

The impact of viral molecular diversity on the clinical presentation and outcome of acute hepatitis B in Italy

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SUMMARY

The incidence of acute hepatitis B (AHB) in Italy, mostly sustained by hepatitis B virus (HBV) genotype D, has significantly decreased in the last two decades, but the new HBV strains introduced through immigrant populations from countries with a higher endemicity constitute a new emergency.

HBV-genotype D still predominates in AHB, the subgenotype D3 being associated with parenteral transmission, and the recently emerging subgenotype A2 with unsafe sexual intercourse. Genetic, phylogenetic and evolutionary analyses are powerful tools for countries with high immigration rates to monitor new viral strains with different aggressiveness and a different response to therapy.

KEY WORDS: Acute hepatitis B, Epidemiology of acute hepatitis B, HBV phylogenetic analysis, HBV genotypes and subgenotypes.

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INTRODUCTION

Hepatitis B virus (HBV) is a major universal health problem with approximately 240 million people chronically infected worldwide. The endemicity level is considered high in countries with an HBV carrier rate over 8% (East Asia, sub-Saharan Africa and the Amazon basin), intermediate in those with a rate ranging from 2 to 8% (Eastern Europe) and low in those with a rate lower than 2% (Western Europe and North

America) (Ott *et al.*, 2012; Rantala *et al.*, 2008). The incidence of acute hepatitis B (AHB) in Italy decreased from 12 cases per 100,000 inhabitants in 1985 to 0.85 per 100,000 in 2012 due to an improvement in socioeconomic conditions, a national mass HBV vaccination campaign started in 1991 and several national campaigns to prevent the spread of human immunodeficiency virus (HIV) (<http://www.iss.it>; Sagnelli E, *et al.*, 2014). Under the universal HBV vaccination program all Italians aged 0-34 years have been vaccinated. Consequently, the incidence rate of AHB per 100,000 inhabitants in 2012 was 0.04% in the age group 0-14 years, 0.35% in the age group 15-24, 0.79% in the age group 25-34 and 1.7% in the age group 35-45 (<http://www.iss.it>; Sagnelli E, *et al.*, 2014). HBV genotypes, subgenotypes and HBsAg serotypes are viral populations sharing a genetic evolutionary history. The endemicity of

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HBV infection is low in Italy and genotype D is still the predominant genotype both in AHB and chronic hepatitis B (CHB) (Coppola *et al.*, 2010; Sagnelli C, *et al.*, 2014). However, new HBV genotypes have been introduced to Italy due to massive immigration in the last two decades from regions with a higher endemicity, such as Africa, Eastern Europe, and East Asia, as shown by the increasing circulation of non-D HBV genotypes in the new cases of AHB (Coppola *et al.*, 2010; Coppola *et al.*, 2013c; Ferraro *et al.*, 2012; Lanini *et al.*, 2012; Petrosillo *et al.*, 2000; Sagnelli C, *et al.*, 2014). The most frequently detected subgenotypes are D3, which is associated with parenteral transmission particularly in intravenous drug users, and the emerging subgenotype A2, which is associated with unsafe sexual exposure (De Maddalena *et al.*, 2007; Sagnelli C, *et al.*, 2014, Zehender *et al.*, 2008; Zehender *et al.*, 2014).

Phylogenetic analysis is a powerful tool widely used in the study of rapidly evolving viruses that establish chronic infections, such as HBV. This review article is a comprehensive analysis of the phylogeny, evolution, clinical presentation and outcome of AHB observed in Italy in recent decades.

HBV structure

In 1963 a new antigen was discovered and named the Australia antigen (AuAg) since it was first detected in Australian aborigines (Blumberg, 2002). Soon afterwards a correlation of this new antigen with acute and chronic hepatitis became evident (Blumberg, 2002) and the independently identified so-called “serum hepatitis (SH) antigen” was found to be identical to the AuAg (Prince, 1968). Subsequently, the terms SH antigen and AuAg were replaced by hepatitis B surface antigen (HBsAg). HBV is a hepadnavirus of the genus *Orthohepadnavirus* and belongs to a group of hepatotropic DNA viruses (Nassal, 1999; Hollinger *et al.*, 2001) that can infect several mammals, such as primates and bats (Piasecki *et al.*, 2013; Schaefer, 2007a,b). The virus consists of a nucleocapsid carrying the hepatitis B core antigen (HBcAg), a DNA polymerase/reverse-transcriptase (Pol/RT), the viral genome, as well as some cellular proteins (Nassal, 1999; Hollinger *et al.*, 2001) and an outer envelope showing pre-S1 anti-

