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# Prevalence of hepatitis D virus infection in Central Italy has remained stable across the last 2 decades with dominance of subgenotypes 1 and characterized by elevated viral replication

Romina Salpini[1], Lorenzo Piermatteo[1], Giulia Torre[1], Stefano D'Anna[2], Sohaib Khan[2], Leonardo Duca[2], Ada Bertoli[2,3], Simone La Frazia[1], Vincenzo Malagnino[4], Elisabetta Teti[4], Marco Iannetta[4], Pierpaolo Paba[3], Marco Ciotti[3], Ilaria Lenci[5], Simona Francioso[5], Caterina Paquazzi[6], Miriam Lichtner[7], Claudio Mastroianni[7], Francesco Santopaolo[8], Giuseppe De Sanctis[9], Adriano Pellicelli[10], Giovanni Galati[11], Alessandra Moretti[12], Katia Casinelli[13], Luciano Caterini[14], Nerio Iapadre[15], Giustino Parruti[16], Iacopo Vecchiet[17], Maurizio Paoloni[18], Massimo Marignani[19], Francesca Ceccherini-Silberstein[2], Leonardo Baiocchi[5], Sandro Grelli[3], Loredana Sarmati[4], Valentina Svicher[1,*]

[1] *Tor Vergata University, Department of Biology, Rome, Italy*
[2] *Tor Vergata University, Department of Experimental Medicine, Rome, Italy*
[3] *Tor Vergata University Hospital, Virology Unit, Rome, Italy*
[4] *Tor Vergata University Hospital, Infectious Diseases Unit, Rome, Italy*
[5] *Tor Vergata University Hospital, Hepatology Unit, Rome, Italy*
[6] *Sant'Andrea Hospital, Clinical Infectious Diseases, Rome, Italy*
[7] *La Sapienza University, Department of Public Health and Infectious Disease, Rome, Italy*
[8] *Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Internal Medicine and Gastroenterology-Hepatology Unit, Rome, Italy*
[9] *"Umberto I" University Hospital, Infectious Diseases Unit,Rome, Italy*
[10] *San Camillo Forlanini Hospital, Liver and Transplant Unit, Rome, Italy*
[11] *University Campus Bio-Medico, Internal Medicine and Hepatology Unit, Rome, Italy*
[12] *San Filippo Neri Hospital, Gastroenterology Unit, Rome, Italy*
[13] *Spaziani Hospital, Infectious Disease Unit, Frosinone, Italy*
[14] *Belcolle Hospital, Infectious Diseases Unit, Viterbo, Italy*
[15] *San Salvatore Hospital, Infectious Diseases Unit, L'Aquila, Italy*
[16] *Pescara General Hospital, Infectious Disesases Unit, Pescara, Italy*
[17] *University "G. d'Annunzio" Chieti-Pescara, Clinic of Infectious Diseases, Department of Medicine and Science of Aging, Chieti, Italy*
[18] *Avezzano General Hospital, Infectious Diseases Unit, Avezzano, Italy*
[19] *Regina Apostolorum Hospital, Department of Gastroenterology and Hepatology, Albano Laziale, Italy*

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ABSTRACT

*Objectives:* Here we investigate Hepatitis D virus (HDV)-prevalence in Italy and its fluctuations over time and we provide an extensive characterization of HDV-infected patients.
*Methods:* The rate of HDV seroprevalence and HDV chronicity was assessed in 1579 hepatitis B surface antigen (HBsAg)+ patients collected from 2005 to 2022 in Central Italy.
*Results:* In total, 45.3% of HBsAg+ patients received HDV screening with an increasing temporal trend: 15.6% (2005-2010), 45.0% (2011-2014), 49.4% (2015-2018), 71.8% (2019-2022). By multivariable model, factors correlated with the lack of HDV screening were alanine-aminotransferase (ALT) less than two times of upper limit of normality (<2ULN) and previous time windows ($P$ <0.002). Furthermore, 13.4% of HDV-screened patients resulted anti-HDV+ with a stable temporal trend. Among them, 80.8% had detectable HDV-ribonucleic acid (RNA) (median [IQR]:4.6 [3.6-5.6] log copies/ml) with altered ALT in 89.3% (median [IQR]:92 [62-177] U/L).

Anti-HDV+ patients from Eastern/South-eastern Europe were younger than Italians (44 [37-54] vs 53 [47-62] years, $P$ <0.0001), less frequently nucleos(t)ide analogs (NUC)-treated (58.5% vs 80%, $P$ = 0.026)

* Corresponding author.
*E-mail address:* valentina.svicher@uniroma2.it (V. Svicher).

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with higher HDV-RNA (4.8 [3.6-5.8] vs 3.9 [1.4-4.9] log copies/ml, $P = 0.016$) and HBsAg (9461 [4159-24,532] vs 4447 [737-13,336] IU/ml, $P = 0.032$). Phylogenetic analysis revealed the circulation of HDV subgenotype 1e (47.4%) and -1c (52.6%). Notably, subgenotype 1e correlated with higher ALT than 1c (168 [89-190] vs 58 [54-88] U/l, $P = 0.015$) despite comparable HDV-RNA.

*Conclusions:* HDV-screening awareness is increasing over time even if some gaps persist to achieve HDV screening in all HBsAg+ patients. HDV prevalence in tertiary care centers tend to scarcely decline in native/non-native patients. Detection of subgenotypes, triggering variable inflammatory stimuli, supports the need to expand HDV molecular characterization.



## Introduction

Hepatitis D virus (HDV) is the smallest known human virus with a genome of ~1.7 kb single-stranded circular ribonucleic acid (RNA). HDV is a satellite virus that can infect only in presence of hepatitis B virus (HBV), its helper virus [1]. Indeed, HDV exploits the HBV surface proteins (collectively defined as hepatitis B surface antigen [HBsAg]) for the release of its progeny and *de novo* entry into hepatocytes [1]. HBV+HDV infection causes the most severe form of viral hepatitis, leading to the development of cirrhosis in 15% of cases within 1-2 years and 70-80% of cases within 5-10 years [2]. The rates of hepatocellular carcinoma (HCC) and hepatic decompensation are also 2-3-fold higher than for HBV mono-infection [3,4].

Recent estimates suggest that 9-60 million individuals may be infected by HDV worldwide, raising the global relevance of HDV infection [5,6]. However, these fluctuating estimates highlight a huge uncertainty about the real prevalence of HDV infection, mostly related to the lack of robust data on large population of HBsAg+ patients and suboptimal screening programs. Indeed, although HDV screening in all HBsAg+ patients are recommended by several international guidelines [7,8], a substantial fraction of HBsAg+ patients remains untested [9]. Factors underlying the lack of HDV screening have not been properly defined yet.

