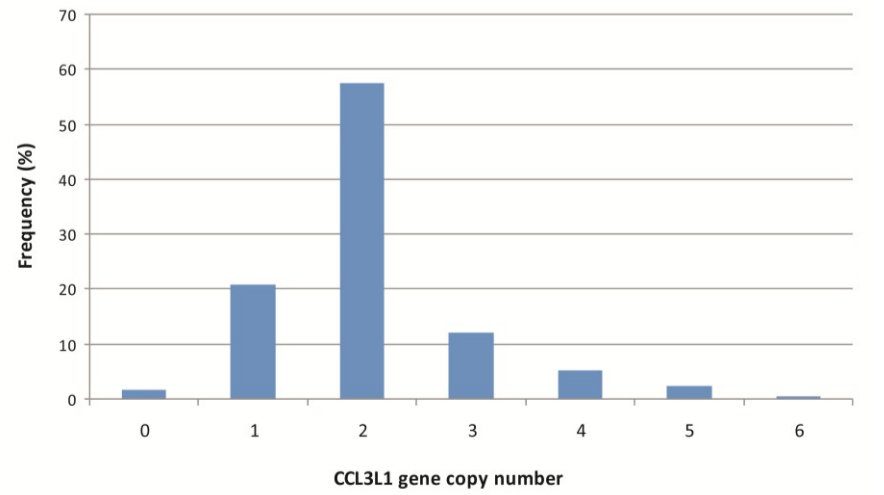


Figure 11. The distribution of CCL3L1 gene copy number among 373 IDUs. The bars show the percentage of IDUs possessing the specific *CCL3L1* copy number.

5.1.9. CCL3L1 gene copy number and HIV serostatus

Based on the population median in CCL3L1 gene copy number, we categorized subjects into two groups – those with a *CCL3L1* copy number from zero to two (low *CCL3L1* copy number) and those with greater than two (high *CCL3L1* copy number). The distribution of CCL3L1 gene copy number differed between HIV seropositive and HIV seronegative IDUs such that HIV seronegative IDUs had a greater proportion of higher gene copy number compared with HIV seropositives (Figure 12).

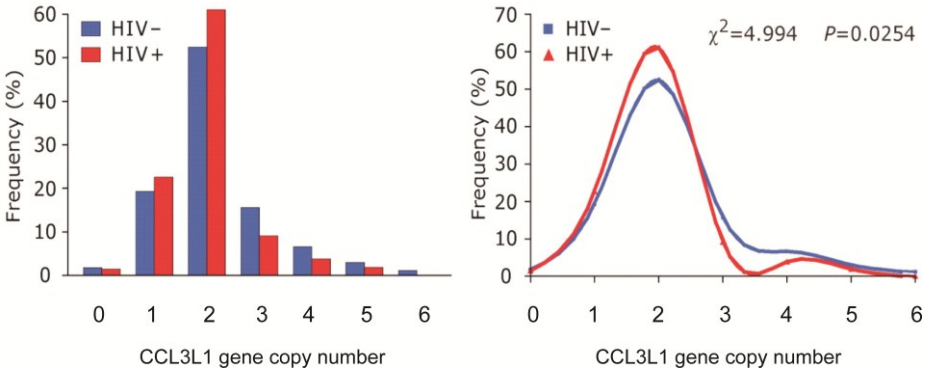


Figure 12. The distribution of CCL3L1 gene copy number in HIV negative and HIV positive IDUs. Left panel bars show the frequency of specific CCL3L1 gene copy number and on the right panel the bars are presented as smooth lines. Blue indicates HIV seronegative IDUs and red indicates HIV seropositive IDUs. The p-value is calculated for the distribution of high and low *CCL3L1* copy number.

In the univariate analysis, a high copy number was associated with the decreased odds of HIV seropositivity (OR = 0.49; 95% CI = 0.29–0.81) (**Article 1, Table 2**).

The results of the stepwise multivariate logistic regression model are presented in **Article 1, Table 3**. For the outcome of HIV seropositivity, in models that did not include the duration of IVDU, both HCV and HBV serostatus were associated with higher odds of HIV seropositivity (**Article 1, Table 3**, models 1 and 4). Conversely, in the model that did not include HCV or HBV serostatus, the increased duration of IVDU was associated with the increased odds of HIV seropositivity (**Article 1, Table 3**, model 5). By contrast, the inclusion of the duration of IVDU in the same model with HCV and HBV led to no associations with each of these highly correlated parameters and HIV seropositivity (**Article 1, Table 3,** models 2 and 3). However, higher *CCL3L1* copy number was associated with decreased odds of HIV seropositivity, independent of the duration of IVDU and HCV and HBV co-infection status (**Article 1, Table 3,** models 3–5).

The associations between the higher than the population median of *CCL3L1* copy number and the reduced risk of acquiring HIV infection as in our study have been demonstrated previously in subjects exposed to HIV via sexual or perinatal route [Gonzalez et al., 2005; Kuhn et al., 2007; Meddows-Taylor et al., 2006; Nakajima et al., 2007]. In addition, the slower disease progression has also been associated with the higher than population mean CCL3L1 copy numbers [Gonzalez et al., 2005; Kulkarni et al., 2008]. However, these findings have not been universally unidirectional. There are recent studies in which no associations between the *CCL3L1* copy number and the susceptibility to HIV or the disease progression has been found in African, Indian and AA exposed or infected by sexual route [Bhattacharya et al., 2009; Larsen et al., 2012; Rathore et al., 2009; Shao et al., 2007; Urban et al., 2009]. These controversial findings may be explained by various population selections. It is possible that these associations may not be present in every population. However, the different origin and risk factors might influence the results.

The debate over proper method for the detection of *CCL3L1* copy number has been vivid. Studies that have tried to replicate the results by real-time PCR have failed and the accuracy of the real-time PCR assay used by Gonzalez et al., 2005 have been questioned [Bhattacharya et al., 2009; Urban et al., 2009]. In addition, Field et al (2009) compared real-time PCR assay with another method to measure the gene copy number – paralogue ratio test (PRT) [Armour et al., 2007; Field et al., 2009; Walker et al., 2009]. Field et al (2009) debated that in the case of real-time PCR (e.i. qPCR) assay rounded results were not HWE. In addition, the primers used in qPCR for *CCL3L1* may also bind a *CCL3L1* pseudogene *CCL3L2* [Gonzalez et al., 2005; Modi, 2004; Walker et al., 2009]. In conclusion, Field et al (2009) recommend to use PRT instead or qPCR (if deviation from HWE is resolved) in large well-powered samples.

However, these results were confronted by He et al (2009), using an argument that the original protocol was modified in many ways (different quencher, non-fixed DNA amounts in qPCR) [He et al., 2009]. He et al (2009) demonstrated that MGB assay underestimated the lower values of copy number but over estimated higher values compared to TAMRA. The PRT used by Fields et al (2009) quantifies two CCL3L genes (*CCL3L1* and *CCL3L3*) but qPCR quantifies three (including also CCL3L2 which seems to not be a pseudogene [Shostakovich-Koretskaya et al., 2009], so the two assays are not comparable. In addition, He et al (2009) stated that HWE used by Fields et al (2009) would be valid if the copy number variation of individual CCL3L gene were both duplicated and distributed randomly on each chromosome, permitting accurate computation of copies per chromosome. But recent studies do not support that [Colobran et al., 2008; He et al., 2009; Perry et al., 2008].

Another study by Sudmant et al (2010) developed a method using whole-genome shotgun sequencing data to accurately determine assay specific duplicated genes and the copy numbers of genes; a very high correlation between CCL3L1 qPCR and the whole-genome sequencing method was shown (r = 0.95) [Sudmant et al., 2010].

It is also suggested that the quality of the DNA plays a major role in order to determine the copy number correctly [Bhattacharya et al., 2009; Urban et al., 2009]. We have paid much attention to the quality of the DNA; the DNA was extracted using the same method throughout the study, the DNA quality was measured by NanoDrop and stored at –80 C° (at the conditions that have been proven to be acceptable for DNA storage). We believe that all this reduced the inter-sample variability and increased reliability of the testing. At the moment the debate is still ongoing and more precise method is searched.

5.1.10. CCL3L1 gene copy number and HCV serostatus

The distribution of CCL3L1 gene copy number did not differ between HCV seropositive and HCV seronegative IDUs (Figure 13).

Despite of no differences in the distribution of *CCL3L1* copy number between HCV seropositive and seronegative IDUs (OR = 1.15; 95% CI 0.62–2.12), we performed step-wise analyses similar to those in HIV infection. As demonstrated in **Article 1**, **Table 4,** the associations between HCV seropositivity and co-variates depend on the inclusion of co-variates. Unlike in HIV infection (**Article 1, Table 3**), the associations between *CCL3L1* copy number and HCV seropositivity differed before and after accounting for the duration of IVDU. A higher *CCL3L1* copy number was associated with the increased odds of HCV seropositivity when the duration of IVDU was included in the model (**Article 1, Table 4,** model 3) but did not associate with HCV serostatus when the duration of IVDU or HIV and HBV serostatus were excluded from the models (**Article 1, Table 4,** models 4 and 5). We concluded that *CCL3L1* copy number was not associated with HCV seropositivity.