gen, pre-S2 antigen and HBsAg, which plays a prominent role in the diagnosis of HBV infection. The genome consists of 3200 base pairs in a partially double-stranded circular DNA containing four overlapping open reading frames encoding surface proteins, the core protein, the polymerase and a multifunctional nonstructural protein called X. The pre-S region of the S gene consists of the pre-S1 and pre-S2 domains and partially overlaps the gene region encoding the polymerase. The enhancer and basic core promoter regions overlap with the X gene.

The pre-S1, pre-S2 and S genes code for three antigens, pre-S1, pre-S and HBsAg. The protein encoded by the pre-core/core gene yields the hepatitis B “e” antigen (HBeAg) through a post-translational modification. The core gene codes for HBcAg, the major structural protein of the nucleocapsid, and the X gene for a potent transactivator of viral and cellular genes (HBxAg) (Schaefer, 2007a,b; He *et al.*, 2013).

The polymerase gene encodes for the viral reverse transcriptase DNA polymerase, viral replication proceeding through a reverse transcription of an intermediate pre-genomic RNA and the subsequent synthesis of viral DNA (Summers *et al.*, 1982; Bartenschlager *et al.*, 1988).

HBV molecular epidemiology

To date, phylogenetic analysis has revealed ten human genotypes of HBV, from A to J, with a sequence divergence larger than 8% (Arauz-Ruiz *et al.*, 2002; Kidd-Ljunggren *et al.*, 2002; Lyra *et al.*, 2005; Mahtab *et al.*, 2008; Miyakawa *et al.*, 2003; Norder, 1995; Norder *et al.*, 2004; Naumann *et al.*, 1993; Olinger *et al.*, 2008; Ribero *et al.*, 2006; Stuyver *et al.*, 2000; Sumi *et al.*, 2003; Tran *et al.*, 2008; Tatematsu *et al.*, 2009) and presenting a peculiar geographical distribution relating in part to environmental factors. They have a different clinical impact (Chu *et al.*, 2002; Dal Molin *et al.*, 2006; Deterding *et al.*, 2008; Flink *et al.*, 2006; Kao, 2002; Ozasa *et al.*, 2006; Panessa *et al.*, 2009; Westland *et al.*, 2003; Takeda *et al.*, 2006; Tseng *et al.*, 2008; Yuen *et al.*, 2008; Svicher *et al.*, 2009; Schaefer, 2007a,b; Sloan *et al.*, 2009; Van Houdt *et al.*, 2007) and different responsiveness to alpha interferon treatment (Dal Molin *et al.*, 2006, Stroffolini *et al.*, 2008). The HBsAg subtypes ayw, adr, adw, and ayr (Kidd-Ljunggren *et al.*, 2002, Okamoto

et al., 1988) have a sequence divergence in the S region of 4-7% (Schaefer 2007a,b; Kidd-Ljunggren *et al.*, 2002; Norder *et al.*, 2004), and for decades have been known to have a different geographical distribution. HBV genotype A is present in North-Western Europe, North America and Central Africa (Norder *et al.*, 2004), genotype D in the Mediterranean area, the Middle East and Southern Asia (Norder *et al.*, 2004, Amini-Bavil-Olyaei *et al.*, 2005), genotypes B and C in Asia (Kao, 2002), genotype E in sub-Saharan Africa (Norder *et al.*, 2004; 1994), genotype F in Central and South America (Norder *et al.*, 2004), genotype G in France and the USA (Stuyver *et al.*, 2000), genotype H in Latin America (Arauz-Ruiz *et al.*, 2002), genotype I in Laos, Vietnam and Eastern India (Tran *et al.*, 2008; Olinger *et al.*, 2008) and north-western China (Yu *et al.*, 2010) and genotype J in Japan (Tatematsu *et al.*, 2009; Zehender *et al.*, 2014). Two genotypes are responsible for the majority of infections in Europe, genotype A (mainly subgenotype A2) in the north-west and genotype D (mainly subgenotypes D1, D2 and D3) in the Mediterranean basin and North-Eastern Europe (Schaefer, 2007a,b; Zehender *et al.*, 2014).

