Research paper

# Phylogenetic analysis of the Belgian HIV-1 epidemic reveals that local transmission is almost exclusively driven by men having sex with men despite presence of large African migrant communities

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## ARTICLE INFO

## ABSTRACT

To improve insight in the drivers of local HIV-1 transmission in Belgium, phylogenetic, demographic, epidemiological and laboratory data from patients newly diagnosed between 2013 and 2015 were combined and analyzed. Characteristics of clustered patients, paired patients and patients on isolated branches in the phylogenetic tree were compared. The results revealed an overall high level of clustering despite the short time frame of sampling, with 47.6% of all patients having at least one close genetic counterpart and 36.6% belonging to a cluster of 3 or more individuals. Compared to patients on isolated branches, patients in clusters more frequently reported being infected in Belgium (95.1% vs. 47.6%; p < 0.001), were more frequently men having sex with men (MSM) (77.9% vs. 42.8%; p < 0.001), of Belgian origin (68.2% vs. 32.9%; p < 0.001), male gender (92.6% vs. 65.8%; p < 0.001), infected with subtype B or F (87.8% vs. 43.4%; p < 0.001) and diagnosed early after infection (55.4% vs. 29.0%; p < 0.001). Strikingly, Sub-Saharan Africans (SSA), overall representing 27.1% of the population were significantly less frequently found in clusters than on individual branches (6.0% vs. 41.8%; p < 0.001). Of the SSA that participated in clustered transmission, 66.7% were MSM and this contrasts sharply with the overall 12.0% of SSA reporting MSM. Transmission clusters with SSA were more frequently non-B clusters than transmission clusters without SSA (44.4% versus 18.2%). MSM-driven clusters with patients of mixed origin may account, at least in part, for the increasing spread of non-B subtypes to the native MSM population, a cross-over that has been particularly successful for subtype F and CRF02_AG.

The main conclusions from this study are that clustered transmission in Belgium remains almost exclusively MSM-driven with very limited contribution of SSA. There were no indications for local ongoing clustered transmission of HIV-1 among SSA.

# 1. Introduction

## 1.1. The value of phylogenetic analysis

Defining the evolutionary relationship between viruses using similarities in molecular sequences is a widely applied method for identification and visualization of HIV-1 transmission networks (Hassan et al., 2017). Results of a recent study provided proof for the strength of these phylogenetic methods by showing their capacity to identify transmission partners even on an individual patient scale (Wertheim et al., 2017). HIV-1 *pol* sequences are routinely generated for assessment of baseline drug resistance and contain sufficient information for the reliable identification of transmission events (Hue et al., 2004). They have been used in numerous nationwide phylogenetic studies intended to better understand the dynamics of local HIV-1 transmission (Kouyos et al., 2010; Leigh Brown et al., 2011; Lunar et al., 2015; Perez-Parra et al., 2016; Brenner et al., 2017; Paraskevis et al., 2017; Parczewski et al., 2017; Temereanca et al., 2017).

## 1.2. The HIV-1 epidemic in Belgium and Western Europe

With an estimated overall HIV prevalence of 1.7 infected persons per 1000 inhabitants, the overall burden of HIV-1 in Belgium is low, but the infection is highly concentrated in specific populations. Recent surveys revealed a HIV-1 prevalence of 6.0% in men having sex with men (MSM) (Vanden Berghe et al., 2011) and between 4.2% and 5.9% in Sub-Saharan Africans (SSA) (Loos et al., 2017). A national HIV surveillance system, with registration of all new diagnoses, is operating since 1985. Care and the continuum of care are well ensured. Of all HIV diagnosed patients living in Belgium in 2011, an estimated 98.2% are linked to care (Van Beckhoven et al., 2015). In 2015, approximately 1000 (95% CI: 800–1200) individuals were newly diagnosed (Sasse et al., 2015). For the same year the number of undiagnosed infections was estimated at 2800 (95% CI: 2200–3500) (Marty et al., 2017). About half of all new diagnoses in Belgium are in MSM. The HIV epidemic in MSM displayed an exponential growth between 1999 and 2010 and has then stabilized for some years with moderate decreases since 2014. The infection in MSM is largely dominated by subtype B infections (Chalmet et al., 2010). Infections in heterosexuals (HET) represent about 40% of all new diagnoses, half of them in SSA. In this subpopulation a large variety of non-B HIV-1 subtypes is seen (Fransen et al., 1996; Snoeck et al., 2004; Dauwe et al., 2015). An increased entrance of these non-B subtypes in the MSM population has been reported recently (Dauwe et al., 2015), in line with observations in other Western European countries (Beloukas et al., 2016; Esbjornsson et al., 2016; Ragonnet-Cronin et al., 2016; Dennis et al., 2017; Vinken et al., 2017). In a study covering the Nordic countries, Esbjornsson et al. also observed increased mixing of MSM and HET in transmission clusters (Esbjornsson et al., 2016). All these observations suggest potential changes in the dynamics of the HIV-1 epidemic in Western Europe, with increasing crossovers between nationalities and risk groups. Because of the large migrant population in Belgium and the high HIV-1 burden in this group, local transmission among migrants is expected. There are indeed indications that a high proportion of migrants acquire their HIV infection post migration in a European country (Loos et al., 2017; Alvarez-del Arco et al., 2017) but there is no information available on the participation of SSA in local transmission networks.

## 1.3. Study objective

The aim of the present study was to define characteristics and drivers of the current HIV-1 epidemic in Belgium by combining phylogenetic cluster analysis with demographic, epidemiological and laboratory data for HIV-1 infections diagnosed between 2013 and 2015.

