# HCV genotype distribution in Flanders and Brussels (Belgium): unravelling the spread of an uncommon HCV genotype 5a cluster

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JANNICK VERBEECK[1], LEEN KWANTEN[1], FRANÇOIS D'HEYGERE[2], ARNE BEGUIN[1], STIJN MICHIELS[1], ISABELLE DESOMBERE[3], GEERT LEROUX-ROELS[3], PHILIPPE LEMEY[1,4], FREDERIK NEVENS[5], AND MARC VAN RANST[1*]

*[1]Laboratory of Clinical Virology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium; [2]Department of Gastroenterology, C.A.Z. Groeninge Kortrijk, Belgium; [3]Center for Vaccinology, Ghent University and Hospital, Ghent, Belgium; [4]Department of Zoology, University of Oxford, Oxford, United Kingdom; [5]Division of Hepatology, University Hospital Gasthuisberg, Leuven, Belgium;*

*Corresponding author. Mailing address: Laboratory of Clinical Virology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, BE-3000 Leuven, Belgium. Phone: +32-16-347908. Fax: +32-16-347900. Email: vanranstmarc@gmail.com

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## ABSTRACT

**Purpose:**

**In order to study the hepatitis C virus (HCV) epidemiology in Flanders, Belgium, the HCV genotype of 2301 patients diagnosed with HCV between 2001 and 2009 was determined.**

**Methods:**

**HCV genotyping was conducted using the VERSANT LiPA 1.0 or VERSANT LiPA 2.0 assay. To explore the transmission history of a remarkable cluster of the rarely found HCV genotype 5a, face-to-face interviews based on detailed questionnaires and maximum likelihood phylogenetic analysis were performed.**

**Results:**

**HCV genotype 1 was the most prevalent genotype in all provinces, followed by HCV genotype 3 in East-Flanders, Antwerp, Flemish-Brabant and Limburg. In Brussels, HCV genotype 4 was the second most prevalent genotype. This observation is due to the immigration of patients from the Middle East and Africa. Remarkably, a cluster of HCV genotype 5a was found in West-Flanders, where it represents the second most prevalent genotype accounting for 26.2% of HCV infections. We could not identify one major transmission source explaining the whole HCV genotype 5a epidemic. Instead, several smaller possible transmission chains were identified and confirmed phylogenetically.**

**Conclusion:**

**Overall, the HCV genotype 5a epidemic in West-Flanders seems to be mainly associated with blood transfusion and unsafe medical practices.**

Keywords: Belgium, epidemiology, HCV genotype 5a, HCV genotype distribution, hepatitis C virus, phylogeny

## INTRODUCTION

The hepatitis C virus (HCV) is a leading cause of chronic liver disease and confronts many regions of the world with a severe disease burden. Worldwide, more than 170 million people are infected with this virus [1]. Since its discovery in 1989, there is a growing interest in different aspects of HCV research, including HCV epidemiology, evolution, host interactions and antiviral defence mechanisms. Despite considerable research efforts, several aspects are still poorly understood.

Phylogenetic analyses have revealed that HCV can be classified in 6 different genotypes and multiple subtypes [2]. These six genotypes have different geographical distributions [3]. HCV genotypes 1a, 1b and 3a are highly prevalent "epidemic" strains that are found globally. These strains spread swiftly around the world during the twentieth century, most likely through infected blood, blood products and injecting drug use, and have relatively low levels of genetic variation. In contrast, other HCV strains are highly divergent but are found in restricted geographic areas. These "endemic" strains reflect long-term transmission at low levels in particular regions. Genotypes 1, 2 and 4 appear to be endemic to regions of West and Central Africa and the Middle East, whereas divergent endemic strains of genotypes 3 and 6 strains are found in South-East Asia [4]. HCV genotype 5a infections are mainly found in the northern part of South Africa, where 40% of the HCV patients carry this genotype [5]. In other regions of the world, HCV genotype 5a infections are scarce, and therefore, little is known about the geographical distribution, evolution and treatment response of this uncommon genotype.

In Belgium, HCV prevalence has been estimated at about 0.8%, representing about 80.000 patients [6, 7]. A study of the epidemiological profile of HCV patients in Belgium conducted between 1992 and 2002 showed that HCV genotype 1 is the most prevalent genotype, followed by HCV genotype 3. HCV genotype 2 and HCV genotype 4 are roughly equally

distributed, and HCV genotypes 5 and 6 are only encountered sporadically [8]. In 2006 however, a remarkable cluster of at least 100 HCV genotype 5a infected patients was identified in the West-Flanders province of Belgium [9].

