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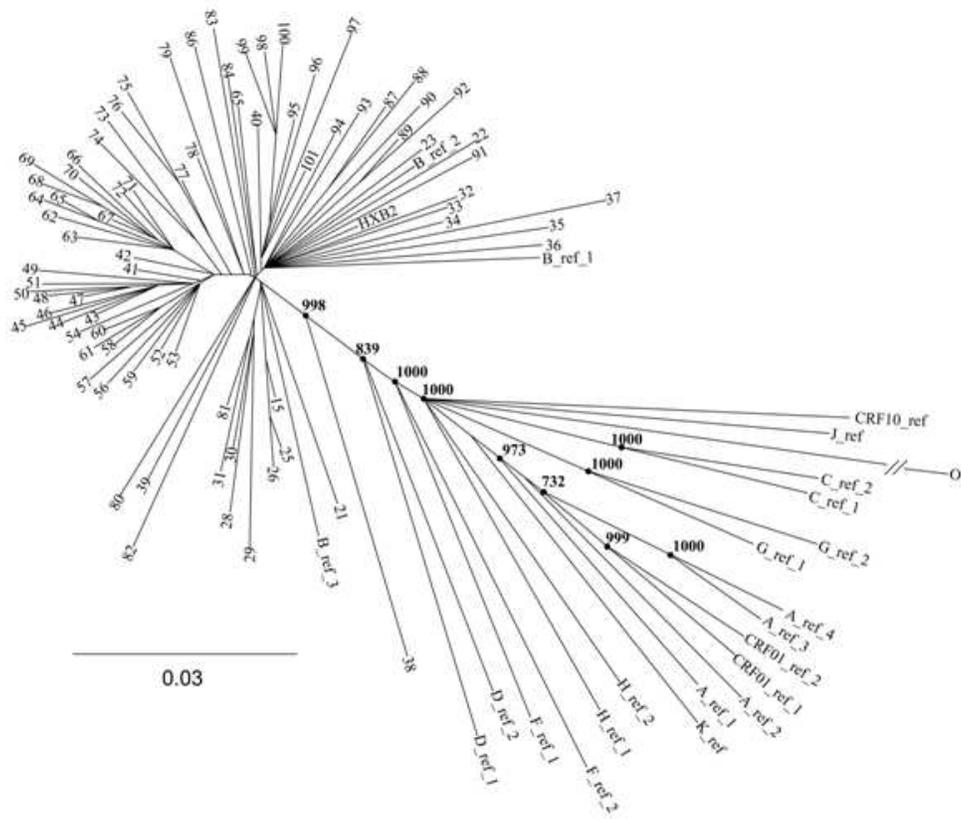


Characteristics of HIV-1 Non-B Subtype Infections in Northwest Poland

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Keywords:	Subtype D, HIV subtypes, phylogenesis, HIV transmission, Poland