Phylogeny and phylogenetic methods

Phylogeny is a branch of molecular biology that allows us to infer knowledge about the taxonomy and evolution of species (Lemey *et al.*, 2009). In recent years a number of methods allowing the inference of phylogenetic trees have been introduced. These methods are based on genetic distances, the maximum-likelihood estimation and Bayesian inference (Felsenstein, 2004; Fitch *et al.*, 1967; Saitou *et al.*, 1987; Yang *et al.*, 1997). Genetic distances and phylogenetic trees (coupled with a correct epidemiological design i.e., cross-sectional studies), inferred via different sequence evolutionary models and model selection criteria, are normally used to assign the genotype (Pol, 2004). In addition, phylogenetic and evolutionary methods have been widely used to define circulating recombinant forms (CRFs). Instead, the coalescent theory and the molecular clock hypothesis are used to study the ancestral relationships of individuals sampled from a population, which can be inferred from a gene genealogy (phy-

logenetic tree) (Bon *et al.*, 2010; Callegaro *et al.*, 2011; Ciccozzi *et al.*, 2012; de Oliveira *et al.*, 2006; Salemi *et al.*, 2008; Zehender *et al.*, 2010). Phylodynamic inference has become a rapidly expanding field in recent years. A phylodynamic analysis employs the coalescence theory, which is usually used to investigate how the genealogy of a pathogen population is influenced by the interaction among the pathogen's demographic history, host immunological milieu and environmental factors (Drummond *et al.*, 2003; Zehender *et al.*, 2012).

In practice, several steps are necessary for a basic genetic analysis before starting the phylogenetic approach:

- a) generation of the data set;
- b) alignment with reference sequences and the manual editing to delete "indels" (insertions/deletions);
- c) determination of the phylogenetic signal.

Phylogenetic and/or phylodynamic analyses constitute the "core" of the data analysis and hypothesis testing. Testing for the best substitution model, inferring phylogeny using different algorithms, and testing the tree's reliability are essential steps in the evolutionary analyses (Bon *et al.*, 2010; Drummond *et al.*, 2003; Ciccozzi *et al.*, 2011).

In 2012, Ferraro *et al.* (Ferraro *et al.*, 2012) described the relationship between new cases of AHB and the effect of immigration. They studied 34 isolates from patients with AHB, and 35 from CHB patients. The phylogenetic analysis of the pre-S/S region showed that 44% of the strains from AHB patients were genotype A, 53% were genotype D and 3% were genotype E. The data emphasized that in Sicily there was a change in the HBV population, with an increase in HBV genotype A and a clustering effect for HBV subgenotype D2, possibly correlated to immigration. In 2012, Lanini *et al.* (Lanini *et al.*, 2012) described a nosocomial outbreak of AHB that occurred in February 2007 in Rome. Six incident cases of HBV infection were reported among the 162 patients admitted to the Oncohematology Unit. The subsequent molecular investigation proved that three of the six incident cases and one prevalent case made up a monophyletic cluster of infection. The environmental investigation found that an identical HBV viral strain was present on a multi-patient

lancing device in use in the unit, and the inferential analysis showed a statistically significant association between ongoing lancing procedures and the infection (Lanini *et al.*, 2012). In this outbreak, the phylogenetic investigation was of great support to the classic epidemiological investigation.

Published data on molecular epidemiology and phylogeny of acute hepatitis B

In a recent investigation on changes in the molecular epidemiology of HBV in Southern Italy, viral genotypes were determined by direct sequencing of the pre-S/S region in 123 consecutive patients with AHB observed in the last ten years and in 123 HBV chronic carriers from the same areas who had been HBsAg positive for more than ten years (Coppola *et al.*, 2010). Genotype D was significantly less frequent in patients with AHB than in those with CHB. In AHB patients, the authors found intravenous drug addiction (IVDA) as the prevalent risk factor for acquiring HBV in patients with HBV genotype D, whereas it was rarely involved in AHB patients with genotypes non-D. Unsafe