# 2. Methods

## 2.1. Study population and data collection

HIV-1 diagnosis and care in Belgium is centralized in 11 Aids Reference Centers and 7 Aids Reference Laboratories, established across the country. Demographic and epidemiological data of all new diagnoses are registered in a national HIV surveillance system. Generalized baseline drug resistance testing of all newly diagnosed, therapy naïve, individuals is operational since 2005.

For the period covered by this study (2013 to 2015), baseline HIV-1 *pol* sequences were available for about 60% of all newly diagnosed persons. The most important reason for missing baseline sequence data is not being therapy naive at the time of first diagnosis in Belgium. All baseline *pol* sequences generated for therapy naive patients diagnosed between 2013 and 2015 were used. Demographic and epidemiological data included gender, age at diagnosis, country of birth, transmission route and country of infection. Transmission risk categories were MSM, HET, intravenous drug users (IVDU), blood transfusion and perinatal infection. Because of the small numbers of IVDU (n = 14), blood transfusion (n = 12) or perinatal infection (n = 9) these categories were excluded for statistical analysis but the two patients being MSM and IVDU were classified in the MSM category because in the phylogenetic tree, both were closely linked with individuals infected through MSM. For the category "country of birth", patients were classified as Belgian, European (born in any European country except Belgium), SSA or "other". For the country of infection we focused on infections in Belgium and infections in Sub-Saharan Africa. Patients reporting infection outside of these countries were grouped as "other". Viral load, CD4$^+$ T cell count and *pol* gene sequence data were obtained from the 7 Belgian Aids Reference Laboratories. Collection of sequence data was facilitated by the use of a network database (Integrated Database Network System, Smartgene, Zug, Switzerland).

## 2.2. Sequencing

Partial *Pol* sequences were generated in one of the 7 Aids Reference Laboratories using population sequencing and either an in-house protocol or the TruGene HIV-1 genotyping kit (TruGene, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). The length of the generated sequences slightly differed according to the sequencing protocol. Therefore, only the 870 nucleotides, representing codons 9 to 99 of the *protease* gene and codons 41 to 239 of the *reverse transcriptase* gene and covered by all methods, were included in the alignment. Alignments were composed in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Gaps resulting from the insertions of three nucleotides at codon positions 33 or 35 of the *protease* gene, observed in 8 of the 1665 sequences, were removed. Positions associated with drug resistance were kept after excluding any influence on the tree topology or cluster identification.

## 2.3. Phylogenetic analysis and cluster identification

A phylogenetic tree was reconstructed using the maximum likelihood (ML) approach implemented in PhyML 3.0 (Guindon et al., 2010) with automatic selection of the best fit evolutionary model of DNA substitution (GTR + G + I) using the Akaike information criterion (AIC). Branch support was obtained by approximate likelihood-ratio test (aLRT, SH-like) (Anisimova and Gascuel, 2006), a likelihood based alternative to the computationally intensive bootstrapping. Sequences clustering with at least one other sequence with aLRT of ≥ 0.97 and a mean pairwise distance ≤ 0.015 were considered as genetically linked. After defining the clusters, all sequences with a distance of > 0.030 to the closest relative were removed as cluster members and considered as individual branched patients. A radial cladogram was drawn using the Interactive Tree of Life web service at http://itol.embl.de/ (Letunic and
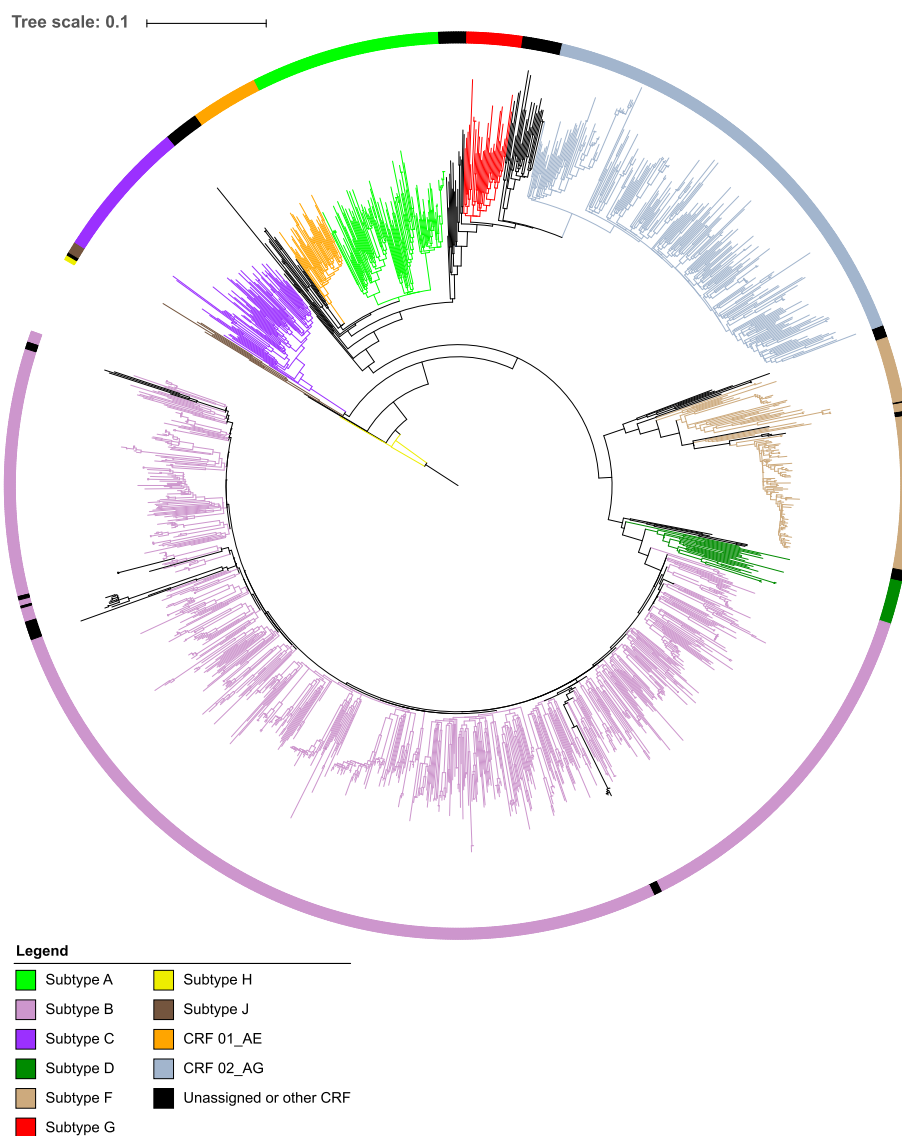
**Fig. 1.** ML phylogenetic tree of 1665 patients newly diagnosed in Belgium between 2013 and 2015. Tree branches colored according to subtype. The tree scale refers to the number of nucleotide substitutions per site.

