JAIDS Journal of Acquired Immune Deficiency Syndromes Publish Ahead of Print
DOI: 10.1097/QAI.0000000000001628

Title: Transmission Networks of Hcv Genotype 1a Enriched with Pre-Existing Polymorphism Q80k Among Hiv-Infected Patients with Acute Hepatitis C in Poland

Running head: HIV/HCV transmission chains in Poland

Miłosz Parczewski[1], Iwona Cielniak[2], Justyna Kordek[1], Bogusz Aksak-Wąs[1], Anna Urbańska[1], Magdalena Leszczyszyn-Pynka[1], Ewa Siwak[2], Monika Bociąga-Jasik[3], Anna Nowak[3], Aleksandra Szymczak[4], Małgorzata Zalewska[4], Władysław Łojewski[5], Anne-Mieke Vandamme[6,7], Nadine Lübke[8], Lize Cuypers[6,9]

1. Department of Infectious, Tropical Diseases and Immune Deficiency, Pomeranian Medical University in Szczecin, Szczecin, Poland.
2. Hospital for Infectious Diseases, HIV Out-Patient's Clinic, Warsaw, Poland.
3. Department of Infectious Diseases, Hepatology and Acquired Immune Deficiencies, Wrocław Medical University, Wrocław, Poland.
4. Department of Infectious Diseases, Jagiellonian University Medical College, Kraków, Poland.
5. Department of Infectious Diseases, Regional Hospital in Zielona Gora, Zielona Góra, Poland.
6. KU Leuven – University of Leuven, Department of Microbiology and Immunology, Rega Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, Belgium.
7. Center for Global Health and Tropical Medicine, Microbiology Unit, Institute for Hygiene and Tropical Medicine, University Nova de Lisboa, Lisbon, Portugal.
8. Institute of Virology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany.
9. Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

Funding: This work was partially funded by internal funds of Pomeranian Medical University (FSN 343-10/14), funds of Fundation Scio et Preaveceo, and partially by the Fonds Wetenschappelijk Onderzoek Vlaanderen (FWO – grants G0E8416N and G0B2317N).

The authors report no conflicts of interest related to this work.

*Correspondence to: Milosz Parczewski MD, Ph.D.

Pomeranian Medical University, Department of Infectious, Tropical Diseases and Immune Deficiency, Arkońska 4 71-455 Szczecin, Poland

Tel. 0048918139456, fax 0049918139449

e-mail: mparczewski@yahoo.co.uk

Abstract

Background: Hepatitis C virus (HCV) resistance-associated variants (RAVs) have been shown to adversely affect treatment response of direct-acting antivirals (DAAs). Identifying pre-existing RAVs and transmission networks among HIV/HCV genotype 1 (G1) infected patients from Poland will assist in shaping surveillance strategies for HCV.

Methods: NS3 and NS5A sequences were obtained from samples of 112 DAA-naive G1 patients (45 G1a, 67 G1b), of which 74 were chronically infected and 38 were diagnosed with acute hepatitis C (AHC). RAVs were identified using geno2pheno, and 98 concatenated NS3/NS5A alignments were constructed to identify transmission clusters using a maximum likelihood approach.

Results: G1a was notably more prevalent compared to G1b among men-having-sex-with-men (MSM) (60.0% vs. 31.3%, p=0.004), AHC cases (46.7% vs. 25.4%, p=0.019) and patients diagnosed with syphilis (52.2% vs. 24.5%, p=0.009). The overall NS3/NS5A RAVs frequency was 14.3% with variants occurring more often in G1a compared to G1b (27.5% vs. 5.2%, p=0.005), mostly for NS3 due to the high prevalence of polymorphism Q80K. NS5A RAVs were only found in 2.9% of sequences. Significant clustering was observed for 73.5% of the Polish sequences, however more common in G1a MSM compared to G1b (50.0% vs. 25.9%, p=0.02). The identified clusters contained sequences originating from up to five Polish cities, located within a mean distance of 370 km.

Conclusions: Close clustering of Polish strains suggests the presence of compartmentalized epidemics of MSM that fuel the spread of G1a variants. Particularly AHC patients form a national transmission network, including clusters enriched with the NS3 Q80K polymorphism.

Key words: Phylogenetic analysis, acute hepatitis C, HIV/HCV coinfection, transmission networks, natural resistance.

Introduction

Hepatitis C virus (HCV) infection remains a key worldwide epidemiological issue with recent estimates indicating ~80 million viraemic infections on a global level[1]. So far, seven HCV genotypes and more than 80 subtypes have been identified with subtypes 1a (G1a) and 1b (G1b) being the most common, irrespective of the HCV mono- or HIV/HCV co-infection status [2]. The HCV G1 distribution varies significantly according to geographical region, with the highest prevalence of G1a reported in North America and Oceania, while G1b is the major subtype in Europe, however particularly predominant in East European countries[3,4].

Recent advances in HCV treatment achieve virus elimination in ~95-100% of cases with the use of direct acting antiviral (DAA) combinations[1]. Treatment outcomes are affected not only by the HCV genotype a patient is infected with, but also by the presence of pre-existing resistance-associated variants (RAVs)[5]. Lower treatment response rates were observed, especially in the setting of the NS3 Q80K polymorphism[6], and for high-level resistance conferring NS5A variants located at codon positions 28, 30, 31, 58 and 93[5,7].

Many RAVs are not natural variants but selected under DAA selective pressure, which continue to evolve after treatment failure, often reverting back to the wild-type variant after a few months or only after years in case of NS5A[8,9]. This delay in reversion may result in transmission of drug resistant strains, however so far infrequently reported[10,11]. This may become especially important in the light of the increasing frequency of diagnoses of recent HCV infection among men-who-have-sex-with-men (MSM) with and without HIV infection, with the latter reported in candidates for HIV pre-exposure prophylaxis[12,13].