Italy has historically represented a large basin of HDV infection in Europe, characterized by high HDV endemicity (HDV seroprevalence >20% among HBsAg+ patients in the 1980s). The introduction of universal anti-HBV vaccination in 1991 has determined a decline in HDV prevalence as a result of the reduction in new HBV infections [10], followed by a stable trend of HDV infection [10]. Initial reports have described an epidemiological changing scenario of HDV infection in Italy, with a declining HDV prevalence among native patients, paralleling an increased circulation among immigrants [10,11].

So far, interferon-alfa treatment has been the only therapeutic strategy against HDV but unfortunately, it was associated with <20% virological response and a high risk of post-treatment virological relapse [12]. More recently, novel anti-HDV compounds have been identified. Among them, the entry-inhibitor Bulevirtide interacts with the Na+-taurocholate cotransporting polypeptide receptor and prevents viral entry into the hepatocytes. Bulevirtide received conditional approval from the European Medicines Agency in July 2020 and was recently introduced in clinical practice in some European countries, including Italy, in March 2023 [13,14].

Furthermore, HDV is endowed with a very high degree of genetic variability that has enabled viral differentiation into eight genotypes (from HDV-1 to HDV-8), characterized by a genetic divergence of >20% over their full-length genome [15]. HDV-genotype 1 represents the most prevalent and geographically widespread HDV-genotype, usually associated with more severe hepatitis compared to other genotypes such as 2 and 4 [16–18]. Notably, HDV-genotype 1 has been recently reclassified into different subgenotypes (from HDV-1a to HDV-1e), differing by at least 16% of their sequence [15,19], whose impact on liver disease progression is largely unknown.

To date, a paucity of studies has analyzed HDV seroprevalence in a large population of chronically HBV-infected patients over an extended period of time, providing an in-depth epidemiological and virological characterization of HDV-infected patients. An improved knowledge on the burden and the characteristics of HDV infection is crucial to evaluate its impact on the health system, and to set up more effective prevention and treatment strategies. This information will also contribute to a better estimate of the basin of patients in which the treatment with the new anti-HDV drugs should be prioritized.

In this light, this study aims at defining the rate of HDV screening and factors correlated with its lack over time and to characterize HDV seroprevalence and its temporal fluctuations in the last 2 decades by analyzing a large cohort of HBV-infected patients from Central Italy. Furthermore, we provide an extensive demographic, clinical and virological characterization of patients with chronic HDV infection by defining the levels of HDV replication, the circulating HDV and HBV subgenotypes and their correlation with the severity of HDV-related liver disease.

## Material and methods

### *Patients*

This study included 1579 consecutive individuals positive to HBsAg for at least 6 months referred to the Virology Unit of Tor Vergata University Hospital from January 1, 2005, to June 30, 2022, for the virological characterization of HBV infection. Patients were followed for HBV infection in 18 Infectious Diseases or Gastroenterology/Hepatology clinical centers in Central Italy. Information regarding patients' demographics (age, sex, country of origin), HBV markers (hepatitis B e antigen [HBeAg] status; serum HBV-DNA and quantitative HBsAg), alanine-aminotransferase (ALT) levels and anti-HBV therapy were collected in an *ad hoc* designed and anonymized database. This database also includes information on HDV screening, based on anti-HDV antibodies, and serum HDV-RNA quantification.

Patients were stratified in four temporal windows: 2005-2010 (N = 180), 2011-2014 (N = 722), 2015-2018 (N = 553), and 2019-2022 (N = 124), to define the proportion of patients undergoing HDV screening over time.

HDV seroprevalence was defined according to anti-HDV antibody positivity, while active HDV infection was defined according to serum HDV-RNA positivity. Severe fibrosis/cirrhosis was diagnosed if liver stiffness measurement was ≥9 kPa in patients with normal ALT, or ≥12 kPa in patients with ALT ≤5 upper limit of normality (ULN), by liver biopsy (when available) (Metavir score ≥F4 or Ishak score ≥ 5) and/or by clinical signs (varices, ascites, encephalopathy) [20]. In anti-HDV+ patients, the recently proposed cut-off of 14 kPa (with 78% sensitivity, 86% specificity, 93% negative predictive value and 64% positive predictive value) was also

evaluated along with FIB-4 to confirm the diagnosis of cirrhosis [8,21,22].

*Serological test*

HBV and HDV serological markers (qualitative detection of HBsAg, HBeAg, Anti-HBe antibodies, and anti-HDV antibodies) were tested by commercially available immunoassays. HBsAg quantification was assessed by Elecsys®HBsAgII assay (Roche, Basel, CH), with a lower limit of detection of 0.05IU/ml.

Serum HBV-DNA was quantified by COBAS®AmpliPrep/COBAS® TaqMan®HBV-Test v2.0 with a lower limit of HBV-DNA quantification of 20IU/ml (Roche Diagnostic Systems Inc, Mannheim, Germany).

The following assays for serum HDV-RNA quantification were used in chronological order: HDV real-time polymerase chain reaction (PCR) assay (LifeRiver Diagnostics, Shanghai ZJ Bio-Tech Co., Shanghai, China) from 2005 to 2018 and HDV qRT-PCR EurobioPlex (Eurobio scientific, Les Ulis, France) from 2019 to 2022.

*HBV sequencing and assessment of HBV genotype/subgenotype*

For patients with HBV-DNA>20IU/ml and with an available serum sample (509 HBV-monoinfected and 58 HBV+HDV coinfected patients), the nucleotide sequence of the genomic HBV region encoding HBsAg (aa 1-226) was obtained by Sanger-based sequencing, as previously described [23] (Supplementary Material).

HBV genotypes and subgenotypes were determined by a phylogenetic tree constructed by Neighbor-Joining method on MEGA6 software. Branching order reliability was assessed by bootstrap analysis of 1000 replicates.

*HDV sequencing and assessment of HDV-genotype/subgenotype*

For patients with active HDV replication and an available serum sample (N = 27), the nucleotide sequence covering a portion of HDV genome (nucleotide 309-1292, thus partially covering the region encoding hepatitis Delta antigen) was obtained by Sanger-based sequencing, following a previously published protocol [15] (Supplementary Material).

The HDV-genotypes and subgenotypes were attributed by phylogenetic tree constructed by Neighbor-Joining method on MEGA6 software according to bootstrap >60%. The reference sequences for each genotype and subgenotype were retrieved from Karimzadeh *et al.* [19]. Branching orders reliability was assessed by bootstrap analysis of 100 replicates. The attribution of subgenotypes was confirmed by calculating pairwise genetic distance on MEGA6 software, considering a percentage of similarity >90% [24].

*Statistical analysis*

Statistical analysis was performed by using IBM SPSS Statistics, v.23.0 (Armonk, New York). Quantitative variables were expressed as median (interquartile range [IQR]) while qualitative variables as counts and percentages. Chi-squared test of independence based on a 2 × 2 or 4 × 2 contingency tables was used for qualitative data, while Mann-Whitney test for continuous data. Correlations with $P$ <0.05 were considered statistically significant.