In this paper, we describe the general epidemiological profile of HCV genotypes in Flanders, Belgium for the period between 2001 and 2009. Next, we focus on the uncommon HCV genotype 5a cluster in West-Flanders. In order to find out how HCV genotype 5a was introduced in the population and to study its local spread, we conducted face-to-face interviews with the infected patients using a detailed questionnaire to learn more about the medical history and the HCV infection of each individual patient. We performed phylogenetic analyses to confirm the links between the patients that were found during thorough investigation of the questionnaires.

## MATERIALS AND METHODS

### Study population

Between January 2001 and August 2009, blood samples of 2301 new patients with chronic hepatitis C were received for HCV genotyping at the University Hospital of Leuven. Only HCV carriers presenting for the first time were included in this study. The study population consists of blood samples from patients attending the medical consultation at the hospital and blood samples from outpatients from other hospitals in Flanders that do not have their own HCV genotyping facilities. Patients with anti-HCV antibodies who are HCV RNA negative are not included. The selected study population is a representative sample of the total HCV population in Flanders since patients from all provinces are included.

Genotyping was performed using the INNO LiPA 1.0 or the INNO LiPA 2.0 (developed by Innogenetics Inc., Ghent, Belgium and distributed by Siemens Healthcare Diagnostics, IL, USA) according to the manufacturer's instructions. Demographic and geographic information (age, gender, city of residence) were collected for all patients.

Patients infected with HCV genotype 5a were invited to participate in our study. In total, 67 patients were willing to participate. Informed consent was obtained from all patients. Face-to-face interviews based on a detailed questionnaire were conducted for all 67 patients in order to explore possible transmission routes. The questionnaire investigated HCV diagnosis, HCV treatment outcome, HCV family history, hospitalisation history, general medical care, blood transfusion history, contact with blood products, blood donations, military history, tattooing, piercing, acupuncture, IDU, visits to general doctors, visits to dentists, travelling to foreign countries, and possible links with South Africa. For the female patients, additional questions concerning pregnancy and childbirth were included. The study was approved by the ethical committee of the University Hospital Leuven.

**Phylogenetic analysis**

In order to reconstruct the phylogenetic relationships between the HCV genotype 5a patients, 98 serum samples were collected from 61 different patients. Viral RNA was isolated from 140 µl serum using the QIAamp Viral RNA Mini kit (Qiagen Benelux B.V., Venlo, the Netherlands) according to the manufacturer's instructions, and eluted in 60 µl elution buffer. One-step reverse transcription PCRs were carried out using the OneStep RT-PCR kit (Qiagen) to amplify a 584 bp fragment from the highly variable E1-E2 region (genome location: nt 554 to nt 1138 according to the numbering of the H77 strain) and a 573 bp fragment from the more conserved NS3-NS4 region (genome location: nt 5058 to nt 5631 according to the numbering of the H77 strain) as described previously [9]. Purified DNA fragments were directly sequenced on both strands using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems).

Multiple nucleotide sequence alignments were created for the E1-E2 region, NS3-NS4 region, and the concatenated E1-NS4 region using the Clustal X program version 1.83 [10]. Alignments were adjusted by hand. For the E1-E2 region, the hypervariable region was excluded from the analysis, since it is impossible to unambiguously align this region. Molecular phylogenies were estimated for each alignment using a maximum-likelihood (ML) approach implemented in PAUP* v4.0b [11]. Model selection for each sequence alignment was performed with the Modeltest 3.7 software [12] under the Aikake Information Criterion. For the E1-E2 region and the concatenated region, the general time reversible model with gamma distributed rate heterogeneity among sites and invariable sites (GTR+I+G) was selected. For the NS3-NS4 region, the transversional model with gamma distributed rate heterogeneity among sites and invariable sites (TVM+I+G) had the smallest AIC score. Substitution model parameters were estimated on a initial neighbor-joining tree and were fixed throughout heuristic search optimization during which the NNI branch-swapping

algorithm was used. Bootstrap analysis was performed using 500 replicates to assess the statistical robustness of the estimated phylogenies.

**Statistical analysis**

Statistical analysis was performed using SPSS 11.5 (SPSS Inc., Chicago, USA). P-values $\leq 0.05$ were considered statistically significant. The Chi-Square test was used for the detection of significant differences between dichotomous variables. For continuous variables, the Mann-Whitney test was used.