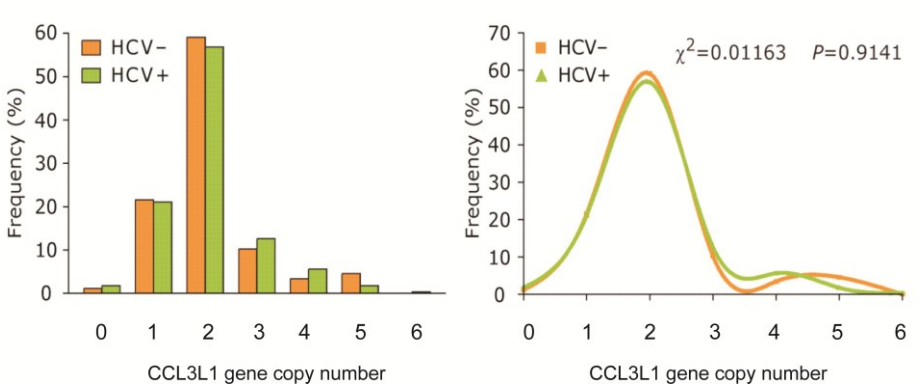


Figure 13. The distribution of CCL3L1 gene copy number in HCV negative and HCV positive IDUs. Left panel bars show the frequency of specific CCL3L1 gene copy number and on the right panel the bars are presented as smooth lines. Orange indicates HCV seronegative IDUs and green indicates HCV seropositive IDUs. The p-value is calculated for the distribution of high and low *CCL3L1* copy number.

To the best of our knowledge, only one study has demonstrated the protective effect of high *CCL3L1* copy number on the susceptibility to chronic HCV infection in Caucasian population [Grunhage et al., 2010]. In addition, they demonstrated a significantly lower *CCL3L1* copy number in HCV/HIV co-infected subjects compared to healthy controls suggesting that lower copy number could be the risk factor for HCV/HIV co-infection.

5.1.11. The distribution of *CCL5* haplotypes and haplotype pairs

The prevalence of IDUs who carried at least one allele of haplotype A was 96.2%, other haplotypes were presented in less than 20% (Figure 14).

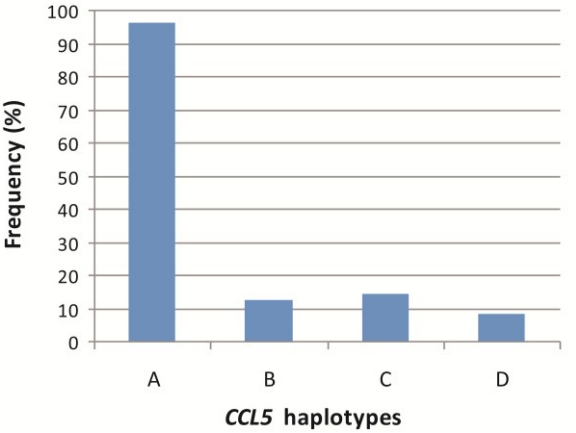


Figure 14. The distribution of *CCL5* haplotypes in IDUs. The bars show the percentage of IDUs who carry at least one specific *CCL5* haplotype.

Accordingly, the most frequent *CCL5* haplotype pair was A/A (67.4%) followed by A/C (11.1%), A/B (9.8%), A/D (7.9%), B/C (2.2%) and B/B, B/D, C/C and C/D were presented in less than one percent (Figure 15).

As far as we are aware, the distribution of *CCL5* haplotype and haplotype pairs (determined in this thesis) in Caucasian populations has not been studied. In North-Indian population, the distribution of these haplotypes was similar to ours [Rathore et al., 2008]. The study that included two SNPs in *CCL5* showed that -471G/-96C (haplotype A) is most prevalent among Africans (50%), African-Americans (60%) and European-Americans (84%) as it is most prevalent in our population. Similar to us, the following haplotype was -471A/-96C (present in haplotype B and C). However, -471A/-96G (haplotype D) was present in low frequency (1–2%) among European-Americans and African-Americans and missing among Africans [Gonzalez et al., 2001].

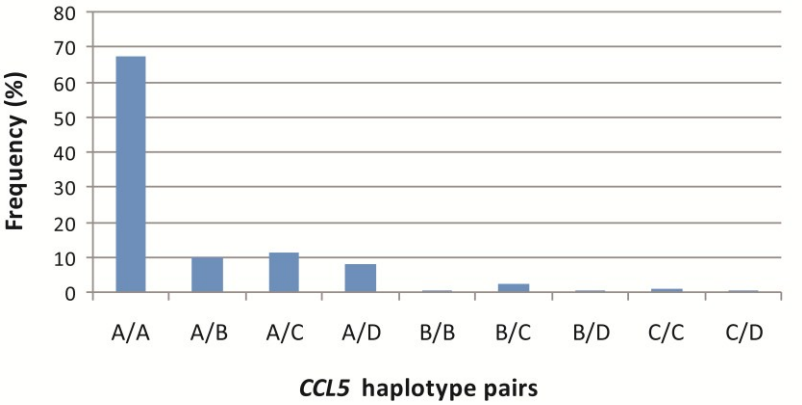


Figure 15. The distribution of *CCL5* haplotype pairs in IDUs.

5.1.12. *CCL5* haplotypes and HIV serostatus

The distribution of *CCL5* haplotypes in HIV seronegative and HIV seropositive subjects was similar suggesting that this haplotype does not influence the susceptibility to HIV infection (data not shown).

5.1.13. *CCL5* haplotypes and HCV serostatus

Similar to the analyses of *CCR5* haplotypes, we started with detecting the relationship between *CCL5* haplotypes and HCV serostatus, which was followed by the analyses of *CCR5* halpotypes and HIV/HCV co-infection, and ended with the inclusion of the haplotype pairs.

In HCV seropositives, the frequency of *CCL5* haplotype C was higher and the frequency of haplotype D was lower than in the HCV seronegatives (16.8% vs 6.9% and 4.5% vs 20.7%, respectively; both $p < 0.05$).

In a univariate logistic regression model we observed that subjects possessing *CCL5* haplotype C had almost three times the increased odds of being HCV seropositive compared with those not possessing *CCL5* haplotype C (Table 11**,** model 1). IDUs with *CCL5* haplotype D had five times the reduced odds of HCV seropositivity compared with those who did not possess *CCL5* haplotype D (Table 11**,** model 2). Both these associations remained significant in a logistic regression model after the adjustment for HIV, HBV serostatus and the duration of IVDU (Table 11**,** model 1 and 2). When both haplotypes were included into the same model, only haplotype D remained significantly associated with the HCV serostatus suggesting the predominant influence of the *CCL5* haplotype D (Table 11, model 3).

Table 11. Associations between *CCL5* haplotype C and D and HCV seropositivity

Models	**Outcome: HCV seropositivity**	
	Unadjusted OR (95% CI)	Adjusted[a] OR (95% CI)
MODEL1		
CCL5 hap C[b]	2.72 (1.12–6.61)	5.19 (1.11–24.17)
MODEL2		
CCL5 hap D[c]	0.19 (0.09–0.40)	0.10 (0.03–0.36)
MODEL3		
CCL5 hap C[b]	2.37 (0.97–5.81)	3.79 (0.80–17.92)
CCL5 hap D[c]	0.20 (0.09–0.43)	0.12 (0.03–0.42)

[a]adjusted for HIV, HBV and the duration of IVDU.
[b]reference group is *CCL5* non-C haplotype
[c]reference group is *CCL5* non-D haplotype
hap – haplotype

To the best of our knowledge, this is the first study looking at associations between *CCL5* polymorphisms or haplotypes and the susceptibility to HCV infection despite the well-described role of CCL5-CCR5 axis in HCV pathogenesis [Zeremski et al., 2007]. Most studies so far have concentrated on the *CCL5* variability and the disease progression. For example, in Caucasian population *CCL5* -471A (present in haplotypes B, C and D) leads to less severe hepatic inflammation and milder portal inflammation [Hellier et al., 2003; Promrat et al., 2003]. The possession of In1.1C and 3'-222C and the combination of -471A/In1.1C/3'222C (first two SNPs are present in haplotype C and D), however, are associated with the worse response to interferon-

ribavirin therapy among patients infected with HCV genotypes 1 and 4 [Wasmuth et al., 2004].

5.1.14. *CCL5* haplotype D and HIV/HCV co-infection status

As seen in Figure 16, HCV monoinfected and HIV/HCV dualinfected IDUs had a lower frequency of haplotype D compared with double negative IDUs (HIV-/HCV-). There were no differences between other groups.