Logistic regression analysis was performed to assess factors significantly associated with the lack of HDV screening, considering the following variables: gender, age, nationality, and ALT. After stepwise elimination for optimized Akaike information criterion, only variables showing a $P$-value <0.200 in univariable analysis were included in multivariable analysis.

## Results

*Patients' characteristics*

This study included a total of 1579 HBsAg+ patients referred to Tor Vergata University from 2005 to 2022 for the virological characterization of HBV infection. Most patients were male (64.7%) and Italian (57.2%), with a median (IQR) age of 47 (35-60) years (Table 1). In total, 75.3% of patients were HBeAg-negative and 56.0% were NUC-treated. Median (IQR) levels of serum HBV-DNA and HBsAg were 3.1 (1.7-4.8) log IU/ml and 3499 (618-11,662) IU/ml, respectively (Table 1). Median (IQR) ALT was 42 (26-78) U/l, indicating that half of the patients had ALT levels >ULN (Table 1). Non-Italian patients were mainly from Eastern/South-eastern Europe (21.5%), followed by Africa (11.2%), Asia (5.6%) and Southern America (0.8%) (Table 1). Notably, a progressive increase in the percentage of non-native HBsAg+ patients was noted over time. In particular, the percentage of HBsAg+ patients from Eastern/South-eastern Europe significantly increased from 10% in 2005-2010 up to 26.6% in 2019-2022, while the percentage of HBsAg+ patients from Africa and Asia increased from 1.7% to 12.1% and from 1.1% to 9.7%, respectively (Table 1).

*Rate of HDV screening*

Overall, 715 (45.3%) HBsAg+ patients were tested for anti-HDV antibodies, highlighting that more than half of patients did not receive any HDV screening despite HBsAg+ status. Patients undergoing anti-HDV screening were more frequently from Eastern/South-eastern Europe (24.6% for screened vs 20.3% for not screened, $P$ = 0.005) and from Africa (15.2% vs 8.5%, $P$ <0.0001). They were younger (median [IQR]: 46 [33-59] vs 49 [35-62] years, $P$ = 0.004) and had higher levels of ALT (47 [27-97] vs 36 [24-67] U/l, $P$ <0.0001) and HBsAg (3988 [749-13,373] vs 2839 [566-9930] IU/ml, $P$ = 0.026) (Supplementary Table 1).

Notably, the rate of HDV screening underwent a significant rise over time. Indeed, the proportion of patients tested for anti-HDV antibodies increased from 15.6% in 2005-2010 to 45.0% in 2011-2014, 49.4% in 2015-2018 and up to 71.8% in 2019-2022 ($P$ <0.0001) (Table 1, Figure 1a), suggesting a higher awareness toward HDV screening in more recent years. By multivariable model, independent factors significantly correlated with the lack of HDV screening were normal/slightly altered ALT (ALT<2ULN) (odds ratio [OR] [95% CI]: 1.61 [1.20-2.15], $P$ = 0.001) and previous time windows (OR [95% CI]: 9.79 [4.48-21.62] for 2005-2010, 2.69 [1.54-4.70] for 2011-2014, 3.10 [1.76-5.46] for 2015-2018, $P$ <0.0001 for all using the time window 2019-2022 as reference) (Table 2).

*HDV seroprevalence over time*

Among the 715 patients receiving HDV screening, 96 (13.4%) resulted positive for anti-HDV antibodies. Notably, the temporal trend of HDV seroprevalence remained over 9% in all the analyzed time windows: 10.7% in 2005-2010, 16.9% in 2011-2014, 10.9% in 2015-2018, and 9% in 2019-2022 ($P$-value = 0.84) (Table 1, Figure 1b). A decline of HDV seroprevalence over time was noted in the setting of Italian patients, characterized by a rate of anti-HDV positivity of 14.3% in 2005-2010, 15.9% in 2011-2014, 9.2% in 2015-2018 and 7.9 % in 2019-2022 ($P$-value = 0.26) (data not shown).

*Characteristics of patients stratified according to anti-HDV status*

Anti-HDV+ patients were prevalently male (63.5%) and slightly older than anti-HDV-negative patients (49 [40-57] vs 45 [32-59] years, $P$ = 0.068) (Table 3). Notably, all anti-HDV+ patients >60

**Table 1**
Characteristics of 1579 HBsAg+ patients stratified according to the different time windows.

| | Overall (N = 1579) | 2005-2010 (N = 180) | 2011-2014 (N = 722) | 2015-2018 (N = 553) | 2019-2022 (N = 124) |
|---|---|---|---|---|---|
| Male, *N (%)* | 1022 (64.7) | 123 (68.3) | 484 (67.0) | 336 (60.8) | 79 (63.7) |
| Country of origin [a], *N (%)* | | | | | |
| Italy | 903 (57.2) | **105 (58.3)** | **467 (64.7)** | **271 (49.0)** | **60 (48.4)** |
| Eastern/South-eastern Europe | 339 (21.5) | **18 (10.0)** | **141 (19.5)** | **147 (26.6)** | **33 (26.6)** |
| *Albania* | *33 (9.7)* | *1 (5.6)* | *11 (7.8)* | *16 (10.8)* | *5 (15.2)* |
| *Moldova* | *20 (5.9)* | *2 (11.2)* | *3 (2.1)* | *11 (7.5)* | *4 (12.1)* |
| *Romania* | *141 (41.7)* | *12 (66.6)* | *63 (44.7)* | *48 (32.7)* | *18 (54.5)* |
| *Ukraine* | *13 (3.8)* | *3 (16.6)* | *5 (3.6)* | *4 (2.7)* | *1 (3.0)* |
| *Undefined* | *132 (38.9)* | *0 (0.0)* | *59 (41.8)* | *68 (46.3)* | *5 (15.2)* |
| Asia | 89 (5.6) | **2 (1.1)** | **32 (4.4)** | **43 (7.8)** | **12 (9.7)** |
| *Middle East* | *9 (10.1)* | *0 (0.0)* | *4 (12.5)* | *4 (15.6)* | *1 (8.3)* |
| *South Asia* | *14 (15.7)* | *0 (0.0)* | *1 (3.1)* | *11 (25.6)* | *2 (16.7)* |
| *East Asia* | *66 (74.2)* | *2 (100.0)* | *27 (84.4)* | *28 (62.2)* | *9 (75.0)* |
| Africa | 177 (11.2) | **3 (1.7)** | **70 (9.7)** | **89 (16.1)** | **15 (12.1)** |
| *North Africa* | *23 (13.0)* | *1 (33.3)* | *9 (12.9)* | *11 (12.4)* | *2 (13.3)* |
| *Sub-Saharan Africa* | *154 (87.0)* | *2 (66.7)* | *61 (87.1)* | *78 (87.6)* | *13 (86.7)* |
| South American | 13 (0.8) | 0 (0.0) | 7 (1.0) | 3 (0.5) | 3 (2.4) |
| Unknown | 60 (3.7) | 52 (28.9) | 5 (0.7) | 0 (0.0) | 1 (0.8) |
| Age, *median (IQR) years* | 47 (35-60) | 50 (38-62) | 48 (35-61) | 45 (32-59) | 47 (34-59) |
| ALT levels, *median (IQR) U/l* | 42 (26-78) | 40 (25-71) | 41 (26-75) | 42 (25-79) | 43 (24-96) |
| HBsAg levels, *median (IQR) IU/ml* | 3499 (618-11,662) | 2038 (154-5428) | 3414 (559-11,453) | 3728 (671-11,841) | 3942 (743-19,033) |
| HBV-DNA levels, *median log (IQR) IU/ml* | 3.1 (1.7-4.8) | 3.8 (1.9-5.6) | 2.9 (1.4-4.2) | 3.4 (2.1-5.1) | 3.2 (2.0-4.9) |
| NUC-treated patients, *N (%)* | 885 (56.0) | 157 (87.2) | 392 (54.3) | 263 (47.6) | 73 (58.9) |
| Patients screened for anti-HDV, *N (%)* | 715 (45.3) | **28 (15.6)** | **325 (45.0)** | **273 (49.4)** | **89 (71.8)** |
| Patients positive for anti-HDV, *N (%)*[b] | 96 (13.4) | 3 (10.7) | 55 (16.9) | 30 (10.9) | 8 (9.0) |
| Patients tested for serum HDV-RNA, *N (%)*[c] | 78 (81.3) | **1 (33.3)** | **43 (78.2)** | **26 (86.7)** | **8 (100.0)** |
| Patients with detectable serum HDV-RNA, *N (%)*[d] | 63 (80.8) | 1 (100.0) | 32 (74.4) | 22 (84.6) | 8 (100.0) |