In Europe, Australia and North America, acute hepatitis C (AHC) transmission networks have been documented, associated with sex-related HCV acquisitions[14-16]. Additionally, among HIV-positive MSM, alarmingly high incidence rates of HCV reinfection after viral cure have been reported, ranging from 7.3-7.4 to 15.2/100 person-years[17]. This raises concerns for the possible introduction of RAVs within a risk population that is actively fueling HCV transmission, potentially resulting in reduced first-line DAA therapy efficacy and/or limited treatment options after DAA failure.

In Poland, around 160 thousand cases are considered HCV-RNA positive[18], with ~30% of people living with HIV being co-infected with HCV. In the last decade, the HIV predominant transmission route shifted from injecting drug use (90% decrease) to MSM (345% increase), with approximately 65% of new infections with known transmission route linked to the group of MSM. A stable increase in the number of new HIV diagnoses per year is observed across the country, with >22.000 cases diagnosed as of December 2017[19]. In the group of HIV/HCV co-infected patients, a decrease of 17.3 years in life expectancy is reported as well as a low likelihood of survival beyond the age of 65, associated with an increased risk of cardiovascular diseases, diabetes, neurocognitive performance and kidney disease, compared to those that are HCV mono-infected[20]. Although availability of DAA therapies has recently increased, access to treatment is not universal yet and is still prioritized for patients with advanced liver fibrosis, at least in Poland. Moreover, data on pre-existing variants conferring resistance to the most used anti-HCV drug classes still remain sparse, with only one Polish study investigating variants located in the NS3 region published so far[21], while no data is available for Polish patients carrying NS5A RAVs.

In this study, we aimed to map the genetic variability of viral proteins NS3 and NS5A in a cohort of HCV G1 patients co-infected with HIV, including patients diagnosed with acute hepatitis C. Moreover, phylogenetic inference was applied to identify clustering patterns within the Polish epidemic, and to investigate the possibility of natural RAVs circulating in the identified networks.

Material and methods

Study group

Samples from 112 patients, all of Caucasian origin and co-infected with HIV-1 and HCV genotype 1 (G1a or G1b), the most prevalent genotype in Poland, were analyzed. Also, this genotype was the most prevalent among patients with acute hepatitis C, with other genotypes accounting for 30.1%[22]. The samples were linked to care in five Polish HIV treatment centres (Warsaw, Wrocław, Cracow, Szczecin, Zielona Góra) and sequenced at the Pomeranian Medical University in Szczecin, Poland. The study protocol was approved by the Bioethical committee of the Pomeranian Medical University, approval number KB-0012/26/17. All samples were gathered from patients with chronic hepatitis C detected at HIV diagnosis (78/112, 66.07%) or from patients with AHC observed after HIV diagnosis (38/112, 33.92%). AHC patients diagnosed with genotype 1 were consecutively enrolled at the clinics participating in the study, while individuals with chronic hepatitis C were selected based on availability of stored left-over samples. AHC was defined as hepatitis C antibody seroconversion from negative to positive with concomitant increase of aminotransferases and detection of HCV-RNA during follow up. The majority (36/38, 94.74%) of the included AHC patients were on stable antiretroviral therapy regimens suppressing their HIV viral load to <50 copies/ml. None of the patients were treated before with pegylated-interferon and ribavirin, nor with any DAA at the time of sample collection.

The clinical data collected for all patients included the following parameters: age, gender, HCV transmission route, lymphocyte CD4 count and HIV-RNA viral load at HIV diagnosis, year of HCV infection for the AHC cases, HBV coinfection status (defined as HBs antigen positive), alanine aminotransferase activity and HCV RNA level (last available measurement prior to or at the date of sampling, with a median time range between the date of sampling and the HCV RNA level measurement being 3.7 months). History of syphilis was assumed positive if any serological test (VDRL, FTA-ABS, TPHA) was ever recorded reactive.

Sequencing methodology

NS3 and NS5A genetic sequencing was performed using the Sanger methodology as previously published[23]. Amplicons obtained by nested PCR were used for sequencing with the BigDye technology on an ABI 3500 platform (Applied Biosystems, Foster City, CA). NS3 and NS5A sequence assembly was performed with the Recall online tool, and variants were scored above a threshold of 15%. The final dataset included 112 patients and consisted of 108 NS3 (108/112 = 96.4%) and 102 NS5A sequences (102/112 = 91.07%), as sequencing experiments, either targeting NS3 or NS5A, repetitively failed for the remaining samples. Of the successfully amplified sequences, 98 cases (87.5%) were characterized by the presence of paired NS3/NS5A sequences. All sequences generated have been submitted to GenBank (pending sequence IDs).

Genotypic drug resistance and phylogenetic analyses

The G1 subtype (a or b), clade I or II (in case of G1a), and NS3 and NS5A RAVs were identified using the rules-based algorithm geno2pheno HCV v.0.92. For interpretation of drug resistance, any variant scored as "reduced susceptibility" or "resistant" to known NS3 or NS5A inhibitors, was included in the analysis. HCV genotype and subtype were confirmed using the Oxford HCV Automated Subtyping Tool , and the COMET subtyping tool .

