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European Recommendations for the Clinical Use of HIV Drug Resistance Testing: 2011 Update

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Abstract

The European HIV Drug Resistance Guidelines Panel, established to make recommendations to clinicians and virologists, felt that sufficient new information has become available to warrant an update of its recommendations, explained in both pocket guidelines and this full paper. The Panel makes the following recommendations concerning the indications for resistance testing: for HIV-1 (i) test earliest sample for protease and reverse transcriptase drug resistance in drug-naive patients with acute or chronic infection; (ii) test protease and reverse transcriptase drug resistance at virologic failure, and other drug targets (integrase and envelope) if such drugs were part of the failing regimen; (iii) consider testing for CCR5 tropism at virologic failure or when a change of therapy has to be made in absence of detectable viral load, and in the latter case test DNA or last detectable plasma RNA; (iv) consider testing earliest detectable plasma RNA when a successful nonnucleoside reverse transcriptase inhibitor-containing therapy was inappropriately interrupted; (v) genotype source patient when postexposure prophylaxis is considered; for HIV-2, (vi) consider resistance testing where treatment change is needed after treatment failure. The Panel recommends genotyping in most situations, using updated and clinically evaluated interpretation systems. It is mandatory that laboratories performing HIV resistance tests take part regularly in external quality assurance programs, and that they consider storing samples in situations where resistance testing cannot be performed as recommended. Similarly, it is necessary that HIV clinicians and virologists take part in continuous education and discuss problematic clinical cases. Indeed, resistance test results should be used in the context of all other clinically relevant information for predicting therapy response. **(AIDS Rev. 2011;13:77-108)**

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Introduction

In 2004, the European HIV Drug Resistance Guidelines Panel presented recommendations for the use of HIV-1 drug resistance testing for treatment management, with special attention to the European setting and to quality control measures in the laboratory[1,2]. As sufficient new information has now become available, especially regarding the new drug classes targeting HIV integrase and entry, the Panel worked on an update in the form of pocket guidelines[3], which were presented to the public during the 7th European HIV Drug Resistance Workshop, 25-27 March, 2009, in Stockholm, Sweden, and further updated in this full paper. The current Panel consists of the members of the original Panel who actively participated in the discussion of the updated guidelines, with the addition of new members who were recruited to maintain a balanced expertise. The current Panel received unrestricted educational grants from several pharmaceutical and diagnostic companies to cover the logistic costs, such as meeting rooms and print costs. None of the Panel members received fees or travel reimbursement for their work.

The current European HIV Drug Resistance Guidelines Panel consists of experts from mainly European countries, 20 academic clinicians and 20 academic virologists from 22 European countries, 12 experts in the field from pharmaceutical or diagnostic companies, one statistician/epidemiologist as well as one representative of an European patient organization. Discussions were undertaken during four on-line and one face-to-face discussion meetings, from January, 2009 to November, 2010. Further e-mail communication was extensive to arrive at the final pocket guidelines and the current full paper. The recommendations as mentioned in this document were voted on by the Panel, with the total number of completed votes being 38. Panel members employed by a commercial company involved in resistance testing or drug development had no voting power to avoid conflict of interest situations, but their expertise contributed significantly to the discussions and the resulting documents. For some countries, clinicians and/or virologists chose to consult their colleagues (also mentioned in the Panel), without increasing the number of votes for that country. The level of consensus in table 1 reflects the result of this voting. The final document was approved by all Panel members. The European guidelines differ from the international and US guidelines[4,5] on issues related to the specific European situation as discussed in this document. They pertain mainly to geographical differences in transmission of resistant virus, differences in prevalence of HIV-1 subtypes and HIV-2, to the implementation of resistance testing throughout Europe, which is often related to cost issues, and to differences in education strategies.

This paper contains scientific support for the decisions of the Panel (See: General concepts of HIV antiviral drug resistance and specific points of interest), recommendations addressed to the clinician designing the best possible long-term therapy strategy for an individual patient (See: Clinical indications for drug resistance testing), recommendations addressed to laboratories regarding resistance testing and proper reporting to the clinician (See: Genotyping or phenotyping; Interpretation systems), and a proposal for quality control measures and cost-effectiveness considerations to the authorities (See: Laboratory quality control requirements for sequencing; Sample storage; Cost issues). The recommendations summarized in table 1 are graded to indicate strength of recommendation and level of evidence, and contain in addition the level of consensus among the Panel. The updates to previous guidelines are mentioned throughout the document and consist mainly of scientific data on transmitted drug resistance, updated evidence and recommendations with regard to resistance to the new drug classes integrase inhibitors and coreceptor antagonists, technical improvements, and cost issues.

General concepts of HIV antiviral drug resistance and specific points of interest

Understanding antiretroviral drug resistance

Resistance reduces therapeutic options

Current therapeutic choices for the treatment of HIV infections have considerably expanded over time but remain limited. While 23 different anti-HIV drugs from five classes are currently available to the patient, these are used in a limited number of combinations of usually three or more drugs[6,7]. Development of drug resistance is both the cause and consequence of a failing antiretroviral therapy (ART)[8]. How fast and which mutations arise depends on the interplay between several factors such as drug potency, genetic barrier, patient adherence to treatment, host genetics, and the contribution of specific mutations to virus drug resistance

Table 1. Recommendations for drug resistance testing in the European setting

Clinical indication	Recommendation to clinicians	Recommendation and evidence level Academic consensus
Drug-naive patient (with acute or chronic infection)	Test earliest sample for PR and RT drug resistance. Testing should not delay treatment of acute infection if immediate treatment is being considered. Await resistance test result before starting treatment in chronically infected.	AII Consensus: 96%
Virologic failure	Test sample taken on the virologically failing therapy. Test envelope and integrase only when EI and/or INSTI respectively were part of the failing regimen.	AI Consensus: 88%
CCR5 antagonist	In addition to resistance testing at virologic failure as mentioned above, for a CCR5 antagonist, test tropism (i) before use as indicated in treatment guidelines, (ii) consider testing upon virologic failure of a CCR5 antagonist, and (iii) consider testing for patients with undetectable viral load for whom a therapy change has to be made and a CCR5 antagonist is considered.	ii. CIII Consensus: 100% iii. CIII Consensus: 58%
Inappropriate treatment interruption of a successful NNRTI-containing therapy	Treatment interruption is generally considered not a good strategy, but if needed, appropriate treatment interruption of a successful NNRTI-containing therapy is described under the treatment guidelines http://www.europeanaidsclinicalsociety.org/. At treatment re-initiation and if resistance history is not available, consider retrospective testing a post-stop sample as soon as viral load rises above resistance testing threshold, if such an early sample is available.	CIII Consensus: 88%
Postexposure prophylaxis	Use genotypic information from the index case to guide PEP. If this genotype is not known, do not delay PEP, but if a sample from the index case is available, genotype index case to change or simplify PEP if needed.	AIII Consensus: 92%
HIV-2	Consider resistance testing when treatment change is needed after therapy failure.	CII Consensus: 96%
Technical issues	**Recommendation to laboratory experts**	**Recommendation and evidence level Academic consensus**
Which assay to use	1. The Panel recommends the use of genotyping in most routine clinical situations. Current genotyping can be performed below a viral load of 1,000 copies/ml. 2. Consider additional phenotyping for new drugs, in heavily pretreated patients, and for HIV-2 where genotyping is not easily interpretable.	1. AI Consensus: 97% 2. CII Consensus: 100%
Interpretation	1. For genotyping, use continuously updated and clinically evaluated resistance interpretation systems and compare the results of different such interpretation systems. Consider discordant interpretations as uncertainty on the resistance profile being scored. 2. Store sequence for future re-interpretation. 3. For phenotyping, use clinical cutoff if available, otherwise use biological cutoff. 4. It is recommended to take into account the clinical context, therapy history, and resistance history.	1. AII Consensus: 97% 2. AIII Consensus: 100% 3. AII Consensus: 97% 4. AIII Consensus: 100%
Laboratory quality control requirements for sequencing proposed to the accreditation authorities	1. Include proper negative and positive controls during extraction/PCR. 2. Editing of the sequence should be traceable. 3. Resistance-related positions should be evaluated by sequencing in 2 directions. 4. Each laboratory should pass at least once a year a proficiency panel test. 5. At least every two months or every 50 samples (whichever comes first), a known genotype should be re-sequenced. 6. Interpretation of the results should be documented.	AIII Consensus: 1. 97% 2. 100% 3. 97% 4. 100% 5. 94% 6. 100%
Laboratory quality control requirements for phenotyping (RVA) proposed to the accreditation authorities	1. Include proper negative and positive controls during extraction/PCR. 2. Sequence recombinant virus used in the test to ensure proper representativeness of genotypically confirmed resistance mutations. 3. Fold resistance values should be expressed versus a reference laboratory strain which should be included in every run (usually a subtype B). 4. Each laboratory should pass at least once a year a proficiency panel test. 5. Interpretation of the results should be documented.	AIII Consensus: 1. 100% 2. 97% 3. 85% 4. 100% 5. 100%
Storage of sample	If resistance testing cannot be performed as indicated, consider storage of recommended plasma (2 ml at -80 °C).	CIII Consensus: 100%

All recommendations are for HIV-1 except when stated otherwise. The recommendations are graded as is usual in guidelines documents for clinical indications: A = recommended, B = strongly consider, C = consider; with indications of the evidence (I = based on at least one prospective randomized study using surrogate markers e.g. viral load, II = based on at least one retrospective study, III = expert opinion based on scientific evidence derived from other clinical and *in vitro* observations); the level of consensus represents only the academic members (expressed as %).

PR: protease; RT: reverse transcriptase; APV: amprenavir; EI: entry inhibitor; CCR5: coreceptor for HIV; INSTI: integrase strand transfer inhibitor; NNRTI: nonnucleoside reverse transcriptase inhibitor; PCR: polymerase chain reaction; PEP: post-exposure prophylaxis; PI: protease inhibitor; RTI: reverse transcriptase inhibitor; RVA: recombinant virus assay.

and replicative capacity[9,10]. Therapy failure with resistance compromising the next line combination is a problem since cross-resistance within each class can be extensive, limiting the use of other drugs from the same class. With every subsequent therapy failure, multiclass resistance may accumulate, reducing chances for prolonged viral suppression.

Virus population dynamics and fitness of resistant virus

Drug resistance is the result of virus population dynamics under drug selective pressure[11]. Virus replication allows the genetic variability of the virus to increase. Residual replication under drug selective pressure has as consequence that random resistance mutations arising in a minority of the virus population give the variant a selective advantage. Drug selective pressure allows such resistant variants to become predominant due to a shift in the virus population. As a result, the majority virus population becomes fitter, replication increases, and further accumulation of resistance mutations is possible.

Major drug resistance mutations are defined as resistance mutations that have a major impact on drug resistance; they often contribute to reduced fitness of the virus in absence of drug[12]. Minor drug resistance mutations have only a minor impact on drug resistance or increase resistance only in presence of a major resistance mutation. Often, minor drug resistance mutations increase the replication capacity of the virus to compensate for the fitness cost of the major resistance mutations and act as compensatory mutations. This terminology of major and minor resistance mutations should not be confused with the terminology of majority and minority resistant variant, a terminology exclusively used to indicate the frequency of a mutation within a single patient (See: Minority drug-resistant variants). Because of this fitness cost of major resistance mutations in absence of drugs, more fit wild-type virus can gradually (within months) replace the resistant mutant in absence of drug selective pressure, for example during treatment interruption[13,14], while some HIV genotypic resistance may still be detectable after a prolonged treatment interruption[15].