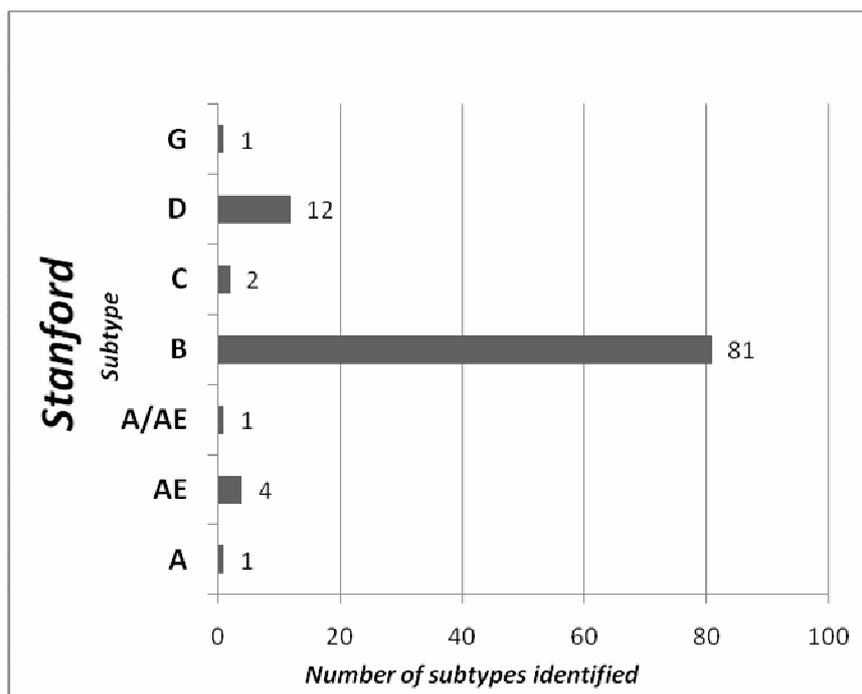
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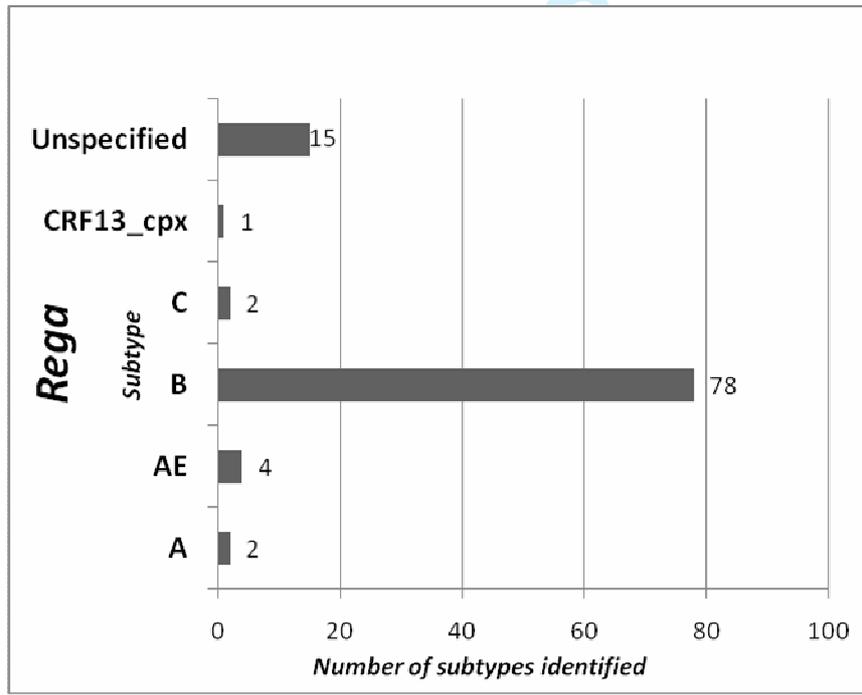
Neighbor-joining tree showing phylogenetic relationships of subtype B isolates to the HIV-1 M group reference strains
25x21mm (600 x 600 DPI)

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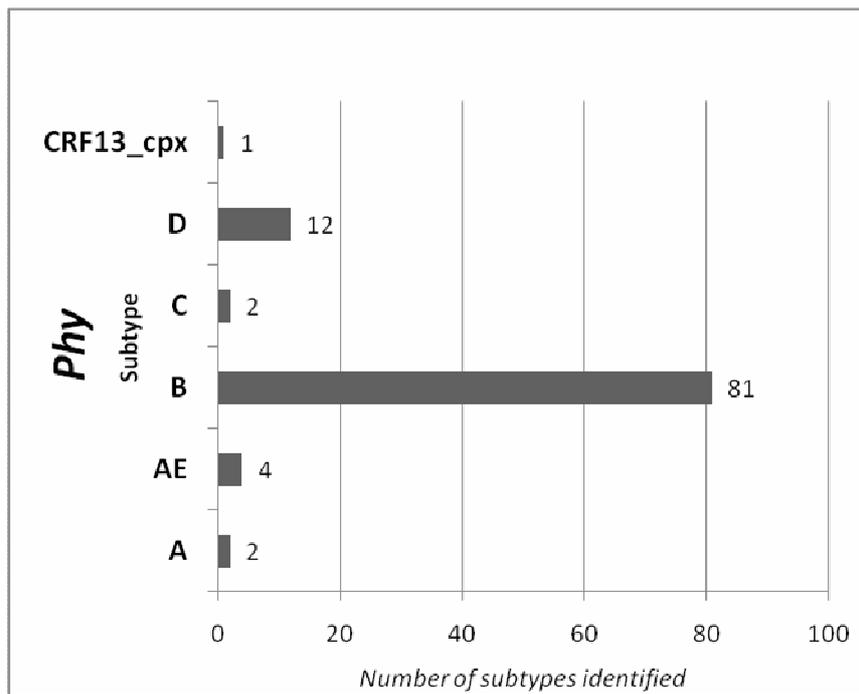
Fig 2