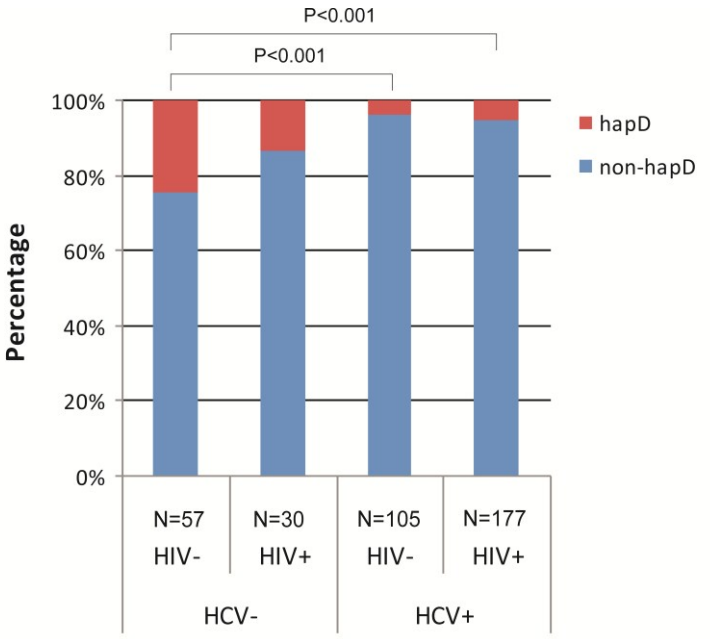


Figure 16. The distribution of *CCL5* haplotype D among IDUs stratified by HIV/HCV co-infection status.

5.1.15. *CCL5* haplotype D-containing haplotype pairs and HCV serostatus

Next we determined which specific haplotype D-containing haplotype pair contributed to the decreased odds of HCV seropositivity. The haplotype pair A/D was overrepresented in subjects who were seronegative for HCV compared with subjects who were seropositive for HCV (3.9% vs 20.7%; $p < 0.001$). IDUs who possessed A/D had the reduced odds of HCV seropositivity (OR = 0.16; 95% CI 0.07–0.35) compared with those who did not have this haplotype pair. This remained significant after the adjustment for co-variates (OR = 0.10; 95% CI 0.03–0.36).

The analyses of *CCL5* haplotype pair A/D showed that IDUs who possessed A/D had approximately 8 times the decreased odds to be HCV monoinfected and HCV/HIV dualinfected (OR = 0.12: 95% CI 0.04–0.39 and OR = 0.13; 95% CI 0.05–0.34, respectively) compared with HIV/HCV double negative IDUs.

5.1.16. *CCR5* HHG*1, *CCL5* haplotype D and HCV serostatus

As both *CCR5* HHG*1 and *CCL5* haplotype D were associated with the decreased odds of HCV seropositivity (paragraph 5.1.4–5.1.7 and 5.1.13), we next conducted a multivariate logistic regression model to evaluate the independent effect of these haplotypes. We observed that both of these haplotypes were associated with the decreased odds of HCV seropositivity (Table 12**,** model 1). In the purpose of not losing the power of the analysis, we adjusted the model for HIV and HBV serostatus but not to the interdependent factor – the duration of IVDU. After the adjustment, both haplotypes remained significantly associated with the susceptibility to HCV (Table 12, model 1), suggesting that the genetic variability of both CCR5 and CCL5 may play a role in the susceptibility to HCV infection. However, in the interaction model the combination of *CCR5* HHG*1 and *CCL5* haplotype D was significant but after the adjustment for HIV and HBV serostatus the association did not remain significant (Table 12, model 2).

Table 12. Associations between *CCL5* haplotype D and *CCR5* HHG*1 on HCV serostatus

	Outcome: HCV serostatus	
Models	Unadjusted OR (95% CI)	Adjusted[a] OR (95% CI)
MODEL1		
CCL5 hap D[b]	0.24 (0.11–0.54)	0.27 (0.11–0.63)
CCR5 HHG*1[c]	0.47 (0.22–0.99)	0.43 (0.19–0.98)
MODEL2 – interaction model		
CCL5 hap D	0.62 (0.21–1.82)	0.51 (0.17–1.57)
CCR5 HHG*1	1.03 (0.37–2.86)	0.77 (0.27–2.21)
CCL5 hap D x *CCR5* HHG*1	0.03 (0.00–0.38)	0.08 (0.01–1.03)

[a]adjusted for HIV and HBV status.
[b]reference group is CCL5 non-hap D
[c]reference group is CCR5 non-hap HHG*1
hap – haplotype; HH – human haplotype

5.2. The effect of *TLR3* rs3775291 on the susceptibility to HIV

To evaluate the relationship between *TLR3* rs3775291 polymorphism and HIV serostatus we recruited 345 IDUs in 2011 as described above (Table 4).

5.2.1. The population characteristics of *TLR3* study

The detailed characteristics of study populations are presented in **Article 3**, **Table 1.** Briefly, similar to other population in this thesis, half of the IDUs were seropositive for HIV, 89% for HCV, 77% for HBV and 40% for all three viruses.

5.2.2. The distribution of *TLR3* rs3775291

The T allele frequency of *TLR3* rs3775291 was 36% among HIV seronegative IDUs (48% in HESNs, 34% in non-HESNs and 35% in unexposed IDUs), 31% among HIV seropositive IDUs and 34% among blood donors. The *TLR3* rs3775291 was in the Hardy-Weinberg equilibrium in all the groups. The frequencies of *TLR3* rs3775291 genotypes in HESNs, non-HESNs and blood donors are presented in **Article 3, Table 2**. Our results of T allele frequency blood donors are similar to prior studies on Caucasian populations [Kindberg et al., 2011; Sironi et al., 2012]. The frequency of T allele among HESNs was higher in our study compared to Sironi et al (2012) but it was not significant.

5.2.3. *TLR3* rs3775291 and HIV serostatus

The number of persons who possessed T allele was significantly greater among HESNs compared to blood donors (80% vs 55%; $p = 0.037$) and HESNs compared to HIV seropositive IDUs (80% vs 53%; $p = 0.031$) (Figure 17, right panel). The possession T allele did not differ between non-HESNs and other groups**.** In addition, the distribution of *TLR3* rs3775291 genotypes did not differ between the study groups and was similar to other Caucasian populations [Kindberg et al., 2011; Sironi et al., 2012].

The control populations in the previous study by Sironi *et al* (2012) and in our study were similar in terms of T allele frequency. However, we found the T allele on a higher frequency in HESNs than Sironi et al (2009) but a different population size and the extent of exposure (20 IDU HESNs vs 83 sexually and 102 IDU exposed HESNs) in these two studies have to be noted. In addition, the definition of HESN differed between our and their study. We defined HESNs based on the frequency of receptive sharing during six last months. Sironi et al (2009) included persons who shared needles more than 3 months (IDU HESNs) and persons who had unprotected sexual episodes more than 4 years (more than 3 episodes a month).

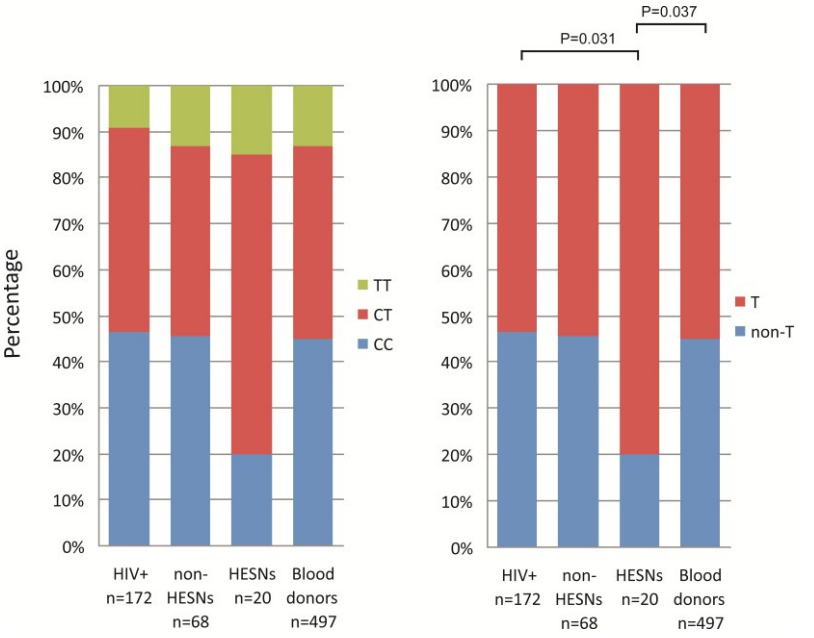


Figure 17. The distribution of *TLR3* rs3775291 genotypes (on the left) and T allele frequency (on the right) among the HIV positive (HIV+), non-heavily exposed HIV seronegative subjects (non-HESNs), heavily exposed HIV seronegative subjects (HESNs) and blood donors. On the left: blue indicates persons possessing CC genotype, red CT persons possessing genotype and green persons possessing TT genotype. On the right: blue indicates persons who do not possess T allele and red indicates persons who possess T allele.

In the univariate analysis, the possession of *TLR3* T allele was associated with the decreased odds of HIV seropositivity in HESNs (OR = 0.29; 95% CI 0.09–0.90) (**Article 3, Table 3**). In the multivariate analysis, the possession of *TLR3* T allele and HCV seropositivity both were significantly associated with HIV seropositivity (**Article 3, Table 3**) indicating the independent effect of these two variables. Our results of *TLR3* T allele being associated with the decreased odds of HIV infection confirm those of Sironi *et al* (2012) demonstrating that TLR3 rs3775291 T allele plays a role in the resistance to HIV infection in HESNs.