Although transmitted resistance mutations can revert to wild-type[16,17], some of them have been reported to persist in plasma for many years after infection[17,18], likely reflecting different population dynamics following infection with a drug-resistant strain in the absence of wild-type virus. This reversion rate is not the same for each mutation and likely depends on the fitness cost of such a mutation in absence of therapy, along with the effect of potential compensatory mutations and the genetic barrier to reversal[19,20]. As a result, drug-naive patients may show only a few or a single drug resistance mutation as majority variant, while it can be assumed that the original virus with more resistance mutations has been archived and remains present as a minority variant. In case these (partially reverted) transmitted drug resistance genotypes remain stable as the dominant population, they can become the source of further transmission of drug-resistant HIV[21-25]. In such a case, the new recipient would not be expected to carry additional minority 'hidden' variants of transmitted drug resistance. In some cases, such onward transmission of drug-resistant variants is causing new epidemic lineages, testifying their fitness both in terms of intra- and inter-patient dynamics[25].

The concept of genetic barrier to resistance is related to both the evolutionary distance and the fitness difference of the resistant virus compared to the wild-type virus[11]. The evolutionary distance is dependent on the number of nucleotide mutations required and on the mutation rate and bias by the enzymes involved in a full replication cycle, in the case of HIV the viral reverse transcriptase, cellular DNA polymerases (expansion of integrated proviral DNA during cell division) and cellular RNA polymerase (production of new viral RNA). The fitness difference is dependent on the difference in potency of the drug with respect to mutant and wild-type virus, and on the genetic background of the virus since the virus is restricted in its movements through genetic space due to epistatic fitness interactions between residues. Only linked mutations, i.e. on the same genome, are contributing to the genetic barrier to resistance.

Resistance remains archived in the body

In the case of HIV, which infects long-lived cells, the history of genotypes remains archived[26,27]. Therefore, wild-type or resistant variants acquired due to transmitted resistance or during the treatment history of the patient are unlikely to completely disappear from the body with currently available drugs, even though they may have become minority variants[28,29]. Such minority variants have been detected, for example, in women after treatment with single-dose nevirapine or in patients undergoing treatment interruption. Since current clinically used resistance assays only detect resistance when it is present in the majority virus population in

the plasma of the patient at the time of testing, such archived resistant variants may be undetectable on resistance tests[30]. It is a matter of discussion as to which resistant strains eventually archived in reservoirs may later reappear and affect future therapeutic attempts. A working hypothesis is that any replicating resistant virus variant in a patient will be archived and may later reappear under appropriate selective pressure.

Minority drug-resistant variants

Because of the population dynamics and/or archived resistance (See: Virus population dynamics and fitness of resistant virus; Resistance remains archived in the body), some resistant variants can be present only as a minority variant of the entire virus population in a patient, therefore remaining undetected with current population sequencing strategies. Non-laborious methodologies for the detection and quantification of minority species became widely available only recently. According to several studies using ultra-deep sequencing, allele specific real-time PCR amplification, or clonal genotypic analysis, minority quasispecies harboring resistance mutations can be detected in naive patients at a higher prevalence than by the use of conventional population sequencing, in both chronically as well as recently infected individuals[31-42], suggesting that such resistant variants can remain present in a minority of the virus population for a long period after HIV infection.

The potential clinical implications of such minority resistant strains in therapy naive as well as in treated patients have been discussed for several years now[30,43,44]. Concerning the first-line regimen, some studies show that minority resistant variants at baseline are associated with increased risk of virologic failure in patients with no or only limited detectable resistance by standard genotyping, with the minority resistant virus often becoming dominant upon virologic failure[8,28,34,36,38,42,45]. However, low abundant resistant variants can also be detected in responders, although in a lower frequency than in nonresponders[37,46]. Other studies, including one from the same authors[33], did not find such an association. For some patients with virologic failure to first-line regimens there was no evidence of minority resistant viruses at baseline[40], suggesting either that resistance was selected upon treatment rather than preexisting at baseline, or that the minority variant was below the detection limit of the technique used. Peuchant, et al. observed that detection by allele specific real-time PCR of minority resistant variants harboring the 103N or 184V mutations in the reverse transcriptase (RT) and the 90M mutation in the protease (PR) did not affect the HIV-1 RNA decline following treatment initiation[38].

There is more evidence that transmitted drug resistance (See: Transmission of drug resistance) present as a minority quasispecies in treatment-naive individuals can affect the outcome of nonnucleoside reverse transcriptase inhibitor (NNRTI)-based first-line treatment, compared to boosted protease inhibitor (PI)-based first-line regimens[41,47]. These findings could be associated with the genetic barrier to resistance (See: Understanding antiretroviral drug resistance). Low genetic barrier drugs (e.g. NNRTI) lose their efficacy after fewer mutations than high genetic barrier drugs (e.g. PI), suggesting that a minority presence of a limited number of preexisting resistance mutations may affect more the low genetic barrier drugs than high genetic barrier drugs[28,39,46]. Detection of transmitted drug resistance in the majority population may alert towards the presence of additional hidden minority variants of other mutations, but only if transmission was from a patient failing treatment in the presence of drug resistance (See: Virus population dynamics and fitness of resistant virus). However, when a transmitted drug-resistant variant was acquired from a drug-naive patient, the new recipient would not be expected to carry additional minority 'hidden' variants of transmitted drug resistance. Whereas in the first case, transmitted drug resistance may be considered a warning against the use of low genetic barrier drugs, this is not so in the case of onward transmission of resistance and such patients would have more options for the first-line regimen. Some treatment guidelines advise against the use of NNRTI in first line when transmission of drug resistance (TDR) was detected. However, when the majority of TDR in a population is acquired from drug-naive patients through sustained epidemics of TDR, such a different first-line treatment strategy for TDR would not be needed. When counseling an individual patient it is, however, difficult to discriminate between the two situations: transmitted drug resistance from a treated patient compared to onward transmission of resistance mutations.

These findings suggest that preexisting minority resistant strains may cause virologic failure to first-line treatment especially in case of a combination with low genetic barrier drugs. Similar arguments can be used for further line treatments[30], albeit a larger number of mutations may have been archived after failing several previous therapies. Goodman, et al.[39] provided evidence correlating > 2,000 copies of RT103N-containing HIV-1

per ml of plasma (copy number of mutant even if minor variant) to therapy failure with NNRTI. In a meta-analysis, Li, et al.[48] came to the conclusion that there is a dose-dependent correlation between the level of minority variants of resistance mutations and an increased risk of therapy failure, and this remained significant down to 0.5% (10-99 copies/ml). Data are, however, lacking for many mutations and drug combinations, such that no cutoff can currently be suggested above which the chances of failure are sufficiently high to recommend minority testing in routine clinical practice. Also, the new methodologies are costly, not available in most of the clinical laboratories performing routine HIV resistance testing, and quality control issues have not been resolved yet. Therefore, recommendations for detection of minority resistant strains can still not be made. Current research, especially using deep sequencing approaches, is expected to improve our understanding of the clinical significance of minority resistant variants during the next few years.

Transmission of drug resistance

Transmission of resistant virus has been documented in all European countries that have surveyed for it, and increasingly, clusters of onward transmission of such resistance mutations are reported[21-25,49-51]. However, the data reported are not always directly comparable due to differences in sampling strategies, technologies used, and criteria to score the transmission of a resistant virus. In the USA, estimates of the proportion of untreated people infected with viral strains displaying resistance towards particular drugs vary between 1-27%, while in Europe the range has been 2-52%[52,53]. More recently, reports have appeared documenting TDR in developing countries along with the expanding access to ART[54-56]. Results from the European CATCH study[57], in which available sequences from drug-naive patients were retrospectively collected and analyzed in a consistent way, the prospective European SPREAD study[58,59], using representative sampling and a uniform technology and analysis plan, and other European studies suggest a relatively stable or declining (during recent years) general prevalence of 9% in Europe with large inter-country differences[60-62]. Such geographic differences affect the decision of whether or not to test for drug resistance prior to initial therapy.

In the majority of drug-naive patients, only one or a few resistance mutations are found[58], as can be expected given the fitness cost of many major drug resistance mutations (See: Understanding antiretroviral drug resistance). How many of these patients are carrying minority variants with more resistance mutations depends on the extent of spread of TDR among drug-naive patients (See: Virus population dynamics and fitness of resistant virus). This is currently being investigated by several research groups. It is therefore still unclear to what extent TDR is limiting treatment options also to drugs for which no resistance can be found.

Some (minor) resistance mutations reported in drug-naive patients are not the result of transmitted resistance but are natural polymorphisms, especially in non-B subtypes. They seem to have little effect on initial treatment responses[63]. Other mutations, such as reversal mutations (e.g. RT215A/C/D/S) which have a sensitive phenotype, are a 'signature' of past drug resistance and can be associated with reduced therapy response[64,65]. Similarly, the recently identified RT210M and RT69S have been proposed to predict the presence of TDR minority species, confirmed by ultra-deep sequencing[66]. These issues are taken into account in dedicated algorithms that score marker mutations for transmission of resistance. A few algorithms have been built to assess genotypes for surveillance testing, which can be used in the setting of transmission. Such algorithms are mainly based on the presence of major resistance mutations and non-polymorphic mutations significantly associated with drug selective pressure[67]. These dedicated algorithms can be consulted to assess the extent of TDR. The Panel encourages to keep collecting timely epidemiological information on the spread of resistant HIV strains across Europe. Considering the feasibility and value of the results, evaluation of such prevalence is preferentially done, using a genotypic population sequencing approach scoring TDR according to an algorithm dedicated to resistance surveillance.

Superinfection

Superinfections with a new strain carrying different resistance patterns, coexisting with or replacing the original virus, have been reported, even after established immune response to the first infection[68-72]. Many reports of new intersubtype recombinants[73] also show that superinfection does occur. Some studies suggest the incidence of superinfection to be similar to the incidence of infection[74], while others report it to be very rare[75]. Incidence of superinfection is related to a high-risk behavior[76]. A sudden rise in viral load during

an otherwise relatively stable viremia in treatment-naive individuals could alert the clinician to a possible superinfection[72].

Treatment interruptions

Structured treatment interruptions, often guided by CD4 cell counts, have failed to achieve expected benefits in chronically suppressed or unsuppressed patients, and were associated with increased mortality in a large randomized trial (SMART)[77-80]. They are not recommended as a part of therapeutic strategies by current treatment guidelines[5,6,81]. However, in routine clinical practice, planned or unplanned interruptions of ART happen for multiple reasons (e.g. drug toxicities, intervening illnesses, surgery, or lack of adherence from the patient). Treatment interruptions are associated with a rebound in viral load[82], immune degradation, and eventually clinical progression[83]. Treatment interruptions generate a risk for emergence of resistance, particularly for drugs with a low genetic barrier and long plasma half-life, such as NNRTI, if no appropriate withdrawal strategy is used. Data derived from the SMART trial[84] showed that the probability of observing NNRTI resistance (i.e. presence of at least one resistance mutation two months after treatment interruption) is 16.4% if all drugs have been stopped simultaneously, 12.5% in the case of staggered interruption (i.e. NNRTI stopped before other drugs), and only 4.2% with switched interruption (i.e. NNRTI interruption protected by a short course of high genetic barrier drugs such as PI). Furthermore, the presence of NNRTI mutations was significantly associated with a lower likelihood of viral re-suppression (69.2 vs. 86.7%; p = 0.05) when resuming ART.

Consistent with past HIV resistance guidelines[1], a plasma sample for resistance testing should be taken under drug pressure, before treatment is interrupted if viral load is detectable or supposed to be detectable. However, treatment interruption might be the cause of drug resistance selection, especially in the context of inappropriate treatment interruption of a successful NNRTI-containing therapy. Therefore, a majority of the Panel – mainly based on the SMART data – believe that in such cases it is useful to obtain a post-interruption plasma sample generally around 1-3 months after treatment stop (See: Virus population dynamics and fitness of resistant virus). However, it is important to emphasize that no clinical trial has yet evaluated the impact on clinical outcome of this sampling approach.