A



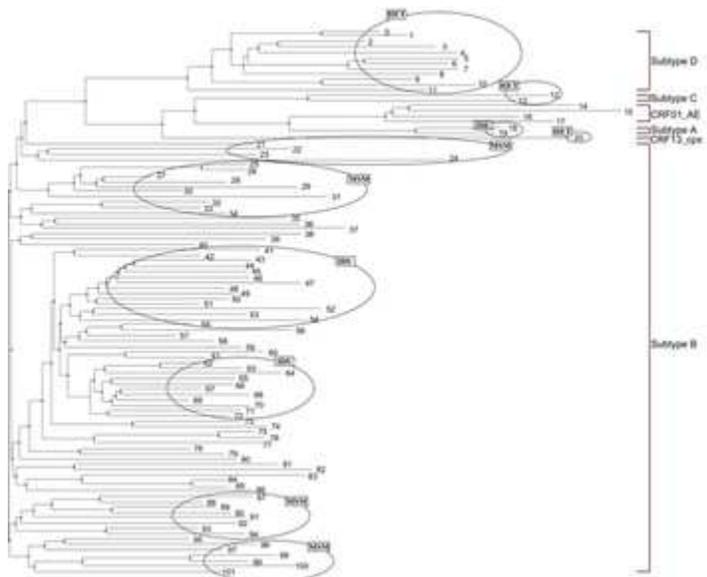
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C

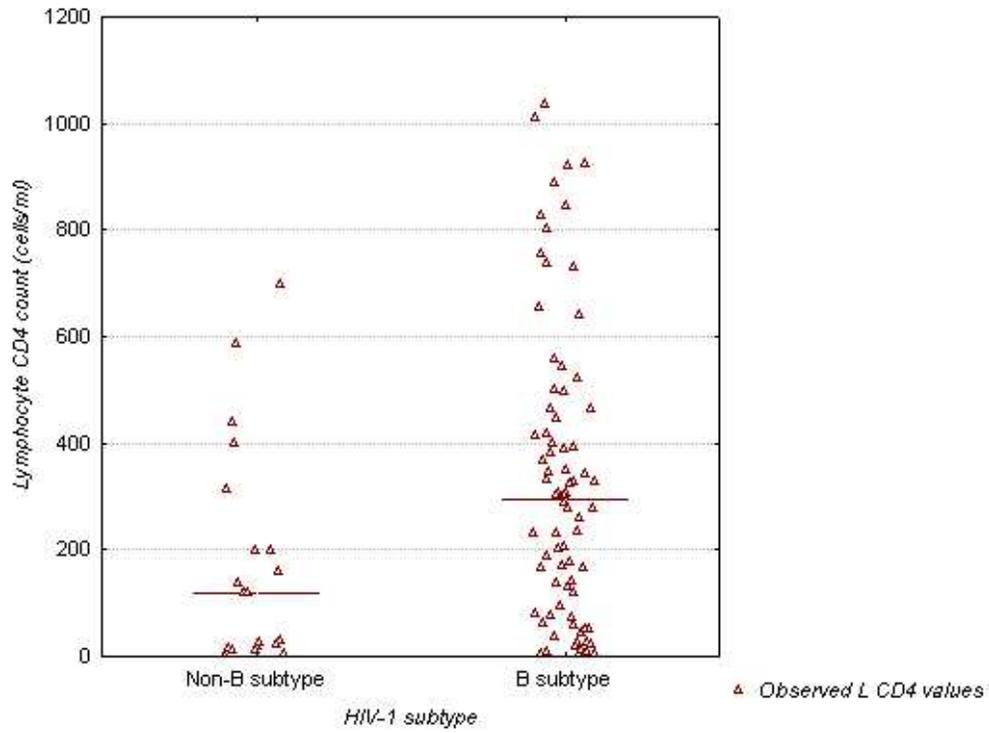
Peer Review

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Phylogenetic tree reconstruction
15x12mm (600 x 600 DPI)

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Baseline CD4+ lymphocyte counts in the analysed groups.
137x103mm (96 x 96 DPI)

Review

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5 1 **Title:** Characteristics of HIV-1 Non-B Subtype Infections in Northwest Poland.
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11 4 Grzegorz Stańczak², Anna Boroń-Kaczmarek¹.

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22 8 **Running head:** HIV-1 Non-B subtypes in Northwest Poland
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Abstract

The number non-B subtype HIV-1 infections in Europe have been increasing even though major regional differences have been observed. This trend was investigated in northwestern Poland using sequence and epidemiological data from a cohort of 102 HIV-1-infected patients from Szczecin, Poland. HIV-1 subtypes were defined by phylogenetic analysis of viral reverse transcriptase- and protease-partial coding regions and results were compared with online subtyping by Stanford and REGA tools. Subtype analysis using on-line subtyping methods produced varying results if compared to phylogenesis, with concordant variant assignment obtained for 98% (100/102) of sequences by Stanford and 85% (87/102) by REGA. In the population studied, non-B subtype infections comprised 21% of the infections and consisted of subtype D (57%, n=12), CRF01_AE (19%, n=4), A and C clades (9.5%, n=2) and the CRF13_cpx recombinant isolate (4.8%, n=1). Patients carrying non-B subtypes were predominantly heterosexuals with high percentage (57%) of women observed in the group. All HIV-1 non-B women were Caucasian with majority (83%) of infections acquired in Poland, however among 12 travelers included in the study a higher proportion of non-B infections was noted (50%, $p=0.01$). Moreover, lower baseline lymphocyte CD4 counts ($p=0.01$), higher baseline HIV-1 viraemia ($p=0.08$) and a more advanced stage of the disease ($p=0.03$) were observed among individuals infected with non-B subtypes. The data indicated that the proportion of HIV-1 non-B subtype infections was higher than previously reported in Poland consisting of a high subtype D prevalence. Furthermore, subtype D transmission occurred primarily between heterosexual Caucasian individuals from this region.