## RESULTS

### General HCV genotype distribution in Flanders, Belgium

In this study, the HCV genotype of 2301 HCV positive patients was determined. HCV genotype 1 was the most prevalent genotype and was found in 1401 (60.9%) of patients. HCV genotype 3 was the second most prevalent genotype with 466 (20.3%) infected patients, followed by HCV genotype 4 (184 patients, 8.0%), HCV genotype 2 (145 patients, 6.3%) and HCV genotype 5 (104 patients, 4.5%). Only one patient was infected with HCV genotype 6. No mixed infections with different genotypes were detected. HCV subtype information was obtained for 80.4% of samples (11.2% 1a, 42.6% 1b, 0.7% 1a/1b, 3.6% 2a/2c, 1.0% 2b, 18.1% 3a, 0.1% 3b, 0.3% 4a/4c/4d, 2.4% 4c/4d, 0.2% 4f and 0.2% 4h).

In total, 1392 (60.5%) patients were male, and 909 (39.5%) were female. For HCV genotypes 1 and 3, significantly more men were infected than women (Pearson Chi-Square tests, $P<0.001$). For the other HCV genotypes, no significant differences according to gender could be found. The mean age ± SD of the total study population was 51.5 ± 15.6 years (range: 5-94). Patients infected with HCV genotype 3 were significantly younger than patients infected with one of the other genotypes (42.4 ± 10.3 years; range: 5-84; Mann-Whitney test, $P<0.001$).

### Description of HCV genotype 5a cluster in West-Flanders, Belgium

Figure 1 displays the HCV genotype distribution for the northern part of Belgium (Flanders and Brussels). For the southern part of Belgium only 37 samples were collected in the different provinces. Since this number is too low to represent the general genotype distribution in the southern part of Belgium, these data were deleted from the dataset for further analyses.

In each province, HCV genotype 1 is the most prevalent. In Antwerp, Limburg, East-Flanders and Flemish-Brabant, HCV genotype 1 is followed by HCV genotypes 3, 4, 2 and 5a respectively. In Brussels, HCV genotype 4 is more prevalent than HCV genotype 3. Remarkably, HCV genotype 5a is a minor genotype in all provinces, except for West-Flanders, were it is the second most prevalent genotype. In this province, more than a quarter of the patients (26.2%) is infected with the uncommon HCV genotype 5a. A Pearson Chi-Square test indicated that the number of HCV genotype 5a patients is significantly higher compared to the number of HCV genotype 5a patients in other provinces ($P<0.001$). The HCV genotype 5a patients are significantly older (mean age 62 ± 14.7; range: 13 - 88; Mann-Whitney test: $P<0.001$).

The data on the treatment response of the HCV genotype 5a patients are not based on the official medical records, but on the information given by the patients during the interview. Therefore, we do not have the exact information on the dosage for each patient. In total, 49 patients received antiviral therapy. Of these, 59.2% (29/49) showed sustained virological response (SVR) after completion of therapy, 14.3% (7/49) were non-responders, and 26.5% (13/49) relapsed after therapy.

**Epidemiological investigation of the HCV genotype 5a cluster in Flanders**

In total, 104 patients were infected with HCV genotype 5a. Of these, 67 patients were willing to be interviewed at their home using a standardized questionnaire to identify potential risk factors. All 67 patients were born and are living in Belgium. Forty-four patients (65.5%) are living in West-Flanders, 4 (6.0%) in East-Flanders, 5 (7.5%) in Flemish-Brabant, 6 (9.0%) in Antwerp, 6 (9.0%) in Limburg, and 2 (3.0%) in Brussels.

Among the HCV genotype 5a patients, 49 (73.1%) received blood transfusion and 24 (35.8%) patients were blood donors before they were diagnosed with HCV. The questionnaire

contained several questions that addressed the possibility of transmission via needles or syringes. None of the patients reported a history of IDU. Thirteen patients underwent acupuncture treatment, most of them at the practice of the family doctor. Two patients have a tattoo, and 22 patients have one or more piercings. Seventeen patients received multiple injections from multi-dose vials, mainly for pain relief. Two patients reported that they received several injections during their military service, and that hygienic procedures were not followed correctly. Vertical transmission does not seem to occur often, only two "mother-child" couples were identified. The same holds for horizontal or familial transmission. We identified only one "husband-wife" couple, one "father-son" couple and two sibling couples. The patients were asked to give an overview of the family doctors and dentists that they visited in the past. The names of three different family doctors appeared on top of the list. These family doctors were mainly medically active between 1960 and 1980. Two of them are also infected with HCV genotype 5a, and performed several deliveries. Patient-to-doctor or doctor-to-patient transmission might have occurred since hygienic procedures were less strict prior to the identification of HCV in 1989. No obvious links between HCV infection and dentistry history were identified. Patients were also requested to provide us with an overview of the surgical procedures they underwent and details on when and where these were performed. Surgeries were executed in several hospitals, at different wards, and in a period spanning more than 50 years. Therefore, it is very difficult to make any links between HCV transmission and surgical history.