Powers and limitations of resistance testing assays

Genotyping versus phenotyping

When the cause of therapy failure is resistance, this results from phenotypic changes in target proteins as a result of virus evolution under the selective pressure of the drugs. Such phenotypic changes are always caused by genotypic changes[11]. It was therefore originally anticipated that phenotyping would give a more accurate estimate of resistance and consequently therapy failure than genotyping. Because of technical limitations and the effect of genetic barriers to resistance, the relationship between genotype and phenotype and both towards therapy response is more complex.

Genotypic drug resistance assays aim to detect mutations that are known to confer phenotypic drug resistance or to compromise treatment response. Therefore, genotypic test results always need to be interpreted based on prior knowledge of the effect of such mutations. Current clinically used assays all involve sequencing the genes whose gene products are targeted by the drugs: PR, RT, integrase, and envelope. Whereas traditionally, most European drug resistance testing laboratories are equipped to perform PR and RT genotyping, either using commercial assays or in-house methods, integrase and envelope (gp41 for enfuvirtide and V3 for maraviroc) genotyping is not so widely available. However, such laboratories either are currently implementing integrase and envelope sequencing in routine clinical practice or have agreements with other laboratories who can offer such services.

Phenotypic assays measure the ability of an HIV-1 isolate to grow *in vitro* in the presence of an inhibitor in comparison with a known susceptible strain. Current clinically used phenotypic assays are all based on recombining the target genes of a patient isolate into the genetic background of a laboratory (subtype B) strain, called a recombinant virus assay. Few laboratories are equipped for such tests, and their availability varies across countries. For details about the methodologies, see reviews such as MacArthur, et al.[85].

Typically, genotypic assays have a faster turnaround time between blood sampling and reporting of one to two weeks, while for phenotypic assays up to four weeks may be needed. Both types of assays start from PCR-amplified target genes. Genotyping then proceeds with sequencing, techniques that, although

demanding, can be performed in many dedicated laboratories. Phenotyping requires further processing of the patient-derived amplicon in higher biosafety levels than genotyping and is performed in only a few specialized laboratories. In general, the cost of genotyping is 50% or less of the price of a phenotype test. None of the current clinically used genotypic or phenotypic drug resistance assays are able to reliably detect minor resistant variants present below 20-30% of the total viral population[30,32], although some specialized genotypic assays selectively detect mutations at a much lower detection limit (See: Minority drug-resistant variants). The most significant challenge for resistance assays is, however, the translation of the results obtained into clinically relevant guidance (See: Interpretation of resistance). Since genotyping relies on prior knowledge of particular mutations and combinations of mutations for their relevance in resistance phenotype or therapy response, this interpretation is complex and needs continuous updating. Phenotypic test results provide a quantitative measure of the impact of all mutations, known and unknown, but the levels of phenotypic drug resistance associated with reduced therapeutic response (clinical cut-off; See: Interpretation of resistance) are difficult to determine for several drugs.

Relatively few clinical studies have made direct comparisons between genotyping and phenotyping. Both types of assays have shown to be correlated with therapy response in retrospective studies and a limited number of prospective studies[86,87]. Among direct comparisons of genotyping to phenotyping, one of the five studies (RealVirFen) showed a significant benefit of genotyping over phenotyping (Narval, Cert, RealVirFen, GenPherex, VIHRES, considering VirtualPhenotype as a genotypic analysis)[1], while none showed a benefit of phenotyping over genotyping. In addition, phenotypic testing did not provide benefit over and above genotypic testing in highly drug-experienced patients enrolled in the ERA trial[88]. However, for heavily pretreated patients for whom interpretation of the genotype is very complicated, phenotyping may improve therapy outcome[89,90].

Possible causes for the apparent greater usefulness of genotyping may be that genotyping allows the detection of mutations such as reversal mutations (RT215A/C/D/S) as 'signature' of past drug resistance[64,65]. These may not by themselves contribute to a significantly reduced phenotypic susceptibility, but may contribute to therapy failure. Due to virus population dynamics (See: Virus population dynamics and fitness of resistant virus), (renewed) selective pressure of drugs to which resistance has been archived may result in a quick shift of the virus population in favor of the archived resistance variant. Some minor PI mutations have been reported to be associated with therapy failure, although they only contribute to phenotypic resistance in the presence of major mutations[91,92]. Finally, genotyping also allows the detection of mutations associated with failure of drugs for which clinically relevant phenotypic cutoffs are within the reproducibility range of the assay (as shown for stavudine[93,94]) or are not sufficiently documented.

Interpretation of resistance

For resistance test results to be useful for clinicians, such results need to be interpreted according to the latest knowledge. Our knowledge of mutations conferring resistance, cross-resistance, or antagonism of reversal mutations and of clinically relevant phenotypic cutoffs is continuously expanding and a great body of literature is supporting several interpretation systems[95]. Information on resistance and resistance mutations against the commonly used PR and RT inhibitors and their clinical relevance has been widely described[96], and their implementation in interpretation systems is well informed, although there is still much scope for achieving closer alignment of interpretation systems based on further analyses linking mutations with virologic response. Data on resistance against enfuvirtide has also been accumulating, although the resistance patterns are less clear and often resistance testing is not available for this drug[97]. Since resistance to enfuvirtide in failing patients is developing very fast, enfuvirtide should be considered as a drug with a low genetic barrier to resistance development, and this is true for all subtypes[97]. Implementation of resistance information on the newer drugs is less confident, and this is described further for maraviroc (See: Tropism testing and testing for resistance against maraviroc) and integrase inhibitors (See: Interpretation of resistance).

Since resistance is not a discrete variable, a first challenge is to interpret genotypic and phenotypic resistance test results into different levels of constraint against the use of particular drugs. Most genotypic interpretation systems consider discrete categories (for example susceptible, reduced susceptibility, resistant), a requirement resulting from the need to give simple and straightforward advice, but also because such systems are more easily designed[98]. Therefore, residual drug activity may remain, even when drugs are scored as resistant[99].

The most frequently used clinically available systems are listed in table 2. These systems are based on two different concepts. Some systems try primarily to predict phenotype under the assumption that phenotype predicts therapy response, such as Geno2Pheno and the VirtualPhenotype. Both use databases of genotypic and correlated phenotypic data, with information on clinical response allowing assessing the predictive power for therapy response of inferred phenotypic cutoffs. They provide a cheap alternative to phenotyping in case genotyping has already been done, but one should keep in mind that they suffer from the same caveat as all genotyping systems: they depend on existing knowledge and may be less reliable in the presence of new (combinations of) mutations. Other systems try primarily to predict therapy failure/response, such as all other systems listed in table 2. The most used systems currently rely on "rules" devised by experts using information extracted from databases of genotypic and correlated phenotypic or treatment response data. The aim of interpretation systems is to predict therapy response/failure, and thus they are validated for their clinical relevance by evaluating their predictive power for therapy response and failure in retrospective and, when possible, prospective studies[99]. Even among systems that have been shown to be correlated with therapy outcome, significant discrepancies in interpretation exist[100,101], in particular for non-B strains[100], although with our increasing knowledge such discrepancies are diminishing.

A new type of genotypic interpretation system is increasingly relying on large databases, where data mining tools allow to devise engines that use genotype, but also other information such as therapy history, CD4 count, and viral load, and that try to model resistance to drug combinations[102-105]. Such systems need large amounts of data to offer reliable advice, and for newer drugs there may still not be sufficient data. To overcome this caveat, some of these engines use rules-based scoring for particular drugs until more data become available. These systems are still rarely being used in routine clinical practice, but they may become more commonly used in the future. Up to that time, rules-based systems need to be continuously updated and clinically evaluated. Such systems provide genotypic susceptibility scores (GSS), some of which are guided by the system itself[106], while others rely on the knowledge of the user to convert levels of resistance into a score of susceptibility. Combinations of drugs are then scored by adding up the scores of the individual drugs (regimen GSS or rGSS), to be able to present the results in the form of expected activity of a regimen. Here again, some systems recommend minimum rGSS scores for particular clinical situations. For example, the Rega algorithm suggests building a regimen with a higher rGSS for treatment-naive patients with evidence of transmitted resistance, with the philosophy that hidden minority resistant variants against the first-line regimen may be present. If this would be the case, the effective rGSS would be lower than that based on observed mutations alone, and to target a higher rGSS would compensate for potential impaired activity[106]. The Rega algorithm also suggests to add a higher GSS weight to fully active boosted PI (1.5) compared to non-boosted PI (1.0), a concept that is gaining support in the field[107]. In this way, when evidence of transmitted resistance is present, the suggestion by the Rega algorithm to build a regimen with a higher rGSS is met simply by using a boosted PI instead of an NNRTI in first-line.

For the interpretation of phenotypic results, appreciation of several parameters is important. Assay reproducibility, often captured as a technical cutoff, indicates what differences in levels of resistance can be reliably measured. Some phenotype assays provide biological cutoffs, indicating the variation in phenotype of isolates from untreated individuals[108] below which a sample cannot be confirmed to have acquired resistance as a result of drug selective pressure. The currently most used variable is the clinical cutoff , the only cutoff developed to provide guidance as to levels of reduced drug susceptibility that compromise therapeutic response to each drug[109,110]. To date, clinical cutoffs are available for most drugs; however, for some drugs there is still a lack of guidance on which is the most relevant cutoff.

Even though our knowledge on genotypic and phenotypic interpretation is continuously increasing, reliable interpretation systems can still be discordant for particular samples, and in general they are designed to score individual drugs, not drug combinations. The Panel recognizes that there is still much room for improvement of interpretation systems. This can be done by analyzing large clinical datasets with improved analysis methods[105,111], by comparing interpretation systems and preferentially the individual rules in retrospective analysis[101,112], and by performing basic research to better understand the relationship between mutations and resistance and therapy response.

Testing at low viral load

Current guidelines state that the goal of therapy is to achieve and maintain HIV-1 RNA suppression below

Table 2. Clinically available genotypic drug interpretation systems update

Interpretation System	Source	Levels	Access
HIVdb v 6.0.10 Stanford, USA (20 Jan 2011)	Experts Rule-based	S/PL/LL/IR/HR	http://hivdb.stanford.edu/ http://www.hiv-grade.de
EuResist 2.0 EuResist Network GEIE (November 2011)	Database (> 6,000 TCE plus additional features)	Quantitative. probability for short-term response with specific drug combinations	http://www.euresist.org
GenoSure MG V12; Monogram Bioscience/LabCorp (October 2010)	Proprietary computational algorithm (>200,000 G/P)	S/RP/R	http://www.monogramhiv.com
ANRS v 19 (HIV-1 and HIV-2) France (July 2010)	Experts Rule-based	S/I/R	http://www.hivfrenchresistance.org/index.html http://hivdb.stanford.edu/pages/asi/ http://www.hiv-grade.de
ResRIS (in Spanish); Spanish AIDS Network (January 2010)	Experts Rule-based	S/I/R	http://www.retic-ris.net
HIV-TRePS 1.0 RDI (2010)	Database (> 60,000 TCE plus additional features)	Quantitative. probability for short-term response with specific drug combinations	http://www.hivrdi.org/treps/
HIV-GRADE 12/2009 Germany (December 2009)	Experts Rule-based	S/I/LS/R	http://www.hiv-grade.de
Rega v 8.0.2 (HIV-1 and HIV-2) Leuven, Belgium (16 June 2009)	Experts Rule-based	S/I/R with drug-GSS weighting factors	http://regaweb.med.kuleuven.be/software/rega_algorithm/ http://hivdb.stanford.edu/pages/asi/ http://www.hiv-grade.de
AntiRetroScan v 2.0 ARCA, Italy (10 January 2009)	Experts Rule-based	100/75/50/25/0 in % activity with drug-GSS weighting factors	http://www.hivarca.net/includeGenpub/AntiRetroScan.htm
Virco®TYPE HIV-1 report vpt-LM 4.3.01 Virco (5 January 2009)	Database (> 59,000 G/P > 8,000 TCE)	Quantitative; lower clinical cut-off at 20% loss of response, upper at 80%	For information: http://www.vircolab.com
Geno2pheno v 3.0 Arevir, Germany (December 2008)	Database (> 1,000 G/P)	S/I/R Quantitative	http://www.genafor.org http://www.geno2pheno.org/index.php
ViroSeqTM v 2.8 Abbott/Celera (August 2008)	Experts Scores-based	S/P/R	No access or information on-line available http://www.abbottmolecular.com http://www.celera.com
TRUGENE® GuideLinesTM Rules 14.0 Siemens (Bayer) (June 2008)	Experts Rule-based	S/I/R	No access or information on-line available http://www.labnews.com/

The here listed version is the one available on January, 2011. These algorithms are all clinically evaluated (most retrospectively, some prospectively), and are regularly updated, please visit the indicated websites.
S: susceptible; PL: possible low level resistance; LL: low level resistance; IR or I: intermediate resistance; HR: high level resistance; R: resistance; RP: resistance possible; TCE: treatment change episodes; G/P: genotype/phenotype.

commercially available assay quantification limits, with recommendations to switch regimens upon virologic failure based on the adverse consequences of higher levels of viremia[5,7]. Replicating HIV continues to evolve if kept under pressure of (partially) failing antiviral therapy (See: Virus population dynamics and fitness of resistant virus). Maintenance of unchanged antiviral therapy in subjects with detectable viral load leads to further resistance accumulation, increase of cross-resistance, and then decreased chances of efficacy of subsequent drugs and regimens[113,114]. A substantial percentage of patients fails therapy with sustained viral loads < 1,000 RNA copies/ml plasma.