Key Words: HIV subtype D; non-B subtypes; HIV transmission, phylogenetic analysis, Poland

INTRODUCTION

The transmission of major (M) and non-major (N) groups of Human Immunodeficiency Virus (HIV) from chimpanzees, along with outlier (O) group viruses of likely gorilla ancestry, were the initial steps in the evolution of the human HIV epidemic [Keele et al., 2006, Van Heuverswyn F et al. 2006]. Furthermore, high reverse transcriptase error rates have resulted in additional diversification into different HIV strains [Rambaut et al., 2004]. Sequence-based research has identified a range of subtypes (clades) and recombinant forms within the most common HIV-1 M group. Subtype C is the most common clade worldwide (approximately 50% of all HIV-1 clades) and is found predominately in Sub-Saharan Africa and the Indian subcontinent whereas clades A, D and CRF01_AE are observed less frequently (12%, 6.5%, and 2.5%, respectively). Circulating recombinant forms (CRFs) including CRF01_AE and CRF02_AG have been reported mainly in East-Asia and West Africa, while novel recombinants continue to be identified worldwide [Taylor, Hammer, 2008]. In present time Europe, subtype B remains the most common clade identified, particularly among Caucasians, however, subtype B represents only 10% of global HIV-1 infections [Paraskevis et al., 2009]. In addition, injection drug use and male homosexual contacts are commonly associated with subtype B infections. occur . In contrast, transmission of non-B subtypes is most common between heterosexuals [Kantor et al., 2005] and in Poland, this pattern is similar with respect to subtype B infections predominating in injection drug users and homosexuals [Chaix et al., 2003, Stańczak et al., 2007]. However, the number of non-B subtype infections among Caucasians has been increasing. The current reported prevalence of non-B subtype infections in Poland is 11.2% with more than half of these belonging to the C and D subtypes and the CRF03_AB; CRF05_DF and CRF01_AE recombinant clades [Stańczak et al., 2007]. Although most antiretroviral studies and observational cohorts have analyzed populations comprised predominantly of clade B-infected individuals, others have indicated that virological and immunological treatment responses are similar regardless of the subtype [Bocket et al., 2005; Holguin et al., 2006].

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5 65 The city of Szczecin, located in northwestern Poland, has an HIV-1 incidence of 0.35/100,000
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7 66 compared to an incidence rate of 0.47/100,000 nationwide [Czarkowski et al., 2007]. In this region,
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9 67 intravenous drug use used to be the predominant HIV transmission route, however, sexually
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11 68 transmitted infections between Caucasians now predominate (immigrants comprise less than 1% of
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13 69 observed HIV infection cases). Since the port of Szczecin is strategically situated on the Polish-
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15 70 German border, HIV infections transmitted from sailors and young homosexual males travelling to
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17 71 Germany are common (internal center data) and HIV infections in Poland (and in the region) are
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19 72 more prevalent among males. However, the number of infected women among newly diagnosed
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21 73 subjects has been increasing recently (from 21% in 2006 to 24% in 2008) (National Institute of Public
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23 74 Health, data available on-line www.pzh.gov.pl/oldpage/epimeld/index_p.html).
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28 75 In this study, HIV-1 transmission data is correlated with demographic and epidemiologic data
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30 76 from a northwestern Polish cohort. HIV isolates obtained from patient plasma samples were
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32 77 subtyped and subjected to phylogenetic reconstruction analyses based on partial protease and
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34 78 reverse transcriptase sequences. This analysis revealed a larger than expected number of non-B
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36 79 subtype isolates, suggesting a change in the epidemiology of HIV infection. Awareness of the
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38 80 infecting strains in respective populations will be critical for the administration of appropriate
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40 81 antiviral interventions.
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METHODS

Population

This study analyzed HIV-1 sequence data from 102 patients (73 male, 29 female) that were followed-up routinely at the Department of Infectious Diseases and Hepatology, Pomeranian Medical University, Szczecin, Poland. With the exception of one patient, all were Caucasian and acquired their infections sexually (n=68) or by injection drug use (n=34). HIV sequencing was performed in patients failing antiretroviral regimens (n=37) and in patients with newly diagnosed infections as a means of identifying new subtype strains or newly transmitted drug resistant strains (n=65). The mean age of the patients was 38.7 years (median 36±9.1, range 23-67 years).