Bork, 2011).

### 2.4. Subtyping and identification of transmitted drug resistance mutations (TDRM)

HIV-1 subtyping was performed using the COMET HIV-1 (Version 1.0) (Struck et al., 2014) and Rega v3.0 genotyping tools (Pineda-Pena et al., 2013). The subtype was attributed in case of a concordant result for both tools and considered as "undefined" in all other cases. Transmitted drug resistance mutations were identified using the World Health Organization (WHO) 2009 surveillance list (Bennett et al., 2009) with additional inclusion of the E138K mutation, a mutation that induces resistance against a non-nucleoside reverse transcriptase inhibitor that became available after 2009.

### 2.5. Infection timing

The recency of infection at diagnosis was estimated using the *BED HIV-1 Incidence EIA* and the *HIV-1 LAg-Avidity EIA* (both from Sedia Biosciences Corporation, Portland, Oregon, USA). These methods have been extensively validated. A concordant prediction of recent infection in both assays was shown to be a reliable predictor for an infection acquired < 4 months before (Verhofstede et al., 2017). For practical reasons infection timing was limited to patients newly diagnosed in 2014.

### 2.6. Statistical analysis

Univariate analyses were performed using Pearson Chi-Square for categorical variables and Mann-Whitney *U* nonparametric test for continuous variables. Variables with a p-value ≤ 0.05 in the univariate analysis were included in the multivariate analysis. If considered appropriate, the categories for a variable were restricted, e.g. by omission of the "other" category, or grouped e.g. for subtype B and F infections. For certain variables (country of birth, CD4 count and viral load) it was assumed that the lack of data for part of the patients was random and a multiple imputation model was used to complete missing data before proceeding to multivariate analysis. Fifty imputed data sets were created and multivariate analysis was run on the pooled dataset. Binary logistic regression was performed using backward selection and a significance level of 0.05. Bonferroni adjustment was applied to correct for multiple testing. All data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 23.0. Released 2015; IBM Corp, Armonk, NY, USA).

## 2.7. GenBank accession numbers

Sequences of one representative of each of the 84 clusters have been deposited into GenBank (accession numbers MG897604-MG897687). All other sequences are available on request.

## 2.8. Ethics statements

The study was approved by the ethical boards of the participating institutions with the Ethical Committee of Ghent University Hospital operating as the central organization (reference number 2014/0717). Apart from the incidence analyses, performed on rest material, all analyses were performed with existing data. Anonymized patient identifiers were used to combine baseline sequences, incidence, demographic and behavior data.

## 3. Results

### 3.1. Study population

The study population comprised 1665 individuals, 658 diagnosed in 2013, 557 in 2014 and 450 in 2015. Mean age at diagnosis was 38 years (IQR 29–45), 76.8% of the patients were male. Mean viral load and CD4 count were 4.80 log c/ml (IQR 4.26–5.38) and 428 cells/mm$^3$ (IQR 234–575) respectively. Of the 1290 patients with registered country of birth, 47.9% were Belgians and 27.1% SSA. Of the 1395 patients with registered transmission risk, 56.9% were MSM and 40.5% HET. The HIV-1 subtype distribution for the 1631 patients with attributed subtype was 53.1% B, 16.2% CRF02_AG, 8.6% F, 8.4% A, 5.2% C, 2.6% CRF01_AE and 5.9% other subtypes or CRF. Overall, 164 patients (9.9%) were infected with a variant containing at least one transmitted drug resistance mutation. The contribution of TDRM against non-nucleoside reverse transcriptase, nucleoside reverse transcriptase and protease inhibitors was respectively 51.3%, 36.0% and 12.7%. The phylogenetic tree colored according to subtype distribution is shown in Fig. 1.

### 3.2. Phylogenetic analysis

Phylogenetic analysis revealed at least one close genetic counterpart for 793 (47.6%) of the 1665 patients while 872 (52.4%) resided on isolated branches in the tree. Of the 793 patients with a close genetic counterpart, 186 (23.6%) were linked with a single other individual, resulting in the formation of 93 pairs, and 607 (76.5%) were members of a transmission cluster of 3 or more individuals. Overall, 84 transmission clusters of 3 or more individuals were identified. These clusters comprised 36.5% of all patients.

### 3.3. Characteristics of patients in transmission clusters

The comparison between patients in clusters of 3 or more and patients on isolated branches is shown in Table 1. This comparison highlights the pronounced involvement of individuals infected in Belgium (95.1%), MSM (77.9%) and native Belgians (68.2%) in clustered transmission. Other characteristics significantly associated with clustering were male gender, subtype B or F infection, high CD4 count at diagnosis, high viral load at diagnosis and being diagnosed early after infection. For multivariate analyses (Table 2), the comparison was restricted to Belgians and SSA for the variables "country of birth" and "country of infection" and to subtype B or F versus the other subtypes for the variable "subtype". Male gender, Belgian origin, being infected with a subtype B or F virus, younger age, higher viral load and higher CD4 count were all identified as independent predictors for being member of a transmission cluster. Transmission risk and presence of TDRM were not withhold as independent predictors in multivariate analysis.