[a] The percentages of HBsAg+ patients from each Eastern European Country or Asia and Africa were calculated on the total number of HBsAg+ patients from Eastern Europe, Asia, and Africa, respectively.
[b] The percentages of anti-HDV+ patients are calculated on anti-HDV-screened patients.
[c] The percentages of patients tested for serum HDV-RNA are calculated on anti-HDV+ patients.
[d] The percentages of patients with detectable HDV-RNA are calculated on patients tested for HDV-RNA. Variables with statistically significant differences by chi-squared for trend across the different time windows (with *P*-value <0.05) are reported in bold.ALT, alanine ammino-transferase, IQR, interquartile range; NUC, nucleos(t)ide analogs.

**Table 2**
Multivariable logistic regression analysis to identify factors significantly correlated with the lack of HDV screening.

| Variables | Univariable analysis[a] | | Multivariable analysis[a] | |
|---|---|---|---|---|
| | Crude OR (95% CI) | *P*-value | Adjusted OR (95% CI) | *P*-value |
| Gender (Male vs Female[b]) | 1.03 (0.80-1.33) | 0.823 | | |
| Nationality (Non-Italian vs Italian[b]) | **0.72 (0.56-0.93)** | **0.011** | 0.87 (0.63-1.21) | 0.41 |
| Age (per 5 years increase) | **1.05 (1.01-1.09)** | **0.007** | 1.04 (0.99-1.09) | 0.11 |
| Time windows[c] | | | | |
| *2005-2010* | **10.41 (4.76-22.73)** | **<0.0001** | **9.79 (4.48-21.62)** | **<0.0001** |
| *2011-2014* | **2.73 (1.57-4.73)** | **<0.0001** | **2.69 (1.54-4.70)** | **<0.0001** |
| *2015-2018* | **3.02 (1.72-5.29)** | **<0.0001** | **3.10 (1.76-5.46)** | **<0.0001** |
| <2xULN ALT | **1.57 (1.18-2.08)** | **0.002** | **1.61 (1.20-2.15)** | **0.001** |

[a] Univariate and multivariate logistic regression analysis was performed on 1579 patients positive for HBsAg for ≥6 months. Variables with *P*-value <0.05 in univariable analysis were included in multivariable analysis. Variables significantly associated with the lack of HDV screening are reported in bold.
[b] Reference group.
[c] Time window 2019-2022 as reference group.ALT<2xULN, ALT lower than 2-fold upper limit of normality; CI, confidence interval; OR, odds ratio.

years were Italian while 68% of patients <40 years were from Eastern/South-eastern Europe (Supplementary Table 2).

Furthermore, a significantly higher proportion of HDV+ patients were from Eastern/South-eastern European countries (42.7% vs 21.6%, *P* <0.0001) while the percentage of Italian patients was comparable in the two groups (52.1% vs 54.3%, *P* = 0.7) (Table 3). Notably, a remarkable increase in the percentage of anti-HDV+ patients from Eastern/South-eastern Europe was observed in the time window 2015-2022 compared to 2005-2014 (60.6% in 2015-2022 vs 31% in 2005-2014, *P* = 0.004), paralleling with a decrease in the percentage of anti-HDV+ Italian patients (36.8% in 2015-2022 vs 62.1% in 2005-2014, *P* = 0.016) (Table 3).

Only 4.2% of anti-HDV+ patients were from Africa (all from Sub-Saharian Countries) while no patients from Asia resulted positive for anti-HDV (Table 3).

Anti-HDV+ patients were also characterized by significantly higher ALT (median [IQR]: 68 [49-129] vs 44 [26-88] U/l, *P* <0.0001) and HBsAg levels (6943 [1770-15,769] vs 3352 [667-12,597] IU/ml, *P* = 0.049) despite lower serum HBV-DNA (1.6 [1.3-3.2] vs 3.4 [2.0-5.2] logIU/ml, *P* <0.0001) (Table 3). This datum was also confirmed in the subset of patients naïve to anti-HBV drugs, confirming the inhibitory effect exerted by HDV on HBV replicative activity (2.1 [1.5-2.8] vs 3.4 [2.4-5.0] logIU/ml, *P* <0.0001) (data not shown) as previously reported [25,26].

A higher percentage of cirrhosis was observed in anti-HDV+ patients (46.0% vs 18.7%, *P* <0.0001) (Table 3). In particular, among the 29 anti-HDV+ cirrhotic patients, 10 (34.5%) had overt clinical signs of decompensated cirrhosis, such as ascites, varices, or encephalopathy while 11 patients (37.9%) received a diagnosis of cirrhosis based on transient elastography with a value >14 kPa. Among them, nine patients had also a FIB-4 score >3.6 (median [IQR]: 7.1 [6.3-11.4]). Finally, eight (27.6%) patients received the diagnosis of compensated cirrhosis on the basis of liver histological examination, showing the typical features of cirrhotic progression.