Sample Collection and Analysis

Plasma samples collected from patients between 1996-2009 were stored at -80°C and analyzed retrospectively. HIV RNA extraction (with subsequent sequencing of the protease and reverse transcriptase genes) was performed using a 2.8 Viroseq HIV-1 Genotyping System (Abbott Molecular, Illinois, USA) and the Trugene Genotyping system (Bayer HealthCare, Tarrytown, USA) according to the manufacturer's protocols. Fasta sequences (*pol* sequence lengths of 1297-1302 base pairs [bp]) obtained in the process were used in this study. All sequences were aligned using the Clustal X2.0.10 software (www.clustal.org) with manual editing by Bioedit (version 7.0.5., Ibis Therapeutics, Carlsbad, USA). Phylogenetic analyses were performed using the Phylip Package version 3.69 (University of Washington, Department of Genome Sciences, USA) with evolutionary distances estimated using DNADIST (Kimura two-parameter method) and bootstrapping carried out using SEQBOOT (1000 replicates). Phylogenetic reconstructions were performed by NEIGHBOR (neighbor-joining method) for tree construction. Trees were visualized with TreeView (version 1.6.6, University of Glasgow, UK) or Tree Figure Drawing Tool (version 1.2.2, University of Edinburgh, UK).

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5 108 For phylogeny-based subtype determination, a selection of reference sequences listed in the HIV
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7 109 Sequence Compendium 2009 (Los Alamos National Laboratory Los Alamos, U.S.A.
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9 110 <http://www.hiv.lanl.gov>, two sequences for each subtype and at least one for each CRF), as well as
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11 111 sequences from Central Europe for two common subtypes (gen-bank IDs AF34733, DQ823362
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13 112 (subtype B), DQ823357, EF545108 (Subtype A)) were used.

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16 113 Tree topology analysis was used to assign subtypes based on a high level of bootstrap
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18 114 support (>700) for each subtype or CRFs examined. Additionally, subtype determinations were
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20 115 performed using two rapid on-line tools: the REGA genotyping 2.0 tool
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22 116 (<http://bioafrica.mrc.ac.za/rega-genotype/html/subtypinghiv.html>) and the Stanford HIV database
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24 117 (<http://hivdb.stanford.edu>) for verification and comparison.
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Statistics

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32 120 Statistical analyses for nominal variables were performed using Chi-square and Fisher's exact
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34 121 tests for small groups using EPI6 Statcalc software (Department of Mathematics, University of
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36 122 Louisiana-Lafayette, Lafayette, LA, USA); for nominal variables, the U-Mann Whitney test was
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38 123 performed using Statistica software (Statsoft, Tulsa, USA). P-values of <0.05 were considered
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40 124 significant; with values in the range of 0.1-0.051 regarded as borderline statistically significant.
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RESULTS

Using phylogeny-based subtyping methods it was possible to assign an HIV subtype to each isolate examined with bootstrap support of 100%. Two phylogenetic trees, drawn separately for B and non-B subtypes are shown (Fig. 1 A,B). Subtype B infections were the most common (n=81; 79%), although the prevalence of non-B strains was 21% (21/102). Of these non-B strain infections, 57% (n=12) were subtype D, 19% were CRF01_AE (n=4), 9.5% were A and C clade infections (n=2); 4.8% were of the CRF13_cpx recombinant variant (n=1). Subtype analysis results using on-line subtyping methods produced varying results. The REGA genotyping tool was unable to assign a subtype to all subtype D and three subtype B isolates. On the other hand, analysis using the Stanford HIV database identified one case to be infected with subtype A/CRF01_AE recombinant (protease– clade A, reverse transcriptase - CRF01_AE) that were assigned as subtype A by both phylogenetic analysis and REGA while a strain classified as CRF13_cpx was classified as subtype G by the Stanford database (Figure 2).

For each patient examined, the route of transmission was identified: 33% (34/102) resulted from male homosexual contacts, 31% (32/102) by injection drug use and 28% (29/102) were heterosexual transmissions. In 7% (7/102) of cases it was not possible to define equivocally the transmission route (patients reported both unprotected heterosexual and homosexual sexual contacts or unprotected sexual contacts combined with injection drug use), therefore a mixed route of infection was assigned.