Therefore, to maintain future treatment options, clinicians must prevent resistance accumulation by detecting virologic failure early, and by quickly changing regimens to fully suppressive combinations. Most commercial HIV-1 genotypic resistance assays claim a limit of detection of 1,000 copies/ml. In persons with detectable viral load < 1,000 copies/ml, testing may be unsuccessful, but if a result is obtained it is considered useful[5,7]. Current genotyping can be adapted to perform well at lower levels of viremia, and several centers routinely perform resistance tests for patients with viral loads down to 300 copies/ml, or sometimes even lower, with high success rates[54,115-120]. As a result, the percentage of resistance tests performed with viral load < 1,000 copies/ml is increasing[53,119]. This reflects the new perception that a resistance test can provide information regarding the degree and type of resistance, also at low viremia.

In the recent paper focused on antiretroviral drug resistance in HIV-1-infected patients with low-level viremia in the UK[119], overall, 1,001 (12.7%) of 7,861 test results were obtained among patients with viral loads < 1,000 copies/ml. The number of tests performed at low viral load as a proportion of all tests increased over the years, from 27 (3.4%) of 787 before 1999 to 178 (21.9%) of 813 in 2006, whereas the total number of tests remained fairly stable. Testing at low viral load was more frequent among patients who had previously achieved a viral load < 50 copies/ml. Other factors associated with having a resistance test at low viral load included clinical center, more recent calendar year of testing, receiving therapy with nucleoside (or nucleotide) reverse transcriptase inhibitors (NRTI) plus a ritonavir-boosted PI, and no previous experience of virologic failure[119].

It is important to mention that multiple drug resistance mutations can be selected and detected also at HIV-1 rebound with low viral load[116,119,121,122]. In addition, resistance mutations have been found to accumulate in 68-93% of patients with persistent viremia between 50-1,000 copies/ml[123]. HIV-1 genotypic resistance testing among patients with viral loads < 1,000 copies/ml did not significantly reduce the likelihood of detecting resistance, compared with testing at higher levels of viremia[119]. Furthermore, several major resistance mutations were as likely to be detected at viral loads < 1,000 copies/ml as they were to be detected at viral loads above this level. These resistance mutations included for NRTI mutations 65R, 184V, and pathway 2 thymidine analog mutations; for NNRTI mutations 103N, 181C, and 190A; and for PR mutations 30N, 46I, and 82A[119].

Successful genotyping in HIV-1-infected patients with detectable viremia between 50-1,000 copies/ml is increasingly reported[115-118], while clinical validation of such results can in part be done, for example, through comparison with previous or follow-up genotyping[54]. Overall, the assessment that a genotypic resistance test is unreliable in samples with low-level viremia should be reconsidered: the reproducibility, to some extent reflecting the reliability, depends on the level of viral load and on the laboratory[124]. Therefore, recommendation of viral load cutoff should be related to the reproducibility of results at such levels, which may differ among laboratories. This level might change according to the expertise, the method used, and in particular the level of input viral RNA per test. Generally, genotyping tests performed below a viral load of 1,000 copies/ml require a larger volume of starting plasma (2-10 ml), concentrating the sample by centrifugation, and performing a nested PCR if necessary.

Overall, although data do not yet exist regarding the utility of HIV-1 genotyping at low viral load in terms of clinical outcomes, the use of HIV-1 genotypic resistance testing among patients with viral loads < 1,000 copies/ml is helpful and relevant in clinical practice to allow timely and optimized therapeutic changes. These may prevent the further evolution of the virus towards selection of mutations causing multiple cross-resistance, and such a strategy may preserve future therapeutic options.

The role of peripheral blood mononuclear cell DNA sequencing

While plasma HIV-1 RNA remains the preferred compartment for genotypic resistance tests, proviral DNA extracted from peripheral blood mononuclear cells (PBMC) represents an attractive alternative. Indeed, DNA is more stable than RNA, can be obtained easily, does not require reverse transcription, and can

be sequenced cheaply and efficiently. The PBMC-derived HIV-1 DNA and circulating HIV-1 RNA represent two different viral compartments: in the same individual, the former is an indicator of the total burden of viral resistance, including a mixture of wild-type and mutated viral strains, transmitted or archived during primary infection[27,125] or archived during chronic, HAART-treated infection[126]. Genotypic information in HIV-1 DNA reflects patient history of virologic failures during drug exposure; conversely, plasma HIV-1 RNA represents the actively replicating virus, originated from productively infected cells. Consistent with this different origin, comparative studies have shown that HIV-1 resistance mutations in HAART-treated individuals who experience virologic failure are detectable earlier in plasma RNA, but for a longer period in peripheral blood cellular DNA[127,128]. It is therefore not surprising that transmitted drug resistance can be more extensive in DNA than in RNA[129,130]. Literature about resistance patterns in DNA and RNA suggest different degrees of discordance between the two compartments[126,128,131]. Some reports demonstrate concordance; for example, a recent work on samples from 253 newly diagnosed individuals found a correlation between RNA- and DNA-derived sequences in the *pol* region, suggesting that genotypic testing could be carried out on either template[132]. Methodological aspects, stage of disease, and virus replication dynamics are likely to account for these different results.

Archived mutations may be associated with subsequent treatment failure in HAART-naive and -experienced subjects[133-135] and for some clinical conditions data exist supporting the use of HIV-1 DNA rather than RNA. This is the case for subjects who experience virologic failure with very low viremia and those with undetectable viral load in whom a therapeutic switch is planned for simplification purposes[136] or toxicity problems. In these settings, genotyping plasma HIV-1 RNA is usually unsuccessful, while mutational pattern in PBMC HIV-1 DNA may help the choice of the new regimen. When switching strategy includes the use of a CCR5-inhibitor, HIV-1 DNA V3-loop sequencing can provide an easy, cheap and reliable alternative compared to last detectable plasma RNA sequencing for assessing viral tropism in patients with undetectable plasma viremia[137-140]. One of the drawbacks of PBMC HIV-1 DNA genotyping is that there is the potential that the DNA sequence detected in this way is a non-viable sequence, archived but not able to replicate, and such variants are probably of little clinical significance.

Since our knowledge is as yet not sufficient to support a clear role of HIV-1 DNA genotyping in clinical practice, the Panel decided not to include specific recommendations on this topic in the guidelines, and to consider it a research subject deserving additional studies, except with regard to tropism testing (See: Tropism testing and testing for resistance against maraviroc).

Resistance testing for new drug classes and for different genomic regions

Tropism testing and testing for resistance against maraviroc

Maraviroc is the second entry inhibitor that has been marketed. It is currently still the only one targeting a cellular coreceptor of HIV, in this case CCR5[141-144]. Tropism testing prior to the use of a CCR5 antagonist is indicated according to treatment guidelines[5,7] and the maraviroc package insert. Specific guidelines concerning tropism testing are reported elsewhere, and extensive argumentations can be found there[140]. However, since tropism testing can be considered a special case of resistance testing, some issues need to be clarified also in resistance testing guidelines.

The two main coreceptors used by HIV-1 to enter the cell are CCR5 and CXCR4[145]. HIV-1 can use CCR5 (R5 variants) or CXCR4 (X4 variants) or both (dual-tropic variants). These variants may also occur in parallel (mixed-tropic). The X4 or dual-tropic variants can occur as minority and may lead to therapy failure in maraviroc-containing regimens. However, these minorities do not always lead to failure, as reported in retrospective analyses of maraviroc studies[146]. Whether there is a single cutoff for X4 or dual-tropic variants below which maraviroc is still active has not been determined yet, and it may vary according to the test used and the antiviral activity of the entire regimen. The specificity and sensitivity to detect minority variants with dual tropism or X4 tropism and thus the predictive power for maraviroc response is also dependent on the assay and subsequent interpretation system. In addition, in many patients, coreceptor tropism of the virus population shifts along the course of disease progression from solely or mainly R5 during early infection to dual/mixed-tropic or X4-tropic[146], although rarely the reverse shift can be observed[147]. Maraviroc treatment can also cause a shift in tropism test result from R5 to dual/mixed X4, and discontinuation of maraviroc can cause the reverse shift[146]. As a consequence, the assessment of tropism of the current replicating virus

needs to be performed on a sample as recent as possible. Moreover, at failure of a maraviroc-containing treatment, tropism testing assesses whether failure was associated with (a potential preexisting minor variant of) dual/mixed X4 virus.

Since, in treatment-failing patients, viral coreceptor tropism determination is mandatory when the use of CCR5 antagonists in the next regimen is considered, the availability of the tropism result along with the information on drug resistance would give the clinician the opportunity to also consider maraviroc. In patients for whom a treatment change is needed, tropism testing allows the clinician to assess whether a CCR5 antagonist can be included in the new therapy. In this context, in case a therapy change is necessary at undetectable plasma viral load, tropism testing may need to be done on either the proviral DNA or the latest plasma sample with sufficient viral RNA; however, neither of these strategies has been clinically validated. The experience with proviral DNA is still limited[137,138,148,149].

Tropism can be assessed using either phenotypic or genotypic assays and this is discussed in detail in Vandekerckhove, et al.[140]. The original Trofile assay (OTA)[150] has been used in the prospective evaluation of maraviroc. However, this assay is not available any more. The currently used enhanced sensitivity Trofile assay (ESTA) has a higher sensitivity in detecting minority X4 variants. Both ESTA and genotypic tropism assessment based on population sequencing of the gp120 V3 loop have been extensively evaluated retrospectively and also prospectively in a limited number of uncontrolled observational studies[151-153]. An R5-tropism result from either the ESTA or V3 loop genotypic sequencing (either population sequencing or deep sequencing[154]) has been found to correlate with favorable outcome in retrospective analyses of clinical trials and cohort studies. Therefore, both approaches can be used, although V3-loop sequencing needs standardization and quality control since it is currently still mainly based on in-house methods in a limited number of laboratories. Such efforts are also needed for the bioinformatics tools that have been developed for the interpretation of genotypic tropism testing[154]. The most frequently used and clinically evaluated bioinformatics tool is the geno2pheno coreceptor interpretation system[154]. For guidance on the use of such tests we refer to the tropism testing guidelines[140].

Apart from tropism shifts, escape from maraviroc selective pressure has been observed in R5-tropic strains, associated with resistance mutations in the V3 loop[155], but mutations outside of the V3 loop have also been observed, although their significance has not been reported yet. Both *in vitro* and *in vivo*, albeit rarely observed, resistance to maraviroc in R5 viruses seems to be associated with inhibitor-bound use of the CCR5 coreceptor[155-157]. Considering the variability of the HIV envelope, and in particular the V3 loop, designing genotypic drug resistance interpretation systems for maraviroc will be challenging.