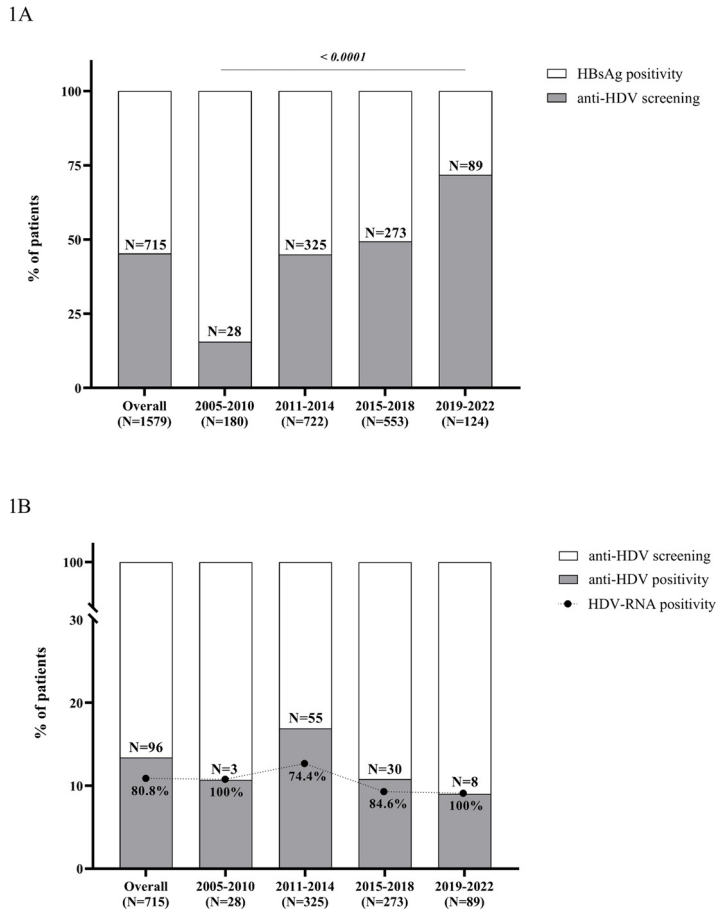


**Figure 1.** Rate of HDV screening and seroprevalence over time. The histogram in panel a reports the percentage of patients undergoing HDV screening (based on the search for anti-HDV antibodies) in the overall population (N = 1579) and according to different time windows: 2005-2010 (N = 180), 2011-2014 (N = 722), 2015-2018 (N = 553), 2019-2022 (N = 124). The numbers in the columns represent the absolute number of screened patients in the overall population and in each time window. Panel b reports the percentage of anti-HDV+ patients in the overall group of patients undergoing HDV screening (based on the search for anti-HDV antibodies) (N = 715) and stratified according to the different time windows: 2005-2010 (N = 28), 2011-2015 (N = 325), 2016-2019 (N = 273), 2020-2022 (N = 89). The numbers in the column represent the absolute number of patients resulting positive to anti-HDV in the overall population and in each time window. The dots represent the percentage of patients with detectable serum HDV RNA: 80.8% in the overall population of anti-HDV+ patients tested for HDV RNA and 100% in 2005-2010, 74.4% in 2011-2015, 84.6% in 2016-2019, 100% in 2020-2022. The percentages under the dots represent the rate of HDV-RNA+ patients calculated on the amount of patients tested for HDV-RNA in the overall population and in each time window. Statistical significance was assessed by chi-squared for trend based on 2 × 4 contingency table.

Anti-HDV+ patients were also characterized by higher rate of positivity to anti-HCV antibodies (7.3% vs 2.9%, $P = 0.030$) (Table 3). Notably, all patients positive for anti-HDV and anti-HCV had an undetectable serum HCV-RNA. Conversely, the percentage of patients with HIV coinfection was comparable between the two groups (4.2% vs 4.7%, $P = 0.822$) (Table 3).

Lastly, by analyzing the distribution of HBV genotypes according to the anti-HDV status, a more marked dominance of HBV genotype-D was revealed in anti-HDV+ with respect to anti-HDV-negative patients (HBV genotype-D prevalence: 82.8% vs 59.5%, $P < 0.001$) (Supplementary Figure 1a), suggesting a preferential circulation of HDV in association with HBV genotype-D in Italy.

Focusing on HBV subgenotypes D, the most prevalent was D3 (Supplementary Figure 1a). A similar distribution of HBV subgenotypes D was revealed across all HBsAg+ patients, independently from the presence of HDV coinfection (Supplementary Figure 1b).

### *HDV infection among Italian and Eastern/South-eastern European patients*

Since Eastern/South-eastern European and Italian patients represent together almost the totality (90%) of HDV-infected patients in our cohort, the virological and clinical characteristics of these two different groups of patients were compared.

Focusing on the 41 anti-HDV+ patients from Eastern/South-eastern Europe, 58.6% were from Romania, 26.8% from Moldova, and 7.3% from Albania and Ukraine, respectively. Furthermore, anti-HDV+ patients from Eastern/South-eastern Europe were significantly younger than Italians (44 [37-54] vs 53 [47-62] years, $P < 0.0001$), and this difference was even more marked in recent years (44 [38-54] vs 58 [53-63] years, $P < 0.0001$) (Table 4). A trend toward a higher female prevalence was noted in anti-HDV+ patients from Eastern/South-eastern Europe (53.7% vs 24%) (Table 4). This difference in gender distribution was particularly pronounced in the time window 2005-2014 in which 66.7% of anti-HDV+ patients from Eastern/South-eastern Europe were female (Table 4).

A lower percentage of NUC treatment was observed in anti-HDV+ from Eastern/Southern-eastern Europe than those from Italy (58.5% vs 80%, $P = 0.026$). Anti-HDV+ from Eastern/Southern-eastern Europe was also characterized by a shorter duration of NUC treatment (2.6 [1.2-4.4] vs 5.4 [2.4-14.6] years, $P = 0.0004$) (Table 4).

These findings support distinct profiles underlying anti-HDV+ patients from Italy and Eastern/South-eastern Europe.

By analyzing virological and biochemical parameters, Eastern/South-eastern European patients had higher levels of HDV-

