

HIV-1 early and late diagnosis in the Emilia Romagna Region (Italy): a three year study

Giuseppina Musumeci¹, Giacomo Magnani², Isabella Bon¹, Serena Longo¹, Alessia Bertoldi¹, Anna Maria Degli Antoni³, Maria Rita Rossi⁴, Alessandro Ruggieri⁵, Vittorio Sambri⁶, Simona Semprini⁶, Laura Sighinolfi⁷, Maria Alessandra Ursitti², Alessandro Zerbini⁸, Vincenzo Colangeli⁹, Leonardo Calza⁹, Alba Carola Finarelli¹⁰, Erika Massimiliani¹⁰, Maria Carla Re¹

¹Department of Experimental, Diagnostic and Specialty Medicine (DIMES), Microbiology Section, University of Bologna, Bologna, Italy;

²Department of Infectious Diseases, S. Maria Nuova IRCCS Hospital, Reggio Emilia, Italy.

³Department of Infectious Diseases and Hepatology, Parma Hospital, Parma, Italy;

⁴Department of Infectious Diseases and Microbiology, Arcispedale S. Anna, Ferrara, Italy;

⁵Malattie Infettive Unit, Piacenza, Italy;

⁶The Greater Romagna Hub Laboratory, Unit of Microbiology, Pievesestina, Cesena and Department of Experimental, Diagnostic and Specialty Medicine (DIMES), Microbiology Section, University of Bologna, Bologna, Italy;

⁷Department of Infectious Diseases, S. Anna Hospital, Ferrara, Italy;

⁸Clinical Immunology, Allergy, and Advanced Biotechnologies Unit, Diagnostic Imaging and Laboratory Medicine Department, IRCCS-Arcispedale Santa Maria Nuova, Reggio Emilia, Italy;

⁹Department of Medical and Surgical Sciences, Section of Infectious Diseases, S. Orsola-Malpighi Hospital, University of Bologna;

¹⁰Public Health Service - Directorate General for Health and Social Policy - Emilia-Romagna Region, Italy

SUMMARY

It is crucial to establish the timing of infection and distinguish between early and long-lasting HIV-1 infections not only for partner notification and epidemiological surveillance, but also to offer early drug treatment and contain the spread of infection.

This study analyzed serum and/or plasma samples with a first positive HIV antibody/antigen result coming from different Medical Centers in the Emilia Romagna Region, North East Italy, using the avidity assay, Western Blotting, RNA viral load, CD4 cell counts and genotyping assay.

From May 2013 to May 2016, we certified 845 new HIV-1 infections, 18.7% of which were classified on the basis of avidity index as recent infections and 81.3% as long-lasting infections, with an estimated conversion time exceeding six months at the time of study. Western Blotting showed reactivity to only one or two HIV-1 proteins in recently infected patients (RIPs), while a complete pattern to gag, env and pol proteins was observed in most long-lasting infected patients (LLIPs). The median age, gender, nationality and risk transmission factors were comparable in RIPs and LLIPs. Phylogenetic analysis performed in available plasma disclosed B strains, non-B subtypes and circulating recombinant forms (CRFs) in both groups of patients, with a major presence of CRFs in non-Italian HIV subjects.

The large number of patients unaware of their HIV status makes it crucial to discover hidden epidemics and implement appropriate targeted public health interventions.

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INTRODUCTION

Standard serological tests able to establish HIV-1 infection show high levels of sensitivity and reproducibility and play an essential role in the diagnosis and prevalence of HIV infection (CDC, 2014; CDC, 2016; Abimiku *et al.*, 2016). Nevertheless, available techniques cannot determine the incidence of new infections or differentiate between recent and chronic infection (Re *et al.*, 2008, Re *et al.*, 2012; Bon *et al.*, 2015; Simmons *et al.*, 2016).

Since the acute phase of HIV infection is short-lived and

the clinical manifestations (Daar *et al.*, 2008) often resemble those of other viral infections, diagnosis may be established long after the onset of infection.