For phylogenetic analysis, only the dataset of 98 paired NS3/NS5A sequences was used, as short fragments are often associated with poor phylogenetic signal. Firstly, joint NS3/NS5A sequences were aligned with the software Clustal X 2.0.10, separately for G1a (n=40) and G1b (n=58), using two reference sequences (G1a HQ850279 to root G1b tree and G1b D90208 to root G1a tree) as outgroup. Alignments were edited in MEGA 7.0 to improve their quality, while sequences containing stop codons were removed. The alignments covered NS3 codons 1-181 for both G1a and G1b, and NS5A codons 6-103 for G1a and 1-103 for G1b.

To investigate clustering patterns, datasets were supplemented with a selection of highly similar sequences obtained from the public database GenBank (see Supplementary Material, http://links.lww.com/QAI/B110 for the Genbank IDs), using the software tool BLAST. More in detail, for each sequence from the initial dataset, 10 sequences with the highest similarity

score and with 100% NS3 and NS5A sequence coverage were selected. After removal of duplicates, the final datasets consisted of 136 G1a and 151 G1b sequences. To construct phylogenetic trees, a maximum likelihood (ML) approach with an approximate likelihood ratio test (aLRT) (online version PHYMLv.3.0) and the use of smart model selection, indicating the general time reversible (GTR) nucleotide substitution model with four gamma categories as the optimal model for both datasets. A neighbor joining tree, supported by 1000 bootstrap replicates, was constructed in Clustal (results not shown), showing a similar topology as the ML tree. For the identification of clusters (≥3 sequences) and transmission pairs, the software Cluster Picker was used. The maximum genetic distance for both datasets was set at 0.08, combined with a clade support of aLRT >0.85, to call a group of sequences a cluster, similar to a previous analysis[24]. The selection of these parameters was in agreement with the clinical information related to exposure risk (disclosed epidemiological information in physician provided clinical notes on the shared sexual partners/parties). All trees were visualized in Figtree v.1.4.3.

Statistics

Statistical comparisons were performed using Fisher's exact and Chi^2 tests for nominal variables, while for continuous variables the Mann-Whitney *U*-test for nonparametric statistics was used, with P values ≤0.05 considered statistically significant. Confidence intervals (CI) and interquartile ranges (IQR) were indicated where appropriate. The commercial software Statistica (13PL, Statasoft Polska, Warsaw, Poland) was used for all statistical calculations.

Results

Clinical group characteristics and HCV genotype distribution

Initially, by the geno2pheno algorithm, 47 sequences were assigned as G1a and 65 as G1b. However, in two cases, the subtype needed to be corrected, according to the concordant results from the two well-known subtyping tools used, so as a final result, infection with G1a was found in 45 patients (40.18%), of whom 37 were infected with clade I (82.2%) and 8 with clade II (17.8%), while G1b infection was observed in 67 individuals (59.82%).

All genotype related differences in group characteristics are outlined in Table 1. Notably, G1a infection was more common among men (41/45, 91.11%) compared to G1b (51/67, 76.12%, p=0.042), with a significantly higher frequency of G1a among MSM (27/45, 60.0%) compared to injection drug users (IDUs) and their respective partners (18/45, 40%, p=0.003). Additionally, G1a was enriched among AHC cases (21/45, 46.67% vs. 17/67, 25.37% for G1b) and individuals with a history of syphilis (18/45, 52.24% for G1a vs. 14/67, 24.45% for G1b, p=0.009), while the opposite was true for patients diagnosed with chronic HCV (24/45, 32.43% for G1a vs. 50/67, 67.57% for G1b, p=0.019). Acute HCV infection was exclusively observed among MSM and associated with a history of syphilis (27/35, 84.38% with a positive syphilis diagnosis vs. 8/35, 14.04% with a negative syphilis serology, p<0.001).

Prevalence of resistance-associated variants (RAVs)

Overall frequencies of HCV RAVs were calculated jointly for the paired NS3/NS5A sequences (n=98, 40 G1a and 58 G1b) and separately for the available NS3 (G1a, n=42 vs G1b, n=66) and NS5A (G1a, n=43 G1b, n=59) sequences. The overall NS3/NS5A RAVs frequency was 14.3% (14/98 strains), and this frequency was significantly higher among G1a infected cases (n=11/40, 27.0%) compared to G1b (n=3/58, 5.2%, p=0.005) (Figure 1a).

In total, NS3 RAVs were found in 15.7% of the patients (17/108), while for NS5A only in 2.9%. The most common variant in the dataset was located in protein NS3, more particularly Q80K, found in 11.1% (12/108) of the analyzed NS3 sequences. One patient infected with G1a had RAVs both in the NS3 and NS5A region. A notably higher frequency of NS3 RAVs among G1a infected individuals (n=13/42, 31.0%) compared to G1b (n=4/66, 6.1% p=0.0005) was demonstrated. The most predominant NS3 was Q80K, exclusive to G1a, observed in 12/42 (28.6%) G1a patients, and especially more prevalent among MSM (n=9, 19.57%) compared to patients with a history of IDU and their partners (n=3, 4.84%, p=0.016) (Figure 1b). Other observed NS3 RAVs were 54S, 55A, 117H, and 168E. Overall, NS3 RAV frequencies (n=7/37, 18.92%), including Q80K polymorphism (n=6/37, 16.22%), were similar among HIV cases with AHC compared to HCV chronically infected patients (n=10/71, 14.08% and n=6/71, (
8.45%, respectively).

NS5A RAVs were only observed in three sequences (2.9%) across the entire dataset, including two G1a (4.7%) and one G1b strain (1.7%), more particularly NS5A variants 28T/V and 31M (Figure 1c). All three patients were identified to be chronically infected with HCV. No further significant differences in the distribution of NS3 and/or NS5A RAVs were noted for gender, transmission route, history of syphilis, diagnosis of AHC, when analyzing both the paired NS3/NS5A sequences or separately for NS3 and NS5A, neither for G1a or G1b.