5.2.4. *TLR3* rs3775291 and HCV serostatus

As seen in Table 13, there were no differences in the distribution of *TLR3* rs3775291 T allele between HCV seropositives and seronegatives (including highly exposed) suggesting that this genotype is not associated with the susceptibility to HCV infection.

Table 13. The distribution of *TLR3* rs3775291 T allele frequency between IDUs groups stratified by HCV and HBV infection and compared to blood donors and HCV or HBV positive IDUs

Populations	*TLR3* rs3775291 T allele frequency % (total n)	p-value
HCV infection		
1. HCV- IDUs vs HCV+ IDUs	62% (39) vs 56% (306)	0.50
2. HCV- IDUs vs blood donors	62% (39) vs 55% (497)	0.50
3. HCV- non-highly exposed IDUs[a] vs HCV+ IDUs	75% (8) vs 56% (306)	0.47
4. HCV- non-highly exposed IDUs[a] vs blood donors	75% (8) vs 55% (497)	0.31
5. HCV- highly exposed IDUs[b] vs HCV+ IDUs	100% (4) vs 56% (306)	0.13
6. HCV- highly exposed IDUs[b] vs blood donors	100% (4) vs 55% (497)	0.13

[a]persons who reported the use of previously used syringes (receptive sharing) less than once a month

[b]persons who reported the use of previously used syringes (receptive sharing) at least once a month

- seronegative; + seropositive

6. GENERAL DISCUSSION

The susceptibility to blood borne viruses (e.g. HIV and HCV) is influenced by several factors of which the leading role is taken by risk behaviour such as needle sharing among IDUs or unprotected sex in general population. Among others, host genetic factors have shown to have an impact to the susceptibility to complex diseases as well as infections diseases. The host genetic factors and population characteristics are likely interrelated. It is known that the ethnicity is a crucial factor in host genetics studies as the genetic background differs between populations (e.g. genetic variation in Caucasians vs. Africans).

6.1. The importance of studies related to HIV and HCV in IDUs

The majority of previous research on HIV infection has been conducted in sexually (homo or hetero) or perinatally infected subjects (see chapter 2.7.3.2). Most likely because it is one of the leading transmission routes of HIV and compared to other risk groups these subjects are easier to reach and monitor. Thus, in these populations the relationships between the genetic host factors and the susceptibility and progression of HIV infection have been relatively well described – several genes have been identified. However, very limited data is available on IDUs, the population that is different from sexually transmitted population for several reasons. Firstly, due to sharing contaminated needles the risk of infection with blood borne viruses is much higher than by any other route. Secondly, bearing in mind that in the case of intravenous transmission the viruses do not pass the mucosal barrier, it is possible that host genetic factors in this transmission route have a different role than in other routes [Shaw and Hunter, 2012]. Furthermore, IDUs who despite the very high risk remain uninfected form an interesting and valuable group for studies on factors that influence the susceptibility to HIV. Thirdly, most of the IDU-epidemics (including the Estonian one) consist of monophyletic viral population, which could be the results of the lack of mucosal barrier and fast spread of one viral strain instead of multiple strains [Avi, 2011]. This unique situation provided us an opportunity to minimise the potential influence of viral heterogeneity commonly seen in sexually transmitted epidemics. Fourthly, the vast majority of subjects in the IDU studies, including ours, are also infected with HCV and in lesser extent with HBV allowing to evaluate the effect of the host genetic factors in the context of co-infection.

All abovementioned population advantages were seen in our study on Estonian IDUs. Vast majority of subjects were young (median age 26–30 years), all were Caucasian and majority were males (approximately 80%). The rate of HIV infection was high (50–56%), more than three quarters (76–89%) had HCV infection and nearly 40% HIV/HCV co-infection. The monophyletic

viral population consisting predominantly of CRF06_cpx viruses has been repeatedly described in Estonia [Avi et al., 2009; Avi et al., 2010; Avi et al., 2011] and was undoubtedly another advantage of our study. Taken together, we have studied a homogenous population where the influence of environmental, viral and risk behaviour factors was similar.

6.2. The pros and cons of study design

Ideally, in order to evaluate the influence of host genetic factors on the susceptibility to blood borne infections in IDUs one would aim to conduct a long-term prospective study. First, to observe the susceptibility to the virus and second, to detect the risk behaviour in more detail over the course of time. A study of such population will be logistically very difficult if not impossible. Thus, we have decided to conduct cross-sectional studies but appreciate that this design could also have problems. In the case of cross-sectional study, the causality cannot be determined, the population size should be relatively large (e.g. in the case of rare outcomes and factors) and in the secondary analysis there is no control over the choice or the methods of data collection. On the other hand, a cross-sectional study will also have several advantages because it enables to identify the new associations, which could be evaluated in more detail and rigorously in the cohort studies in the future. Following the advantages of cross-sectional study, design should be noted: (i) studies are relatively easy to conduct in a short period of time; (ii) they are less resource consuming; and (iii) there is a higher chance to complete the data collection than in prospective studies. Bearing the abovementioned in mind, we have chosen the cross-sectional study design for four reasons. First, as known IDUs population is hard to recruit and even more complicated to follow prospectively. Second, the data collection had to be manageable. Third, the study budget was limited. Finally, we looked at the genetic factors that had not been described in IDU population and because of that the cross-sectional study design met our needs to evaluate the associations at the beginning.

6.3. The role of *CCR5-CCL5* variability in HCV infection

Although several host genetic factors have been evaluated in the context of HIV infection the genes encoding CCR5 and its ligands are by far the most commonly targeted. This is not surprising as the homozygocity of 32 bp deletion in *CCR5* is the only genetic polymorphism that offers complete protection against R5-tropic HIV infection [Samson et al., 1996]. CCR5 and its ligand CCL5 genes have also been studied in HCV infection, especially looking at the response to therapy. Mostly the different SNPs in these genes have been evaluated and shown that *CCR5* polymorphisms have an influence on the HCV disease outcome (see chapter 2.8.3.2).

Until now, less attention has been paid to *CCR5* haplotypes. To the best of our knowledge, for the first time we have shown that persons with *CCR5* HHG*1, the haplotype upon which *CCR5*-Δ*32*-containing HHG*2 arose, had the decreased odds of HCV seropositivity independent of other factors (HCV, HBV serostatus and duration of IVDU) associated with HCV susceptibility. In addition, we saw that *CCL5* variability was associated with HCV susceptibility so that the *CCL5* haplotype D (-471A/-96G/In1.1C) had a protective effect against HCV seropositivity. So far, only HHG*2/HHG*2 (homozygocity of *CCR5*-Δ32) had been associated with an increased risk of HCV infection, but this finding may reflect the resistance to HIV rather than the increased susceptibility to HCV infection [Woitas et al., 2002]. We did not find associations between the *CCR5*-Δ*32* homozygocity and the susceptibility to HCV.

Although the mechanism behind these associations is unknown, the reasons why CCR5 and CCL5 are important in HCV infection may lie in the overall immune response and the role of CCR5-CCL5 in HCV pathogenesis. First, the CCR5 is one of the chemokine receptors that is responsible for recruiting a variety of immune cells (e.g. monocytes, DCs, NK) to the sites of inflammation, and induces Th1 response in order to control infection and eliminate antigens. Together with that, the interaction between CCR5 and CCL5 has an effect on T cell activation [Dairaghi et al., 1998; Manes et al., 1999; Nguyen and Taub, 2002; Xiao et al., 1999]. Second, specifically in HCV infection, the lack of Th1 response leads to the development of chronic HCV infection in which the CCR5-CCL5 axis could play a crucial role [Lechner et al., 2000a; Zeremski et al., 2007; Woitas et al., 1997]. In addition, HCV itself interacts directly with CCR5 and CCL5 resulting in the decreased chemotactic responsiveness to CCL5 in CD4+ and CD8+ cells and keeps CCR5 expressing HCV-specific T cells away from the infection site in order to prevent viral clearance [Lechner et al., 2000b; Losana et al., 2002; Nattermann et al., 2004; Solari et al., 1997; Soo et al., 2002; Thimme et al., 2001]. This all suggest that CCR5 and CCL5 play an important role in the immune responses and through that the haplotypes of CCR5 and its ligands' genes might influence HCV infection.

Thus, one potential mechanism behind our findings might be the influence of *CCR5* and *CCL5* haplotypes on their expression and by that modulating the Th1 response in order to eliminate the viruses. Despite the fact that some SNPs and group of haplotypes have been shown to influence the receptor expression (e.g. minor alleles in *CCL5* promoter SNPs enhance CCL5 production and CCR5 HHA-HHE have higher transcription activity), the mechanism how *CCR5* HHG*1 and *CCL5* haplotype D affect their expression is largely unknown [An et al., 2002; Lin et al., 1999; McDermott et al., 1998; Mummidi et al., 2000]. On the other hand, the associations determined by us might not be mediated directly through CCR5 and CCL5.

In addition to the associations between *CCR5* HHG*1 and HCV, we observed that *CCR5* HHF*2 had a protective effect against HIV but the

association was weak and lost its significance after the adjustment for co-variates (HCV, HBV serostatus and the duration of IVDU). Thus, we could not demonstrate that *CCR5* haplotypes had an independent effect on HIV susceptibility in IDUs population.