**Table 3**
Characteristics of HBsAg+ according to HDV screening result and across the different time windows.

| | Overall screened population | | *P*-value[a] | Anti-HDV + | | *P*-value[a] |
|---|---|---|---|---|---|---|
| | Anti-HDV - (N = 619) | Anti-HDV + (N = 96) | | 2005-2014 (N = 58) | 2015-2022 (N = 38) | |
| Male, *N (%)* | 400 (64.6) | 61 (63.5) | 0.977 | 37 (63.8) | 24 (63.2) | 0.950 |
| Country of origin, *N (%)* | | | | | | |
| Italy | 336 (54.3) | 50 (52.1) | 0.752 | 36 (62.1) | 14 (36.8) | **0.016** |
| Eastern/South-eastern Europe | 134 (21.6) | 41 (42.7) | **<0.0001** | 18 (31.0) | 23 (60.6) | **0.004** |
| *Albania* | *29 (3.4)* | *3 (7.3)* | ***0.039*** | *1 (5.5)* | *2 (8.7)* | *1.000* |
| *Moldova* | *10 (1.6)* | *11 (26.8)* | ***0.002*** | *3 (16.7)* | *8 (34.8)* | *0.291* |
| *Romania* | *90 (14.5)* | *24 (58.6)* | *0.149* | *13 (72.3)* | *11 (47.8)* | *0.201* |
| *Ukraine* | *5 (0.8)* | *3 (7.3)* | *0.393* | *1 (5.5)* | *2 (8.7)* | *1.000* |
| Asia | 37 (6.0) | 0 (0.0) | **0.006** | 0 (0.0) | 0 (0.0) | |
| *South Asia* | *29 (3.4)* | *0 (0.0)* | ***<0.0001*** | | | |
| *East Asia* | *8 (1.3)* | *0 (0.0)* | *0.606* | | | |
| Africa | 104 (16.8) | 4 (4.2) | **0.001** | 3 (5.2) | 1 (2.6) | 1.000 |
| *North Africa* | *15 (2.4)* | *0 (0.0)* | *1.000* | *0 (0.0)* | *0 (0.0)* | *1.000* |
| *Sub-Saharan Africa* | *89 (14.4)* | *4 (100.0)* | *1.000* | *3 (100.0)* | *1 (100)* | *1.000* |
| South American | 7 (1.1) | 0 (0.0) | 0.602 | 0 (0.0) | 0 (0.0) | |
| Unknown | 1 (0.2) | 1 (1.0) | | 1 (1.7) | 0 (0.0) | |
| Age, *median (IQR) years* | 45 (32-59) | 49 (40-57) | 0.068 | 48 (39-56) | 51 (43-58) | **<0.0001** |
| *[min-max] years* | [4-85] | [18-78] | | [18-78] | [23-75] | |
| ALT levels, *median (IQR) U/l* | 44 (26-88) | 68 (49-129) | **<0.0001** | 67 (46-126) | 51 (43-58) | 0.368 |
| *[min-max] U/l* | [6-3355] | [11-2000] | | [11-2000] | [17-849] | |
| Cirrhosis, *N (%)*[b] | 55 (18.7) | 29 (46.0) | **<0.0001** | 17 (44.7) | 12 (48.0) | 0.617 |
| HBsAg levels, *median (IQR) IU/ml* | 3352 (667-12,597) | 6943 (1770-15,769) | **0.049** | 5500 (873-16,369) | 8080 (2089-17,096) | 0.912 |
| *[min-max] IU/ml* | [2-134,651] | [3-67,588] | | [3-65,882] | [5-67,588] | |
| HBV-DNA levels, *median log (IQR) IU/ml* | 3.4 (2.0-5.2) | 1.6 (1.3-3.2) | **<0.0001** | 1.5 (1.3-3.2) | 1.9 (1.1-3.0) | **0.005** |
| NUC-treated patients, *N (%)* | 304 (49.2) | 67 (69.8) | **< 0.0001** | 45 (77.6) | 22 (57.9) | **0.04** |
| Patients with detectable serum HDV-RNA, *N (%)*[c] | | 63 (80.8) | | 33 (75.0) | 30 (88.2) | 0.637 |
| HDV-RNA levels, *median log (IQR) IU/ml* | | 4.6 (3.6-5.6) | | 4.4 (3.6-4.9) | 5.0 (3.5-5.8) | 0.285 |
| Coinfections | | | | | | |
| Anti-HCV+, *N (%)* | 18 (2.9) | 7 (7.3) | **0.03** | 5 (8.6) | 2 (5.3) | 0.7 |
| HCV-RNA levels, *median log (IQR) IU/ml* | 6.0 (5.1-6.1) | 0.0 (0.0-0.0) | 0.190 | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | |
| HIV +, *N (%)* | 29 (4.7) | 4 (4.2) | 0.822 | 2 (3.4) | 2 (5.3) | 0.647 |
| HIV-RNA levels, *median (IQR) copies/ml* | 667 (20-15,466) | 2192 (1111-232,677) | 0.984 | <20; 463,162[d] | 29; 2192[d] | |
| CD4 T-cells, *median (IQR) cells/µl* | 530 (348-695) | 562 (157-977) | 0.902 | 158; 1011[d] | 154; 966[d] | |

[a] Statistically significant differences were assessed by chi-squared test of independence based on a 2 × 2 contingency table for qualitative data, and Mann-Whitney test for continuous data.
[b] Data available for 357 patients: 294 anti-HDV-negative and 63 anti-HDV+.
[c] Calculated on 77 anti-HDV+ patients for whom serum HDV-RNA was tested.
[d] The single values of the two patients are reported.ALT: alanine ammino-transferase, IQR: interquartile range; NUC: nucleos(t)ide analogs.

RNA (median [IQR]: 4.8 [3.6-5.8] vs 3.9 [1.4-4.9] log copies/ml, $P = 0.016$) and HBsAg (median [IQR]: 9461 [4159-24,532] vs 4447 [737-13,336] IU/ml), $P = 0.032$), despite a similar degree of HBV replication (median [IQR] serum HBV-DNA: 405 (30-1444) and 132 (20-500) IU/ml, $P = 0.68$) (Table 4). The association of patients from Eastern/South-eastern Europe with higher levels of HDV-RNA has been confirmed in drug naïve (Median [IQR]: 5.0 [3.6-5.8] versus 3.4 [0.0-4.6] IU/ml, $P = 0.03$) and a trend was observed in NUC-treated patients (median [IQR] HDV-RNA: 4.6 [3.5-5.8] vs 4.0 [2.5-5.9] IU/ml, $P = 0.07$) (data not shown). Nevertheless, the levels of ALT were comparable between Eastern/Southern-eastern European and Italian patients (median [IQR] ALT: 67 (30-175) vs 78 (55-122) U/l, $P = 0.363$), also after stratification according to NUC treatment (median [IQR]: 78 (58-145) vs 62 (30-164) U/l, $P = 0.342$ for NUC-treated patients and 70 (52-114) vs 84 (59-147) U/l, $P = 0.764$ for drug-naïve patients). Similarly, the prevalence of cirrhosis was comparable between the two groups of patients (41.3% vs 50.0%, $P = 0.609$). These results support limited differences in terms of cytolytic activity and liver injury between Italian and Eastern/Southern-eastern European anti-HDV+ patients.

*Proportion of patients with active viral replication*

Among the 96 anti-HDV+ patients, 19 had never been tested for quantitative HDV-RNA, highlighting an incomplete characterization of HDV infection in a not negligible proportion of patients despite HDV seropositivity. Among the remaining 78 anti-HDV+ patients tested for quantitative HDV-RNA, 80.8% had a detectable HDV-RNA with median (IQR) levels of 4.6 (3.6-5.6) log copies/ml, unveiling an active HDV replication in most anti-HDV+ patients (Table 1, Figure 1b).