Phylogenetic inference

In total, 72 out of 98 NS3/NS5A paired sequences (73.47%) formed 10 clusters and three pairs of sequences. For G1a, 32 out of 40 Polish strains (80.0%) formed four clusters and two pairs, including three clusters consisting exclusively of sequences derived from MSM and one cluster consisting of 10 sequences which were all associated with IDU transmission (Figure 2a). For G1b, six clusters and one pair including 40 sequences (69.0%) were counted, of which both the pair and four clusters consisted of sequences obtained from IDU-infected individuals and their heterosexual partners, while a large cluster of 14 sequences was found to be associated with MSM transmission (Figure 2b).

In G1a, the occurrence of both pairing and clustering among sequences derived from MSM patients was notably more common compared to G1b (n=20/40, 50.0% for G1a infected MSM vs. n=15/58, 25.86% for G1b MSM, p=0.02). Six sequences harboring the NS3 Q80K polymorphism (50% of all sequences with Q80K in the study) clustered within a G1a-MSM cluster, all were derived from patients with documented AHC infection during HIV follow-up and were diagnosed in Warsaw and Wrocław (Table 2). Five transmission clusters included cases diagnosed at a variety of clinical centers with a mean intercity distance of 370 km. Patients characterized by NS5A RAVs were not shown to cluster closely together in the trees, nor for G1a or G1b.

Discussion

In the current study, we analyzed HCV G1a and G1b NS3 and NS5A genetic sequences obtained from individuals co-infected with HIV, to investigate resistance patterns associated to DAA treatment failure. Transmission networks were constructed to reflect clustering patterns as well as transmissibility of natural RAVs in association with specific clinical characteristics. A high frequency of infections with G1a was observed among MSM with documented AHC after diagnosis of HIV and after being virologically suppressed with antiretroviral treatment. In this group of patients, sequences characterized by the NS3 Q80K polymorphism clustered together. On the other hand, NS5A RAVs were absent among patients with AHC and also rarely observed (<5%) in the overall study group, supporting to start treatment with NS5A inhibitors at an early stage. Phylogenetic inference revealed that the HIV/HCV co-infected population in Poland is facing two separate epidemics, dependent on the HCV genotype 1 subtype. While the more isolated G1b epidemic is probably related to the spread of autochthonous Polish strains, the HCV subtype 1a epidemic in the MSM population may be fueled due to mixing with strains from other European epidemics, transmitted through sexual contact between MSM.

In our dataset, RAVs in the NS3 region were found in 31% and 6.1% of all G1a and G1b sequences, respectively. The high frequency in G1a patients can be explained by the presence of NS3 polymorphism Q80K, which is known to be virtually absent in G1b[25]. This finding was consistent with previously published cohorts which reported prevalence rates ranging from <10% to ~50%, depending on the geographical region[26]. The Q80K polymorphism is specifically associated to G1a clade I and is only of key importance for patients treated with second-wave protease inhibitor (PI) simeprevir, due to significant reduction of response rates in combinations containing this agent and pegylated interferon/ribavirin or sofosbuvir[27], compared to a small or even no impact for other PIs[28]. In our study, other NS3 variants associated with PI resistance were detected on amino acid positions 54, 55, 117 and 168, with variants on position 117 only affecting susceptibility to boceprevir or telaprevir, agents which are nowadays no longer used[5]. Variant D168E reduces susceptibility to grazoprevir, paritaprevir and provides full resistance to simeprevir[29]. A RAV on NS3 position 168, reported for <1% of the DAA naive population in general, was previously reported to be common among G1b infected patients experiencing virologic failure on treatment with

faldaprevir or asunaprevir[30]. In general, the presence of naturally occurring NS3 non-Q80K variants was infrequent in our study (<10%), which is in line with reports of other European and Asian cohorts[3,31].

Among the analyzed NS5A sequences, RAVs were rarely detected, more particularly a prevalence of 4.7% for G1a and 1.7% for G1b. NS5A variants M28T/V, which are associated with antiviral resistance to daclatasvir, elbasvir, ledipasvir and ombitasvir[32,33], and L31M conferring resistance to daclatasvir and elbasvir[34,35], were observed in the dataset. The frequency of these RAVs was in agreement with previous reports, where pre-existing M28T/V variants were found among 6% of G1a patients and in 7% of G1b infected patients[3,28]. However, these prevalence rates are still lower compared to the population of HIV infected patients diagnosed with acute HCV infection (24%) or compared to a large European HCV database (28%)[36,37]. Variant L31M was previously reported to be even less prevalent, more particularly present in 1.2% of Japanese patients and 8% according to a study using public sequences[36,38]. The NS5A variant Y93H, related to increased therapy failure among individuals treated with NS5A inhibitor containing regimens, including asunaprevir and daclatasvir, or grazoprevir and elbasvir was not detected, which is consistent with a Dutch report presenting a low prevalence for this variant in patients recently infected with HCV[37], however in general higher for HCV1b infected patients [39].

Clustering of more than three sequences, supported by a high aLRT value and a short evolutionary distance, was commonly observed in the studied dataset, with five clusters among IDUs and their respective heterosexual partners, and four MSM-transmission clades identified. Only in one cluster, two cases with the history of IDU were linked to MSM risk behavior, indicating that in general HCV epidemics are restricted to particular transmission groups. Also, as G1a predominated among MSM, clustering was more common for this subtype. Additionally, in the analyzed cohort of MSM, no IDU was reported, however use of oral or intranasal chem-sex agents was common, in agreement with the reported broad use of recreational drugs in the MSM community, previously associated with HCV acquisition[40]. It should be also noted that other sexual practices, such as the use of sexual toys, anal douches or lubricants might additionally facilitate HCV transmission[40,41]. Of note, similarly to another European cohort, association between acute hepatitis C and prior diagnosis of syphilis among HIV-infected MSM was observed in our study[42].