Clustering of sequences with concordant transmission routes was noted as indicated in Figure 3. Infections with clade C, D or CRF13_cpx variants were acquired exclusively through heterosexual contact while subtype A- or CRF01_AE-infected patients acquired their infections as a consequence of injection drug use. Two patients presented with a variant CRF01_AE infection acquired through heterosexual contact (from Cambodia and Germany). A significant number of patients infected as a consequence of heterosexual exposure were infected primarily with non-B subtypes (versus B subtype-infected patients) ($p < 0.01$, 95% CI [0.04-0.16], OR: 0.04) with a significantly higher number of women presenting with this type of infection ($p = 0.01$, 95%CI [0.06-0.06],

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4 151 OR: 0.2). All women with non-B subtype infections were Caucasian and the majority (83%) of
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6 152 infections were acquired in Poland. Of these, only two reported commercial sex work with the
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9 153 remaining admitting to casual, infrequent heterosexual contacts. A total of 12 cases reported risky
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11 154 sexual behavior outside of Poland; four patients were professional sailors reporting exposure in
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13 155 Germany, one in the UK, Cambodia and Vietnam, respectively. Among travelers, non-B subtype
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16 156 infections (four CRF01_ AE cases, one from each clade D and CRF13_cpx cases) were statistically
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18 157 greater (n=6, 50%, p=0.01, Fisher's exact test) compared to the rest of the cohort.
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21 158 Most of HIV-diagnosed patients in the study (61/102, 59.8%) were either symptomatic or in
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23 159 the AIDS stage. Baseline lymphocyte CD4 counts below 350 cells/ μ l were noted in 64 (62%)
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25 160 individuals. Significantly lower baseline lymphocyte CD4 counts (p=0.01), higher baseline HIV-1
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27 161 viremia (borderline statistical significance, p=0.08) and a more advanced stage of the disease (AIDS
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29 162 cases notably more common, p=0.03) were observed among individuals infected with non-B
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31 163 subtypes. Patient characteristics are described in Table I, with distribution of lymphocyte CD4 counts
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34 164 and viral load levels among subtype B and non-subtype B groups presented in Figures 4 and 5.
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DISCUSSION

This study assessed the HIV subtypes present in a cohort of patients from Szczecin, Poland. HIV strains were subtyped based on protease and reverse transcriptase sequences using three distinct typing tools. Subtyping results from the two on-line rapid tools (REGA genotyping 2.0 and the Stanford HIV database) were compared with phylogenetic analysis. This comparison revealed that the REGA tool was not able to classify equivocally subtype D strains in all cases, however, it classified correctly the CRF13_cpx recombinant strain assigned to subtype G by the Stanford database. The subtyping analysis carried out using REGA matched the A, CRF01_AE and C clades in accordance with the phylogenetic analysis results, while the Stanford analysis inappropriately classified one subtype A sample as an A/CRF01_AE recombinant (Figure 2A-C). However, the Stanford database tool correctly assigned all B clade samples (100%) compared to the 96.2% rate observed using the REGA tool. The failure to identify subtype B strains in three cases using the REGA tool was probably due to shorter sequence lengths. As described previously [Holguín et al., 2008], also in this study misclassification of some non-B subtypes was observed when using the rapid on-line tools, which reinforces the need to use phylogenetic analyses to confirm subtyping results.

To date, the HIV subtype distribution in Poland was similar to that reported for Western Europe, with subtype B strains being the cause of the majority of infections [Hemelaar et al., 2006, Stańczak et al., 2006]. However these data were based on data from rapid subtyping tools in the absence of phylogenetic analyses. In the population described in this study, the number of non-B subtypes was higher than the published national average reported between 1996-2004 (21% versus 11.2%) [Stańczak et al., 2006]. Of the non-B variants identified in this study, subtype D was the most common (57%). Furthermore, among the patients examined, there were a surprisingly higher percentage of C and D clades among Polish women infected as a consequence of heterosexual exposures (75%).

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5 191 Until this study was carried out, it was believed that non-B transmissions between heterosexual
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7 192 Caucasians occurred only infrequently [Böni et al., 1999; Ramirez-Piedad et al., 2009, Thomson et al.,
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9 193 2007], however, the number of new infections resulting from non-B clades is increasing in this
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11 194 population [Holguín et al., 2008]. In this study all but one infection with subtype D was related to
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13 195 heterosexual transmission among Caucasians and such spread of this non-B clade strain has not
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16 196 been reported to date. The data presented in this report also suggest that the number of HIV
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18 197 infections resulting from non-B variants in Poland is increasing. Although the HIV epidemic initially
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20 198 was a result of subtype B infections, the rise in cases resulting from non-B variants in the recent years
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22 199 reflects a change in the epidemiology of this disease [Stańczak et al. 2006] and this trend parallels
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25 200 what has been observed in Western Europe [Geretii 2006].
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28 201 Non-B subtypes have also been identified across Europe with variable prevalence, however,
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30 202 European data on non-B subtype cases resulting from sexual contact are commonly seen in
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32 203 populations with a high immigrant population or among individuals who are epidemiologically linked
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34 204 [Böni et al., 1999]. For example, in Germany, the circulating recombinant forms, CRF02_AG and
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36 205 CRF01_AE, are most commonly observed in the context of smaller subtype C and D infections [Sagir
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38 206 et al., 2007]. In the UK, the highest non-B prevalence was observed among black African and black
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40 207 Caribbean patients [Hemelaar et al., 2006], while in Spain, a high frequency of subtype G was
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42 208 reported [Holguín, Alvarez, Soriano, 2002].
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46 209 The high frequency of non-B subtypes observed in the studied cohort might be related to the
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48 210 fact that the study center was located in a major port city. Sailors traveling the world might have
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50 211 imported non-B variants which spread in the local population. Indeed, the CRF13_cpx variant was
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52 212 identified in a male sailor traveling from Africa who reported heterosexual contacts in this continent,
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54 213 however, only in one subtype D case exposure outside Poland was reported, while review of the
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56 214 remaining medical records of subtype D patients revealed that the infections were acquired in
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58 215 northwestern Poland. The surprising dominance of non-clade B infections (17/21, 81%) in
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60 216 heterosexuals, particularly women (12/21, 57%) was a novel finding. The majority of the women in