Serological tests identifying biological markers of recent infection are fast becoming an alternative method to cohort studies and mathematical models (Chawla *et al.*, 2007, Murphy and Parry, 2008; Simmons *et al.*, 2016). To understand the current state of HIV infection dynamics, it is necessary to calculate the incidence of infection in the population (Suligo *et al.*, 2003; Re *et al.*, 2008). This will be essential to understand the necessary prevention programs and to improve clinical studies and vaccine trials designed to reduce transmission rates. People who recently acquired HIV infection belong to a high-risk category for transmission to others because of the high viral load present in the early phases of infection.

It is crucial to establish the timing of infection and distinguish between early and long-lasting HIV-1 infection

Key words:

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Corresponding author:

Maria Carla Re

E-mail: mariacarla.re@unibo.it

not only to determine the incubation period and for partner notification and epidemiological surveillance (World Health Organization, 2014), but also for decision-making on drug treatment, since it is easier to achieve immune recovery and decrease the viral load if antiretroviral therapy is started in the early phases of infection. Moreover, identifying and classifying recent HIV infections is a priority not only to characterize recent HIV infections but also to discover *hidden epidemics* and implement appropriate targeted public health interventions (Moschella *et al.*, 2014).

On the basis of previous studies (Re *et al.*, 2010; Suligoi *et al.*, 2011; Simmons *et al.*, 2016) on the serological methods used to identify a recent infection, we focused our attention on the avidity test, based on evidence that the bond strength between IgG antibodies and the antigen increases in proportion to the time elapsing from the date of seroconversion and able to work on a single sample of serum taken at a single point in time. Moreover, the avidity test distinguishes HIV patients with an infection acquired in the last six months and patients, so-called *late presenters*, with a long-lasting infection (Orchi *et al.*, 2013).

Even if acute, early, recent, chronic and late-stage infections have been clearly defined (Suthar *et al.*, 2015), we report the data obtained by avidity assay (able to measure the strength of the binding between IgG antibody and the corresponding antigen) in two groups of patients: the first defined as recently infected patients (RIPs) and comprising acute, early and recent infections, and the second defined as long-lasting infected patients (LLIPs) comprising chronic and late stage infections.

This study has as its objectives to determinate the incidence, in the analyzed period, of cases with recent HIV infections, compare them with population arriving late at diagnosis, as regard the immunological status, viral load and HIV-1 subtypes circulation and to characterize the HIV-1 types and subtypes responsible for HIV infection in our region. So, we have analyzed 845 serum samples collected from May 2013 to May 2016 resulting HIV-1 positive for the first time with any previous HIV test or with a previous negative result and 161 available plasma samples for phylogenetic analysis of HIV-1 *pol* gene. We report our results in RIPs and LLIPs including sociodemographic characteristics, immunological (CD4 count, CD4/CD8 ratio) and virological markers (HIV RNA viral load, HIV-1 subtyping).

MATERIALS AND METHODS

Samples. Within regional surveillance system, focused on HIV new diagnosis, serum and/or plasma samples were collected from May 2013 to May 2016 by different Microbiology or Infectious Diseases Units in the Emilia Romagna Region (Figure 1) from patients met the following criterion: a first positive enzyme-linked-immunosorbent HIV-1 assay with any previous positive result or a previous negative result. The samples were codified by serial numbers certifying the center of origin, gender, age and nationality and all available data were also reported to regional surveillance system, operative since 2009.

HIV-1 serology. Each sample was tested by a fourth generation assay (ARCHITECT HIV Ag/Ab Combo, Abbott, Chicago, Illinois, USA) and confirmation test (Genius HIV1/2 Confirmatory Test, BioRad, Manes-la-Coquette, France) according to the manufacturers' instructions. Immunoblot strips were read and interpreted by Genius reader software (BioRad). Results were reported on the basis of combined type specific band profiles [reactivity to one or more specific HIV proteins (gp140, gp160, gp41, p31 and p24)].