Testing for resistance to integrase inhibitors

HIV-1 integrase is one of the three virally encoded enzymes essential for replication. Raltegravir is so far the only integrase inhibitor approved for the treatment of HIV-1 infection[158-160]. However, elvitegravir is an experimental integrase inhibitor currently in the last step of clinical development[161]. Information about raltegravir resistance mutations mainly derives from registrational clinical trials. Three signature raltegravir resistance mutations were identified in integrase in phase II[158] and phase III[159-160] studies: 155H, 148C/H/K/R and, less frequently, 143R/C. Mutations 155H and 148K/R/H were mutually exclusive and conferred phenotypic resistance to raltegravir *in vitro*, with mutations at codon 148 resulting in measurably larger reductions in susceptibility than 155H. This is probably why 155H can be selected early in the course of raltegravir resistance, but is later replaced by genotypes that include mutations at codon 148[162]. It is interesting that amino acids at codons 148 and 155 are both located around the catalytic DDE motif of the integrase[163]. At this time, factors influencing the preferred selection of any of these mutations as well as its clinical implications are unclear[164].

Much of the current information on elvitegravir resistance derives from analysis carried out on patients experiencing virologic failure in phase II trials conducted in highly antiretroviral-experienced patients[165]. The most common resistance-associated integrase mutations seen in ritonavir-boosted elvitegravir-failing patients were 92Q, 138K, 148R/K/H, and 155H. Other resistance-associated changes include 147G and 66I/A/K. This shows that extensive cross-resistance exists between elvitegravir and raltegravir, despite the different structure of these compounds[166].

Given that the HIV PR, RT, and integrase share the same polyprotein precursor, and that integrase is cleaved by the viral protease, it has been hypothesized that interactions between changes at these proteins may occur, complicating the interpretation of resistance changes[167]. However, a recent study could not

confirm these findings[168]. Naturally occurring polymorphisms at resistance-related positions in the integrase are more frequent in some subtypes and in HIV-2 than in subtype B[168-173]. Variants other than the abovementioned resistance-related ones seem, however, not to impair the activity of integrase inhibitors. It should be noted that the catalytic domain of the integrase of HIV-1 group M subtypes and of HIV-2 is highly conserved[167,169,174].

Only two case of transmission of HIV-1 variants harboring primary (major) integrase inhibitor resistance mutations has been reported so far[175-176], but more cases are expected to appear in the future[177]. Therefore, baseline testing is not currently recommended. However, polymorphisms in integrase and the presence of minority variants with resistance mutations, mainly 148R, have been reported in integrase inhibitor-naive individuals, although their presence has not consistently been associated with subsequent virologic failure[178,179]. Studies assessing whether these polymorphisms might influence the genetic barrier and/or drive the selection of specific resistance pathways are warranted.

The current evidence suggests that the first-generation integrase inhibitors raltegravir and elvitegravir display a low genetic barrier to resistance, with only one major mutation required to produce significant loss of drug susceptibility and therapy failure. This explains the importance of ensuring activity of accompanying agents to reach and sustain complete viral suppression, as recently shown in the SWITCHMRK trial[180]. Second-generation drugs within this family (e.g. dolutegravir), however, seem to require more than one change to significantly compromise their activity[164].

Discontinuation of raltegravir in patients who fail therapy with selection of 155H or 148R/H/K is typically, but not always, associated with disappearance of these mutations within six months, suggesting that these changes impair viral fitness[181]. However, there is evidence for a compensatory role of some mutations, for example 140S for 148R/H changes[182], and clinical evidence does not support a residual therapeutic benefit of maintaining raltegravir in patients with resistance-associated mutations to the drug[183], while maintaining raltegravir would increase the risk for cross-resistance to next-line integrase inhibitors.

Evaluation of "other" genomic regions for clinical management

Antiretroviral drug resistance assays usually target PR and the N-terminal half of the RT gene. With the introduction of the new entry and integrase inhibitors, for two additional regions, parts of *env* and *integrase*, there is an increasing demand for testing these genes. However, several studies have shown that mutations and polymorphisms in Gag cleavage sites and the RT C-terminus, respectively, may modulate HIV-1's susceptibility to PI and RT inhibitors *in vitro*. Mutations in Gag can confer resistance to the experimental drug class of maturation inhibitors[184-188], restore decreases in viral fitness derived from PR mutations[189,190] and decrease PI susceptibility in the presence or absence of mutations in the PR gene (Gag 431V or 437V, 449F/H, 451T, 452S, 453A)[191-195]. Mutations in the RT connection (348I, 369I, 399G/D, 335C/D, 360I/V, 365I, 371V, 376S) and RNAseH domains (509L) are often co-selected with thymidine-associated mutations during NRTI therapy[196-200]. Such substitutions increase NRTI resistance further, particularly in the presence of thymidine-associated mutations and/or 184V, but do not seem to have an independent clinical impact[201,202]. Some mutations (348I, 369I, 376S and 399G/D) also confer decreased susceptibility to NNRTI.

The clinical relevance of these *in vitro* findings, however, remains unclear. The overall phenotypic impact of mutations in Gag and the RT C-terminus is usually small[191,203]. Large studies evaluating the impact of Gag mutations on virologic outcomes are lacking[191,204]. With the possible exception of 348I[197], C-terminus mutations in RT typically emerge after well-known RT resistance mutations appear, and thus most often do not change the susceptibility score of the patient virus in phenotypic assays[205]. Thereby, the additional information conveyed by C-terminus mutations regarding NRTI or NNRTI susceptibility is usually small. The systematic interpretation of the clinical significance of mutations in Gag or RT C-terminus is further complicated by the fact that they are not included in validated drug interpretation algorithms. Finally, there is little evidence that detection of mutations in Gag and the RT C-terminus could substantially improve the clinical management of HIV-1-infected subjects, compared with current standards. Although connection domain mutations were associated with increased risk of virologic failure to NNRTI-based regimens[197,206-208], they were not clearly associated with reduced phenotypic susceptibility or virologic response to etravirine in the DUET trials[209]. It might be interesting to perform Gag resistance testing in selected subjects who, despite having adequate adherence to ART, experience virologic failure to PI in the absence of resistance mutations in PR. Similarly, C-terminus resistance testing could provide information

whether prior NRTI experience could have compromised the use nevirapine. However, such strategies are still a research area.

Given the abovementioned considerations, the Panel believes that at present there is not enough evidence to recommend systematic genotypic or phenotypic evaluation of mutations in Gag or the RT C-terminus to inform treatment decisions. The Panel also acknowledges the need for continued research to further understand the clinical implications of mutations in Gag and the RT C-terminus, in particular, the percentage of patients whose treatment outcome is affected by substitutions in Gag or RT connection and RNAseH domains.

HIV diversity and antiretroviral drug resistance

Resistance and genetic diversity of HIV-1 in Europe

Compared to the USA where HIV-1 subtype B is responsible for over 95% of infections, European countries have a varying but much higher and still rising prevalence of different HIV-1 non-B subtypes[63,210], especially in countries with historical ties to Africa, countries with high immigration rates from endemic regions, and in some Central, Eastern, and Southern European countries where the epidemic is driven by particular non-B subtypes[211-214]. The SPREAD study[215] reports an overall prevalence of 30% non-B strains, but with only limited data from Central and Eastern Europe.

It may be relevant to be aware of the subtype when performing resistance testing, both for technological and interpretation reasons. Not all resistance assays perform equally well on strains from different subtypes[216-218], although continuous efforts are made to improve assays in this respect. Since treatment response is usually measured using virologic criteria, an additional complexity is added since also not all viral load assays perform equally well across subtypes[219-221].

Although, in general, similar drug resistance mutations are found in the different subtypes, and so far clinical data suggest that treatment responses do not differ too much between subtypes, some differences in resistance pathways according to subtype have been reported[63]. For example, in patients failing a nelfinavir-containing therapy, subtype B strains most commonly develop the 30N marker mutation in PR, whereas those infected with subtypes A, C, or G develop more frequently the 90M mutation[222-225]. Similarly, 106M in RT upon failure with a NNRTI is more often observed in subtype C[226,227], while 210W is less often observed upon failure with NRTI in subtype F[228]. Non-B subtype strains also have a higher prevalence of secondary, or minor, PI resistance mutations[229,230] and new variants at NNRTI resistance-related positions[224]. A new mutation 89V/I was identified, associated with PI therapy failure and phenotypic drug resistance in subtypes G and F but not in subtype B[231,232]. All HIV-1 non-B subtypes display natural polymorphisms in positions associated with HIV-1 drug resistance in subtype B, especially in the PR region. In addition, some non-B subtypes are not polymorphic at drug resistance-related positions, but have a different wild-type amino acid compared to subtype B[229,233,234]. In some cases the resistant variant derived from subtype B studies is the wild-type variant in other subtypes, while therapy response seems not affected[63]. Similar observations have been made for integrase inhibitor drug resistance; the mutations seem the same, but their prevalence and impact on the phenotype can be different among different subtypes[235,236]. While substantial information has been gathered on the impact of subtype on drug resistance, such information is still lacking for tropism testing.

Currently, all available interpretation algorithms use a subtype B sequence as a reference also when dealing with HIV-1 drug resistance testing of non-B subtypes. It seems logical to introduce a subtype-specific reference sequence for each subtype. However, due to the explosive growth of HIV-1 genetic diversity, frequent recombination and sometimes lack of phylogenetic signal to determine the HIV-1 subtype from the PR or RT sequences only, a subtype-specific reference sequence approach in HIV-1 drug resistance testing of non-B subtypes is not realistic. Thus, in HIV-1 drug resistance testing of patients infected with non-B subtypes, use of a subtype B reference remains the standard, and subtype-specific issues need to be dealt with in the interpretation algorithms.

However, discordance between genotypic drug resistance interpretation systems is subtype-dependent[100]. In case different mutations are observed in different subtypes, simple knowledge of such mutations can elicit their inclusion in interpretation algorithms. It is more difficult to take into account potential differences in resistance levels when the different subtypes have similar mutations[237]. Therefore, the main question remaining is the interpretation of results[238]. Until interpretation systems can properly deal with this subtype issue, the question arises whether for HIV-1 drug

resistance testing of patients infected with non-B subtypes, natural polymorphisms at resistance-associated positions should be subtracted from the final score. The Panel could not reach consensus on this important question since the opinions in favor and against were almost equally distributed. The major argument for the subtraction of natural polymorphisms from the final score was that the available phenotypic studies dealing with drug-naive patients infected with non-B subtypes showed that such natural polymorphisms are very frequent and do not have an important impact on baseline resistance[239-241]. Those who were against subtraction of natural polymorphisms from the final score most frequently quoted the lack of basic understanding of resistance mechanisms in non-B subtypes and lack of clinical data for implementation of such strategy.

To increase our knowledge, the Panel supports the collection across Europe of epidemiological, genotypic (resistance and tropism) and therapy response data in all subtypes, and recommends to differentiate between the different (non-B) subtypes. It is preferable to organize this at a pan-European level. To facilitate such data collection, subtype information is best added to HIV-1 drug resistance testing reports. Researchers should, however, be aware that subtyping methods have their own problems, which will not be discussed here.

Drug resistance in HIV-2

HIV-2 emerged from a different simian reservoir, sooty mangabeys, compared to HIV-1 which originated from a chimpanzee virus[242,243]. So far, eight distinct HIV-2 groups (A to H) have been described. Only groups A and B are responsible for epidemics in West Africa, where the number of infected people is estimated to reach more than one million[244]. Most cases in North America and Europe were identified in immigrants from endemic regions or natives who have lived in or have had sexual partners from those regions. It is important to distinguish HIV-1 from HIV-2 since the natural history, transmission, and response to therapy are quite different[245]. The highest prevalence of HIV-2 outside Africa is found in Portugal (up to 3.2 % of HIV diagnosis[246]). Since HIV-1/HIV-2 coinfected patients often arise as superinfection of one type with the other, special attention has to be paid to such patients[247] (See: Dual infections with HIV-1 and HIV-2).