**Table 1**

Results of univariate analysis for comparison between patients on isolated branches in the phylogenetic tree and patients in phylogenetic clusters of ≥3. For each parameter only the patients with available information for that parameter were included. Pearson Chi-Square test was used for categorical variables and Mann-Whitney $U$ nonparametric test for continuous variables.

| | All | Position in phylogenetic tree | | p-Value |
|---|---|---|---|---|
| | | Isolated branch | Clustered ≥ 3 | |
| | N = 1479 | N = 872 | N = 607 | |
| Gender (n = 1479), n (%) | | | | |
| Male | 1136 (76.8) | 574 (65.8) | 562 (92.6) | < 0.001 |
| Female | 343 (23.2) | 298 (34.2) | 45 (7.4) | |
| Age at diagnosis (n = 1479), mean (IQR) | 38 (29–45) | 38 (30–46) | 37 (28–44) | 0.005 |
| Transmission risk (n = 1206), n (%) | | | | |
| MSM | 698 (57.9) | 295 (42.8) | 403 (77.9) | < 0.001 |
| HET | 508 (42.1) | 394 (57.2) | 114 (22.1) | |
| Country of birth (n = 1139), n (%) | | | | |
| Belgium | 535 (47.0) | 226 (32.9) | 309 (68.2) | < 0.001 |
| Europe | 145 (12.7) | 74 (10.8) | 71 (15.7) | |
| Sub-Saharan Africa | 314 (27.6) | 287 (41.8) | 27 (6.0) | |
| Other | 145 (12.8) | 99 (14.5) | 46 (10.1) | |
| Country of infection (n = 880), n (%) | | | | |
| Belgium | 613 (69.7) | 224 (47.6) | 389 (95.1) | < 0.001 |
| Sub-Saharan Africa | 154 (17.5) | 150 (31.8) | 4 (1.0) | |
| Other | 113 (12.8) | 97 (20.6) | 16 (3.9) | |
| Subtype (n = 1451), n (%) | | | | |
| B | 762 (52.5) | 343 (40.6) | 419 (69.0) | < 0.001 |
| F | 138 (9.5) | 24 (2.8) | 114 (18.8) | |
| 02_AG | 237 (16.3) | 189 (22.4) | 48 (7.9) | |
| A | 115 (7.9) | 99 (11.7) | 16 (2.6) | |
| C | 74 (5.1) | 68 (8.1) | 6 (1.0) | |
| 01_AE | 34 (2.3) | 34 (4.0) | 0 (0) | |
| Other | 91 (6.3) | 87 (10.3) | 4 (0.7) | |
| CD4$^+$ T cells (cells/mm$^3$) (n = 1024), mean (IQR) | 428 (234–575) | 376 (188–516) | 487 (317–624) | < 0.001 |
| Viral load (log copies/ml) (n = 1430), mean (IQR) | 4.80 (4.26–5.38) | 4.73 (4.20–5.33) | 4.93 (4.41–5.49) | < 0.001 |
| Infection stage at diagnosis (n = 456), n (%) | | | | |
| Early | 179 (39.3) | 81 (29.0) | 98 (55.4) | < 0.001 |
| Late | 277 (60.7) | 198 (71.0) | 79 (44.6) | |
| Transmitted drug resistance (n = 1479), n (%) | | | | |
| Yes | 157 (10.6) | 81 (9.3) | 76 (12.5) | 0.047 |
| No | 1322 (89.4) | 791 (90.7) | 531 (87.5) | |

n: number; IQR: Interquartile Range; MSM: men having sex with men, HET: heterosexual.

Of the 607 individuals in transmission clusters, 419 were subtype B infections (64 clusters), followed by subtype F (114 individuals, 4 clusters), CRF02_AG (48 individuals, 10 clusters), subtype A (16 individuals, 3 clusters), C (6 individuals, 2 clusters) and "other" (4 individuals, 1 cluster).

Drug resistance mutations were found in 76 clustered patients (12.5%). In the majority of them (83.9%) only one resistance mutation was detected. Nucleoside reverse transcriptase inhibitor revertant mutations at position 215 (T215E/D; 4 clusters, 35 patients) and the non-nucleoside reverse transcriptase inhibitor mutation K103N (2 clusters, 16 patients) were the most represented. Multiclass resistance was found in two clusters, with a combination of the protease mutations I54V and N88D and nucleoside reverse transcriptase mutations L210W and T215S in one cluster (7 patients) and a combination of the nucleoside reverse transcriptase revertant mutation T215D and the non-nucleoside reverse transcriptase mutation Y181C in another cluster (3 patients).