Detection of avidity index. For each serum sample, two aliquots were prepared: one by a 1/10 dilution with phosphate-buffered saline (PBS) and one with 1 M guanidine (G) as denaturing agent. Both aliquots were shaken and incubated for 10 min at room temperature and then tested as previously described (Suligoi *et al.*, 2003; Re *et al.*, 2012) using a fourth-generation automated anti-HIV enzyme immunoassay (EIA). Sample to cut-off ratios (S/CO) were calculated and the avidity index of HIV antibodies was computed as (S/CO of the G aliquot)/(S/CO of the PBS aliquot). A cut-off of 0.80 was selected according to previous observations. Serum samples with an AI of <0.80 were classified as "recent infections" and those with an AI of ≥ 0.80 were classified as "established infections" (Re *et al.*, 2010; Suligoi *et al.*, 2011).

Peripheral blood CD4 and CD8 lymphocyte count. Peripheral blood CD4 and CD8 lymphocytes were counted by flow cytometry (FACScan, Becton and Dickinson, Mountain View, CA, USA) using commercial available monoclonal antibody (Becton and Dickinson).

HIV-RNA quantification. For HIV-1 RNA quantification, all the whole blood samples were centrifuged at 2500 rpm for 20 minutes and plasma was stored at -80°C until use

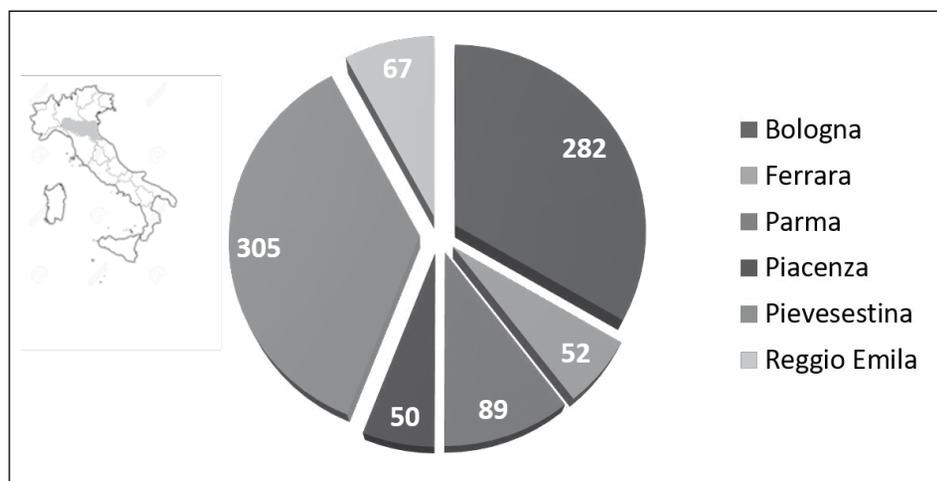


Figure 1 - Number of serum samples from different microbiology and/or Infectious Diseases Units in the Emilia Romagna region.

and analyzed by COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The amount of HIV-1 RNA was expressed as copy number per mL of plasma and the lowest detection limit of the assay was 20 copies/mL.

HIV-1 subtyping. HIV-1 subtypes were determined from HIV-1 *pol* gene sequences obtained with the Trugene HIV-1 genotyping system. Sequences of the protease and RT genes from 161 available plasma samples were aligned with representative sequences of HIV-1 group M subtypes and circulating recombinant forms (CRFs) accessible from the Los Alamos database (<http://www.hiv.lanl.gov>) using BioEdit v. 7.0.0. Phylogenetic distances were then elucidated by constructing neighbor-joining trees based on Kimura's two-parameter matrix, and the robustness of these relationships was tested by the bootstrap method using 100 replications. These analyses were conducted using applications provided in the MEGA 4.1 software (www.megasoftware.net) (Tamura *et al.*, 2007). Determination of subtype or CRF was considered definitive only if the bootstrap value linking a given sample exceeded 70% for the regions analyzed. Accurate identification of CRFs and their breakpoint locations was performed with the bootscan analysis using software SimPlot 3.5.1 (<http://sray.med.som.jhmi.edu/SCROftware>).