Therapeutic decisions are not easy to make when treating HIV-2 patients. Fewer drugs are available since the virus has a natural resistance to NNRTI (nevirapine, efavirenz and etravirine) and enfuvirtide[248]. Although HIV-2 is susceptible *in vitro* to other antiretroviral agents, such as all NRTI, most PI, and integrase inhibitors[249], their resistance pathways are not yet fully characterized[248,250-254]. Since drugs are being developed to combat HIV-1, it is not surprising that antiviral effects of currently approved antiretroviral agents seem to be lower in HIV-2 than in HIV-1. The proportion of patients reaching undetectable viremia, maintaining virus suppression for prolonged periods, and especially achieving satisfactory CD4 recovery are poorer in HIV-2 than in HIV-1 carriers[255,256]. In addition, drug resistance also seems to develop faster in HIV-2 than in HIV-1[257]. Thus, although HIV-2 is thought to be less pathogenic than HIV-1, once advanced immunodeficiency has occurred the chances to restore a good clinical condition with ART are lower in HIV-2- than in HIV-1-infected patients. Follow-up is also hampered by the limited availability of technologies such as viral load assays and resistance assays. There are no commercial assays either for testing viral load or drug resistance in HIV-2. In addition, viral load in progressing patients is lower in HIV-2- compared to HIV-1-infected patients, and often progression even occurs in absence of a measurable viral load. Therefore, HIV-2 therapy response has to be evaluated using both viral load and CD4 cell count as prognostic markers.

Although the positions that are involved in drug resistance in HIV-2 are mostly the same as in HIV-1, with some exceptions[252,258], the different mutational pathways known for HIV-1 arise at very different frequency in HIV-2. The most worrying is that the multi-NRTI resistance pathways associated with RT mutations 151M and 65R are selected much more often in HIV-2 compared to HIV-1[259]. However, it may be that tenofovir is still active in presence of HIV-2 with 151M, as is the case for HIV-1[260].

With respect to PI, the susceptibility of HIV-2 seems to vary widely for the different compounds[261]. The HIV-2 protease shares only 50% AA sequence identity with the HIV-1 protease[262]. Kinetic inhibition assays have shown that lopinavir, saquinavir, tipranavir, and darunavir exhibit the highest potency, although usually weaker than against the HIV-1 protease[263]. Phenotypic tests confirmed that only the IC_{50} of saquinavir, lopinavir, and darunavir (but not tipranavir) are in the same range as observed in HIV-1[264]. While *in vitro* HIV-2 seems to have a reduced susceptibility to atazanavir (up to sevenfold), clinical evidence suggests that the activity of atazanavir is satisfactory as long as it is boosted with ritonavir. As for fosamprenavir, its efficacy

is clearly lower than in HIV-1. This lower potency of PI against HIV-2 is proportional to the lower *in vitro* affinity of the inhibitor against the HIV-2 protease as compared to HIV-1[263]. It is interesting to know that besides a lower potency against HIV-2, the genetic barrier for resistance in HIV-2 may also be lower than in HIV-1. The most striking example is lopinavir, for which HIV-2 is more prone to select mutation I47A, as it only requires one nucleotide change at this position, compared to two in HIV-1[265,266].

Integrase inhibitors exert a potent inhibitory effect on HIV-2 replication *in vitro*[172,267]. Raltegravir has been successfully used in salvage therapy in heavily pretreated patients, with at least a good short-term response[254,268]. In most cases, resistance was easily selected, reflecting both the low genetic barrier to resistance to raltegravir and the lack of enough active drugs in the optimized backbone regimens. Most mutations selected in integrase were similar to the ones found in HIV-1, including all the major mutations: 155H, 148K/R and 143R, plus 140S, 92Q, 97A[269,270]. Interestingly, some mutations seem to be HIV-2 specific: 91R, 147G, 153G, 175M and 183I[174,271] and the combination 91R+175M, selected *in vitro* under raltegravir pressure, confers phenotypic resistance to the drug (13-fold increase in IC_{50}). However, the real impact of these mutations on the efficacy of raltegravir *in vivo* remains to be elucidated. Further studies are also needed to evaluate the efficacy of raltegravir in first-line therapy in HIV-2 patients, where the low genetic barrier to raltegravir resistance might be protected by other fully active drugs in the regimen.

Transmission of HIV-2 antiretroviral-resistant strains has so far rarely been reported[272], but as antiretroviral agents are increasingly being used in this population, it must be expected to increase. Thus, for the time being it does not seem necessary to recommend universal drug resistance testing before initiating ART in HIV-2-infected individuals. However, the amount of data regarding HIV-2 resistance in patients failing therapy is of such a volume that it can be helpful to make appropriate decisions for rescue interventions, and it seems therefore appropriate to consider drug resistance testing in this setting. When interpreting HIV-2 drug resistance, a dedicated algorithm needs to be used (Table 2).

Dual infections with HIV-1 and HIV-2

Dual infections by HIV-1 and HIV-2 have been described since a long time[267]. One must be aware, however, that double serology does not necessarily mean dual infection. Double serology is relatively common when a Western Blot test based on a viral lysate is used to confirm an HIV infection in an HIV-2-infected patient, since cross-reactivity is frequent[274]. The diagnosis of a dual infection can only be made if nucleic acids from both viruses can be found in the plasma or cells of a patient, or if both viruses can be grown in viral cultures from a patient's sample. The diagnosis is not easy, since HIV-2 RNA is not found in plasma in early stages of infection, and proviral DNA is sometimes difficult to amplify in coinfected patients[275]. In the case of a dual infection, disease progression is driven, in most cases, by HIV-1. Consequently, all the recommendations for the HIV-1-infected patient must be taken into account. However, when choosing a therapeutic regimen, the selected drugs must be active against both viruses. A good regimen against HIV-1, but with only partial efficacy against HIV-2, can have a short-term benefit, suppressing HIV-1, but resistance will be easily selected by HIV-2, impairing the long-term prognosis.

Clinical indications for drug resistance testing

Rationale for drug resistance testing

Information on HIV drug resistance will help the clinician to design the best possible long-term therapeutic strategy for an individual patient. Resistance test results can identify drugs with reduced activity towards the patient virus, resulting in advice on the exclusion of such drugs in an optimal next therapy. There is currently not sufficient support to use resistance testing as advice on the inclusion of particular drugs in a regimen, even though new analysis methods are focused on predicting therapy response rather than therapy failure and they intend to suggest which combinations to use[276,277]. Compared to the previous version of the Guidelines[2], the current Guidelines also include the rationale to determine for each drug whether resistance is the cause of failure. As a result, the Guidelines have changed such that also for drugs with no second compound in this class, resistance testing should be recommended at failure. This is because failures are being tested at lower viral loads, when resistance may not have developed to all drugs in the regimen. The clinician needs to know which drugs remain susceptible and can be safely reused. This strategy has the advantage that the clinician immediately

has a better picture of the situation to make a next decision. Even though it is possible in such situations to store samples and retrospectively perform resistance testing when reuse is being considered, a resistance test result is more easily stored than samples. However, this will result in additional costs, and not all centers may be able to carry this cost. On the other hand, storage of samples is also not always affordable, and recovery of stored samples can sometimes be problematic. Drug resistance testing is also valuable to provide epidemiological data throughout Europe. The recommendations below are for all individuals infected with HIV, including pregnant women and pediatric patients.

Drug-naive patients

Although mainly retrospective data on PR and RT drug resistance are available, several studies have shown that resistance in drug-naive patients, whether recent or chronically infected, may reduce the efficacy of the initial and/or subsequent regimens[47,278-284]. Moreover, patients in which one of the drugs of their first-line combination therapy was compromised with TDR also have a higher risk of developing further resistance even to those drugs in their regimen that were originally fully active[8] and a higher risk to a faster disease progression[284]. Since only a limited number of fully suppressive regimens exist to construct a successful lifelong treatment, the rationale to test for drug resistance in naive patients is that an optimal choice of the first regimen is crucial and should take into account transmitted resistance. Resistance genotyping is considered cost-effective for both recent and chronic infection when levels of TDR are > 1-5% (See: Cost issues)[285-287]. Transmitted resistance for PR and RT inhibitors has been observed in most European countries (5-10%; See: Transmission of drug resistance), while for the newer drug classes, transmitted resistance seems currently not to be a problem (See: Interpretation of resistance). Because of reduced fitness in absence of drugs, transmitted resistance mutations may revert to wild-type virus (See: Virus population dynamics and fitness of resistant virus). Therefore, testing of the earliest sample may be more representative of potential TDR. Early or retrospective testing in drug-naive patients is an acceptable strategy despite the risk of superinfection.

The Panel recommends testing for PR and RT drug resistance in both recent (or acute) and in chronic infection (Table 1). Compared to the 2006 update of the Guidelines[2], these recommendations are now also valid for pregnant women and pediatric patients. The Panel recommends testing the earliest sample available, but taking care with the interpretation of the results when superinfection is suspected (e.g. patients with high-risk behavior; See: Superinfection). When superinfection is suspected, both the earliest and the most recent sample before starting ART may be tested. However, in people with high-risk behavior, possible superinfection should always be kept in mind when judging the result of early or retrospective resistance testing. Acute or recent infection is defined as documented seroconversion within the previous six months[6]. Treatment of acute or recent HIV-1 infection is considered to be optional as indication relies only on theoretical considerations. However, in certain situations, such as the presence of AIDS-defining events or severe illness, treatment is indicated[5,7]. If a clinician decides to treat, treatment initiation cannot await resistance test results; rather the initial therapy can be revised if necessary once test results are available. For chronic infection, starting therapy is usually not an urgent issue, and resistance test results can be awaited before starting therapy.

Most clinically used algorithms do not differentiate their interpretation according to whether the patient is treatment-naive or not (See: Interpretation of resistance). In case there is evidence for TDR, e.g. through using TDR-surveillance algorithms[67], it is suspected that major drug resistance mutations have reverted but are still present as minority variants. Such minority variants can compromise the first regimen (See: Minority drug-resistant variants). In such cases, resistance test results may not reflect the full extent of transmitted resistance and may therefore call for a therapeutic choice taking this 'hidden' resistance into account (See: Virus population dynamics and fitness of resistant virus; Minority drug-resistant variants; Transmission of drug resistance; Interpretation of resistance). In this respect, one could argue to add a warning against the use of drugs with a low genetic barrier to resistance (See: Interpretation of resistance). Alternatively, one could suggest probing for minority variants carrying drug resistance mutations. The same warning against the use of drugs with a low genetic barrier to resistance might be valid when resistance testing before start of therapy has not been performed in regions with high prevalence of TDR. However, such differential interpretation is still in an exploratory phase, and measuring minority variants can currently also not be recommended (See: Minority drug-resistant variants).

Drug-exposed patients: virologic failure

Virologic failure is judged according to the European treatment Guidelines[6]. These currently define virologic failure as confirmed plasma HIV-1 RNA > 50 copies/ml six months after starting therapy (initiation or modification) in patients that remain on ART. Sufficient prospective data support that drug resistance testing at virologic failure improves virologic response to next-line therapy[1]. Since virologic failure can be due to the presence of resistance mutations, resistance testing allows assessing the contribution of resistance to virologic failure. However, therapy failure can also be caused by other factors, such as weak drug potency, lack of drug adherence, and pharmacokinetic issues. Therefore, a result indicating susceptibility to all drugs in the regimen may raise concerns regarding these other issues, and especially regarding adherence[9].