Clustering among HCV sequences harboring the NS3 Q80K polymorphism was reported previously for various countries like the UK and the Netherlands[10,41]. It should be noted, that in our study one of the transmission clusters (cluster 2), consisted of eight G1a clade I sequences, all acquired from individuals with AHC and of which six sequences (75%) harbored the NS3 Q80K polymorphism. It may be hypothesized, that for the two remaining sequences reversion to the wild-type variant occurred, similarly as observed by Newsum *et al.*, where probable reversions of Q80K to the wild-type Q80Q were noted[10] among DAA unexposed cases. However, such reversions were impossible to confirm due to the lack of longitudinal sampling in our study. It should be noted that, sexual transmission events of RAVs, including Q80K, have been rarely reported for individual cases[15,11]. However, the founder effect that dominates the history of Q80K strains reflects the occurrence of many former transmission events, as all sequences characterized by polymorphism Q80K cluster together in a large clade[10,26]. Here, we show that transmission clusters, including the ones harboring Q80K, were not limited to sequences from patients located in a single city, but were in half of the cases collected in clinical centers distant from each other (e.g. >350 km distance between Warsaw and Wrocław and even >500 km between Warsaw and Szczecin), indicating countrywide span of the identified transmission networks. The key limitation of this study was the non-random sampling of the sequences, since they were selected based on sample availability, as well as on the patients' history of an acute or recently acquired HCV infection. Moreover, at the time of the study completion, no systematic sequencing of HCV was performed, resulting in a rather low number of viral sequences. It should be stated, though, that the increase in the number of acute HCV infections among MSM living with HIV is a recent phenomenon and so far, there is no systematic surveillance for DAA resistance in this group. Also, the use of deep sequencing methodologies might have allowed the identification of variants present at lower thresholds as well as to obtain longer fragments characterized by higher phylogenetic signal. However this technology was not available and moreover the clinical impact of minor variants on treatment response is still being debated[43], hence why our findings resulted from the use of Sanger population sequencing are still significant without using of next-generation sequencing.

Finally, it should be stressed that G1a, present in 40.2% of the samples and including 46.7% of the AHC cases, has only been identified infrequently in Poland. More particularly, G1a has been reported in only 2.5% of the HCV mono-infections and in 17.8% among the HIV/HCV G1 infected cases[20,44], and was virtually absent in a former dataset focusing on the global epidemiology of HCV genotype distribution, where only G1b infections were observed in the country. In Poland, G1a cases were previously observed among young individuals, however not linked to IDU transmission, in contrast to what has been reported for other regions. Current analysis indicates enrichment of G1a among MSM with possible expanding epidemics of these strains. In Western Europe, G1a was the most prevalent subtype among MSM enrolled in HIV pre-exposure prophylaxis programs, both in HIV-positive and –negative men[41].

Conclusion

The identification of transmission clusters among HIV/HCV infected patients, especially for the ones that recently acquired HCV through MSM contact, suggests that sexual transmission may not only fuel the HCV epidemic, but also promote the spread of DAA resistance-associated variants. Such a phenomenon is of primary importance for long persisting or highly prevalent variants such as polymorphism Q80K in the NS3 region as well as several NS5A variants. Efforts for continued surveillance of variants affecting susceptibility to DAAs should be extended, especially for MSM, as transmission of RAVs known to confer resistance to the currently used drug regimens, may adversely affect HCV treatment options in the future.

Acknowledgements

This work was partially funded by internal funds of Pomeranian Medical University (FSN 343-10/14), funds of Fundation Scio et Preaveceo, and partially by the Fonds Wetenschappelijk Onderzoek Vlaanderen (FWO – grants G0E8416N and G0B2317N). Parts of the data described in this manuscript, will be presented as an oral talk at the 16th European AIDS Conference (EACS) in Milan, Italy, October 26th 2017.