Statistical analysis. Patients' clinical characteristics were described as median (IQR) or frequency (%) as appropriate. Statistical significance was assumed for *P* values below 0.05. All statistical analyses were performed using SPSS for Windows 20.0 version (IBM Corporation, Armonk, NY, USA).

RESULTS

During the three-year period (2013-2016) 845 HIV-1 infections were diagnosed for the first time in the different Medical Centers of Emilia Romagna, Italy. As shown in *Table 1*, data concerning nationality, age, gender, and risk of transmission were available for most subjects included in the study. Most patients were Italian (73%) with a higher number of males (79%) and a median age of 40 years (IQR: 31-47). The majority of first HIV diagnoses were associated with sexual intercourse, due to homo-bisexual (50%) or heterosexual contacts (40%), confirming the principal route of HIV-1 transmission (UNAIDS/WHO, 2015).

Table 1 - Baseline characteristics of HIV-1 subjects enrolled in the study.

Number of serum samples from subjects with a first positive HIV-1 diagnosis	845
Nationality known	74% (628/845)
Italian nationality %	73% (458/628)
Non-Italian nationality %	27% (170/628)
Gender, male % (n°)	79% (670/845)
Subjects' age (years) median (IQR)	40 years (IQR 31 - 47)
Risk transmission factor known % (n°)	73% (628/845)
Heterosexual %	41% (263/629)
Homo-Bisexual %	50% (311/629)
Drug user %	3% (21/629)
Other %	7% (33/629)

Serological tests

(*Elisa, avidity index and Western Blotting*)

Elisa test. All the sera were first analyzed by immune-enzymatic assay. The median CMIA values were significantly ($p=0.0001$) lower in patients with recent infections (66 OD; IQR 9-59) in comparison with long-lasting infections (553 OD; IQR 422-759) (data not shown).

Avidity test. Avidity test was performed in all samples showing a settled HIV positivity (by immune-enzymatic test) without a previous record of EIA and/or WB assay. Among the 845 serum samples, 158 out 845 sera showed an avidity index <0.80 suggesting an HIV infection acquired in the last six months, while 687 out 845 sera showed an avidity index ≥ 0.80 suggesting a late diagnosis. In particular, the percentage of recent infections ranged from 10% (Medical Centre in Piacenza) to 21% (Medical Centre in Bologna) as shown in *Figure 2*. On the other hand, most HIV-1 first diagnoses referred to long-lasting infections (ranging from 90% to 79%).

Western Blotting analysis. Antibody patterns were studied by immunoblotting analysis in all available serum samples [114 serum samples from RIPs (recently infected