Since HCV genotype 5a is most frequently found in South Africa, we asked the patients if they had ever visited that country and whether they had any direct contact with blood or blood products during their stay in South Africa. Eight patients visited South Africa but none of them had contact with blood products, which excludes the possibility that one of the patients was infected in South Africa through blood products or surgery. Five patients had travelled to

Congo. Two of them were active in a medical setting and performed surgical procedures during their stay. Two other patients were there on holiday and reported no contact with blood products. One patient was active in a military setting in Congo, and had a knee surgery during his stay between 1959 and 1961.

**Phylogenetic reconstruction**

Serum samples from 61 HCV genotype 5a infected patients were collected for phylogenetic analysis. Thirty-one of these patients completed the questionnaire, enabling us to investigate possible transmission routes in detail for this subset of patients. Figure 2 shows the phylogenetic tree for the concatenated dataset. Only one viral strain per patient was included for clarity purpose. Each patient is identified by a unique identification number. Numbers in bold represent patients of whom both a questionnaire and a serum sample were available. In general, the phylogenetic signal of the dataset is rather low, since the main branches of the tree are not supported by high bootstrap values. However, some smaller clusters with higher bootstrap values can be recognized. Remarkably, for most of these clusters, possible common infection sources were identified during detailed inspection of the questionnaires. The strains indicated in green originate from patients that visited the same family doctor for several years. The doctor himself is also infected with HCV genotype 5a, but unfortunately, we were not able to collect a serum sample. This sub-cluster (indicated by the dashed rectangle) harbours some additional strains from patients that live in the same rural area. We do not have a questionnaire from these patients and therefore can not confirm whether these patients also visited this family doctor. The strains indicated in orange correspond to a “mother-son” couple where vertical transmission occurred. The two patients who received multiple injections during their military service at the same military base, cluster together with a bootstrap value of 99 (strains indicated in blue). Three patients that do not live in West-

Flanders also cluster with a high bootstrap value. The corresponding strains are indicated in red. They all received blood transfusion in the same year and in the same geographical region. One of the patients was also a blood donor in this geographical region before he/she was diagnosed with HCV, and delivered multiple blood donations during almost 10 consecutive years. The strains shown in yellow are also associated with blood transfusion procedures. Two of the three patients (BE03048920 and BE04458951) were blood donors in the same geographic region and in the same year as the third patient (BE02218930) received a blood transfusion.

The small clusters that can be observed for the concatenated gene region, were also present in the phylogenetic trees of the separate E1-E2 region and NS3-NS4 region (data available on request).

## DISCUSSION

This study aimed at evaluating the HCV genotype distribution in Flanders and Brussels (Belgium) and at investigating the mechanisms underlying the occurrence and spread of an uncommon HCV genotype 5a cluster which was described in a previous study [9].

The global genotype distribution in our patient group indicates that HCV genotype 1 is dominant, followed by HCV genotype 3, HCV genotype 4, HCV genotype 2, HCV genotype 5 and HCV genotype 6. This distribution pattern largely corresponds to the results obtained during a previous survey performed in drug users from Belgium in 2005 [13]. Since the study population in this study was limited to drug users, the reported prevalence of HCV genotype 3 was higher than in our study. Compared to the results reported by Gérard and colleagues [8], the proportion of HCV genotype 3 patients in Belgium seems to be growing over the last decade. This phenomenon is also observed in other European countries [14-17] and is probably due to the increase of injecting drug use (IDU) contamination, which has become the most common way of new HCV transmission since blood screening became available in 1991 [18]. Patients infected with HCV genotype 3 were significantly younger than patients infected with one of the other HCV genotypes in our dataset. This also fits in the IDU scenario. Since HCV genotype 3 has recently been associated with accelerated fibrosis progression [19], the growing share of this genotype may have important consequences for the management of patients infected with this genotype.