Twenty-six of the 84 transmission clusters had 7 or more members

**Table 2**

Model of independent risk factors associated with phylogenetic clustering based on multivariate logistic regression analysis with Bonferroni adjustment. All variables with a p-value ≤ 0.05 in the univariate analysis were retrieved for multivariate analysis. A multiple imputation model was used to adjust for missing data. For subtype and country of birth the categories were limited to the most informative.

| Risk factors | Multivariate analysis | | |
|---|---|---|---|
| | OR | p value | 95% confidence interval |
| Gender | | | |
| Male | 2.078 | < 0.001 | 1.385–3.118 |
| Female | 1 | – | Ref |
| Age at diagnosis | 0.972 | < 0.001 | 0.960–0.984 |
| CD4$^+$ T cells | 1.001 | < 0.001 | 1.001–1.002 |
| Viral load | 1.475 | < 0.001 | 1.255–1.733 |
| Subtype | | | |
| B or F | 4.559 | < 0.001 | 3.190–6.515 |
| Other | 1 | – | Ref |
| Country of birth | | | |
| Belgium | 2.808 | < 0.001 | 1.781–4.426 |
| Sub-Saharan Africa | 1 | – | Ref |

OR: Odds Ratio; Ref: reference category.

and were considered as large clusters with presumed high impact on ongoing local transmission. The characteristics of these 26 large clusters are represented in Table 3. Nineteen (73.1%) had only Belgian members and 16 (61.5%) were men-only. With regard to the transmission risk, 8 (30.8%) were MSM-only and 18 were mixed MSM and HET. Of these 18 mixed transmission risk clusters however, 8 were men only. The overall gender ratio was 344 men over 32 women. The 32 women were distributed over 10 clusters including a large women-dominated cluster (cluster 1410; 10 females, 1 male), 3 clusters with at least one quarter of female members and 4 clusters with a lower female contribution (Table 3).

### 3.4. Contribution of SSA in transmission clusters

Twenty seven (7.7%) of the 350 SSA were member of a transmission cluster. They were distributed over 18 clusters of which 13 with only a single Sub-Saharan African member (Table 4). Nine of the 18 clusters with SSA (50.0%) were mixed risk clusters, 6 (33.3%) were MSM only and 3 (16.7%) were HET only. In line, the majority of SSA in clusters (18/27; 66.7%) were MSM versus 8/27 (29.6%) HET and 1/27 (3.7%) unknown or other risk. This contrasts sharply with the overall risk distribution for the SSA in the study with 78.3% HET, 12.0% MSM and 9.7% other or unknown risk. About half (55.6%) of clusters with at least one SSA member were subtype B clusters, 6 (33.3%) were CRF02_AG and 2 (11.1%) were subtype F infections. For comparison, of the 66 clusters without SSA members, 54 (81.8%) were subtype B and 12 (18.2%) were non-B clusters.

### 3.5. Characteristics of paired patients

Significant differences were observed between patients in pairs and patients in transmission clusters. Paired patients were more frequent females (23.1% versus 7.4%; p < 0.001), HET (35.6% vs. 21.8%; p < 0.001), of SSA origin (23.8% vs. 6.0%; p < 0.001), infected in Sub-Saharan Africa (13.7% vs. 0.5%; p < 0.001) and infected with a non-B virus (42.2% vs. 31.0%; p < 0.001) compared to the patients in transmission clusters. The TDRM prevalence in paired patients was lower than the TDRM prevalence in clustered patients (4.3% vs. 12.5%; p < 0.001).

Significant differences were also observed between patients in pairs and patients on single branches. Compared to the isolated patients, pairs were more frequently males (76.9% vs. 65.8%; p = 0.003), MSM (59.4% vs. 41.4%; p < 0.001), of Belgian origin (55.0% vs. 32.9%; p < 0.001), infected in Belgium (76.1% vs. 47.4%; p < 0.001) and infected with a subtype B virus (57.8% vs. 40.6%; p < 0.001). TDRM prevalence was lower in pairs (4.3% vs. 9.3%; p = 0.026).

**Table 3**

Patient composition for the 26 large transmission clusters (≥7 members).

| Cluster ID | N | Gender (n) | | Transmission risk (n) | | | Country of birth (n) | | | Country of infection (n) | | | Subtype |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Male | Female | MSM | HET | U/O | Belgium | Sub-Saharan Africa | U/O | Belgium | Other | U/O | |
| 1304 | 94 | 90 | 4 | 68 | 15 | 11 | 64 | 1 | 29 | 70 | 3 | 21 | F |
| 1313 | 32 | 31 | 1 | 25 | 3 | 4 | 15 | 0 | 17 | 19 | 1 | 12 | B |
| 1443 | 30 | 28 | 2 | 20 | 7 | 3 | 23 | 1 | 6 | 19 | 0 | 11 | B |
| 1340 | 24 | 24 | 0 | 16 | 0 | 8 | 9 | 0 | 15 | 13 | 0 | 11 | B |
| 1449 | 14 | 10 | 4 | 7 | 5 | 2 | 4 | 1 | 9 | 10 | 1 | 3 | B |
| 1307 | 14 | 12 | 2 | 8 | 3 | 3 | 8 | 0 | 6 | 10 | 0 | 4 | B |
| 1306 | 12 | 12 | 0 | 8 | 4 | 0 | 11 | 0 | 1 | 10 | 2 | 0 | B |
| 1410 | 11 | 1 | 10 | 0 | 11 | 0 | 8 | 1 | 2 | 9 | 0 | 2 | F |
| 1354 | 10 | 10 | 0 | 9 | 0 | 1 | 3 | 0 | 7 | 8 | 0 | 2 | B |
| 1434 | 10 | 10 | 0 | 5 | 2 | 3 | 3 | 0 | 7 | 3 | 1 | 6 | B |
| 1357 | 10 | 9 | 1 | 3 | 6 | 1 | 5 | 0 | 5 | 7 | 0 | 3 | B |
| 1447 | 9 | 9 | 0 | 8 | 0 | 1 | 2 | 0 | 7 | 7 | 1 | 1 | B |
| 1309 | 9 | 9 | 0 | 7 | 1 | 1 | 6 | 0 | 3 | 7 | 0 | 2 | B |
| 1346 | 9 | 9 | 0 | 8 | 1 | 0 | 5 | 0 | 4 | 6 | 0 | 3 | B |
| 1414 | 9 | 9 | 0 | 8 | 0 | 1 | 1 | 0 | 8 | 5 | 1 | 3 | 02_AG |
| 1456 | 8 | 7 | 1 | 6 | 1 | 1 | 1 | 1 | 6 | 1 | 1 | 6 | B |
| 1303 | 8 | 3 | 5 | 0 | 6 | 2 | 5 | 1 | 2 | 3 | 0 | 5 | 02_AG |
| 1424 | 7 | 7 | 0 | 4 | 1 | 2 | 0 | 5 | 2 | 3 | 0 | 4 | B |
| 1341 | 7 | 7 | 0 | 5 | 2 | 0 | 3 | 0 | 4 | 4 | 0 | 3 | B |
| 1310 | 7 | 7 | 0 | 6 | 0 | 1 | 5 | 0 | 2 | 4 | 0 | 3 | B |
| 1311 | 7 | 7 | 0 | 5 | 0 | 2 | 5 | 0 | 2 | 5 | 0 | 2 | B |
| 1334 | 7 | 7 | 0 | 4 | 1 | 2 | 5 | 0 | 2 | 4 | 0 | 3 | B |
| 1342 | 7 | 7 | 0 | 5 | 0 | 2 | 4 | 0 | 3 | 5 | 0 | 2 | B |
| 1364 | 7 | 7 | 0 | 5 | 0 | 2 | 2 | 0 | 5 | 3 | 0 | 4 | B |
| 1403 | 7 | 5 | 2 | 4 | 3 | 0 | 4 | 0 | 3 | 5 | 1 | 1 | A |
| 1438 | 7 | 7 | 0 | 4 | 1 | 2 | 2 | 0 | 5 | 5 | 0 | 2 | B |