sexual intercourse was identified as a risk factor for three quarters of the patients with HBV genotype non-D and for less than one quarter of those with genotype D (Coppola *et al.*, 2010). In these AHB patients, the prevalence of non-D genotypes significantly increased during the observation period from 11.1% in 1999-2003 to 41.1% in 2004-2008, thus paralleling the increase in the prevalence of patients with unsafe sexual intercourse. Similarly, the authors found a progressive decline in IVDA, paralleled by the decrease in the prevalence of HBV-genotype D, from 88.3% in 1999-2003 to 11.7% in 2004-2008 (Coppola *et al.*, 2010). More recently the authors (Sagnelli C, *et al.*, 2014) examined 53 AHB patients in the same Italian region and found that HBV-genotype D was still prevalent (64.1%), followed by genotype A (26.4%), E (3.8%), and F (5.7%). Among patients with HBV-genotype D, sub-genotype D3 predominated (70.6%) and two principal clades were identified by the Bayesian tree dated 1994. One of these clades included six isolates, and the viral flow showed a major exchange of HBV (40%) from intravenous drugs users to subjects who had acquired

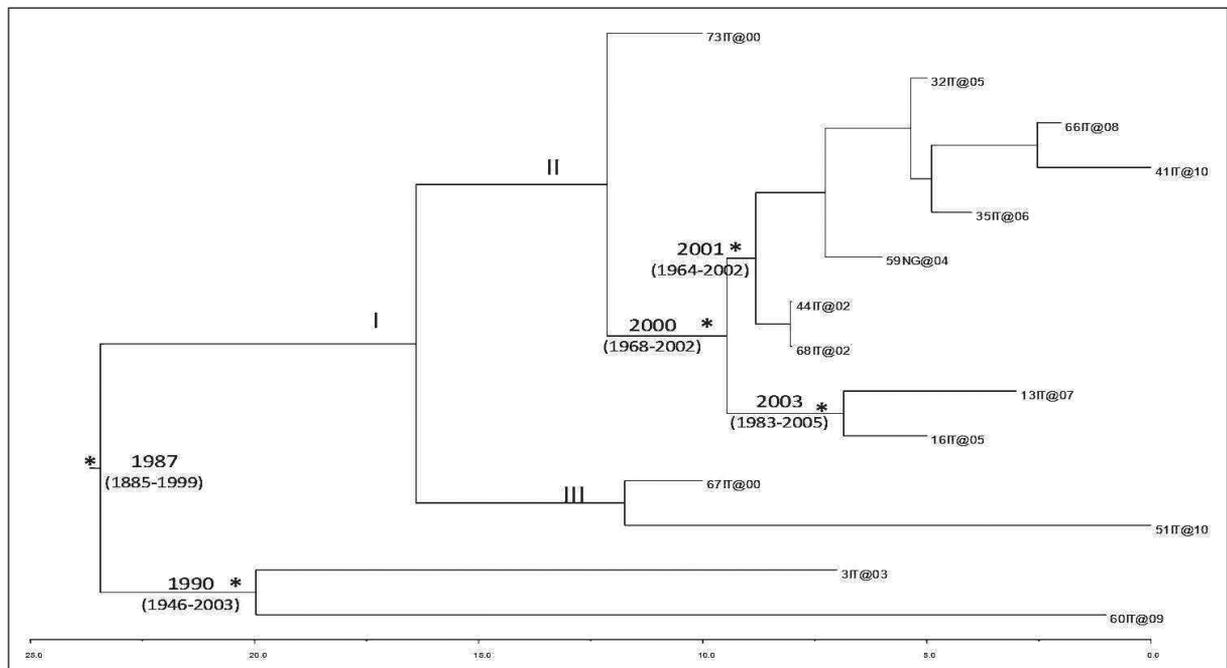


FIGURE 1 - Bayesian time-scaled tree of HBV-subgenotype A2 sequences. The time of the most recent common ancestor, with the credibility interval based on 95% highest posterior density interval (95% HPD), is shown in years. The clades are indicated in Roman numerals. The years are indicated at the base.