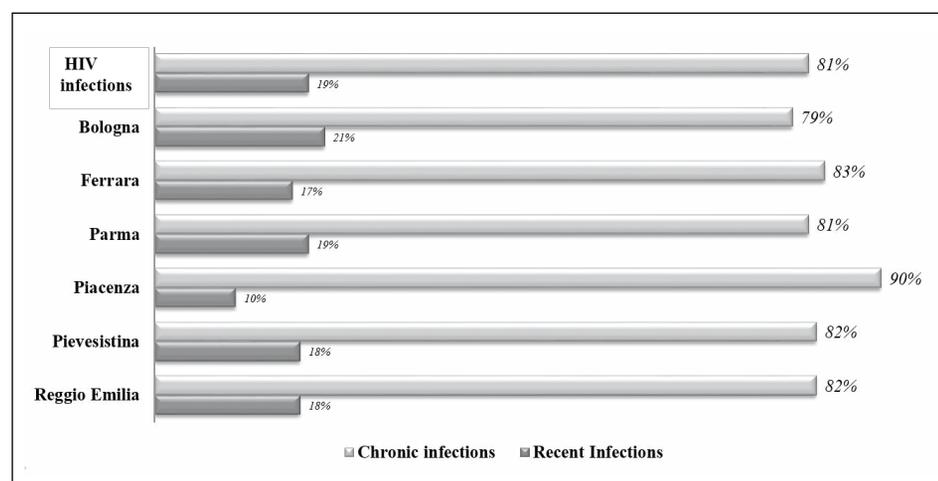


Figure 2 - Percentage of recent infection versus long-lasting infections in Emilia Romagna Medical Center enrolled in the study.

Table 2 - WB reactivity in available serum samples from recently HIV-infected patients (RIPs) and long-lasting HIV-infected patients (LLIPs) (on the basis of the avidity index).

HIV-1 Western blot pattern	RIPs	LLIPs
Reactivity to 1 or 2 HIV proteins	44.7% (51/114)	4.6% (23/496)
Reactivity to 3 HIV proteins	21% (24/114)	9% (45/496)
Reactivity to 4 or 5 HIV proteins	34.2% (39/114)	86.7% (430/496)

patients) and 496 serum samples from LLIPs (long-lasting infected patients)]. As described in the Materials and Methods, we reported the antibody response to specific HIV proteins (gp140, gp160, gp41, p31 and p24). A significantly ($p < 0.001$) higher percentage of recently infected patients showed reactivity to only one or two HIV-1 proteins in comparison with long-lasting infected patients (44.7% versus 4.6%). In addition, a reactivity to three HIV proteins was still found more frequently in RIPs compared with LLIPs (21% versus 9%). The majority of subjects with a chronic infection showed a complete reactivity pattern with a clear reactivity to all four or five HIV-1 proteins in comparison with subjects with recent infections (86.7 versus 34.2%) (Table 2). No patients showed a reactivity against HIV-2 specific proteins (HIV-2 gp36 and gp105).

Age, nationality, gender and transmission route in RIPs and LLIPs. Serum samples, divided on the basis of avidity index into recent and chronic infections, were further stratified by age, gender, nationality and risk transmission factor. The median age, gender, nationality and risk transmission factor were comparable in the two groups. In particular, median age was 38 years (IQR 29-44) for RIPs versus 40 years (IQR 31-48) for LLIPs. In both groups, the majority of patients were males (84% and 78%: recent and long-lasting infections, respectively). In addition, most first diagnoses regarded Italian patients (82% in recently infected individuals and 70% in long-lasting infected patients) and the total number of HIV first diagnoses were similar between Italian and non-Italian patients.

The main route of transmission was homo-bisexual intercourse followed by heterosexual contacts both in RIPs (66% and 26%) and LLIPs (46% and 45%). Other factors correlated to intravenous drug abuse or unknown causes were recorded in the remaining cases.

Recent and long-lasting infections are correlated with immunological and virological parameters

CD4 levels and RNA viral load. Virological and immunological values showed significant differences between the two groups. In particular, the overall viral load showed higher HIV-1 RNA values [median 1.7×10^6 copies/ml (IQR 1.8×10^4 - 2.2×10^5)] in RIPs in comparison with LLIPs [3.4×10^5 (IQR 2.0×10^4 - 2.2×10^5)]. CD4 cell counts (cell/ μ L) were significant higher ($p = 0.029$) in RIPs (549 cells/ μ L; IQR 318-760) in comparison to LLIPs (308 cells/ μ L; IQR 71-483) and also the CD4/CD8 ratio was significantly ($p = 0.02$) higher in HIV RIPs respect to LLIPs.