N: total number; n: number; MSM: men having sex with men; HET: heterosexual; U/O: unknown/other.

**Table 4**
Patient composition for the 18 clusters with at least one Sub-Saharan African representative.

| Cluster ID | N | Gender (n) | | Transmission risk (n) | | | Country of birth (n) | | | Country of infection (n) | | | Subtype |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Male | Female | MSM | HET | U/O | Belgium | Sub-Saharan Africa | U/O | Belgium | Other | U/O | |
| 1424 | 7 | 7 | 0 | 4 | 1 | 2 | 0 | **5** | 2 | 3 | 0 | 4 | B |
| 1325 | 3 | 3 | 0 | 3 | 0 | 0 | 0 | **2** | 1 | 2 | 1 | 0 | 02_AG |
| 1439 | 6 | 6 | 0 | 5 | 0 | 1 | 1 | **3** | 2 | 4 | 0 | 2 | B |
| 1304 | 94 | 90 | 4 | 68 | 15 | 11 | 64 | **2** | 28 | 70 | 3 | 21 | F |
| 1521 | 3 | 2 | 1 | 1 | 2 | 0 | 0 | **2** | 1 | 0 | 1 | 2 | 02_AG |
| 1456 | 8 | 7 | 1 | 6 | 1 | 1 | 1 | **1** | 6 | 1 | 1 | 6 | B |
| 1302 | 3 | 1 | 2 | 0 | 3 | 0 | 2 | **1** | 0 | 2 | 1 | 0 | 02_AG |
| 1303 | 8 | 3 | 5 | 0 | 6 | 2 | 5 | **1** | 2 | 3 | 0 | 5 | 02_AG |
| 1328 | 3 | 3 | 0 | 3 | 0 | 0 | 0 | **1** | 2 | 0 | 0 | 3 | 02_AG |
| 1410 | 11 | 1 | 10 | 0 | 11 | 0 | 8 | **1** | 2 | 9 | 0 | 2 | F |
| 1449 | 14 | 10 | 4 | 7 | 5 | 2 | 4 | **1** | 9 | 10 | 1 | 3 | B |
| 1540 | 3 | 3 | 0 | 2 | 1 | 0 | 0 | **1** | 2 | 1 | 0 | 2 | B |
| 1327 | 6 | 6 | 0 | 5 | 0 | 1 | 3 | **1** | 2 | 5 | 0 | 1 | 02_AG |
| 1340 | 24 | 24 | 0 | 16 | 0 | 8 | 9 | **1** | 14 | 13 | 0 | 11 | B |
| 1417 | 5 | 5 | 0 | 4 | 1 | 0 | 2 | **1** | 2 | 2 | 0 | 2 | B |
| 1432 | 4 | 4 | 0 | 4 | 0 | 0 | 1 | **1** | 2 | 2 | 2 | 0 | B |
| 1436 | 5 | 5 | 0 | 4 | 1 | 0 | 3 | **1** | 1 | 5 | 0 | 0 | B |
| 1443 | 30 | 28 | 2 | 20 | 7 | 3 | 23 | **1** | 6 | 19 | 0 | 11 | B |

Clusters are ordered according to the contribution of Sub-Saharan African members (numbers in bold)
N: total number; n: number; MSM: men having sex with men; HET: heterosexual; U/O: unknown/other.

*3.6. Characteristics of patients on isolated branches in the phylogenetic tree*

Compared to the genetically linked patients, patients on individual branches were more frequently female (34.2% vs. 11.1%; p < 0.001), HET (55.3% vs. 25.1%; p < 0.001), SSA (41.8% vs. 10.4%; p < 0.001), infected outside of Belgium (52.4% vs. 9.2%; p < 0.001) and infected with a non-B subtype (59.4% vs. 33.5%; p < 0.001). They had a lower CD4 count (mean 376 cells/mm$^3$, IQR 188–516 vs. mean 485 cells/mm$^3$, IQR 300–630; p < 0.001) and were less frequently diagnosed early after infection (29.0% of the 279 patients with information on the infection time vs. 53.5% of the 245 patients with known infection time; p < 0.001). Although a significant lower number of patients on individual branches reported infection in Belgium still 47.6% acquired the infection locally and a high number of these individuals were of Belgian origin (32.9%) and/or MSM (42.8%).