HBV by unsafe sexual practices. All 14 patients with HBV genotype A observed in this study denied IVDA and carried HBV sub-genotype A2, suggesting a recent introduction and spread of this viral strain through unsafe sexual practices (Sagnelli C, *et al.*, 2014). A subsequent investigation on the same patients allowed the reconstruction of the time-scaled Bayesian tree of the 14 HBV-A2 isolates, representative of the mean evolutionary rate of the HBV-A2 sub-genotype polymerase sequences (Figure 1). The estimation of the time of the tree's root gave a mean value of 16 years ago, corresponding to 1987 (95% HPD: 1885-1999). The Bayesian tree showed one main clade (Clade I) dating back to the year 2000 (95% HPD 1968-2002) and further divided into two subclades (II and III). Subclade II included two statistically supported clusters. The first included seven isolates and dated back to the year 2001 (95% HPD: 1964-2002) and the second included two isolates dating back to 2003 (95% HPD: 1983-2005). These observations, although preliminary, indicate that phylogenetic and evolutionary methods may provide useful information on the HBV strains recently introduced to countries with low endemicity by immigrant populations from hyperendemic geographical areas.

HBV infection and transmission

HBV DNA can have high titers in the blood, and any mucosal or parenteral exposure to infected blood is a risk for the acquisition of HBV infection. HBV can be detected in the blood, saliva, breast milk, vaginal secretions, semen, and ascitic fluid (Chlumsky *et al.*, 1982; Karayianis *et al.*, 1985; Villarejos *et al.*, 1974), but the major risk factors for acquiring HBV infection include the transfusion of unscreened blood, undergoing renal dialysis, sharing or re-using syringes among injection drug users, tattooing, sexual promiscuity, working in healthcare, being a long-term household or intimate non-sexual contact of an HBsAg-positive subject, and living in a correctional facility (Sagnelli E, *et al.*, 2014).

Since the screening of blood donors for HBV markers became mandatory in Italy in 1972, the current risk of post-transfusion transmission of HBV is extremely low. Due to the effective HBV universal vaccination program started in 1991,

all Italian citizens aged from 0 to 34 years have been vaccinated, and the contribution of intravenous drug addiction to the spread of HBV has dramatically declined. HBV transmission in men having sex with men has also declined in countries where awareness campaigns and action have been undertaken to contain the spread of HIV infection.

Etiological diagnosis of AHB

HBsAg and the corresponding antibody anti-HBs, HBeAg and the corresponding antibody anti-HBe, the antibodies to the HBV core antigen IgG anti-HBc and IgM anti-HBc and HBV DNA are all serological markers of HBV infection. HBsAg in serum indicates an acute or chronic HBV infection and the HBsAg titer and HBeAg can be used as surrogate markers of viral replication, whereas the detection of HBV DNA reveals the presence of HBV and its titer measures the HBV load and viral replication. In addition, the detection of a high titer of serum IgM anti-HBc allows the diagnosis of AHB. The persistence of HBeAg for more than ten weeks indicates a high likelihood of transition to chronic HBV infection, as does the persistence of HBsAg and HBV DNA for more than six months.

The impact of host, lifestyle, environmental and HBV genetic factors on the clinical presentation and outcome of AHB

After an incubation period of 45-180 days some constitutional symptoms such as fever, fatigue, anorexia, nausea and body ache may develop during a pre-icteric phase lasting for 2-7 days. The appearance of jaundice and dark urine signals the start of the icteric phase that may last a couple of weeks or longer in some adults. Jaundice occurs in less than 10% of children under five years and in more than 50% of older children and adults. Occasionally, extrahepatic features may complicate the clinical presentation, reflecting immune complex-related damage such as acute necrotizing vasculitis, membranous glomerulonephritis, and papular acrodermatitis in children (Chen *et al.* 1988; Dienstag, 1981; Yoffe *et al.*, 1990; Gianotti, 1966; Lisker-Melman *et al.*, 1989; Piccinino *et al.*, 1978, Johnson *et al.*, 1990; Venkateshan *et al.*, 1990; Trepo *et al.*, 2001).

The risk of progressing to chronicity is inversely correlated to the age of the patient at the time of infection. HBV infection becomes chronic in nearly 90% of babies born of an HBeAg-positive mother, in 20-30% of children infected from the first to the fifth year of age, in 6% of those aged 5-15 years and in 1-5% in older patients (Berk *et al.*, 1978; Lavanchy, 2004; Lavanchy, 2005; Liaw *et al.*, 2009). Thus only 1-5% of adults develop CHB, which leads in 10-20% of these patients to severe chronic hepatitis B, liver cirrhosis or hepatocellular carcinoma. Patients who do not progress to chronicity recover from AHB and clear HBV infection.