6.4. The role of *CCL3L1* copy number in HIV infection

Beside SNPs, there is also a gene copy number variation, which is a rarer polymorphism than SNPs but covers larger genomic regions. In recent years, there has been a great attention on the CCL3L1 gene copy number in HIV infection research. It is due to CCL3L1 being a ligand for CCR5 and the most potent inhibitor of R5-tropic HIV strains [Aquaro et al., 2001; Menten et al., 1999]. We demonstrated that a higher than population median *CCL3L1* copy number had a protective effect against HIV susceptibility independent from other factors that strongly influence HIV acquisition such as the duration of intravenous drug use. This is in concordance with the previous findings in other risk groups infected with HIV via sexual route or by blood products (hemophiliacs) [Gonzalez et al., 2005; Nakajima et al., 2007].

The potential mechanism behind the protection of higher *CCL3L1* copy number against HIV infection could be viral entry-dependent. The higher *CCL3L1* copy number is associated with the increased mRNA and CCL3/CCL3L1 protein levels and a lower percentage of CD4+CCR5+ cells [Gonzalez et al., 2005; Meddows-Taylor et al., 2006; Townson et al., 2002; Urban et al., 2009]. This indicates that when there is a high level of CCL3L1 then a competition between CCL3L1 and HIV occurs in order to bind with CCR5. In addition, higher amount of CCL3L1 could down-regulate CCR5 expression on the cell surface and by that directly influence the viral entry into the cells (ref). However, a recent study showed that CCL3L1 mRNA did not correlate with gene copy number while the ratio of CCL3L1:CCL3 correlated significantly [Carpenter et al., 2012]. Carpenter *et al* suggested that the majority of CCL3L protein is CCL3, but at the moment there is no specific antibody to prove that.

On the other hand, the viral entry-independent mechanism could also potentially explain the associations between gene copy numbers and the susceptibility to HIV infection. For example, the entry-independent might include T cell regeneration and activation induced cell death, the formation of immunological synapse and cell-mediated immunity (CMI) [Castellino et al., 2006; Lillard et al., 2003; Molon et al., 2005]. Dolan *et al* showed that certain *CCL3L1-CCR5* genotypes (non-detrimental *CCR5* haplotypes and *CCL3L1* copy number higher than population median) alter CMI (measured as delayed-type hypersensitivity responses) in both healthy and HIV infected subjects and these genotypes are similar to those that influence the risk of HIV transmission and the disease progression. Still, analyses demonstrated that the *CCL3L1-*

CCR5 genotypes influence the HIV course independently of their effects on viral load and CMI. Thus, Dolan *et al* indicated that the effect of *CCL3L1-CCR5* genotype on HIV disease course includes the viral entry-independent processes (such as CMI) and suggested that their effect might be even greater than entry-dependent processes. Based on these results, it could be speculated that similar processes are affecting also the viral transmission but how and in which extent is still to be explored.

6.5. The detection of gene copy number

Still, a vivid debate behind the controversial results of the associations between *CCL3L1* copy number and HIV infection is ongoing and the accuracy of qPCR to measure CCL3L1 copy numbers has been raised [Bhattacharya et al., 2009; Field et al., 2009; Shrestha et al., 2010a; Urban et al., 2009]. Actually, this problem is not specific only to *CCL3L1* but also other genes with the copy number variation. The gene copy number variation is a form of structural variation in the genome where the duplication or deletion of DNA segments occurs. Because of the varying length of DNA segments (1–400 kb) and a high level of sequence identity (>90%) it is difficult to measure the copy number compared with the detection of SNPs.

Many different assays have been developed to determine a copy number variation starting with fluorescence in situ hybridization and ending with next-generation sequencing (NGS). Today, several NGS-based methods have been developed [Duan et al., 2013]. These methods vary by the NGS reads (single-end or pair-end), software and algorithms. Although NGS technology is a powerful tool, it still produces short sequences that complicate the mapping processes. In addition, the results of Duan et al (2013) showed that more efficient software and improved algorithms are needed. They compared six different NGS-methods and produced the recommendations for choosing the method based on the researchers priorities. However, all NGS-methods are developed using full genome data, thus, today's NGS-technology is still rather resource consuming especially in the association studies where hundreds of subjects should be evaluated. On the other hand, the locus-specific NGS methods could be developed but in this case the bottleneck would be the amplification processes. Bearing in mind the features of copy number variation, it is hard to design appropriate primers to amplify the right regions including all copies. Nevertheless, the progression of technology and accelerated development of software, the NGS-based methods for the copy number variation will soon be used widely in the detection of gene copy numbers.

The determination of *CCL3L1* copy number was carried out by qPCR the method that was available at the moment we conducted our experiments. We have not measured the copy number with other methods like PRT or NGS-based methods. Still, Sudmant et al (2010) have shown a high correlation

between *CCL3L1* qPCR and whole-genome shotgun sequencing data to accurately determine assay specific duplicated genes and the copy numbers of genes (r = 0.95). This suggests that the qPCR results and findings of Gonzalez et al and ours in this study on *CCL3L1* copy number are correct allowing to drawn reliable conclusions.

6.6. Study limitations

Some limitations of the current thesis should be acknowledged. First, the duration of IVDU, a crucial factor in the acquisition of HIV and HCV infection was available only for two thirds of the CCL3L1, CCR5 and CCL5 study population missing from IDUs from prison due to a technical error. However, populations from prison and the syringe exchange programme were similar in terms of demographic and risk behaviours, and in the multivariate analysis they did not influence the principle findings (Article 2; Table 4). Thus, we believe that the duration of IVDU is similar between the populations, and associations in one population can be extrapolated to the whole population. Another population bias was that all IDUs recruited from prisons were HIV positive and the rate of HIV/HCV co-infection was higher than in IDUs from the syringe exchange programmes (Results; Table 6). This may be due to the fact that HIV negative populations cannot be recruited from prisons. Nevertheless, we believe that despite these small discrepancies, these two populations are similar in terms of demographics and risk behaviour. Therefore, the patients from two sources likely do not influence the associations between CCR5 and its ligands CCL3L1 and CCL5 genetic variability and HIV/HCV serostatus. In addition, due to the high rate of HIV/HCV co-infection the number of HCV monoinfected or uninfected subjects was relatively small and accordingly the power of several analyses was thus diminished. Finally, the HIV negative group was relatively small in all studies, especially HESNs group in TLR3 study but it has to be noted that this group among IDUs is very rare because most of the highly exposed IDUs become infected with HIV suggesting that factors that protect a person from HIV infection are very rare. In spite of that, the associations between the evaluated host genetic factors and HIV and HCV infections remained significant in both the uni- and multivariant analysis. Regardless of these limitations, we believe that the results presented here adequately describe the role of host genetic factors in the acquisition of HIV and HCV infection.

6.7. Future research

The currents thesis revealed associations between host genetic polymorphisms and HIV and HCV acquisition. Still, there are several questions that we were not able to address using this study design and methods. Firstly, how the *CCR5* haplotypes and especially haplotype pairs influence the expression of CCR5 on

different cell types (CD4+, CD8+, naïve and memory T cells etc.). So far the studies have evaluated only single SNPs or the cluster of haplotypes. It will be most interesting to find how *CCR5* haplotype pairs are related to the receptor expression, especially because the expression of CCR5 may be impacted by the partner allele. Secondly, how the opioids influence the expression of CCR5 and how are they influenced by *CCR5* haplotypes and haplotype pairs. There are suggestions that opioids (e.g. cocaine), widely used by Estonian IDUs [Uuskula et al., 2010], increase the expression of CCR5. How this process is influenced by different *CCR5* haplotypes is largely unknown.

Another issue that was not evaluated in the current study but is crucial is the relationship between host genetic factors and HIV disease progression in IDU population. As discussed above, IDUs are a unique population and there is a lack of knowledge about many processes in the disease course. However, it is a challenge to recruit this population especially into the prospective studies. Since 2009, an Estonian HIV database has been established containing at present data on more than 3000 HIV positive patients from different risk groups (including IDUs). In addition, this database contains biosamples from HIV positive subjects. This will enable evaluating the effect of host and viral genetic as well as immunological factors on HIV disease progression in IDUs. In addition, finding out the relationship between antiretroviral regimens and host genetic factors would give a practical value allowing physicians to select the most appropriate antiretroviral regimen.