In the Brussels capital region, HCV genotype 4 is the second most prevalent genotype. This HCV genotype is most common in the Middle East and in Northern and Central Africa [20]. Egypt has the highest HCV prevalence worldwide, and more than 90% of the patients are infected with HCV genotype 4 [21]. Recent studies indicate that HCV genotype 4 prevalence has increased in European countries due to the immigration of HCV genotype 4-infected patients [22-27]. A study on the emergence of HCV genotype 4 in The Netherlands revealed

three epidemiological profiles, one associated with immigration from the Middle East and Africa, a second profile linked to injecting drug use, and a third one consisting of men who have sex with men (MSM) that are co-infected with HIV [28]. Brussels is a geographical region with a relatively high immigration rate, which could be a possible explanation for the rise in HCV genotype 4 prevalence in this region. Moreover, HCV genotype 4 is increasing in the IDU population in Belgium (personnel communication, Frederik Nevens).

Another remarkable finding is the fact that HCV genotype 5a is the second most prevalent genotype in West-Flanders, whereas it is only found sporadically in other Flemish provinces. In order to study the epidemiology of HCV genotype 5a in Flanders, a thorough investigation of possible transmission routes and risk factors was conducted using a detailed questionnaire. For the patients infected with non-genotype 5a strains, epidemiological information is lacking. Therefore, we are unable to discuss risk factors associated with transmission for these HCV genotypes, and we can not compare the HCV genotype distribution in different risk groups which is a drawback of our study. A survey in HCV patients using a simple questionnaire during the consultations as suggested by De Maeght and colleagues [29] can provide information on the risk factors and transmission profiles of HCV in Belgium.

The main transmission route for this HCV genotype seems to be blood transfusion, as 73.1% of the HCV genotype 5a patients received one or more transfusions before they were diagnosed with HCV. Moreover, 24 patients (35.8%) were blood donors before they were diagnosed with HCV, and they may have unconsciously contributed to the spread of the HCV genotype 5a virus. In our previous study addressing the origin and spread of HCV genotype 5a, we hypothesized that transmission of HCV genotype 5a in Belgium was mainly associated with contaminated blood products, and that HCV has been circulating in Belgium since mid 1800 [9]. A study on the epidemiology of HCV genotype 5a in central France suggests that this uncommon genotype was transmitted by iatrogenic route before 1972 in this region, and

later on via transfusion to the whole district [30]. In Syria, transmission via blood transfusion, IDU and tattooing was identified in 30% of the HCV genotype 5a patients, while no obvious transmission mechanism could explain the infection in the remaining portion of patients [31]. In southeast Spain, the origin of infection was unknown for 80% of the patients, 16% acquired infection by blood transfusion and 4% of patients were IDUs [32].

In contrast to HCV genotypes 1, 3 and 4 for which epidemiological data are well established, the epidemic spread of HCV genotype 5a remains unknown. Since HCV genotype 5a is the predominant genotype in South Africa [5], it may well be that this genotype originated in that part of the world and was subsequently introduced in Belgium. However, our study reveals no direct epidemiological links with South Africa. Although HCV genotype 5a is not commonly found in Congo, we hypothesized in a previous study that this region could harbour the original source of this genotype [9]. One general surgeon and one urologist were professionally active in Congo, so it is possible that they had contact with HCV infected patients during their stay.

Since HCV genotype 5a infections are rare, they theoretically represent an excellent marker to follow the epidemiological spread. Tracing the predominant transmission routes mainly depends on information obtained from the patients themselves, and is therefore subjected to the obvious disadvantages of this kind of studies, such as recall bias. However the results of our phylogenetic analysis confirm some findings of the face-to-face interviews. Based on the combined results of both the epidemiological and phylogenetic study, small and localized clusters of transmission have been identified. When, where and how HCV genotype 5a originated and started spreading in the human population, still remains an unsolved issue. Our results suggest that several independent transmission events have led to the local spread of this genotype, and that transfusion and unsafe medical practices are the main transmission routes for HCV genotype 5a in Flanders, Belgium.