The Panel maintains its recommendation to test for drug resistance to PR and RT inhibitors in virologically failing therapy when treatment change is being considered (Table 1). Compared to the 2006 update of the Guidelines[2], testing of the envelope and integrase genes should be done, but only when entry inhibitors (EI) and integrase strand transfer inhibitors (INSTI) were part of the regimen. In case such resistance testing is not done or is not available, it should be assumed that during virologic failure of an INSTI- or EI-containing regimen, resistance to the respective drugs was preexisting or has developed (See: Interpretation of resistance). Lack of INSTI and EI resistance testing when failing under such drugs still allows the clinician to install a proper new regimen since no drugs with cross-resistance are currently available. However, in absence of resistance information, the judgment of the clinician on which drugs are actually failing is impaired. It will be more difficult to assess whether any of the drugs can be safely reused in a later stage or whether there are still other factors associated with this treatment failure. In addition, when new drugs within these classes are approved, information about any resistance pattern may be crucial for the potential choice of new drugs within these classes.

Resistance mutations often confer a replication disadvantage in absence of drug, resulting in shifting back to wild-type virus during treatment interruption (See: Virus population dynamics and fitness of resistant virus). Due to the risk of missing resistance mutations related to the failing therapy in absence of drug-selective pressure, the Panel recommends testing a sample taken when on therapy. Resistance test results should be considered together with treatment and resistance history.

CCR5 antagonists

While resistance testing for entry inhibitors is recommended when failing such drugs virologically, failure of a CCR5 inhibitor-containing regimen can also be associated with a (potentially preexisting) dual/mixed X4-tropic virus population (See: Tropism testing and testing for resistance against maraviroc). Whether this is the case needs to be assessed using a tropism test in addition to a resistance test. Moreover, for a clinician to be able to consider maraviroc for virologically failing patients, they need information on the tropism of the patient's virus before this drug can be included in a next-line therapy (See: Tropism testing and testing for resistance against maraviroc). When change is being considered at an undetectable viral load, the only accessible viral genome available in the patient's blood at that time is proviral DNA in PBMC (See: The role of peripheral blood mononuclear cell DNA sequencing).

Therefore, the Panel suggests considering tropism testing upon failing of CCR5 antagonists (Table 1). Since tropism and resistance can be estimated from the same virus genetic fragment, genotyping for CCR5 antagonists can serve both resistance testing and tropism testing (See: Genotyping or phenotyping). In this way, the genotypic tropism test result can reach the clinician together with resistance test results from the other drugs in the regimen, whereas a phenotypic tropism test result will almost always come later. The Panel also suggests considering tropism testing at undetectable viral load when treatment change is needed and a CCR5 inhibitor is considered, albeit with a consensus of only 58%. In this situation, proviral DNA should be used (See: Tropism testing and testing for resistance against maraviroc). Alternatively, or if no result can be obtained for DNA, the last sample with detectable viral load can be used to test tropism on viral RNA as explained by Vandekerckhove, et al.[140]. With respect to resistance testing for maraviroc, this is as explained in Drug-exposed patients: virologic failure; and Genotyping or phenotyping.

Inappropriate treatment interruption of a successful NNRTI-containing therapy

Treatment interruption is generally not considered a good strategy and resistance testing during treatment

interruption can give misleading results (See: Treatment interruptions). Therefore, the Panel does not recommend resistance testing during treatment interruption since no studies have shown the utility of this therapeutic strategy. However, appropriate treatment interruption of a successful NNRTI therapy, if needed, is described under the treatment Guidelines[6]. In general, at re-initiation after interrupting a successful treatment, if last treatment is resumed, this may safely be done without resistance testing, except in cases of inappropriate interruption of a combination of drugs with different pharmacokinetics, where accumulation of resistance to the drug with the longest half-life can occur during interruption. This recommendation deals with NNRTI, which have a longer half-life than most drugs and a low genetic barrier to resistance and thus can be considered as an exception where separate guidance is needed. Therefore, a majority of the Panel believes that in such cases it is useful to obtain a post-interruption plasma sample. Expected resistance reversal (overgrowth of more susceptible virus) in absence of drugs supports testing the sample within two months after treatment interruption. However, it is important to emphasize that no clinical trial has yet evaluated the impact on clinical outcome of this sampling approach. Treatment interruption of an NNRTI-containing regimen that failed virologically should be treated as any other virologic failure. In general, in situations when restarting treatment after an interruption with no information on resistance history or no on-therapy sample from previous failure stored, clinicians may choose to consider results of a resistance test on an off-treatment sample to help guide the next therapy. Interpretation may, however, be very difficult since, due to resistance reversal, some resistant variants may not be detected any more in the majority population, although they have been archived in the body, and it is currently assumed that all archived resistance can reemerge.

Whereas the previous version of the Guidelines considered treatment interruption[2], the current Guidelines only deal with inappropriate treatment interruption of a successful NNRTI-containing therapy. In such cases, at treatment re-initiation and if resistance history is not available, the Panel suggests considering retrospective testing of a post-stop sample as soon as viral load rises above the resistance testing threshold, if such an early sample is available. Test results need to be interpreted in view of treatment and resistance history, if available. If resistance testing is not performed, the Panel suggests storing the earliest viraemic plasma sample for later testing.

Post-exposure prophylaxis

The recommendation to test for drug resistance in cases of post-exposure prophylaxis (PEP) still holds[2] (Table 1). Treatment should be initiated without delay. If treatment history and/or resistance information are available from the source patient, the PEP therapy should be individualized accordingly. If resistance data for the source are lacking, genotypic resistance testing on a sample from the index case can be done, when available, and the results can be taken into account to modify the treatment if necessary. The purpose of PEP is to prevent or abrogate infection in the recipient by treating with a powerful combination for a period of four weeks. Any resistance result should therefore return to the clinician within a period useful to change or simplify the prophylaxis itself. Hence the recommendation to genotype as soon as possible, but preferentially within one week since it is generally believed that changing therapy according to resistance test results after one week is not expected any more to help preventing infection. There are, however, no solid scientific data to make a clear timeline of when such resistance data are not useful any more in PEP context.

HIV-2

Treatment options for HIV-2 patients are more limited than for HIV-1 patients, and our knowledge on drug resistance in HIV-2 is lagging behind what is known for HIV-1 (See: Drug resistance in HIV-2). Therefore, the Panel is more careful in expressing recommendations for drug resistance testing for HIV-2-infected patients. There are, however, sufficient retrospective data indicating an association of some mutations with therapy failure. Similar as in the 2006 update[2], the Panel suggests considering resistance testing when treatment change is needed after therapy failure. In the case of HIV-2, therapy failure should be judged always on CD4 evolution together with viral load. Amprenavir/fosamprenavir, NNRTI and enfuvirtide resistance testing is not recommended because HIV-2 has a natural resistance to these drugs and they should therefore not be used. In-house genotypic and phenotypic systems are available in specialized laboratories; if available, consider both genotyping and phenotyping. The genetic barrier towards resistance for some NRTI and for most PI is lower than for HIV-1. As a result, unboosted PI are less useful for HIV-2 treatment, and HIV-2 patients are much more difficult

to treat than HIV-1 patients. Recent data suggest that HIV-1 integrase inhibitors are also potent inhibitors of HIV-2 integrase, with good therapy response data (See: Drug resistance in HIV-2), and information on resistance mutations to raltegravir is increasing fast. Therefore, integrase resistance testing may be useful for HIV-2 patients failing this drug, as soon as interpretation systems can include such information. However, at this time, the Panel did not formulate particular recommendations in this respect.

For HIV-1/HIV-2 coinfected patients, resistance testing should be done as indicated for HIV-1 and HIV-2 respectively, and the results of both tests must be taken into account when selecting a new regimen (See: Dual infections with HIV-1 and HIV-2). To avoid resistance development for HIV-2, the clinician should take care to install a regimen that is active against both viruses.

Technical considerations for laboratories

Genotyping or phenotyping

Although genotyping and phenotyping provide complementary information, genotyping has a more extensive clinical validation, better accessibility, lower cost, and faster turn-around time (see also 8). Consequently, additional phenotyping results in a substantial additional cost, which can currently only be considered when genotypic guidance is insufficient. For new drugs and for HIV-2, there is not always sufficient knowledge on resistance mutations. In such cases, both phenotyping and genotyping are useful until reliable genotypic interpretation systems have been developed. The Panel therefore recommends the use of genotyping in most routine clinical situations, and to consider additional phenotyping only for new drugs, in heavily pretreated patients, and for HIV-2 where genotyping is not easily interpretable.

Current genotyping can be performed below a viral load of 1,000 copies/ml (See: Testing at low viral load). Some centers can reliably genotype down to a viral load of 300 copies/ml (or sometimes even lower, depending on the protocol), so at which cutoff samples can be sent for genotyping depends on the experience and protocols of the respective laboratories. Clinicians should, however, be aware that if a genotype can be obtained, the closer the viral load is to the test cutoff, the higher the risk that the genotype will not be representative for the actual replicating virus, since at those levels stochastic distributions play a role. At the lowest viral loads close to the viral load cutoff, it may even be possible that virus production from infected cells is seen in a patient in whom further replication of the produced virus is blocked by potent treatment, and that is technically not a virologically failing patient.

Tropism testing requires separate assay recommendations, as described by Vandekerckhove, et al.[140]; however, samples for resistance genotyping can also be used for tropism genotyping. Maraviroc resistance interpretation is, however, still problematic.

Interpretation systems

Interpretation of resistance is crucial. Since therapy response is dependent on many more factors than resistance only, resistance test results should be interpreted in the context of all factors important for therapy response. In a first approach, all genotypic and phenotypic resistance interpretation systems translate the test result into different levels of constraint against the use of particular drugs (See: Interpretation of resistance). Database-driven systems increasingly include other information in their guidance; for example, the Euresist engine allows to include therapy experience, CD4 count, and viral load in addition to genotype[105] (Table 2). It is important that clinical virologists and clinicians learn how to use such systems, taking into account on which knowledge the guidance is based (See: Interpretation of resistance). Such engines may report advisable combinations instead of constraints against particular drugs, usually as a ranking of which therapy combinations would have the highest potency for the patient evaluated. A trained clinical virologist or clinician has learned to use interpretation system results as constraints against particular drugs, and then adds additional variables on top of the interpretation of resistance genotypes when formulating the expert advice. It will require adaptation to take full advantage of the more complex guidance engines where part of that information is built into the system.

For genotypic interpretation systems, the Panel recommends laboratories to use continuously updated and clinically evaluated resistance interpretation systems and to compare the results of the different available interpretation systems. Interpretation systems, both genotypic and phenotypic, should be able to discriminate between boosted and non-boosted PI. Drug resistance in divergent subtypes and HIV-2 is currently still more difficult to interpret than in HIV-1 subtype B. Interpretation of a resistance test result for new drugs

requires special attention from an expert. When interpretation systems are not concordant, the expert needs to make careful statements in the advice to the clinician, and give more consideration to the more recently updated systems and to systems that have been demonstrated to provide good prediction of treatment response. Interpretation system developers should update their system regularly and compare its performance with other available systems. The laboratory should store the sequences for future re-interpretation, for example when reviewing the resistance history in the context of later resistance test results.

A genotypic report for clinicians should include a list of observed drug-related resistance mutations, an interpretation of the results with indications of which expert system was used (identifying the algorithm and version), and expert advice. For phenotypic interpretation, the laboratories are recommended to consider the clinical cutoff of the assay, if available, otherwise to use the biological cutoff (See: Interpretation of resistance). A phenotypic report should include a list containing the observed fold-reduced susceptibility towards clinically used drugs, and an interpretation of the results with indications of whether clinical cutoff was used, and expert advice.