An over-reaction of the immune system may lead to a fulminant form in nearly 1% of the cases, (Berk *et al.*, 1978; Lavanchy, 2005), a clinical form characterized by a high fatality rate that in most cases requires liver transplantation. Fulminant hepatitis is rare in infants and young children and when present seems to be associated with the HBeAg-negative status of the mother (Chen *et al.*, 2004).

The spectrum of the clinical presentation and outcome of AHB is varied, mostly depending on viral and host factors. Host factors such as female sex or being a young adult have been considered predictors of a more severe clinical presentation (Yao, 1996). Also certain lifestyles (alcohol intake, drug use) and concomitant viral infections (HDV, HCV or HIV) have been associated with a more severe clinical course (Garfein *et al.*, 2004; Sagnelli *et al.*, 2002; Sagnelli *et al.*, 2009). Virological factors have also been associated with a peculiar clinical presentation and disease outcome (Kumar *et al.*, 2007; Kusakabe *et al.*, 2009; Wai *et al.*, 2005; Coppola *et al.*, 2013a,b; Coppola *et al.*, 2014). Differences in the distribution of HBV genotypes among patients with a fulminant or normal course of the disease have been reported, HBV-Ae being more frequent in the normal form (Coppola *et al.*, 2014) and the Bj subgenotype and genotype B more frequent in patients with fulminant hepatitis (Imamura *et al.*, 2003; Sakamoto *et al.*, 2006). In addition, the HBV-subgenotype C2 basal core promoter and pre-core variants have been identified as associated with the progression to fulminant hepatitis (Hayashi *et al.*, 2008). In addition, HBV-genotype D in the USA has been found to be twice as frequent in patients

with a fulminant course compared to those with a normal course (Hayashi *et al.*, 2008). The pre-core stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were found to be significantly more frequent in patients with a fulminant than in those with a normal clinical course (Coppola *et al.*, 2014), which is in full accordance with previous investigations showing fulminant HBV infection associated with a G1896A pre-core stop codon mutation (Aye *et al.*, 1994; Carman *et al.*, 1991; Chu *et al.*, 1996; Ehata *et al.*, 1993; Hasegawa *et al.*, 1991; Liang *et al.*, 1991; Kosaka *et al.*, 1991; Kojima *et al.*, 1991; Omata *et al.*, 1991;) or with two mutations in the core promoter region, A1762 T and G1764 A (Kaneko *et al.*, 1995; Sato *et al.*, 1995). A multivariate analysis in a case-control study on 50 patients with fulminant and 50 with a self-limiting AHB, pair matched by sex and age, showed the G1896A mutation, a serum HBV DNA level higher than 5.23 log copies/mL and a total bilirubin serum value higher than 10.35 mg/mL (odds ratio, 7.81; 95% confidence interval, 1.77-34.51) all to be independently associated with a fulminant outcome (Wai *et al.*, 2005). The M204V/I variant was also associated with the severe forms of AHB in an Italian study (Coppola *et al.*, 2013c). The study of the impact of HBV genetic variants on the clinical presentation and outcome of AHB is still at its early stages, but the published data, although fragmentary and preliminary, strongly suggest that their role is important and that this topic deserves further investigation.

CONCLUSION

Phylogenetic and evolutionary methods have found little application in the studies on AHB to date, but the studies on HBV genotypes in AHB carried out in Italy from 2001 and the data from some preliminary experiences with phylogenetic and evolutionary methods as well as the steady increase in immigration from countries with a high or intermediate endemicity strongly warrant a wider use of these methods and emphasize the need for nationwide investigations to monitor future changes in HBV epidemiology. Indeed, the introduction and wide spread of new HBV genotypes in Italy, as

recently demonstrated for HBV-subgenotype A2 in the Campania region, is a warning for clinicians and epidemiologists to investigate AHB also with genetic, phylogenetic and evolutionary methods both for the treatment and evaluation of vaccination coverage for newly introduced HBV genotypes, an important issue that should also be addressed by the national healthcare authorities.

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