7. CONCLUSIONS

1. HCV seropositivity depends on *CCR5* haplotypes. More specifically, persons with *CCR5* HHG*1 or haplotype pair HHE/HHG*1 are more likely protected from HCV infection compared to those not having this haplotype/ haplotype pair. These data suggest that *CCR5* haplotypes/haplotype pairs, in addition to being involved in the responses to the therapy in chronic HCV infection, are also associated with the susceptibility to the HCV infection. The *CCR5* haplotypes have no independent effect on the susceptibility to HIV infection in IDUs.
2. In Caucasian IDUs, similar to other risk groups of blood-borne infections and ethnic groups, the median CCL3L1 gene copy number affects the susceptibility to HIV infection such that IDUs possessing a higher gene copy number than the population median have significantly decreased odds of HIV positivity to those with lower than population median copy number. This suggests that the effect of *CCL3L1* copy number is independent of the route of transmission. The potential mechanism behind this association might be that the higher CCL3L1 expression blocks the binding of HIV to the receptor and/or diminishes the expression of CCR5 onto the cell surface. In addition, the entry-independent pathway might also contribute to this process. There is no association between *CCL3L1* copy number and the susceptibility to HCV infections.
3. The *CCL5* haplotype D (-471A/-96G/In1.1C) is protective against HCV, highlighting the role of *CCL5* variability in CCL5-CCR5 axis on the susceptibility to HCV in IDUs. However, in contrast to previous studies describing the associations between the *CCL5* variability and the susceptibility to HIV in the multiethnic sexually exposed or infected, we were not able to demonstrate the same in IDUs suggesting that this effect is not universal and may not exist in the case of intravenous transmission.
4. *TLR3* rs3775291 influences HIV seropositivity in HESNs such that T allele gives protection against HIV infection. The protective effect of *TLR3* T allele suggests the immunologically mediated protection from HIV infection through TLR3 by recognising the virus. There are no associations between TLR3 polymorphism and susceptibility to HCV infections.

8. SUMMARY IN ESTONIAN

Inimese geneetiliste faktorite mõju HIV-i ja C-hepatiidi viirusesse nakatumisele süstivate narkomaanide hulgas

Süstitavate narkootikumide kasutamine on maailmas toonud kaasa vere teel levivate viiruste, nagu inimese immuunpuudulikkuse viirus (HIV) ja C-hepatiidi viirus (HCV), kiire leviku. Praeguseks elab maailmas umbes 35 miljonit inimest, kes on nakatunud HIV-i, ja 130 miljonit, kes on nakatunud HCV-sse. Kui HCV puhul umbes 20% inimestest suudab ise viirusest vabaneda, siis HIV infektsiooni ei suuda inimese immuunsüsteem elimineerida.

Inimesi, kes ei nakatu HIV-i isegi peale mitmeid ekspositsioone viirusega, nimetatakse kõrgelt eksponeeritud HIV seronegatiivseteks isikuteks (KESN). Selliseid isikuid võib leida (i) kontamineeritud süstlaid jagavate süstivate narkomaanide (SN) seast; (ii) inimeste seast, kes said epideemia algusaastatel HIV-positiivse vere ülekandeid; (iii) HIV-positiivsetel emadelt sündinud laste seast; ja (iv) HIV-positiivsete isikute seksuaalpartnerite seast. Kuna siiani pole täpselt teada, mis põhjustel need inimesed ei nakatu, pakub see uurijatele suurt huvi.

HIV-i nakatumist mõjutavad paljud faktorid, nagu näiteks nakatumistee, nakataja viirushulk, teised kaasnevad seksuaalsel teel levivad haigused ja inimese immunoloogilised faktorid. Lisaks sellele mõjutavad nakatumist ka inimese geneetilised faktorid, millest tuntuim on 32-aluspaariline deletsioon HIV ko-retseptorit CCR5-te kodeerivas geenis. Nimetatud mutatsiooni homosügootsus annab resistentsuse HIV R5-troopsetele viirustele. Samuti on tuvastatud erinevates geenides mitmeid polümorfisme, mis mõjutavad HIV-i nakatumist. Siiani on inimese geneetiliste faktorite uuringud läbi viidud peamiselt populatsioonides, kus nakatumine on toimunud kas seksuaalsel teel või ülekandel emalt lapsele. Vähe on teada geneetiliste faktorite mõju kohta SN-de seas, populatsioonis, mis on valdav endise Nõukogude Liidu riikide HIV epideemiates, kaasa arvatud Eestis.

Eestis sai HIV-1 epideemia alguse 2000. aastal, kui Euroopas haruldane HIV-1 rekombinantne vorm CRF06_cpx sisenes SN-de populatsiooni. Epideemiat iseloomustas HIV-1 kiire levik peamiselt noorte meessoost SN-de seas ja kõrge HCV levimus. Kuna tegemist on homogeense populatsiooniga, siis annab see võimaluse kirjeldada inimese geneetiliste faktorite mõju HIV-i ja HCV-sse süstimise teel nakatumisele.

Uurimistöö eesmärgid

Töö üldine eesmärk oli kirjeldada inimese immuunsüsteemi kodeerivate geenide ning HIV-i ja/või HCV-sse nakatumise vahelisi seoseid europiidsesse rassi kuuluvatel SN-del.

Uuringu alaeesmärgid olid:

1. Kirjeldada CCL3L1 geeni koopiaarvu ning HIV-i, HCV-sse ja/või HBV-sse nakatumise vahelisi seoseid SN-de seas.
2. Kirjeldada *CCR5* haplotüüpide/haplotüübi paaride ning HIV-i ja/või HCV-sse nakatumise vahelisi seoseid SN-de seas.
3. Kirjeldada *CCL5* haplotüüpide/haplotüübi paaride ning HIV-i ja/või HCV-sse nakatumise vahelisi seoseid SN-de seas.
4. Kirjeldada *TLR3* polümorfismi ning HIV-i ja/või HCV-sse nakatumise vahelisi seoseid SN-de seas.

Uuritavad ja metoodika

Uuring viidi läbi neljas osas:

1. CCL3L1 uuring, mis määras CCL3L1 geeni koopia arvu ja selle seoseid HIV-i ja/või HCV-sse ja HBV-sse nakatumisega. Uuritavateks olid 374 SN-i, kes olid kaasatud süstlavahetuspunktidest ja Eesti vanglatest 2006–2007 aastal. Uuritavad olid valdavalt noored mehed (keskmine vanus 26 aastat, mehi 80%).
2. CCR5 uuring, mis kirjeldas *CCR5* haplotüüpe ja haplotüübi paare ning nende seoseid HIV-i ja/või HCV-sse nakatumisega. Uuritav valim koosnes valdavalt eeltoodud SN-dest. Lisaks kaasati uuringusse 500 HIV-, HCV- ja HBV-negatiivset veredoonorit.
3. CCL5 uuring, mis keskendus *CCL5* haplotüüpidele ja haplotüübi paaridele ning nende seostele HIV-i ja/või HCV-sse ja HBV-sse nakatumisega. Uuritavateks olid valdavalt eelmainitud SN-d.
4. TLR3 uuring, mis määras *TLR3* rs3775291 polümorfismi ja selle seoseid HIV-i ja/või HCV-sse ja HBV-sse nakatumisega. Uuritavateks olid 2011. aastal süstlavahetuspunktist kaasatud 345 SN-i ning 497 eelnevalt kirjeldatud veredoonorit.

Uuring viidi läbi TÜ Tervishoiu Instituudi, Tervise Arengu Instituudi, Tartu Vangla ja TÜ Mikrobioloogia Instituudi koostööna.

Kõikides uuringutes eraldati isikute verest inimese genoomne DNA. *CCL3L1* koopia arv määrati *Real-Time PCR*-ga nagu kirjeldatud Gonzalez *et al* (2005) poolt. *CCR5, CCL5* ja *TLR3* polümorfismid määrati kasutades *Real-Time PCR Allelic Disclimination Assay*'d või *RLFP-PCR*-i. *CCR5* haplotüüpide aluseks oli Gonzalez *et al* (1999) poolt kasutuses olev evolutsioonipõhine klassifikatsioon. *CCL3L1* koopia arvu ja *TLR3* polümorfismi määramine viidi läbi TÜ Mikrobioloogia Instituudis ning *CCR5* ja *CCL5* polümorfismide määramine professor Sunil K. Ahuja laboris Texase Ülikoolis San Antonios USAs.

Statistilises analüüsis kasutati haplotüüpide/haplotüübi paaride jaotuse võrdlemisel Fisheri täpset testi ja hii-ruut testi ning mudelite puhul ühe- ja mitmemõõtelist logistilist regressiooni.

Peamised tulemused ja arutelu

Ootuspäraselt nägime, et SN-de1, kes olid HCV- ja HBV-positiivsed, olid suuremad šansid olla ka HIV-positiivsed, ning SN-del, kes olid HIV- ja HBV-positiivsed, olid suuremad šansid olla HCV-positiivsed. Samuti tõstis pikaajaline süstitavate narkootikumide kasutamine HIV-i ja HCV-sse nakatumise šansse, samas ei mõjutanud SN-de vanus ega sugu HIV-i ja HCV-sse nakatumist. Neid faktoreid, mis mõjutasid HIV-i ja HCV-sse nakatumist, kasutati mudelites kaasuvate faktoritena (HCV, HBV serostaatus, süstitavate narkootikumide kasutamise kestvus).

Kõikide uuringus määratud geenide polümorfismid ja nendest tulenevate haplotüüpide esinemissagedus sarnanes varem kirjeldatud europiidsesse rassi kuuluvate populatsioonide andmetega. *CCR5* haplotüüpidest oli sagedaseim HHE (56% doonoritel ja 55% SN-l), *CCL5* haplotüüpidest A (96% SN-l), *CCL3L1* koopia arvu populatsiooni mediaan oli 2 ja *TLR3* rs3775291 T-alleeli sagedus 34%.