Resistance test results should still be interpreted as different levels of constraint against the use of drugs with evidence of resistance, since therapy response is dependent on many more factors than resistance only. Proper interpretation can only be done in view of treatment history and previous resistance test results. The laboratory is therefore encouraged to request and take into account the complete treatment and resistance history when writing the expert advice, and the clinician should include in his considerations also the clinical context, including the therapy and resistance history. Interpretation should also be done in the context of adherence. Lack of adherence can result in suboptimal therapy response in absence of resistance, but lack of adherence is also correlated with resistance development[9]. For drugs with a low genetic barrier, such as lamivudine, emtricitabine, and the current NNRTI, absence of resistance in the face of virologic failure should alert the expert to poor adherence.

Another important issue is drug levels. Drug levels and the inhibitory quotient (IQ) are correlated with therapy response[288,289]. Insufficient drug levels may place the patients at higher risk of treatment failure and development of resistance mutations, and intermediate resistance may be overcome by high drug levels, as in the case of boosted PI. However, integrating drug level monitoring with resistance test results has now been abandoned by most experts, and guidance in drug level monitoring has become a concern of treatment guidelines[5-7]. Discussion with a HIV pharmacology expert may nevertheless be beneficial in a multidisciplinary approach of selecting the optimal therapy for an individual patient, along with interpretation of resistance testing results.

The Panel recommends clinicians and virologists to discuss with each other resistance test results of complex cases in the context of all other factors that can influence therapy response, and to remain aware of the difficulties associated with interpretation of HIV drug resistance. Interpretation of resistance test results for new drugs requires special attention from an expert.

Laboratory quality control requirements

Laboratories engaging in drug resistance genotyping for clinical practice should comply with a set of minimal quality control requirements. Quality controls for HIV-1 genotyping have revealed large performance differences between laboratories, in particular in scoring resistance mutations in samples containing mixtures[290]. For HIV-2 genotyping, however, quality assurance programs are still lacking.

The Panel estimates that the following minimal quality control requirements should be mandatory.

For genotyping: (i) inclusion of proper negative and positive controls during each extraction/PCR; (ii) editing of the sequence should be traceable; (iii) resistance-related positions should be evaluated by sequencing in both directions; (iv) participation in and passing, at least once a year, an external proficiency Panel test; (v) a sample with a known genotype should be re-sequenced at least every two months or every 50 sequences, whichever comes first; (vi) interpretation of the results should be documented. In addition it is advisable to compare consecutive sequences of the same patient and samples tested simultaneously (for example phylogenetically or using a BLAST approach), which may allow to identify contamination or superinfection.

For phenotyping: (i) inclusion of proper negative and positive controls during each extraction/PCR; (ii) sequence recombinant virus used in the test or have a quality control system in order to ensure proper representation of genotypically confirmed resistance mutations; (iii) fold resistance values should be expressed versus a reference laboratory strain, which should be included in every run (currently still subtype B

in order to allow comparability between tests; See: Resistance and genetic diversity of HIV-1 in Europe); (iv) participation in and passing, at least once a year, an external proficiency Panel test; (v) interpretation of the results should be documented.

The proficiency Panels should preferably contain plasma samples, including some with low viral load, different subtypes, and samples with mixtures at resistance-related positions. Mutants present as mixtures identified in a standard sequencing assay should be scored. At least 99% of all resistance-related mutations (excluding sample with mixtures) should be reported correctly compared to peer group consensus, and at least 50% of all resistance-related mutations present as mixtures. Proficiency Panels should only test the performance of laboratories, compared to peers that were using an approved test.

Sample storage

Considering the limited resources for resistance testing in some European countries, the issue of cost-effectiveness weighs heavily on the discussion to perform resistance testing, especially for drug-naive patients. Where laboratory resources are too limited and where current rates of transmission of resistance are very low in patients at risk, laboratories might have no choice than to prioritize resistance testing for failing patients. An acceptable alternative can be to store the earliest plasma sample available for later retrospective testing. Therefore, the Panel suggests, if resistance testing cannot be performed as indicated, to consider storage of the recommended plasma sample. However, it may be that storage of samples in centers with many patients also becomes an untenable financial burden. In this context it is worth noting that genetic information can be more easily stored. Storing samples in general is a useful strategy, allowing retrospective testing with updated techniques and knowledge. Prioritizing resistance testing requires sufficient knowledge of the local epidemiology and of cost-effectiveness studies (See: Cost issues).

To guarantee optimal results from resistance testing, the sample should be plasma, obtained before starting, stopping, or changing therapy, with a viral load above the detection limit of the resistance test (See: Testing at low viral load). Storage conditions should be such that the integrity of the virus RNA is maintained. Storing a 2 ml plasma sample at –80 °C will serve this purpose. Transportation should be performed by specialized carrier services.

Implementation throughout Europe

Cost issues

In the current economic situation, cost-effectiveness becomes one of the main criteria demanded by government health strategies to guide decisions on implementation of resistance testing in clinical practice. Issuing guidelines that are not economically feasible will result in such guidelines being ignored in clinical practice. All cost-effectiveness studies rely on many parameters, such as clinical evidence, surveillance data, and on what cost a society is ready to bear. Resistance testing in different circumstances may be cost-effective in most European countries, but possibly less so in some Eastern and perhaps also some Southern European countries, and even less in developing countries. Cost effectiveness studies calculate the cost per quality adjusted life-year gained (1 QALY = 1 life-year in good health), comparing these with other health costs generally accepted in the community. Both increase in quality of life (even without life-years gained) and in life expectancy will contribute to judging a medical intervention to be cost-effective.

Resistance reduces therapeutic options, and resistance testing can indicate which drugs are affected, but also, if done on a sample under drug selective pressure, which of the drugs in that regimen are not affected by resistance. In absence of resistance testing, and until proper models are available to predict resistance development or therapy response without genotypic information[291-293], it should be assumed that resistance to all drugs of the virologically failing regimen has developed. In this scenario, the consequences of virologic failure without drug resistance testing are more rapid reduction in clear treatment options, increased direct and indirect health costs associated with the need to start earlier with usually more costly next-line treatment for patients, and hence the need to develop new anti-HIV drugs. The average annual cost of a second-line and salvage ART regimen can reach up to eight-times that of a first-line regimen[294]. Furthermore, with the loss of each line of therapy, therapeutic options become more and more limited, reducing ultimately the lifespan of these patients, which significantly increases the potential years of life lost due to premature death[295]. In addition, not only the quality of life, but also the costs of healthcare are higher in patients that have progressed to AIDS.

Optimization of antiretroviral treatment regimens over two years, taking into account genotypic drug resistance

testing as compared to expert opinion, led to a reduction of healthcare costs in a Swiss cohort patients with treatment failure[286,296]. In the analysis from the societal perspective, including healthcare costs and productivity of the patient, the benefits were six weeks of quality adjusted life expectancy per person (two weeks when only considering healthcare costs). At a willingness to pay € 630 or more per QALY gained, genotypic drug resistance testing-guided therapy at virologic failure is the preferred treatment option (> 50% probability of being cost-effective). Using the widely used societal threshold of € 37,000 (US$ 50,000) per QALY gained, genotypic drug resistance testing at virologic failure is cost-effective with a probability of 88%. This is comparable to other accepted healthcare costs, and more cost-effective than antibiotic prophylaxis against *M. avium* complex infection in patients with AIDS. Therefore, in Switzerland, and countries with similar epidemiological and socioeconomic parameters, genotypic drug resistance testing for treatment optimization in HIV-1-infected patients with virologic failure is a cost-effective use of healthcare resources and beneficial to the society at large.

Only one recent (2005) cost-effectiveness study has been performed for treatment-naive patients, modeling industrialized countries[286]. Taking into account the current level of TDR (close to 10%), a strategy of genotypic resistance testing at initial diagnosis of HIV-1 infection increased quality-adjusted life expectancy per person by one month. The cost-effectiveness ratio for resistance testing remained less than € 37,000 (US$ 50,000) per QALY gained, unless the prevalence of resistance was around 1%. An analysis dating back to 2001 judged genotyping in such a situation cost-effective down to 5% TDR[287]. Genotypic drug resistance testing at diagnosis in a country with around 10% of TDR is estimated to be more cost-effective than combination ART and than prophylaxis for *M. avium* complex infection in AIDS patients, and only marginally less effective than resistance genotyping after virologic failure.

The cost-effectiveness of resistance testing in drug-naive patients does not only depend on the cutoff of acceptable costs, but also on the prevalence of transmitted drug resistance. Therefore, the Panel encourages resistance testing laboratories to take part in surveillance studies that document both the prevalence but also the trend of TDR in particular risk groups. Since ART is now more widely used in developing countries, most often without viral load and drug resistance-guided therapy adjustments, the risk of introducing resistant virus from those regions will undoubtedly increase. On the other hand, in high-income countries, the increasing rate of treatment success will contribute to a reduction of TDR, while continued spread of TDR among drug-naive patients might contribute to an increase of TDR. The Panel also encourages more thorough cost-effectiveness studies, adapted to different socioeconomic situations.

European diversity

The implementation of resistance testing is not uniform throughout Europe, since reimbursement policies are country and in some cases region dependent. In addition, the prevalence of TDR may vary according to region and risk group. Accepted costs for health interventions seem to increasingly depend on local policies, yet the same standards should be valid for all European citizens. The Panel therefore encourages the authorities to take action in order to reach agreements on accepted costs and to facilitate the implementation of pan-European standards. The WHO has made suggestions on how resistance testing can be implemented when confronted with limited financial resources[297] and the present Guidelines should help in the optimal use of limited financial resources. Local authorities that are concerned about cost-effectiveness should realize that proper studies can only be done with timely epidemiological information and resistance test results that are made available to surveillance programs.

In countries where transmission, or the risk for transmission, of HIV is criminalized, resistance test results can be used in court against individuals, and this can be counterproductive for implementation strategies. To avoid this, laboratories and clinical centers should take care not to disclose parameters that could be used to identify patients or transmission events, unless subpoenaed by a court in the context of a legal investigation. In such cases, appropriate guidelines for dealing with samples and sequences need to be followed[298].

Continuous education is essential to remain aware of the powers and limitations of HIV drug resistance testing. HIV resistance expertise contributes greatly to therapy response[86,87]. Education of clinicians and virologists is difficult to organize at a pan-European level; however, the Panel recommends HIV clinicians and virologists to take part in continuous education programs and to discuss with each other on a regular basis.

Conclusions

As outlined above, several arguments have stimulated the Panel to specifically draft European recommendations. First of all it is important to keep the Guidelines updated, even though for some indications the recommendations have not changed, but are supported by more scientific evidence, as is the case for testing in drug-naive patients. A major motivation was to document the high prevalence of non-B subtypes in Europe, along with its consequences for performance of resistance assays and interpretation of results. Another important European issue is the prevalence of drug resistance in drug-naive populations, which varies according to geographic area as documented here. Cost issues weigh heavily on the implementation of resistance testing, and many considerations are differently appreciated in the different countries. Since there is no uniform implementation strategy in Europe, the current document is essential in national efforts to help achievement of this implementation.

The clinical implications of resistance data depend on many factors. Clinicians receiving resistance test results should be aware of the difficulties associated with interpretation of the data, and should not rely only on resistance test results when suggesting a new therapy. Resistance information should be integrated into the clinical judgment, which includes taking into account factors such as the power of the combination chosen, therapy history, resistance history, regimen convenience and tolerability, drug adherence and drug levels, drug toxicities and interactions, and availability of potentially active drugs. Virologists and clinicians should discuss together difficult cases. Clinicians should seek expert advice in order to optimize the use of resistance data.

The European HIV Drug Resistance Guidelines Panel

The current guidelines were written as follows. At the onset of the effort for revising the guidelines, all academic Panel members were invited to be active in the writing committee. Those of the Panel that volunteered were actively involved in setting up the on-line and face-to-face discussions. This writing committee had several conference calls to prepare and summarize these discussions. The writing committee members had equal voting power as other academic Panel members (with the exception that some writing committee members were consultant of another academic Panel member and their joint opinion counted as a single vote). The full guidelines document as presented here was written by the writing committee, using information that resulted from these discussions. These writing committee members are individual authors of this document, with the rest of the Panel mentioned as group author.

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