## 4. Discussion

*4.1. General findings*

With this nationwide viral phylodynamics study we aimed to broaden our insight on the HIV-1 epidemic in Belgium. The study comprised 1665 HIV-1 infected individuals newly diagnosed between 2013 and 2015, representing at least 60.0% of all patients diagnosed in Belgium during that period. Results from phylogenetic analysis were combined with demographic, epidemiological and laboratory data. Despite the short time frame of sample collection we found that 47.6% of the 1665 patients were genetically linked to at least one other individual and 36.5% were member of a transmission cluster. The proportion of patients contributing to transmission clusters closely approaches the 33.4% recorded in 2010 in a study that covered only a small part of the country (Chalmet et al., 2010). Comparable high frequencies of transmission clustering have been reported for other countries where the HIV epidemic is primarily MSM driven (Bezemer et al., 2010; Brenner et al., 2011; Chan et al., 2015; Brenner et al., 2017; Patino-Galindo et al., 2017). The results of our study confirmed the high contribution of MSM in clustered transmission. Importantly, despite the presence of large populations of SSA in the country, SSA were rarely observed in HIV-1 transmission clusters.

*4.2. Transmitted drug resistance*

The overall prevalence of baseline drug resistance was 9.9%, a figure that is comparable with the results of previous national surveys (Muyldermans and Sasse, 2014). There was no difference in prevalence of drug resistance between clustered patients and patients on individual branches. Also in line with previous reports, the non-nucleoside reverse transcriptase inhibitor mutation K103N and nucleoside reverse transcriptase inhibitor revertant mutations at position 215 (T215S/E/D) were the most frequently present in transmission clusters (Ruelle et al., 2013; Pineda-Pena et al., 2014). Moreover, a phylogenetic link between our cluster 1449 and a Belgian transmission cluster with K103N described by Ruelle et al. in 2013 and between our cluster 1334 and a cluster with multi-drug resistance described by Pineda-Pena et al. in 2014, was evidenced (results not shown) (Ruelle et al., 2013; Pineda-Pena et al., 2014). These findings illustrate the long term persistence and continuing expansion of transmission clusters.

*4.3. Cluster identification and characteristics of patients involved in clustered transmission*

Selection of appropriate criteria for the identification of transmission clusters is complex and largely depends on the research question asked (Hassan et al., 2017). Because the aim of this study was to characterize the drivers of local transmission, highly stringent cluster identification criteria were applied in order to increase the chance of targeting clusters resulting from local transmission events. The observation that 90.0% of the patients with a genetic link and 95.1% of the patients in transmission clusters report being infected in Belgium supports the relevance of the chosen strategy.

In analogy with other Western European countries (Kouyos et al., 2010; Leigh Brown et al., 2011; Abecasis et al., 2013; Audelin et al., 2013; Frentz et al., 2013; Esbjornsson et al., 2016; Perez-Parra et al., 2016; Parczewski et al., 2017), the clustered HIV-1 transmission in Belgium remains predominantly MSM driven. The vast majority of cluster members (68.2%) were born in Belgium with a small contribution of individuals from other European countries (15.7%). Because of the large population of SSA in Belgium and the high HIV-1 burden in these migrants (Loos et al., 2017), local spread of HIV-1 within the African communities has been anticipated. No evidence however was found to support this assumption. Of the 350 SSA

included, only 7.7% were member of a cluster. These SSA were divided over multiple clusters, many of them with a majority of native Belgians. Strikingly, the majority of SSA in clusters were MSM (66.7%). No evidence was found for the clustered heterosexual transmission among SSA. Some potential bias must however be considered. The migrant population is slower to engage in care than the native population (Van Beckhoven et al., 2015) and more likely to have undiagnosed infection (Loos et al., 2017; Marty L., 2017) so it may be that the selection of SSA is not fully representative. It is also possible that clustered transmission among SSA occurs but to a low extent, which will hamper its detection when analyzing samples collected during a relatively short time frame. In that sense, it is important to consider the higher contribution of SSA in phylogenetic pairs. We found indications that at least part of these paired transmissions took place in Belgium (results not shown). Another factor that needs to be taken into account is that the per-contact probability of HIV transmission in heterosexuals is lower than in homosexuals. This will reduce the chance for expansion of heterosexual transmission clusters (Patel et al., 2014).

### 4.4. Observation of mixed transmission risk clusters

The observation of clusters with a mixture of MSM and HET has been reported before (Kouyos et al., 2010; Ruelle et al., 2013; Hue et al., 2014) but recently, Esbjörnsson et al. found increased mixing of MSM and HET in clustered transmission in the Nordic Countries (Esbjornsson et al., 2016). We also observed a significant number of mixed clusters but noticed that in the majority of them the number of MSM clearly outweighed the number of HET and in many only men were represented. This observation suggests that misreporting of transmission risk may be an important reason for the presence of mixed clusters. Hue et al. calculated that in the UK at least 6.0% of the male HET with subtype B infection were in fact incorrectly reported MSM infections (Hue et al., 2014). The most important reason for misreporting was fear or embarrassment to disclose sex with other men. In line with this hypothesis is the more recent observation of under-reported male-to-male sex in Dutch and Flemish HIV-1 infected donors (van de Laar et al., 2017). Another explanation for the presence of mixed transmission risk clusters may be the involvement of bi-sexual individuals, as was demonstrated for the epidemic in Greenland (Bruhn et al., 2014). Along the same line, IVDU may serve as go-between risk categories.