Ainuke *CCR5* haplotüüp, mille sagedus oli oluliselt erinev HIV-negatiivsete ja HIV-positiivsete SN-de vahel, oli HHF*2 (23,5% vs 15,0%; $p<0,05$). SN-del, kes omasid HHF*2, oli peaaegu 2 korda väiksemad šansid olla HIV-positiivne kui SN-del, kellel see haplotüüp puudus (OR=0,57; 95% CI 0,34–0,98, p=0,041). Kuid pärast mudeli kohandamist kaasuvatele faktoritele (HCV ja HBV staatus, süstitavate narkootikumide kasutusaeg) ei olnud seos enam oluline. Siiski, varasemates uuringutes on näidatud HHF*2 kaitsvat rolli HIV-i nakatumisel ülekandel emalt lapsele. Võrreldes *CCR5* haplotüüpide jaotuvust HCV-positiivsete ja -negatiivsete SN-de vahel, selgus, et HHG*1 oli sagedasem HCV-negatiivsete seas kui -positiivsete seas (20,7% vs. 7,5%; $p<0,001$). Ühemõõtmeline logistiline regressioonanalüüs näitas, et SN-del, kes omasid HHG*1, olid väiksemad šansid olla HCV-positiivne (OR = 0,37; 95% CI = 0,16–0,82), mis jäi oluliseks ka peale kohandamist kaasuvatele faktoritele (OR = 0,07; 95% CI 0,03–0,20). Analüüsidest selgus, et HHG*1 sisaldav haplotüübi paar HHE/HHG*1 on sarnaselt HHG*1 seotud HCV-sse nakatumisega. CCR5 haplotüüpidele sarnane analüüs viidi läbi *CCL5* haplotüüpide mõju hindamisel. Selle tulemusel selgus, et *CCL5* haplotüüp D olemasolu vähendas šansse olla HCV-positiivne (OR = 0,20; 95% CI 0,09–0,43), mis jäi oluliseks ka peale kohandamist kaasuvatele faktoritele (OR = 0,12; 95% CI 0,03–0,42). Meie teada on see esimene uuring, mis mainitud seoseid näitas. Varasemalt on Woitas *et al* (2005) HCV-sse nakatumist seostanud CCR5-Δ32 homosügootsusega, kuid hilisemad uuringud ei ole sellist seost näidanud, ja arvatakse, et Woitas *et al* tulemus peegeldas resistentsust pigem HIV-le kui HCV-le. Meie tulemused viitavad nii CCR5 kui CCL5 geneetilise mitmekesisuse olulisusele HCV infektsioonis, aga ei ole teada, mis mehhanismi läbi.

CCL3L1 koopia arv oli suurem HIV-negatiivsete SN-de seas võrreldes HIV-positiivsetega. Ühemõõtmeline logistiline regressioonanalüüs näitas, et SN-del, kellel oli *CCL3L1* koopia arv kõrgem kui populatsiooni mediaan, olid suuremad šansid olla HIV-negatiivsed (OR = 0,49; 95% CI 0,29–0,81). Antud seos jäi

oluliseks ka peale mudeli kohandamist kaasuvatele faktoritele. Sellist seost on varem kirjeldatud isikutel, kes on eksponeeritud seksuaalselt või perinataalselt. Samas nägime, et *CCL3L1* koopia arv ei ole seotud HCV staatusega.

TLR3 rs3775291 vähemalt ühe T-alleeli esinemine erines KESN-de, HIV-positiivsete ja doonorite vahel (vastavalt 80% vs 53% vs 55%; kõigil $p<0,05$). Ühemõõtmeline logistiline regressioonanalüüs näitas, et KESN-idel, kellel oli vähemalt üks T-alleel, olid väiksemad šansid HIV-i nakatuda kui KESN-idel, kellel seda alleeli ei olnud (OR = 0,29; 95% CI 0,09–0,90). Seos jäi oluliseks ka peale kaasuvatele faktoritele kohandamist. Meie tulemused kinnitasid varasemaid tulemusi *TLR3* rs3775291 T-alleeli HIV-i eest kaitsva efekti kohta.

Järeldused

1. CCL3L1 geeni koopia arv mõjutab HIV-i nakatumist SN-de seas. Sarnaselt teiste erinevat päritolu riskirühmadega, on populatsiooni mediaanist suurem *CCL3L1* koopia arv seotud HIV infektsiooni eest kaitsva efektiga, viidates, et *CCL3L1* mõju ei sõltu nakatumisviisist. Võimalik mehhanism võib olla seotud kõrgema CCL3L1 ekpressioonitasemega, mille tõttu CCL3L1 blokeerib HIV-i seondumise tema ko-retseptori CCR5-ga ja/või vähendab CCR5 ekspressiooni raku pinnal. *CCL3L1* koopia arv ei ole seotud HCV-sse nakatumisega.
2. *CCR5* haplotüübid mõjutavad HCV seropositiivsust, nii et *CCR5* haplotüüpi HHG*1 või haplotüübi paari HHE/HHG*1 omavatel isikutel on väiksemad šansid olla HCV seropositiivsed võrreldes isikutega, kes neid ei oma. Need tulemused viitavad, et lisaks *CCR5* haplotüüpide rollile kroonilise HCV ravivastuses, on need olulised ka nakatumisel. *CCR5* haplotüübid ei ole seotud HIV nakatumisega SN-de seas.
3. *CCL5* haplotüüp D (-471A/-96G/In1.1C) omab HCV-sse nakatumisel kaitsvat efekti. See tõstab esile CCL5 geneetilise mitmekesisuse olulisuse HCV-sse nakatumise kontekstis. Samas *CCL5* haplotüübid ei ole seotud HIV-i nakatumisega nagu on eelnevalt demonstreeritud seksuaalsel teel eksponeeritute või nakatunute kohortides.
4. *TLR3* rs3775291 T-alleel omab kaitset HIV-i nakatumise eest KESN-ide seas. Kaitse võib olla vahendatud läbi TLR3, mis tuvastab viiruse genoomi olemasolu inimese rakus. *TLR3* polümorfism ei ole seotud HCV-sse nakatumisega.

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PUBLICATIONS

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List of publications:

1. **Huik, K**; Avi, R; Uibopuu, H; Pauskar, M; Margus, T; Karki, T; Krispin, T; Kool, P; Rüütel, K; Talu, A; Abel-Ollo, K; Uusküla, A; Carillo, A; He, W; Ahuja, SK; Lutsar, I (2014). Association between HIV-1 tropism and CCR5 human haplotype E in a Caucasian population. JAIDS (accepted)
2. **Huik, K**; Avi, R; Pauskar, M; Kallas, E; Jõgeda, EL; Karki, T; Marsh, K; Des Jarlais, D; Uusküla, A; Lutsar, I (2013). Association between TLR3 rs3775291 and resistance to HIV among highly exposed Caucasian intravenous drug users. Infection, genetics and evolution, 20, 78–82.
3. **Huik, K**; Avi, R; Carrillo, A; Harper, N; Pauskar, M; Sadam, M; Karki, T; Krispin, T; Kongo, UK; Jermilova, T; Rüütel, K; Talu, A; Abel-Ollo, K; Uusküla, A; Ahuja, SK; He, W; Lutsar, I (2013). CCR5 Haplotypes

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4. Soeorg, H.; **Huik, K**.; Parm, U.; Ilmoja, ML.; Metelskaja, N.; Metsvaht, T.; Lutsar, I. (2013). Genetic Relatedness of Coagulase-Negative Staphylococci from Gastrointestinal Tract and Blood of Preterm Neonates with Late-Onset Sepsis. The Pediatric Infectious Disease Journal, 32(4), 389–393.
5. Avi, R; **Huik, K**; Pauskar, M; Ustina, V; Karki, T; Kallas, E; Jõgeda, EL; Krispin, T; Lutsar, I (2013). Transmitted Drug Resistance Is Still Low in Newly Diagnosed Human Immunodeficiency Virus – 1 CRF06_cpx Infected Patients in Estonia in 2010. Aids Research and Human Retroviruses, x
6. Soeorg, H.; Tamm, E.; **Huik, K.;** Pauskar, M.; Mägi, D.; Pruudel, K.; Vainomäe, L.; Moosar, L.; Kirss, K.; Torm, S.; Närska, M.; Pütsepp, A.; Nurm, H.; Pruunsild, K.; Jänes, A.; Zilmer, K.; Lutsar, I. (2012). Group A rotavirus genotypes circulating prior to implementation of a National Immunization Program in Estonia. Human Vaccines, 8(4), 465–469.
7. Avi, R; **Huik, K**; Pauskar, M; Ustina, V; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2011). Emerging Transmitted Drug Resistance in Treatment Naive Human Immunodeficiency Virus – 1 CRF06_cpx Infected Patients in Estonia. Scandinavian Journal of Infectious Diseases, 43(2), 122–128.
8. **Huik, K**.; Sadam, M.; Karki, T.; Avi, R.; Krispin, T.; Paap, P.; Rüütel, K.; Uusküla, A.; Talu, A.; Abel-Ollo, K.; Lutsar, I. (2010). CCL3L1 copy number is a strong genetic determinant of HIV seropositivity in Caucasian intravenous drug users. The Journal of Infectious Diseases, 730–739.
9. Avi, R.; **Huik, K**.; Sadam, M.; Karki, T.; Krispin, T.; Ainsalu, K.; Paap, P.; Schmidt, J.; Nikitina, N.; Lutsar, I. (2010). Characterization of Integrase Region Polymorphisms in HIV-1 CRF06_cpx Viruses in Treatment Na?ve Patients in Estonia. Aids Research and Human Retroviruses, 26(10), 1109–1113.
10. Avi, R; **Huik, K**; Sadam, M; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2009). Absence of Genotypic Drug Resistance and Presence of Several Naturally Occurring Polymorphisms of Human Immunodeficiency Virus-1 CRF06_cpx in Treatment-Naive Patients in Estonia. Journal of Medical Virology, 81(6), 953–958.