References

1. Hepatitis C virus prevalence and level of intervention required to achieve the WHO targets for elimination in the European Union by 2030: a modelling study. *Lancet Gastroenterol Hepatol.* 2017;2(5):325-336.
2. Simmonds P, Becher P, Bukh J, et al. ICTV Virus Taxonomy Profile: Flaviviridae. *J Gen Virol.* 2017;98(1):2-3.
3. Welzel T, Bhardwaj N, Hedskog C, et al. Global Epidemiology of HCV Subtypes and Resistance-associated Substitutions Evaluated by Sequencing-Based Subtype Analyses. *J Hepatol.* 2017;23(17):30148-30144.
4. Blach S, Zeuzem S, Manns M, et al. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *The Lancet Gastroenterology & Hepatology.* 2017;2(3):161-176.
5. EASL Recommendations on Treatment of Hepatitis C 2016. *J Hepatol.* 2017;66(1):153-194.
6. Nguyen LT, Gray E, Dean J, et al. Baseline prevalence and emergence of protease inhibitor resistance mutations following treatment in chronic HCV genotype-1-infected individuals. *Antivir Ther.* 2015;20(8):865-869.
7. Sarrazin C, Dvory-Sobol H, Svarovskaia ES, et al. Prevalence of Resistance-Associated Substitutions in HCV NS5A, NS5B, or NS3 and Outcomes of Treatment With Ledipasvir and Sofosbuvir. *Gastroenterology.* 2016;151(3):501-512.
8. Krishnan P, Tripathi R, Schnell G, et al. O057 : Long-term follow-up of treatment-emergent resistance-associated variants in NS3, NS5A and NS5B with paritaprevir/r-, ombitasvir- and dasabuvir-based regimens. *J Hepatol.* 2015;62:S220.
9. Yoshimi S, Imamura M, Murakami E, et al. Long term persistence of NS5A inhibitor-resistant hepatitis C virus in patients who failed daclatasvir and asunaprevir therapy. *J Med Virol.* 2015;87(11):1913-1920.
10. Newsum AM, Ho CK, Lieveld FI, et al. The hepatitis C virus nonstructural protein 3 Q80K polymorphism is frequently detected and transmitted among HIV-infected MSM in the Netherlands. *Aids.* 2017;31(1):105-112.
11. Franco S, Tural C, Nevot M, et al. Detection of a sexually transmitted hepatitis C virus protease inhibitor-resistance variant in a human immunodeficiency virus-infected homosexual man. *Gastroenterology.* 2014;147(3):599-601.
12. Hullegie SJ, van den Berk GE, Leyten EM, et al. Acute hepatitis C in the Netherlands: characteristics of the epidemic in 2014. *Clin Microbiol Infect.* 2016;22(2):17.
13. Boesecke C, Grint D, Soriano V, et al. Hepatitis C seroconversions in HIV infection across Europe: which regions and patient groups are affected? *Liver Int.* 2015;35(11):2384-2391.
14. Matthews GV, Pham ST, Hellard M, et al. Patterns and characteristics of hepatitis C transmission clusters among HIV-positive and HIV-negative individuals in the Australian trial in acute hepatitis C. *Clin Infect Dis.* Mar 15 2011;52(6):803-811.
15. Bartlett SR, Jacka B, Bull RA, et al. HIV infection and hepatitis C virus genotype 1a are associated with phylogenetic clustering among people with recently acquired hepatitis C virus infection. *Infect Genet Evol.* 2016;37:252-258.
16. Sexual transmission of hepatitis C virus among HIV-infected men who have sex with men--New York City, 2005-2010. *MMWR Morb Mortal Wkly Rep.* 2011;60(28):945-950.
17. Ingiliz P, Martin TC, Rodger A, et al. HCV reinfection incidence and spontaneous clearance rates in HIV-positive men who have sex with men in Western Europe. *J Hepatol.* 2017;66(2):282-287.
18. Walewska-Zielecka B, Religioni U, Juszczyk G, et al. Anti-hepatitis C virus seroprevalence in the working age population in Poland, 2004 to 2014. *Euro Surveill.* 2017;22(2):1560-7917.

19. Rosinska M, Janiec J, Niedzwiedzka-Stadnik M. Increase of new HIV diagnoses among men who have sex with men in Poland, 2000 to 2011. *Euro Surveill.* 2013;18(48):20642.

20. Leszczyszyn-Pynka M, Ciejak P, Maciejewska K, et al. Hepatitis C coinfection adversely affects the life expectancy of people living with HIV in northwestern Poland. *Archives of Medical Science.* 2016;12(1).

21. Zabek P, Opoka-Kegler J, Baka M, Dyda T, Stanczak GP, Stanczak JJ. Prevalence of hepatitis C virus mutants resistant to protease inhibitors among Polish HCV genotype 1-infected patients. *Przegl Epidemiol.* 2013;67(3):411-413.

22. Cielniak I, Siwak E, Firląg-Burkacka E, Weber-Kaniuk I, Święcki P, Horban A. Characteristics of new dynamic epidemic of Acute Hepatitis C among HIV infected individuals from Warsaw Outpatient Clinic *16th European AIDS Clinical Society Conference, Milan, 25-27 October 2017, abstract number PE16/11* 2017.

23. Walker A, Bergmann M, Camdereli J, Kaiser R, Lubke N, Timm J. A genotype independent, full-genome reverse-transcription protocol for HCV genotyping and resistance testing. *J Clin Virol.* 2017;91:42-48.

24. Jacka B, Applegate T, Krajden M, et al. Phylogenetic clustering of hepatitis C virus among people who inject drugs in Vancouver, Canada. *Hepatology.* 2014;60(5):1571-1580.

25. Cuypers L, Li G, Libin P, Piampongsant S, Vandamme AM, Theys K. Genetic Diversity and Selective Pressure in Hepatitis C Virus Genotypes 1-6: Significance for Direct-Acting Antiviral Treatment and Drug Resistance. *Viruses.* 2015;7(9):5018-5039.

26. Cuypers L, Vrancken B, Fabeni L, et al. Implications of hepatitis C virus subtype 1a migration patterns for virus genetic sequencing policies in Italy. *BMC Evol Biol.* 2017;17(1):017-0913.

27. D'Offizi G, Camma C, Taibi C, et al. Clinical and virological predictors of sustained response with an interferon-based simeprevir regimen for patients with chronic genotype 1 hepatitis C virus infection. *New Microbiol.* 2017;40(1):19-26.

28. Poveda E, Wyles DL, Mena A, Pedreira JD, Castro-Iglesias A, Cachay E. Update on hepatitis C virus resistance to direct-acting antiviral agents. *Antiviral Res.* 2014;108:181-191.

29. Jensen SB, Serre SB, Humes DG, et al. Substitutions at NS3 Residue 155, 156, or 168 of Hepatitis C Virus Genotypes 2 to 6 Induce Complex Patterns of Protease Inhibitor Resistance. *Antimicrob Agents Chemother.* 2015;59(12):7426-7436.