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## References


[1] WHO (1999) Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. Journal of viral hepatitis 6 (1):35-47

[2] Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A (2005) Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42 (4):962-973

[3] Cristina J (2005) Genetic diversity and evolution of hepatitis C virus in the Latin American region. J Clin Virol 34 Suppl 2:S1-7

[4] Pawlotsky JM (2003) Hepatitis C virus genetic variability: pathogenic and clinical implications. Clinics in liver disease 7 (1):45-66

[5] Chamberlain RW, Adams NJ, Taylor LA, Simmonds P, Elliott RM (1997) The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. Biochemical and biophysical research communications 236 (1):44-49

[6] Beutels M, Van Damme P, Aelvoet W, Desmyter J, Dondeyne F, Goilav C, Mak R, Muylle L, Pierard D, Stroobant A, Van Loock F, Waumans P, Vranckx R (1997) Prevalence of hepatitis A, B and C in the Flemish population. European journal of epidemiology 13 (3):275-280

[7] Van Damme P, Thyssen A, Van Loock F (2002) Epidemiology of hepatitis C in Belgium: present and future. Acta Gastroenterol Belg 65 (2):78-79

[8] Gerard C, Delwaide J, Vaira D, Bastens B, Servais B, Wain E, Bataille C, Daenen G, Belaiche J (2005) Evolution over a 10 year period of the epidemiological profile of 1,726 newly diagnosed HCV patients in Belgium. Journal of medical virology 76 (4):503-510

[9] Verbeeck J, Maes P, Lemey P, Pybus OG, Wollants E, Song E, Nevens F, Fevery J, Delport W, Van der Merwe S, Van Ranst M (2006) Investigating the origin and spread of hepatitis C virus genotype 5a. Journal of virology 80 (9):4220-4226

[10] Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73 (1):237-244

[11] Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, MA, USA

[12] Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14 (9):817-818

[13] Mathei C, Wollants E, Verbeeck J, Van Ranst M, Robaeys G, Van Damme P, Buntinx F (2005) Molecular epidemiology of hepatitis C among drug users in Flanders, Belgium: association of genotype with clinical parameters and with sex- and drug-related risk behaviours. Eur J Clin Microbiol Infect Dis 24 (8):514-522

[14] Roman F, Hawotte K, Struck D, Ternes AM, Servais JY, Arendt V, Hoffman P, Hemmer R, Staub T, Seguin-Devaux C, Schmit JC (2008) Hepatitis C virus genotypes distribution and transmission risk factors in Luxembourg from 1991 to 2006. World J Gastroenterol 14 (8):1237-1243

[15] Chlabicz S, Flisiak R, Kowalczuk O, Grzeszczuk A, Pytel-Krolczuk B, Prokopowicz D, Chyczewski L (2008) Changing HCV genotypes distribution in Poland--relation to source and time of infection. J Clin Virol 42 (2):156-159

[16] Bourliere M, Barberin JM, Rotily M, Guagliardo V, Portal I, Lecomte L, Benali S, Boustiere C, Perrier H, Jullien M, Lambot G, Loyer R, LeBars O, Daniel R, Khiri H, Halfon P (2002) Epidemiological changes in hepatitis C virus genotypes in France: evidence in intravenous drug users. Journal of viral hepatitis 9 (1):62-70

[17] Christofidou M, Lambropoulou-Karatza C, Dimitracopoulos G, Spiliopoulou I (2000) Distribution of hepatitis C virus genotypes in viremic patients with beta-thalassemia. Eur J Clin Microbiol Infect Dis 19 (9):728-730

[18] Kleter B, Brouwer JT, Nevens F, van Doorn LJ, Elewaut A, Versieck J, Michielsen PP, Hautekeete ML, Chamuleau RA, Brenard R, Bourgeois N, Adler M, Quint WG, Bronkhorst CM, Heijtink RA, Hop WJ, Fevery J, Schalm SW (1998) Hepatitis C virus genotypes: epidemiological and clinical associations. Benelux Study Group on Treatment of Chronic Hepatitis C. Liver 18 (1):32-38

[19] Bochud PY, Cai T, Overbeck K, Bochud M, Dufour JF, Mullhaupt B, Borovicka J, Heim M, Moradpour D, Cerny A, Malinverni R, Francioli P, Negro F (2009) Genotype 3 is associated with accelerated fibrosis progression in chronic hepatitis C. Journal of hepatology 51 (4):655-666

[20] Smith DB, Pathirana S, Davidson F, Lawlor E, Power J, Yap PL, Simmonds P (1997) The origin of hepatitis C virus genotypes. The Journal of general virology 78:321-328

[21] Ray SC, Arthur RR, Carella A, Bukh J, Thomas DL (2000) Genetic epidemiology of hepatitis C virus throughout egypt. The Journal of infectious diseases 182 (3):698-707