Other administrative and professional activities:
A council member of *European Society* for Translational Antiviral Research

Teaching work:
Information regarding the teaching work carried out at universities: Supervising seminars and practicals of medical microbiology.

Supervision:

1. Merit Pauskar, master's degree, 2013, (sup) Kristi Huik, Radko Avi, HIV-1 transmitted drug resistance in newly diagnosed HIV-infected patients in Estonia in 2010, University of Tartu, Faculty of Medicine, Department of Public Health
2. Ene-Ly Jõgeda, master's degree, 2013, (sup) Kristi Huik, Jaanus Remme, Radko Avi, The prevalence and genotypic distribution of GBV-C and its associations with HIV infection among Estonian intravenous drug users, University of Tartu, Faculty of Science and Technology
3. Ene-Ly Jõgeda, bachelor's degree, 2011, (sup) Kristi Huik, Impact of HLA-G polymorphisms in HIV infection among injecting drug users, University of Tartu, Faculty of Science and Technology
4. Helen Uibopuu, master's degree (MSci), 2010, (sup) Irja Lutsar, Kristi Huik, Radko Avi, The diversity of HIV-1 CRF06_cpx env V3 region and its associations with genetic diversity of CCR5 and its ligands, University of Tartu, Faculty of Medicine
5. Kaisa Tiivoja, master's degree (M.P.), 2008, (sup) Kristi Huik, Tõnu Krispin The prevalence of CCR5Δ32 polymorphism among HIV-1-seropositive intravenous drug users in Estonia, University of Tartu, Faculty of Medicine, Department of Pharmacy

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1999–2002	Vändra Gümnaasium

Teenistuskäik:

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2006–2014	Tartu Ülikool, mikrobioloogia instituut, laborant
2007–2009	OÜ Gvander, proviisor
2007–2007	Riiamäe apteek, proviisor

Teaduslik ja arendustegevus:
Peamised uurimisvaldkonnad: HIV-1 infektsioon, inimese geneetiliste ja immunoloogiliste faktorite mõju HIV ja HCV infektsiooni nakatumisele ning haiguse progressioonile, HIV-1 ravimresistentsus ja mitmekesisus.

Publikatsioonide loetelu:

1. **Huik, K**; Avi, R; Uibopuu, H; Pauskar, M; Margus, T; Karki, T; Krispin, T; Kool, P; Rüütel, K; Talu, A; Abel-Ollo, K; Uusküla, A; Carillo, A; He, W; Ahuja, SK; Lutsar, I (2014). Association between HIV-1 tropism and CCR5 human haplotype E in a Caucasian population. JAIDS (accepted)
2. **Huik, K**; Avi, R; Pauskar, M; Kallas, E; Jõgeda, EL; Karki, T; Marsh, K; Des Jarlais, D; Uusküla, A; Lutsar, I (2013). Association between TLR3 rs3775291 and resistance to HIV among highly exposed Caucasian intravenous drug users. Infection, genetics and evolution, 20, 78–82.
3. **Huik, K**; Avi, R; Carrillo, A; Harper, N; Pauskar, M; Sadam, M; Karki, T; Krispin, T; Kongo, UK; Jermilova, T; Rüütel, K; Talu, A; Abel-Ollo, K; Uusküla, A; Ahuja, SK; He, W; Lutsar, I (2013). CCR5 Haplotypes Influence HCV Serostatus in Caucasian Intravenous Drug Users. PLoS ONE, 8(7), e70561

4. Soeorg, H.; **Huik, K**.; Parm, U.; Ilmoja, ML.; Metelskaja, N.; Metsvaht, T.; Lutsar, I. (2013). Genetic Relatedness of Coagulase-Negative Staphylococci from Gastrointestinal Tract and Blood of Preterm Neonates with Late-Onset Sepsis. The Pediatric Infectious Disease Journal, 32(4), 389–393.
5. Avi, R; **Huik, K**; Pauskar, M; Ustina, V; Karki, T; Kallas, E; Jõgeda, EL; Krispin, T; Lutsar, I (2013). Transmitted Drug Resistance Is Still Low in Newly Diagnosed Human Immunodeficiency Virus – 1 CRF06_cpx Infected Patients in Estonia in 2010. Aids Research and Human Retroviruses, x
6. Soeorg, H.; Tamm, E.; **Huik, K.;** Pauskar, M.; Mägi, D.; Pruudel, K.; Vainomäe, L.; Moosar, L.; Kirss, K.; Torm, S.; Närska, M.; Pütsepp, A.; Nurm, H.; Pruunsild, K.; Jänes, A.; Zilmer, K.; Lutsar, I. (2012). Group A rotavirus genotypes circulating prior to implementation of a National Immunization Program in Estonia. Human Vaccines, 8(4), 465–469.
7. Avi, R; **Huik, K**; Pauskar, M; Ustina, V; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2011). Emerging Transmitted Drug Resistance in Treatment Naive Human Immunodeficiency Virus – 1 CRF06_cpx Infected Patients in Estonia. Scandinavian Journal of Infectious Diseases, 43(2), 122–128.
8. **Huik, K**.; Sadam, M.; Karki, T.; Avi, R.; Krispin, T.; Paap, P.; Rüütel, K.; Uusküla, A.; Talu, A.; Abel-Ollo, K.; Lutsar, I. (2010). CCL3L1 copy number is a strong genetic determinant of HIV seropositivity in Caucasian intravenous drug users. The Journal of Infectious Diseases, 730–739.
9. Avi, R.; **Huik, K**.; Sadam, M.; Karki, T.; Krispin, T.; Ainsalu, K.; Paap, P.; Schmidt, J.; Nikitina, N.; Lutsar, I. (2010). Characterization of Integrase Region Polymorphisms in HIV-1 CRF06_cpx Viruses in Treatment Na?ve Patients in Estonia. Aids Research and Human Retroviruses, 26(10), 1109–1113.
10. Avi, R; **Huik, K**; Sadam, M; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2009). Absence of Genotypic Drug Resistance and Presence of Several Naturally Occurring Polymorphisms of Human Immunodeficiency Virus-1 CRF06_cpx in Treatment-Naive Patients in Estonia. Journal of Medical Virology, 81(6), 953–958.

Muu teaduslik organisatsiooniline ja erialane tegevus: *European Society for Translational Antiviral Research* nõukogu liige.

Õppetöö:
Andmed kõrgkoolis tehtud auditoorse õppetöö kohta: Meditsiinilise mikrobioloogia seminaride ja praktikumide läbi viimine.

Juhendamine:

1. Merit Pauskar, magistrikraad, 2013, (juh) Kristi Huik, Radko Avi, HIV-1 ülekanduva ravimresistentsuse esinemine 2010. aastal HIV-diagnoosi saanud isikutel Eestis, Tartu Ülikool, Arstiteaduskond, Tervishoiu instituut, Epidemioloogia ja biostatistika õppetool
2. Ene-Ly Jõgeda, magistrikraad, 2013, (juh) Kristi Huik, Jaanus Remme, Radko Avi, GBV-C esinemine ja mõju HIV infektsiooni nakatumisele Eesti süstivate narkomaanide populatsioonis, Tartu Ülikool, Loodus- ja tehnoloogiateaduskond
3. Ene-Ly Jõgeda, bakalaureusekraad, 2011, (juh) Kristi Huik, HLA-G polümorfismide mõju HIV infektsiooni nakatumisele süstivate narkomaanide populatsioonis, Tartu Ülikool, Loodus- ja tehnoloogiateaduskond
4. Helen Uibopuu, magistrikraad (teaduskraad), 2010, (juh) Irja Lutsar, Kristi Huik, Radko Avi, HIV-1 CRF06_cpx env V3 piirkonna mitmekesisus ja selle seos CCR5 ja tema ligandide genotüüpidega, Tartu Ülikool, Arstiteaduskond
5. Kaisa Tiivoja, magistrikraad (M.P.), 2008, (juh) Kristi Huik, Tõnu Krispin CCR5Δ32 polümorfismi esinemine HIV-1 positiivsete süstivate narkomaanide seas Eestis, Tartu Ülikool, Arstiteaduskond, Farmaatsia instituut

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
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5. **Ants Peetsalu.** Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
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8. **Ruth Mikelsaar.** Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
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10. **Katrin Gross.** Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
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67. **Tamara Vorobjova.** Immune response to *Helicobacter pylori* and its association with dynamics of chronic gastritis and epithelial cell turnover in antrum and corpus. Tartu, 2001.
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