30. Berger KL, Scherer J, Ranga M, et al. Baseline Polymorphisms and Emergence of Drug Resistance in the NS3/4A Protease of Hepatitis C Virus Genotype 1 following Treatment with Faldaprevir and Pegylated Interferon Alpha 2a/Ribavirin in Phase 2 and Phase 3 Studies. *Antimicrob Agents Chemother.* 2015;59(10):6017-6025.

31. Cao Y, Bao Y, Xia W, et al. Resistance-associated mutations to HCV protease inhibitors naturally pre-existed in HIV/HCV coinfected, treatment-naive patients. *Clin Res Hepatol Gastroenterol.* 2016;40(5):597-604.

32. Liu R, Curry S, McMonagle P, et al. Susceptibilities of genotype 1a, 1b, and 3 hepatitis C virus variants to the NS5A inhibitor elbasvir. *Antimicrob Agents Chemother.* 2015;59(11):6922-6929.

33. Krishnan P, Beyer J, Mistry N, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. *Antimicrob Agents Chemother.* 2015;59(2):979-987.

34. Sulkowski M, Hezode C, Gerstoft J, et al. Efficacy and safety of 8 weeks versus 12 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin in patients with hepatitis C virus genotype 1 mono-infection and HIV/hepatitis C virus co-infection (C-WORTHY): a randomised, open-label phase 2 trial. *Lancet.* 2015;385(9973):1087-1097.

35. Kosaka K, Imamura M, Hayes CN, et al. Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1. *J Viral Hepat.* 2015;22(2):158-165.
36. Patino-Galindo JA, Salvatierra K, Gonzalez-Candelas F, Lopez-Labrador FX. Comprehensive screening for naturally-occurring Hepatitis C virus resistance to direct-acting antivirals in the NS3, NS5A and NS5B genes in worldwide isolates from viral genotypes 1-6. *Antimicrob Agents Chemother.* 2016;8:02776-02715.
37. Christiansen MT, Hullegie SJ, Schutten M, et al. Use of whole genome sequencing in the Dutch Acute HCV in HIV study: focus on transmitted antiviral resistance. *Clin Microbiol Infect.* 2017;23(2):28.
38. Iio E, Shimada N, Abe H, et al. Efficacy of daclatasvir/asunaprevir according to resistance-associated variants in chronic hepatitis C with genotype 1. *J Gastroenterol.* 2017;52(1):94-103.
39. Cuypers L, Li G, Neumann-Haefelin C, et al. Mapping the genomic diversity of HCV subtypes 1a and 1b: Implications of structural and immunological constraints for vaccine and drug development. *Virus Evol.* 2016;2(2).
40. Ireland G, Higgins S, Goorney B, et al. Evaluation of hepatitis C testing in men who have sex with men, and associated risk behaviours, in Manchester, UK. *Sex Transm Infect.* 2017;27(052876):2016-052876.
41. Hoornenborg E, Achterbergh RCA, Schim Van Der Loeff MF, et al. Men who have sex with men starting pre-exposure prophylaxis (PrEP) are at risk of HCV infection: evidence from the Amsterdam PrEP study. *Aids.* 2017;1(10):0000000000001522.
42. Sanchez C, Plaza Z, Vispo E, et al. Scaling up epidemics of acute hepatitis C and syphilis in HIV-infected men who have sex with men in Spain. *Liver Int.* Oct 2013;33(9):1357-1362.
43. McPhee F, Hernandez D, Zhou N. Effect of minor populations of NS5A and NS5B resistance-associated variants on HCV genotype-3 response to daclatasvir plus sofosbuvir, with or without ribavirin. *Antivir Ther.* 2016;23(10).
44. Panasiuk A, Flisiak R, Mozer-Lisewska I, et al. Distribution of HCV genotypes in Poland. *Przegl Epidemiol.* 2013;67(1):11-16.

Figure captions

Figure 1. Total resistance-associated variants (RAVs) frequencies shown per HCV subtype (a), for the HCV NS3 region (b) and NS5A region (c).

Resistance-associated variants are listed separately for NS3, NS5A as well as for the sequences used to construct the concatenated NS3/NS5A alignments. Sample size/the total number of sequences included in each category and subtype is indicated on the left. For G1a, the observed prevalence of RAV is indicated in light blue, for G1b in red, and for the total of both subtypes in dark blue.

** Frequencies calculated only for the paired NS3/NS5A sequences (n=40 for G1A, n=58 for G1B)*

Figure 2. Maximum likelihood trees for joint NS3/NS5A sequences for G1a (a) and G1b (b).

For the reconstruction of the trees, highly similar sequences to the 98 Polish sequences were selected by BLAST and added to the concatenated NS3/NS5A alignment. Black tips represent sequences from the public database Genbank. Polish patient sequences from the study were colored by the probable route of infection (as self-reported by the patient): blue - men who have sex with men, red - injection drug users (IDUs), green - heterosexual partners of IDUs. Identified clusters are shaded in gray, with the indicated cluster ID corresponding to those listed in Table 2. Observed NS3 and/or NS5A resistance-associated variants are annotated at the tips of the tree. Clusters were identified with a support of aLRT values >0.85, and a maximum genetic distance of 0.08. Reference sequences to root the phylogenetic tree (G1a: HQ850279 and G1b: D90208) were removed from the final visualization. The genetic distance is indicated by the bar at the bottom. Maps on the right indicate the Polish city where patient follow-up took place, with putative reconstructed intercity transmission networks. City codes: SZ-Szczecin, WA-Warsaw, WR-Wrocław, ZG-Zielona Góra, KR-Kraków.