[22] Ansaldi F, Bruzzone B, Salmaso S, Rota MC, Durando P, Gasparini R, Icardi G (2005) Different seroprevalence and molecular epidemiology patterns of hepatitis C virus infection in Italy. Journal of medical virology 76 (3):327-332

[23] Delwaide J, Reenaers C, Gerard C, Vaira D, Bastens B, Servais B, Bekhti A, Bataille C, Wain E, De Leeuw P, Daenen G, Mesureur T, Sente JM, Belaiche J (2006) HCV genotype 4 in Belgium: three distinct patterns among patients from European and African origin. Eur J Gastroenterol Hepatol 18 (7):707-712

[24] Payan C, Roudot-Thoraval F, Marcellin P, Bled N, Duverlie G, Fouchard-Hubert I, Trimoulet P, Couzigou P, Cointe D, Chaput C, Henquell C, Abergel A, Pawlotsky JM, Hezode C, Coude M, Blanchi A, Alain S, Loustaud-Ratti V, Chevallier P, Trepo C, Gerolami V, Portal I, Halfon P, Bourliere M, Bogard M, Plouvier E, Laffont C, Agius G, Silvain C, Brodard V, Thiefin G, Buffet-Janvresse C, Riachi G, Grattard F, Bourlet T, Stoll-Keller F, Doffoel M, Izopet J, Barange K, Martinot-Peignoux M, Branger M, Rosenberg A, Sogni P, Chaix ML, Pol S, Thibault V, Opolon P, Charrois A, Serfaty L, Fouqueray B, Grange JD, Lefrere JJ, Lunel-Fabiani F (2005) Changing of hepatitis C virus genotype patterns in France at the beginning of the third millenium: The GEMHEP GenoCII Study. Journal of viral hepatitis 12 (4):405-413

[25] Schroter M, Zollner B, Schafer P, Reimer A, Muller M, Laufs R, Feucht HH (2002) Epidemiological dynamics of hepatitis C virus among 747 German individuals: new subtypes on the advance. Journal of clinical microbiology 40 (5):1866-1868

[26] van Asten L, Verhaest I, Lamzira S, Hernandez-Aguado I, Zangerle R, Boufassa F, Rezza G, Broers B, Robertson JR, Brettle RP, McMenamin J, Prins M, Cochrane A, Simmonds P, Coutinho RA, Bruisten S (2004) Spread of hepatitis C virus among European injection drug users infected with HIV: a phylogenetic analysis. The Journal of infectious diseases 189 (2):292-302

[27] van de Laar TJ, Langendam MW, Bruisten SM, Welp EA, Verhaest I, van Ameijden EJ, Coutinho RA, Prins M (2005) Changes in risk behavior and dynamics of hepatitis C virus infections among young drug users in Amsterdam, the Netherlands. Journal of medical virology 77 (4):509-518

[28] de Bruijne J, Schinkel J, Prins M, Koekkoek SM, Aronson SJ, van Ballegooijen MW, Reesink HW, Molenkamp R, van de Laar TJ (2009) Emergence of hepatitis C virus genotype 4: phylogenetic analysis reveals three distinct epidemiological profiles. Journal of clinical microbiology 47 (12):3832-3838

[29] De Maeght S, Henrion J, Bourgeois N, de Galocsy C, Langlet P, Michielsen P, Reynaert H, Robaeys G, Sprengers D, Orlent H, Adler M (2008) A pilot observational survey of hepatitis C in Belgium. Acta Gastroenterol Belg 71 (1):4-8

[30] Abergel A, Ughetto S, Dubost S, Bonny C, Aublet-Cuvelier B, Delarocque-Astagneau E, Bailly JL, Bommelaer G, Casanova S, Delteil J, Deny P, Laurichesse H, Odent-Malaure H, Roussel J, Peigue-Lafeuille H, Henquell C (2007) The epidemiology and virology of hepatitis C virus genotype 5 in central France. Alimentary pharmacology & therapeutics 26 (10):1437-1446

[31] Antaki N, Haddad M, Kebbewar K, Abdelwahab J, Hamed O, Aaraj R, Alhaj N, Haffar S, Assil M, Ftayeh M, Assaad F, Doghman D, Ali T, Nasserelddine M, Ali A, Antaki F (2009) The unexpected discovery of a focus of hepatitis C virus genotype 5 in a Syrian province. Epidemiol Infect 137 (1):79-84

[32] Jover R, Perez-Serra J, de Vera F, Alamo JM, Munoz C, Yago C, Martinez-Ramirez R, Vidal JV (2001) Infection by genotype 5a of HCV in a district of southeast Spain. Am J Gastroenterol 96 (10):3042-3043

## FIGURE LEGENDS

**Fig 1** Geographic distribution of the HCV genotypes in Flanders and Brussels (Belgium). The pie charts are scaled according to the number of samples analysed in each province.