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5 217 this group never admitted to sexual promiscuity (19/21) and HIV diagnosis was unexpected for both
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7 218 patients and clinicians. Their unknown infection status combined with low social awareness of HIV
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9 219 risk factors in Poland [Izdebski 2003] and infrequent HIV testing among heterosexual individuals
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11 220 might explain the high number of HIV patients observed in this population. As a result, there were a
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13 221 large proportion of symptomatic/AIDS patients, with late diagnoses more common among individuals
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15 222 bearing non-B variants compared to subtype B-infected patients. This result was consistent with the
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17 223 reported tendency for late testing in Poland and Europe [Bander et al., 2009; Adler et al., 2009].

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20 224 Since antiretroviral treatment initiation is recommended immediately in case of the late HIV
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22 225 diagnosis, it is important to determine the virus subtype due to its possible influence on disease
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24 226 transmission and progression and tropism [Geretii 2006]. This is especially important in subtype D
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26 227 infection cases, as observed in this study. It has been demonstrated that in clade D-infected Kenyan
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28 228 women the risk of death is more than two-fold higher compared to clade A-infected individuals.
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30 229 Moreover, depletion of CD4+ lymphocytes was more rapid in subtype D-infected patients, suggesting
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32 230 that disease progressed more rapidly in these patients than in non- D infected patients [Baeten
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34 231 2007]. This phenomenon might be related to the higher rates of the CXCR4 or mixed tropisms
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36 232 observed among individuals with subtype D infections which and faster progression to AIDS [Kaleebu
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38 233 2007]. Indeed, time to AIDS (defined as CD4 \leq 200 cells/ μ l) among Ugandans with subtype D
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40 234 infections was shorter compared to subtype A-infected individuals with a significantly higher risk of
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42 235 death [Kiwauka 2007]. The nature of HIV progression rates in subtype D-infected individuals of
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44 236 Caucasian origin remains to be defined.

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47 237 A limitation of the current study relates to the subtyping methods used. The HIV-1
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49 238 genotyping platforms available at the time of the study (2.8 Viroseq HIV-1 and Trugene Genotyping
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51 239 systems) are used for carrying out HIV drug resistance analyses. Although both tools have been
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53 240 shown to be highly efficient for B clade sequencing, their detection thresholds for non-B variants is
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55 241 lower [Beddows 2003]. Therefore, the possibility that some variants may have gone undetected
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57 242 exists, however, only these detection methods were available. Moreover, the non-B subtype viral
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5 243 loads observed may be inaccurate since most viral load assays are optimized for HIV-1 B clades . This
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7 244 limitation is currently being overcome by the real-time PCR platforms optimized for an array of the
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9 245 most common HIV-1 subtypes and CRFs.

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11 246 This study demonstrated that HIV subtypes associated with infections of Caucasians from
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13 247 northwestern Poland were genetically diverse. It is likely that HIV non-B subtypes were acquired from
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15 248 travelers visiting African and West European countries that spread the infection primarily via
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17 249 heterosexual contacts. The dissemination of subtype D strains among Caucasians was identified in
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19 250 symptomatic and AIDS-stage patients, a large majority of which were women. In general, non-B
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21 251 subtype-infected individuals tended to be diagnosed with symptomatic infections or AIDS and these
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23 252 patients had higher baseline viral loads and lower CD4+ lymphocyte counts compared to subtype B-
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25 253 infected patients. This distribution is likely related to the low HIV testing frequency in Poland
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27 254 [European Centre for the Epidemiological Monitoring of HIV/AIDS, 2007], especially among
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29 255 populations traditionally not associated with risky sexual or drug use-related behavior.