Table 1. Clinical and laboratory characteristics listed by HCV genotype (G1a and G1b).

This table includes demographic data, information about the self-reported HCV transmission route, history of acute hepatitis C or syphilis during follow-up for HIV, as well as a selection of laboratory and serological parameters. P-values with statistical significance (≤0.05) are indicated in bold.

Abbreviations: ALT- alanine aminotransferase, HBsAg – hepatitis B surface antigen, HBV – hepatitis B virus, HCV- hepatitis C virus, HIV – human immunodeficiency virus, IDU – intravenous drug use, IQR - interquartile range, MSM – men-who-have-sex-with-men.

	Genotype 1a, n (%)	***Genotype 1b, n (%)***	***P value***	***Total, n (%)***
Age at HCV diagnosis*	37 (34-41)	37 (32-41)	0.65	37 (33-41)
Gender*				
Male	41 (91.11)	51 (76.12)	**0.042**	92 (82.14)
Female	4 (8.89)	16 (23.88)		20 (17.86)
Reported HCV infection route*				
Intravenous drug use (IDU)	18 (40)	41 (61.19)	**0.004**	59 (52.68)
Heterosexual partners of IDUs	0	5 (7.46)		5 (4.46)
men-who-have-sex-with-men *(MSM)*	27 (60)	21 (31.34)		48 (42.86)
Observed acute hepatitis C*				
Yes	21 (46.67)	17 (25.37)	**0.019**	38 (33.93%)
No	24 (32.43)	50 (67.57)		74 (66.07%)
History of syphilis during follow-up*				
Yes	18 (52.24)	14 (25.45)	**0.009**	32 (35.96)
No	16 (28.07)	41 (71.93)		57 (64.04)
Active HBV coinfection (HbsAg positive) *				
Yes	1 (2.63)	3 (5.26)	0.53	91 (95.79)
No	37 (97.37)	54 (94.74)		4 (4.21)
HIV-1 viral load at diagnosis, log	5.04 (4.46-	4.64 (4.34-5.23)	0.2	4.79 (4.35-

copies/ml (IQR) *	5.48)			5.35)
Nadir lymphocyte CD4 count, cells/ul, median (IQR) *	302 (161-386)	224 (103-353)	0.18	255 (104-371)
Median ALT (IQR) at HCV diagnosis, IU/ml	65 (43-163)	52 (25-78)	**0.03**	72 (47-135)
Median HCV-RNA, log IU/ml (IQR)	5.95 (5.55-6.32)	5.9 (5.41-6.38)	0.62	5.93 (5.45-6.36)

[*] *Age, gender, transmission route and history of acute hepatitis C are available for 112 patients, history of syphilis is available for 89 (79.46%) cases, HBV history for 95 (84.82) cases, HIV viral load for 72 (64.28%) cases, and lymphocyte $CD4^+$ T cell counts for 88 (78.57%) patients.*

Table 2. Characteristics of the identified transmission clusters.

Clusters are consecutively numbered, consistent with the numbering used in Figure 2, followed by the number of sequences included in the cluster. For G1a, the clade (I or II) is added. Observed percentages of resistance associated variants (RAVs) (both for NS3 and NS5A) are calculated for the total number of sequences included in each cluster. The HCV transmission route mentioned in the table is self-reported by the patient. City of diagnosis refers to the clinical center where the HIV/HCV coinfection was first diagnosed. Evolutionary distances (number of nucleotide substitutions per site per year) and statistical support for the clusters are calculated based on the maximum likelihood model, and listed in the last two columns of the table.

Abbreviations: aLRT - approximate likelihood ratio test. *[#]IDU - injection drug use associated transmissions, MSM - men who have sex with men, HET - heterosexual partners of IDU. *City codes: SZ-Szczecin, WA-Warsaw, WR-Wrocław, ZG-Zielona Góra, KR-Kraków.*

Cluster ID	Cluster size, number of sequences	HCV subtype/clade	Observed NS3 RAVs, (%)	Observed NS5 RAVs, (%)	Transmission route[#] (%)	City of diagnosis[*] (%)	Documented acute hepatitis C, (%)	Evolutionary distance within the cluster	Cluster aLRT support
1	10	1a/I	0	28T (10%)	IDU (100%)	SZ (90%) WR (10%)	0%	0.062	0.998
2	8	1a/I	Q80K (75%)	0	MSM (100%)	WA (75%) WR (25%)	100%	0.137[±]	0.932
3	4	1a/II	0	0	MSM (100%)	WA (100%)	100%	0.042	0.87
4	6	1a/II	0	0	MSM (100%)	WA (16.6%)	83.4%	0.042	0.98

						SZ (83.4%)			
5	4	1b	0	0	IDU (75%), HET (25%)	SZ (100%)	0%	0.056	0.865
6	8	1b	0	0	IDU (100%)	SZ (100%)	0%	0.077	0.986
7	14	1b	0	0	MSM (100%)	KR (85.7%) WA (14.3%) SZ (7.1%) WR (7.1%)	100%	0.076	1
8	3	1b	0	0	IDU (66.6%) MSM (33.3%)	SZ (100%)	0%	0.064	0.976
9	4	1b	0	0	IDU (75%) HET (25%)	SZ (100%)	0%	0.58	0.995
10	5	1b	0	0	IDU (80%), HET (20%)	SZ (80%) WR (20%)	0	0.041	0.988

[±] *For this cluster, three smaller clusters with evolutionary distances of 0.02, 0.067 and 0.028 were joint as all patients were epidemiologically linked, therefore the reported overall evolutionary distance is higher than the threshold of 0.85 mentioned in the Methods section.*