Most patients with active HDV replication (89.3%) presented altered ALT (median [IQR]: 92 [62-177] U/L) and 55.3% were cirrhotic. Notably, patients with a serum HDV-RNA >5000 copies/ml were characterized by significantly higher ALT levels than patients with a serum HDV-RNA <5000 copies/ml, highlighting a correlation between the extent of HDV replicative activity and the degree of liver disease activity (data not shown).

*Circulating HDV-genotypes*

HDV sequences were obtained for 27 patients with detectable serum HDV-RNA and phylogenetic analysis revealed the circulation of HDV-genotype 1 in all of them (Supplementary Figure 2).

Since emerging data have highlighted the differentiation of HDV-genotype 1 into different subgenotypes [19], their circulation was explored in this set of patients. Phylogenetic analysis successfully attributed HDV subgenotype one for 19 of 27 patients. Remarkably, two HDV subgenotypes 1 were identified: 1c (N = 10, 52.6%) and 1e (N = 9, 47.4%) (Supplementary Figure 3). Notably, subgenotype 1e was significantly correlated with higher ALT levels than 1c (168 [89-190] vs 58 [54-88] U/l, $P = 0.015$) despite comparable serum HDV-RNA (4.1 [3.6-5.0] vs 4.6 [4.2-5.9] log copies/ml, $P = 0.174$). Furthermore, 80% of patients harboring subgenotype 1e

**Table 4**
Characteristics of anti-HDV+ patients stratified according to Italian and Eastern European nationality and stratified according to the different time windows.

| | Anti-HDV+ patients | | *P*-value[a] | 2005-2014 | | *P*-value[a] | 2015-2022 | | *P*-value[a] |
|---|---|---|---|---|---|---|---|---|---|
| | Italian (N = 50) | Eastern/South-eastern European (N = 41) | | Italian (N = 36) | Eastern/South-eastern European (N = 18) | | Italian (N = 14) | Eastern/South-eastern European (N = 23) | |
| Male, *N (%)* | 38 (76.0) | 19 (46.3) | 0.212 | 28 (77.8) | 6 (33.3) | **0.002** | 10 (71.4) | 13 (56.5) | 0.491 |
| Age, *median (IQR)* | 53 (47-62) | 44 (37-54) | **<0.0001** | 53 (45-60) | 40 (35-53) | 0.143 | 58 (53-63) | 44 (38-54) | **<0.0001** |
| ALT levels, *median U/l (IQR)* | 67 (30-175) | 78 (55-122) | 0.363 | 63 (29-194) | 70 (56-120) | 0.815 | 68 (37-152) | 78 (52-140) | 0.697 |
| Cirrhosis, *N (%)*[b] | 16 (50.0) | 12 (41.3) | 0.609 | 13 (54.2) | 4 (30.8) | 0.300 | 3 (37.5) | 8 (38.1) | 0.667 |
| HBsAg levels, *median IU/ml (IQR)* | 4447 (737-13,336) | 9461 (4159-24,532) | **0.032** | 3560 (452-11,612) | 10,169 (5098-26,677) | 0.111 | 7958 (1416-14,252) | 9229 (2392-27,839) | 0.887 |
| HBV-DNA levels, *median IU/mL (IQR)* | 405 (30-1444) | 132 (20-500) | 0.681 | 32 (20-2989) | 39 (20-869) | 0.769 | 767 (10-388,402) | 70 (12-390) | 0.332 |
| Patients with detectable HDV-RNA, *N (%)* | 29 (74.4) | 31 (91.2) | 0.073 | 19 (70.3) | 11 (84.6) | 0.451 | 10 (83.3) | 20 (95.2) | 0.252 |
| HDV-RNA levels, *median log cp/ml (IQR)* | 3.9 (2.0-4.7) | 4.8 (3.6-5.8) | **0.016** | 4.1 (3.5-4.9) | 4.7 (4.3-5.8) | 0.131 | 4.5 (3.3-5.9) | 5.3 (3.6-5.8) | 0.530 |
| NUC-treated patients, *N (%)* | 40 (80.0) | 24 (58.5) | **0.026** | 28 (77.8) | 13 (72.2) | 0.475 | 12 (85.7) | 11 (47.8) | **0.035** |
| NUC treatment duration, *median years (IQR)* | 5.4 (2.4-14.6) | 2.6 (1.2-4.4) | **0.0004** | 5.0 (1.7-14.1) | 1.2 (1.2-2.1) | **0.039** | 5.8 (3.2-16.4) | 2.6 (1.3-12.8) | 0.289 |

[a] Statistically significant differences were assessed by chi-squared test of independence based on a 2 × 2 contingency table for qualitative data, and Mann-Whitney test for continuous data.
[b] Data available for 61 patients. In detail for 32 Italian patients and 29 Eastern/Southern-eastern European patients.ALT: alanine ammino-transferase; IQR: interquartile range.

showed 2xULN ALT versus 25% of patients with subgenotype 1c ($P = 0.05$) (Supplementary Table 3).

No statistically significant differences between HDV subgenotypes were observed for the other demographic and virological parameters (Supplementary Table 3).

## Discussion

This study, based on a large cohort of patients with chronic HBV infection (N = 1579), depicts a scarcely declining scenario for HDV infection in the setting of tertiary care centers in Italy, with an HDV seroprevalence persisting over 9% among HBsAg+ patients across the last 2 decades, in line with other studies from Western European Countries [10,27–29]. Overall, these data remark the importance of diagnosing HDV infection and promoting their linkage to care, limiting viral spreading, reducing a subsequent risk of liver disease progression, and in turn minimizing the impact of HDV infection on the National Health System. This is also critical considering a recent study, based on data from Italian Surveillance System of Acute Viral Hepatitis. This study shows that sexual transmission (and not intravenous drug injection) represents, so far, the most common route of acquiring acute HDV infection, thus highlighting a changing scenario in the mode of HDV transmission [30].

Our study also highlights that the awareness to request HDV screening has increased over time. However, complete HDV screening in all HBsAg+ patients has not been achieved even in the most recent temporal window with almost 30% of patients still not tested for anti-HDV antibodies despite HBsAg-positivity. In particular, the correlation between the lack of screening and the presence of normal/slightly altered transaminases suggests that in a relevant proportion of patients, the diagnosis of HDV infection could be delayed until liver disease has already progressed, with a subsequent negative impact on the patient's clinical outcome and therapeutic response.

In this light, a potential strategy to achieve the endpoint of a complete and early HDV screening in all HBsAg+ patients (as recommended by the current European Association for the Study of the Liver and Asian Pacific Association for the Study of the Liver guidelines) relies on the application of reflex testing, the algorithm that implies the automatic testing of anti-HDV antibodies in all individuals resulting positive to HBsAg [31].

In this regard, a recent study in Spain showed that the implementation of reflex testing resulted in a 5-fold increase in the absolute number of anti-HDV+ patients [9]. Notably, this study showed that 60% of patients, positive for anti-HDV by reflex testing, had no risk factor for HDV infection [9]. Furthermore, by applying mathematical models, another study showed that the introduction of reflex testing in diagnostic practice (by favoring an early HDV diagnosis) could determine a 15% reduction in liver complications over a time window of 5 years [32]. This evidence reinforces the rationale of a screening strategy based on systematic anti-HDV testing in all HBsAg+ patients, rather than according to risk factors.