**Fig 2** Phylogenetic maximum likelihood tree for the concatenated gene region of HCV genotype 5a strains. Each strain is indicated by a unique patient number. Strain names printed in bold correspond to patients from whom both a serum sample and a questionnaire were available. Possible inter-patient transmission clusters are indicated by a colour code (see text for further details). Numbers at the nodes represent bootstrap values. Only values of >50 are shown.

**Figure 1**

Figure 1

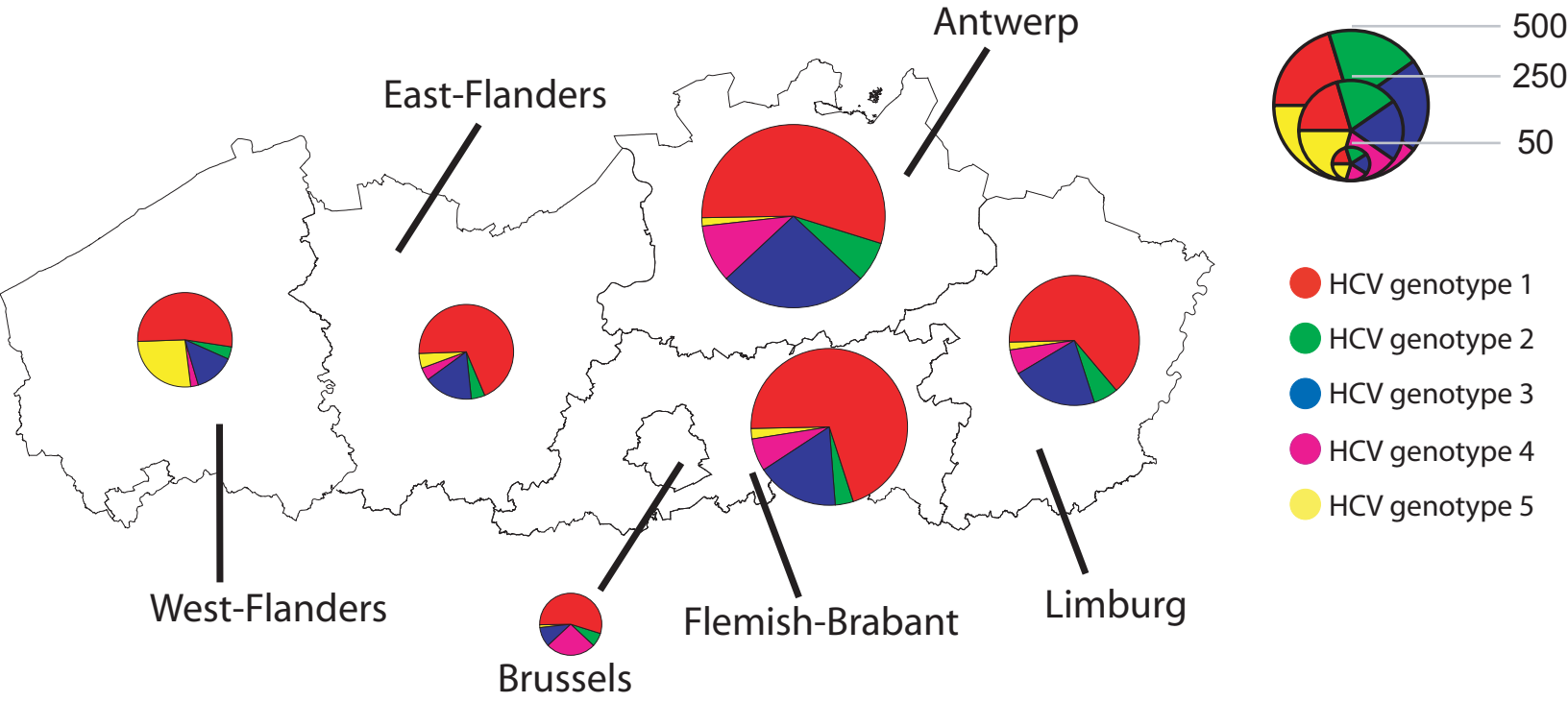

**Figure 2**

Figure 2