Phylogenetic analysis. Plasma samples for phylogenetic analysis of HIV-1 *pol* gene (RT and PR) were only available from 35 RIPs and 126 LLIPs (Table 3). Sequences, amplified directly from plasma revealed that subtype B virus strains were predominant in both groups [20 out 35 recently infected patients (57.1%) and 85 out 126 (67.4%) long-lasting infected patients]. Phylogenetic analysis also disclosed non-B subtypes [F1 (7 cases), C (2 cases), G (1 case)] in a higher percentage (28.5% versus 17.4%) of serum samples from RIPs in comparison with LLIPs [A1 (9 cases), C (5 cases), F1 (5 cases), G (2 cases), K (1 case)]. HIV recombinant forms were detected in both groups with a similar percentage (14.3 % and 15.7 %), recording

Table 3 - Phylogenetic analysis in plasma from recently HIV-infected patients (RIPs) and long-lasting HIV-infected patients (LLIPs).

	HIV subtype B viral strains	HIV subtype non B viral strains	HIV CRFs viral strains
Plasma samples from HIV -1 RIPs (total number 35)	20 (57.1%)	10 (28.6%) F1 (7 cases) C (2 cases) G (1 case)	5 (14.3%) CRF 01_AE (2 cases) CRF 31_BC (1 case) CRF 09_CPX (1 case) CRF 20_BG (1 case)
Plasma samples from HIV-1 LLIPs (total number 126)	85 (67.4%)	22 (17.5%) A1 (9 cases) C (5 cases) F1 (5 cases) G (2 cases) K (1 case)	19 (15.1%) CRF 02_AG (9 cases) CRF 01_AE (2 cases) CRF 46_BF (2 cases) CRF 36_CPX (2 cases) CRF 06_CPX (1 case) CRF 03_AB (1 case) CRF 15_01B (1 case) CRF 28_BF (1 case)

CRF 01_AE (2 cases), CRF 31_BC (1 case), CRF 09_CPX (1 case), CRF20_BG (1 case), in plasma from RIPs and CRF 02_AG (9 cases), CRF 01_AE (2 cases), CRF 46_BF (2 cases), CRF 36_CPX (2 cases), CRF 06_CPX (1 case), CRF 03_AB (1 case), CRF 15_01B (1 case), CRF 28_BF (1 case) in plasma from LLIPs. Interestingly, even if the prevalence of subtypes B and non B were similar among Italian and non-Italian patients, HIV recombinant forms were more frequently detectable (71%) in patients from abroad (Nigeria, Russia, Dominican Republic, Mali, Colombia and Morocco) in comparison with Italian patients (29%).

DISCUSSION

Early HIV-1 diagnosis offers several advantages: it maximizes the benefit of HIV care, offers timely initiation of therapy and reduces morbidity and mortality (Shrestha *et al.*, 2008). Since the risk of transmission from patients with acute and early infection appears to be much higher than that from HIV-1 patients with established infection (Cohen *et al.*, 2011), to define the presence of HIV-1 early and late diagnosis in our region we studied serum samples HIV-1 positive for the first time and collected in the last three years. In addition, we also reported the characteristics of subjects enrolled in the study (age, gender, nationality) and focused our attention on the relationship between early diagnosis and immunological (CD4 count, CD4/CD8 ratio) and virological markers (HIV RNA viral load, HIV-1 subtyping).

Firstly, we certified 845 new HIV-1 infections, 18.7% of which were classified on the basis of avidity index as recent infections, and 81.3% as long-lasting infections, with an estimated seroconversion time exceeding six months at the time of study.

Recent infections were characterized by a low, clearly positive, optical value at Elisa test and by an incomplete Western blotting (WB) pattern. As previously described (Guan *et al.*, 2007; Re *et al.*, 2010; Cohen *et al.*, 2011; Bon *et al.*, 2015; Liu *et al.*, 2016), fourth generation assays - simultaneously detecting HIV p24 antigen and antibodies - have led to major improvements in the identification of HIV early infection, whereas WB still shows a low profile reactivity against specific HIV proteins. Nevertheless, WB analysis must not be underestimated even when it does not reflect the international criteria for positivity as it is highly specific but not sensitive enough particularly during the earliest phase of HIV seroconversion (Cohen *et al.*, 2011). Moreover, RIPs show a higher level of viral replication, certifying that individuals with recently acquired HIV infection may be more infectious than individuals with established infection and might be the source of a significant proportion of ongoing transmission (Buskin *et al.*, 2014; Touloumi *et al.*, 2013), despite good CD4 cell counts.