Another diagnostic criticism that emerged from our study is that a not negligible proportion of HDV-seropositive patients (20%) has not been tested for quantitative HDV-RNA despite anti-HDV positivity, thus missing the diagnosis of HDV active infection. To overcome this issue, a double reflex testing strategy, based not only on anti-HDV testing in all HBsAg+ individuals but also on HDV-RNA quantification in all patients resulted positive for anti-HDV, has been recently proposed to optimize the diagnosis of chronic HDV infection and, in turn, to improve patients' clinical management including treatment initiation [31].

Our study shows an active HDV replication in most HDV-seropositive patients (80%, similar to that observed in other European studies [33]), reinforcing the importance of completing HDV diagnostic flowchart by assessing HDV-RNA quantification. In line

with a recent study [34], our data also highlight that an active HDV infection is characterized by a remarkable prevalence of liver inflammation (90%) and cirrhosis (55.3%). This is also in keeping with previous studies showing the prognostic role of active viral replication in promoting liver disease progression [4,33] and in acting as an independent factor significantly correlated with the onset of HCC [4,35]. Again, this highlights the importance of testing all HBsAg+ patients and fully suppressing viral replication by pharmacological treatment to slow down the progression toward advanced liver disease phases.

It should be noted that the prevalence of anti-HDV positivity and chronic HDV infection, observed in our study population, can be influenced by the fact that these patients, followed in tertiary care centers, were characterized by more active liver disease, and thus were selected for HDV screening. Another aspect that should be considered is represented by the fact that the rate of HDV-RNA detectability could have been influenced by the different analytical performances of the assays used over time for serum HDV-RNA quantification. Given the critical role of serum HDV-RNA quantification in the diagnosis of chronic HDV infection and in ensuring adequate monitoring of virological response to anti-HDV drugs, this highlights the urgent need to set up studies aimed at assessing the robustness and reproducibility of the currently available assays for the quantification of this parameter in clinical practice.

Our study shows distinct profiles underlying anti-HDV+ patients from Eastern/South-eastern Europe and Italy. Indeed, anti-HDV+ patients from Eastern/South-eastern Europe are typically younger than Italian patients (44 [37-54] vs 53 [47-62] years, $P < 0.0001$, respectively), are less frequently treated with NUC (58.5% vs 80%, $P = 0.026$) and for a shorter duration (2.6 [1.2-4.4] vs 5.4 [2.4-14.6] years, $P = 0.0004$). Such distinct characteristics can also explain the reason why Eastern/South-eastern European patients tend to have higher levels of serum HDV-RNA and HBsAg. In this regard, it is known that older patients, usually in the advanced phases of chronic HBV infection, can be characterized by a more restricted HBV intrahepatic reservoir [36,37] that in turn could constrain HDV replicative activity. *Ad hoc* designed studies are necessary to better unravel this interesting issue. Nevertheless, the levels of ALT and the prevalence of cirrhosis were comparable between the two groups of patients, supporting limited differences in cytolytic activity and liver injury in Eastern/Southern-eastern European and Italian patients.

In our study, HDV seroprevalence is remarkably lower in patients from Africa and Asia than that observed in patients from Italy and Eastern/South-eastern Europe. In particular, among the 108 African patients tested for anti-HDV, only four resulted in anti-HDV+. All of them were from Sub-Saharan Countries (one from Togo, one from Gambia, and two from Senegal), known to be characterized by a higher HDV seroprevalence (HDV seroprevalence ranging from 10-30%) according to [5]. Conversely, no anti-HDV+ patients were from Asia. Nevertheless, it cannot be excluded that the lower HDV seroprevalence in patients from Africa and Asia can be explained by the circulation in these patients of different HDV serotypes that could elicit the production of antibodies not capable of fully recognizing the HDV antigens commonly used in the assays for anti-HDV testing [38]. Given the high degree of HDV genetic variability, this could raise the need to optimize the diagnostic performances of the assays for anti-HDV recognition by maximizing the repertoire of Delta antigens with different genetic profiles.

Lastly, the phylogenetic analysis of HDV sequences revealed the circulation of two HDV subgenotypes 1 in Italy (1e, 52.6% and 1c, 47.4%). Notably, our results show that subgenotype 1e can be correlated with an increased liver inflammation compared to HDV subgenotype 1c, suggesting that the genetic variability of the different subgenotypes could potentially modulate HDV pathogenic properties despite comparable replicative activity. In keeping with our findings, a recent study has shown that genotype 1 circulating in Europe correlates with higher progression to cirrhosis than genotypes circulating in other geographic areas [35]. This topic deserves further investigation in longitudinal studies based on larger sample size.

In our study, phylogenetic analysis failed to attribute the subgenotype in nine patients, suggesting the potential circulation of recombinant forms or still unclassified subgenotype 1.

This study also showed a predominant circulation of HBV genotype-D in anti-HDV+ patients compared to anti-HDV-negative ones, suggesting a preferential co-evolution of HDV with HBV genotype-D in Italy. Although it has been demonstrated that the different HBV genotypes can efficiently support HDV-genotype 1 morphogenesis even if with heterogenous kinetics [39], higher infectivity of HDV-genotype 1 has been observed with HBV genotype-D derived HBsAg [40].

In this light, our findings on the detection of different subgenotypes-1, potentially triggering variable inflammatory stimuli, coupled with their high genetic variability (up to 16%) [15], support the need to expand HDV molecular epidemiology and its impact on modulating the pathogenesis of HDV-related disease.

In conclusion, the prevalence of HDV infection may still represent a relevant clinical issue among HBsAg carriers from tertiary care centers, because of its scarcely declining seroprevalence over time and its frequent association with advanced liver disease. Despite this scenario, diagnostic approaches are still suboptimal with relevant gaps remaining to achieve complete and early diagnosis, which could finally reduce the morbidity and mortality of HDV-infected patients. Lastly, the variegated molecular epidemiological scenario, characterized by the circulation of different HDV subgenotypes 1, associated with variable inflammatory levels, suggests the existence of viral mechanisms underlying HDV pathogenic potential, that deserves further in-depth investigations.

## Declaration of Competing Interest

The authors have no competing interests to declare.

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## Ethical approval

Approval by the Ethics Committee was deemed unnecessary because, under Italian law, biomedical research is subjected to previous approval only in the hypothesis of clinical trials on medicinal products for clinical use (art. 6 and art. 9, leg. decree 211/2003 D.L.196/2003). The research was conducted on data previously anonymized, according to the Italian Data Protection Code requirements (D.L.101/2018 and 139/2021). Data were collected in full compliance with the Italian law on personal data protection, and each patient signed an informed consent for participation in the anonymous use of their clinical data for scientific purposes.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.11.005.

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