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34 256 In light of the increased dissemination of non-B subtypes and its impact on treatment
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36 257 responses and disease progression, it will be important to carry out subtype analyses of strains
37
38 258 isolated from respective patients and a careful review of epidemiological data will be necessary as
39
40 259 described previously [Hemelaar et al., 2006]. Regional changes in HIV subtype distribution and
41
42 260 analysis of mutation patterns associated with transmission will play a significant role in clinical
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44 261 management.

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SEQUENCE DATA

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53 264 HIV-1 non-B subtype sequences were submitted to the Gen Bank and may be accessed with
54
55 265 the following IDs: Subtype A: GU906861, GU906877; Subtype C: GU906868, GU906870; Subtype D:
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57 266 GU906857, GU906858, GU906860, GU906863, GU906864, GU906865, GU906866, GU906867,
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59 267 GU906871, GU906872, GU906873, GU906874; CRF01_AE: GU906859, GU906862, GU906869,
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268 GU906876; CRF13_cpx: GU906875.

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4 393 **Figure Legends**
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7 394 Fig. 1. Neighbor-joining analysis. Neighbor-joining tree showing phylogenetic relationships of subtype
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9 395 B (A) and non-subtype B (B) isolates to the HIV-1 M group reference strains. Bootstrap values >700
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11 396 are listed at the base of each branch. Subtype for each reference isolate are indicated on external
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13 397 nodes. From the final version of the phylogenetic tree reference CRFs sequences for the variants not
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15 398 observed in this study were removed.
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20 400 Fig. 2. HIV subtype characterization. HIV-1 M subtype and CRF assignment by phylogenetic analysis
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22 401 (A), the REGA genotyping tool 2.0 (B) or the Stanford HIV drug resistance database (C).
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27 403 Fig. 3. Phylogenetic tree reconstruction. Each patient was coded with a unique identifier and their
28
29 404 respective isolates analyzed based on HIV-1 protease and reverse transcriptase sequences. HIV-1
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31 405 variants are indicated to the right. Bootstrap values (1000 sets) are indicated if above 700. Branch
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33 406 length reflects evolutionary distance. Clusters of patients with similar exposure modes are circled.
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35 407 MSM, Men who have sex with men; IDU, Injection drug users; HET, heterosexual transmissions.
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39 409 Fig. 4. Baseline CD4+ lymphocyte counts in the analysed groups. Each symbol represents the number
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41 410 of CD4+ cells/ μ l from either HIV-1 B or non-B subtype-infected individuals.
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47 412 Figure 5. Baseline HIV-1 viral loads in the analysed groups Each symbol represents the number of
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49 413 copies/ml from either HIV-1 B or non-B subtype-infected individuals.
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414 Table I. Subtype-based characteristics of the study population.

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	Subtype A	Subtype B	Subtype C	Subtype D	CRF13_cpx	CRF01_ AE	Non-B (total)	Entire group
Number of individuals	2	81	2	12	1	5	21	102
Female (%)	50%	21%	100%	58%	0	50%	57%	28%
HIV infection stage at diagnosis (%)								
Asymptomatic	50%	44%	100%	17%	0	0	24%	40%
Symptomatic	0	31%	0	42%	100%	0	29%	30%
AIDS	50%	25%	0	42%	0	100%	48%	29%
CD4 T cell category at diagnosis								
>500 cells/ μ l (%)	0	20%	100%	0	0	0	10%	20%
200-499 cells/ μ l	50%	40%	0	25%	0	0	19%	40%
<200 cells/ μ l	50%	40%	0	75%	100%	100%	71%	40%
Median baseline lymphocyte CD4 count, cells/ μ l (IQR)	198	295.5 (85.5- 461)	644 (616- 672)	119 (25.5- 237,5)	140	17 (14-18)	119,5 (18-229)	234.5 (62-418)

Median baseline viral load ¹ , copies/ml (IQR)	Not available	95,300 (21,200-435,000)	34,450 (25,675-43,225)	370,000 (180,000-480,000)	Not available	390,000 (96,500-590,000)	275,000 (76,900-507,500)	100,000 (24,800-480,000)
Mean age at the diagnosis, years (range)	29 (18-40)	38.2 (23-67)	29 (28-30)	44.6 (32-60)	45	34.8 (21-54)	40.9 (26-60)	38.7 (23-67)
Transmission route								
IDU	100%	35%	0	0	0	50%	19%	31%
Heterosexual	0	15%	100%	100%	100%	50%	81%	28%
MSM	0	42%	0	0	0	0	0	33%
Mixed ²	0	9%	0	0	0	0	0	7%

416 ¹Baseline viral loads available for 51/102 patients (50%). 39/81 (48%) patients presented with subtype B infections and 12/21 (57%) patients presented with
417 non-B clade infections.

418 ²Mixed route of transmission was assigned for individuals with probable infections from exposures resulting from either intravenous drug use (IDU)/men
419 having sex with men (MSM) or from MSM/heterosexual exposure

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