In addition, our data showed that a most of the newly diagnosed cases were subjects with a long-lasting infection, confirmed also by virological and immunological data. This group of patients are characterized by high viral load, albeit more contained in comparison with RIPs, accompanied by a lower CD4 cell counts.

The median age of HIV-infected patients was higher than expected and not significantly different in RIPs and LLIPs, even if compatible with previous reports (Re *et al.*, 2012; D'Arminio Monforte, 2011), emphasizing a low risk perception in our population. Moreover, about 90% of HIV-1 positive adults became infected through the exposure of

mucosal surfaces to the virus and only 10% were infected by percutaneous or intravenous inoculations or unknown causes, confirming that the principal route of transmission is sexual contact (Cohen *et al.*, 2011).

Our second aim focused on the HIV-1 types and subtypes responsible for HIV infection in our region. From its discovery and for at least three decades, the HIV epidemic in the Western World has been dominated by subtype B infections (Gilbert *et al.*, 2007; Magiorkinis *et al.*, 2016) followed by non B subtypes and recombinant forms. The broad genetic diversity of HIV can have important public health implications since subtypes and CRFs can show different properties that can affect their fitness, transmissibility, and response to therapy (Sanarico *et al.*, 2015).

Phylogenetic analysis in available plasma showed a major presence of HIV subtype B, as predominant in most infections with no significant difference in RIPs and LLIPs and independently of patients' nationality.

HIV subtype non B and CRFs were similarly distributed in both groups. Among HIV non B subtypes, strains F, A and C were more frequently recorded. HIV non B subtypes and, in particular, HIV-1 C might be correlated with an increased risk of virological failure (Hägglom *et al.*, 2016) and a poor immunological response. Interestingly, CRFs, in particular CRF02_AG, were predominant in non-Italian patients, confirming a phenomenon resulting from an increased immigration flow from African countries (Paolucci *et al.*, 2014, Santoro and Perno, 2016; Tatarelli *et al.*, 2016). Genotyping data, leading to a characterization of HIV circulating in Italy, have crucial importance since CRF02-AG-infected subjects might develop specific mutations such as K20I, K70R and L89M closely correlated with a poor antiretroviral response (Santoro and Perno, 2016).

In conclusion, we disclosed a large number of patients unaware of their HIV status with a late presentation at diagnosis and consequently a late access to treatment, making it crucial to promote HIV testing with the help of medical physicians and specialists. The elevated age, route of transmission, and high levels of viral replication observed independently from recent or late diagnosis emphasize the need for HIV screening to limit HIV transmission. A late HIV diagnosis is the most important predictor of HIV-related morbidity and mortality. Presenting late for care was associated with a high risk of clinical progression and has important individual, public health and economic consequences. A substantial proportion of people who die from AIDS are late presenters (Adler *et al.*, 2010, Monforte *et al.*, 2011).

Very early administration of antiretroviral therapy may limit the size of the latent pool of HIV-infected CD4 T cells. Today considerable progress has been made in understanding HIV transmission events and different susceptibility to antiretroviral molecules in HIV-B and non B infections, the latter frequently associated with a poor immunological response and virologic failure (Maserati *et al.*, 2014; Antinori *et al.*, 2015; Andreoni *et al.*, 2015).

HIV testing must be offered not only in the so-called specific groups (such as IDUs, MSM, persons with STDs, foreigners, prison inmates, etc.) and in pregnancy (according to current Italian legislation) but it is urgent for clinicians of all disciplines to incorporate HIV screening much more frequently (on a confidential basis and with patients' informed consent) as part of their standard diagnostic procedures (Castilla *et al.*, 2002; Orchi *et al.*, 2013).

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