### 4.5. Cross over of non-B subtypes to the native population

Cross over of non-B subtypes to the native population has been documented in several Western European countries (von Wyl et al., 2011; Brand et al., 2012; Chaix et al., 2013; Dauwe et al., 2015; Fabeni et al., 2015; Ragonnet-Cronin et al., 2016) and increasing trends of non-B infections have been reported (Neogi et al., 2014). These observations fuel the idea of increased involvement of migrants in local transmission. In our population especially clustered transmission of subtype F but also CRF02_AG was observed. The recent introduction and fast spread of subtype F, especially among MSM, was also seen in Italy (Lai et al., 2014; Lai et al., 2010), Spain (Thomson et al., 2012; Delgado et al., 2015; Paraskevis et al., 2015), Switzerland (Castro et al., 2010) and France (Frange et al., 2012). Supplementary phylogenetic analyses of the subtype F variants detected in our population revealed a genetic link with Brazilian (Op De Coul et al., 2000) and Romanian (Gao et al., 1998; Sanabani et al., 2006) strains (data not shown). A South American origin has been established before for subtype F variants found in Italy (Lai et al., 2014), Spain (Delgado et al., 2015) and Belgium (Vinken et al., 2017). Cross over of CRF02_AG to native MSM was observed in Italy (Giuliani et al., 2013), Sweden (Esbjornsson et al., 2016) and France (Chaillon et al., 2017). Why the introduction of subtype F and CRF02_AG has been so successful in our Belgian population is not clear but it is likely due to chance and availability. Due to the high

number of migrants from Central and Western Africa, CRF02_AG is overall the most frequently detected non-B subtype in the country. CRF02_AG was, next to subtype B, the most represented subtype in clusters with at least one member born in Sub-Saharan Africa and clusters with mixed patient origin probably constitute the most important source of non-B subtype introduction in the native population. In the UK, where subtype C accounted for 34.3% of all infections between 2002 and 2010 (UK Collaborative Group on HIV Drug Resistance, 2014), cross over of subtype C to the native MSM was most frequently observed (Ragonnet-Cronin et al., 2016) and in Greece, where subtype A accounted for 51.3% of the infections between 2006 and 2012 (Davanos et al., 2015), clustered MSM transmission of mainly subtype A has been reported (Antoniadou et al., 2014).

### 4.6. Characteristics of patients on isolated branches in the phylogenetic tree

While most phylogenetic studies concentrate on the characterization of members of transmission clusters, individuals on isolated branches in the phylogenetic tree may even so hold important information. In our study they were, as expected, more frequently female, SSA, infected abroad, infected with a non-B subtype and diagnosed with a longstanding infection. We assume that the majority of these infections are dead ends with regard to transmission and infections that could not have been prevented by local prevention initiatives. However, almost half (47.6%) of the 471 patients on individual branches with known country of infection reported being infected in Belgium. Of these locally infected individuals 65.6% of the 215 with known transmission risk were MSM and 29.0% of the 279 with information on the infection time were diagnosed early after infection. This high number of recently infected patients belonging to the most at risk population and likely infected in Belgium but for whom the infection source was not sampled, was unexpected. Possible explanations are that the source partners were diagnosed before 2013, are still undiagnosed or have not yet entered care. Considering the good continuum of care in Belgium and the high percentage of patients successfully treated (Van Beckhoven et al., 2015) the risk for transmission from patients diagnosed before 2013 is assumed to be low. The involvement of undiagnosed infections however cannot be excluded. Using a back calculation model and the HIV surveillance data collected between 2006 and 2015, the number of individuals living with undiagnosed HIV in Belgium was estimated at 2800 (95% CI 2200–3500) (Marty et al., 2017). From a prevention perspective, it will be important to further explore the characteristics of individual branched patients, ideally using phylogenetic data obtained in real time and combined with information on patients' behavior.

### 4.7. Limitations

Our study has some limitations. Although we were able to include the majority of patients for whom a baseline drug resistance analysis has been performed, the selection comprised only about 60.0% of all newly diagnosed individuals registered in the national surveillance database during the study period. An important reason for missing patients is that all individuals seeking medical care in Belgium and diagnosed with HIV infection are registered, including those with an HIV infection already established abroad and those with a history of antiretroviral therapy. For obvious reasons, the latter are not included in the baseline drug resistance testing program. Another potential bias may result from the fact that data on transmission risk or country of infection are not always available and that the reliability of these data is difficult to evaluate. We assume however that the number of patients with trustworthy data is high enough to minimize eventual errors resulting from misreporting.

We used only a small region of the viral genome for phylogenetic analysis. Hue et al. however, clearly demonstrated the suitability of this part of the HIV-1 *pol* region for phylogenetic analysis and

reconstruction of transmission events (Hue et al., 2004).

Paired patients were not considered as clusters in this study because it was clear from the comparison between paired and clustered patients and between paired and individual branched patients that paired patients represent a heterogeneous group. Although the importance of pairs as sources of further ongoing transmission is unclear, it is assumed that a large part of the pairs reflect transmission among regular partners with infection diagnosed as part of partners testing (Lubelchek et al., 2015). In many phylogenetic studies however, pairs are classified and analyzed as transmission clusters and this may introduce bias (Brown et al., 2009; Yerly et al., 2009; Frange et al., 2012; Hue et al., 2014; Ragonnet-Cronin et al., 2016).

### 4.8. Conclusions

In conclusion, this study combined phylogenetic, demographic, epidemiological and laboratory data to show that local HIV-1 transmission in Belgium remains almost exclusively driven by native MSM despite the overall heterogeneous composition of the HIV-1 infected population with regard to patient origin and transmission route. Participation of SSA in clustered transmission was rare and in majority also MSM driven. Transmission clusters of mixed patient origin may constitute opportunities for the cross over of non-B subtypes to the native MSM population and this is an evolution that needs to be monitored.

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### Competing interests

The authors have no competing interests to declare.

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